PI3′-Kinase Inhibition Forestalls the Onset of MEK1/2 Inhibitor Resistance in BRAF-Mutated Melanoma

Marian M. Deuker1, Victoria Marsh Durban1, Wayne A. Phillips2, and Martin McMahon1

ABSTRACT

Phosphatidylinositide 3′(PI3′)-lipid signaling cooperates with oncogenic BRAFV600E to promote melanomagenesis. Sustained PI3′-lipid production commonly occurs via silencing of the PI3′-lipid phosphatase PTEN or, less commonly, through mutational activation of PIK3CA, encoding the 110-kDa catalytic subunit of PI3′-kinase-α (PI3Kα). To define the PI3K catalytic isoform dependency of BRAF-mutated melanoma, we used pharmacologic, isoform-selective PI3K inhibitors in conjunction with melanoma-derived cell lines and genetically engineered mouse (GEM) models. Although BRAFV600E/PIK3CAH1047R melanomas were sensitive to the antiproliferative effects of selective PI3Kα blockade, inhibition of BRAFV600E/PTENNull melanoma proliferation required combined blockade of PI3Kα, PI3Kδ, and PI3Kγ, and was insensitive to PI3Kβ blockade. In GEM models, isoform-selective PI3K inhibition elicited cytostatic effects, but significantly potentiated melanoma regression in response to BRAFV600E pathway–targeted inhibition. Interestingly, PI3K inhibition forestalled the onset of MEK inhibitor resistance in two independent GEM models of BRAFV600E-driven melanoma. These results suggest that combination therapy with PI3K inhibitors may be a useful strategy to extend the duration of clinical response of patients with BRAF-mutated melanoma to BRAFV600E pathway–targeted therapies.

SIGNIFICANCE: Although BRAFV600E pathway–targeted therapies elicit melanoma regression, the onset of drug resistance limits the durability of response. Here, we show that combined treatment with PI3K inhibitors significantly forestalled the onset of MEK1/2 inhibitor–resistant disease in BRAF-mutated GEM melanoma models. These results provide a conceptual framework for the combined deployment of BRAFV600E plus PI3K pathway–targeted inhibitors in the treatment of a subset of patients with BRAF-mutated melanoma. Cancer Discov; 5(2); 143–53. ©2014 AACR.
INTRODUCTION

Over the past 15 years, key genetic lesions that initiate melanomagenesis, promote disease progression, and remain necessary for melanoma maintenance have been identified (1, 2). Approximately 50% of melanomas express mutationally activated BRAFV600E, leading to constitutive activation of the MAP kinase pathway (3). The importance of this pathway in melanoma maintenance is highlighted by the ability of BRAFV600E pathway–targeted inhibitors to elicit dramatic tumor regression in patients with BRAF-mutated, advanced melanoma (4–6). Although the response rate of such patients is high, the depth and durability of response is limited by the onset of drug-resistant disease that is largely refractory to additional BRAFV600E pathway–targeted therapy (7, 8). Therefore, it is critical to identify signaling pathways that contribute to de novo or acquired drug resistance, and to determine whether pharmacologic blockade of these pathways can increase the response rate or the durability of response to BRAFV600E pathway–targeted therapies. Although multiple mechanisms of acquired drug resistance have been documented, the extent to which parallel inhibition of signaling pathways will enhance melanoma patient responses remains unclear (9, 10).

Using genetically engineered mouse (GEM) models, we previously demonstrated that either PTEN silencing or mutationally activated PIK3CAH1047R cooperates with BRAFV600E to elicit metastatic melanoma. However, BRAFV600E/PIK3CAH1047R melanomas grew more slowly than similarly elicited BRAFV600E/PTENnull melanomas (11). In addition, although a pan-class I phosphatidylinositol 3′-kinase (PI3K) inhibitor (BMK120) significantly potentiated the ability of a BRAFV600E inhibitor (LGX818) to induce regression of autochthonous BRAFV600E/PTENnull melanomas, BMK120 was largely ineffective as a single agent (11). Given the frequency of alterations in PI3′-lipid signaling in BRAF-mutated melanoma (12–15), we wished to explore the role of PI3K signaling in melanoma progression and maintenance, as well as the therapeutic implications of targeting this pathway using isoform-selective inhibitors. Our studies reveal that the difference in growth rate between BRAFV600E/PIK3CAH1047R and BRAFV600E/PTENnull melanomas is likely due to the strength of PI3K pathway activation. However, potent blockade of PI3K signaling in either BRAFV600E/PIK3CAH1047R or BRAFV600E/PTENnull melanomas elicited largely cytostatic effects. Finally, and most interestingly, combined blockade of BRAFV600E and PI3K signaling significantly enhanced the depth and durability of the response of BRAFV600E/PIK3CAH1047R or BRAFV600E/PTENnull melanoma to the MEK1/2 inhibitor GDC-0973. These data provide a scientific rationale for the clinical development of such regimens for patients with BRAF-mutated melanoma in which the PI3K pathway is activated by either PTEN silencing or PIK3CA mutation.

RESULTS

PTEN is reported to have both phosphatase-dependent and phosphatase-independent tumor-suppressor activities (16–18). To address whether differences in growth rate between BRAFV600E/PIK3CAH1047R and BRAFV600E/PTENnull melanoma reflect a role for phosphatase-independent tumor-suppressor activities of PTEN, we compared the growth rate of BRAF-mutated melanomas in TycrereBraf+/+ mice that were homozygous for the Pik3canull allele or either heterozygous or homozygous for the conditional Cre-activated Pik3ca+/− (Pik3ca hereafter) allele (Fig. 1A). As shown previously (11), BRAFV600E/PTENnull melanomas grew more rapidly than BRAFV600E/PIK3CAH1047R melanomas arising in heterozygous Pik3ca+/− mice (Fig. 1A). However, BRAFV600E/PIK3CAH1047R melanomas arising in homozygous Pik3ca−/− mice grew significantly more rapidly than BRAFV600E/PTENnull melanomas, suggesting that differences in the growth rate between BRAFV600E/PIK3CAH1047R and BRAFV600E/PTENnull melanomas are likely due to the magnitude of PI3K pathway activation. In addition, cell lines derived from BRAFV600E/PTENnull/CDKN2A Null (B10C) or BRAFV600E/PIK3CAH1047R/H1047R/CDKN2A Null (BPC) melanomas grew more rapidly in vitro than did a cell line derived from a BRAFV600E/PIK3CAH1047R/CDKN2A Null (BPC) melanoma (M.M. Deuker and M. McMahon, unpublished data).

To determine the PI3K isoform dependence of BRAF-mutated melanoma, we treated BPC and B10C melanoma...
**PI3K Inhibition Forestalls MEK Inhibitor Resistance**

**RESEARCH BRIEF**

**A**

Tumor volume (mm³)

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**B**

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**G**

**H**

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**I**

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cell lines with pharmacologic inhibitors of PI3K (Supplementary Table S1). BPC melanoma cells treated with BYL719 (19), a selective inhibitor of PI3-kinase-α (PI3Kα), displayed a robust reduction in cell proliferation over a 72-hour time period and colony-forming ability over a 10-day period (Fig. 1B and C). In contrast, BYL719 treatment of B10C melanoma cells elicited only a modest reduction in short-term cell proliferation and had no effect on long-term colony formation (Fig. 1B and C). Indeed, there was a greater than 10-fold difference between the concentration of BYL719 required for 50% inhibition of proliferation (GL50) of BPC versus B10C cells (Fig. 1D). In addition, the BP.C melanoma cell line derived from homozygous Pik3caH1047R mice displayed similar sensitivity to BYL719 as did the BPC cells (Supplementary Fig. S1A and S1B). Thus, BRAFV600E/PIK3CAH1047R melanoma cells display the predicted geno-type–drug response phenotype relationship. In contrast, BRAFV600E/PTENH1107R melanoma cells appear not to depend solely on PI3Kα for their proliferation.

To examine the effects of PI3Kα blockade on signal pathway activity, extracts of BPC or B10C melanoma cells treated with BYL719 (5 μM/L) were subjected to immunoblot analysis (Fig. 1E). In BPC cells, BYL719 elicited a complete and sustained inhibition of phosphorylated (p) AKT (pS473) over 72 hours. We also noted diminished phosphorylation of downstream pathway components of PI3K→AKT signaling, including PRAS40 and 4E-BP1 (Fig. 1E). In contrast, BYL719-treated B10C cells displayed only a partial and transient inhibition of pAKT with almost no effect on pPRAS40 or p4E-BP1.

Because BRAFV600E and PI3K signal cooperatively through mTOR to regulate melanoma cell proliferation (20), we investigated whether PI3Kα inhibition would enhance the effects of BRAFV600E inhibition in BPC or B10C melanoma cells. Although single-agent BRAFV600E (LGX818; ref. 21) or PI3Kα (BYL719) inhibition potently suppressed BPC melanoma cell proliferation, combined treatment elicited a significantly greater inhibition of cell proliferation at 24, 48, and 72 hours (Fig. 1F). Furthermore, although inhibition of PI3Kα suppressed pPRAS40, pRPS6, and p4E-BP1 in BPC melanoma cells, combined inhibition of both BRAFV600E and PIK3CAH1047R signaling elicited a more robust inhibition of these phosphorylation events (Fig. 1G). Similar observations were made in the independently derived BP.C melanoma cell line (Supplementary Fig. S1C). In contrast, whereas BRAFV600E inhibition (LGX818) potently suppressed B10C cell proliferation, the addition of BYL719 did not significantly enhance the anti-proliferative effects of BRAFV600E inhibition at any time point (Fig. 1F). In B10C cells, LGX818 inhibited pERK but had little effect on pPRAS40 or p4E-BP1 (Fig. 1G).

Because we have previously shown that selective inhibition on the proliferation of human BRAFV600E/PTENH1107R melanoma-derived cells (22). Seven weeks after initiation, mice were randomized to receive vehicle, LGX818, BYL719, or combined LGX818 plus BYL719 treatment, with melanoma size measured weekly. Pharmacodynamic analysis of pAKT inhibition in BRAFV600E/PIK3CAH1047R melanomas indicated the need to dose BYL719 twice daily to achieve maximal target inhibition (Supplementary Fig. S1D).

Single-agent BYL719 initially elicited modest melanoma regression (<30%), followed by a prolonged cytostatic effect (Fig. 1H). In contrast, single-agent LGX818 elicited profound melanoma regression. Importantly, the combination of BYL719 plus LGX818 promoted significantly more potent melanoma regression than that observed with LGX818 monotherapy (Fig. 1H). Analysis of the best overall response by waterfall plot indicated that only 2 of 8 mice treated with BYL719 displayed >30% melanoma regression, which qualifies as a partial response (PR) by modified RECIST 1.1 guidelines (Fig. 1; ref. 23). In contrast, 7 of 7 mice treated with LGX818 exceeded the 30% regression threshold, as did 8 of 8 mice receiving BYL719 plus LGX818. Finally, combined treatment with LGX818 plus BYL719 provided significantly superior melanoma regression compared to single-agent LGX818 therapy (Fig. 1I).

Analysis of glioblastoma, breast cancer, or prostate cancer models suggests that PI3-kinase-β (PI3Kβ/PI3KCB) is the predominant driver of PI3′-lipid production in PTENH1107R tumors (24, 25). Consequently, we tested the effects of PI3Kβ selective inhibition on the proliferation of human BRAFV600E/PTENH1107R melanoma-derived cells. Two structurally distinct PI3Kβ selective inhibitors were used: GSK2636771 (GSK771) and KIN193 (26–28). Perhaps surprisingly, even at the highest concentration tested (10 μM/L), GSK771 failed to reach the GL50 of BRAFV600E/PTENH1107R human melanoma cells (Fig. 2A and Supplementary Fig. S2A). Furthermore, even at 5 μM/L, GSK771 elicited only a minor reduction of pAKT in BRAFV600E/PTENH1107R melanoma cells, with no effects on the phosphorylation of downstream PI3K pathway components (Fig. 2B and Supplementary Fig. S2B). Although KIN193 displayed enhanced—but still modest—antiproliferative activity compared with GSK771, the antiproliferative activity of KIN193 on PI3Kα-dependent BPC cells (Supplementary Fig. S2A) suggests that at higher concentrations, the antiproliferative activity of KIN193 is likely due to inhibition of PI3Kα.

In addition, although 5 μM/L KIN193 modestly suppressed pAKT in some human-derived BRAFV600E/PTENH1107R melanoma cells (Fig. 2B and Supplementary Fig. S2B), the ability of 5 μM/L KIN193 treatment to robustly suppress pAKT in PI3Kα-dependent BPC cells (Supplementary Fig. S2B) further underscores the fact that this activity is likely due to inhibition of PI3Kα. B10C melanoma cells also displayed proliferative and biochemical resistance to PI3Kβ inhibition (Supplementary Fig. S2A and S2B). Interestingly, although both KIN193 and GSK771 treatment suppressed pAKT activation in M249 BRAFV600E/PTENH1107R melanoma cells, this inhibition did not result in reduced phosphorylation of...
PI3K Inhibition Forestalls MEK Inhibitor Resistance

Figure 2. BRAFV600E/PTENNull human melanoma-derived cells are insensitive to PI3K-selective inhibition but sensitive to PI3K-sparing class I PI3K inhibition. A, WM793, 1205Lu, or WM9 melanoma cells were cultured in the presence of the indicated concentrations of KIN193 (pink) or GSK771 (green) for 72 hours before being fixed and stained with Crystal Violet. Crystal Violet staining was quantified as described above. Values indicated are normalized to DMSO control and error bars represent SEM. B, lysates of WM9 melanoma cells treated for 24 hours with the indicated concentrations of KIN193 or GSK771 were analyzed by immunoblotting. C, WM793, 1205Lu, or WM9 melanoma cells were cultured in the presence of the indicated concentrations of BYL719 (pink) or BYL719 plus GSK771 (1 μmol/L, green) for 72 hours before fixation and staining with Crystal Violet. Crystal Violet staining was quantified as described above. Values indicated are normalized to DMSO control and error bars represent SEM. D, WM793, 1205Lu, or WM9 melanoma cells were cultured in the presence of the indicated concentrations of GDC-0032 (blue) or GDC-0941 (pink) for 72 hours before fixation and staining with Crystal Violet. Crystal Violet staining was quantified as described above. Values indicated are normalized to DMSO control and error bars represent SEM. E, lysates of WM9 melanoma cells, treated for 24 hours with DMSO (D), GDC-0941 (2.5 μmol/L), GDC-0032 (2.5 μmol/L), or a 2-fold dilution series from 10 μmol/L to 31.25 μmol/L as indicated by gradient, were analyzed by immunoblotting. F, WM793, 1205Lu, or WM9 melanoma cells were cultured in the presence of the indicated concentrations of IPI145 (pink), BYL719 (purple), BYL719 + 2.5 μmol/L IPI145 (blue), or GDC-0032 (green) for 72 hours before fixation and staining with Crystal Violet. Crystal Violet staining was quantified as described above. Values indicated are normalized to DMSO control, and error bars represent SEM. G, lysates of WM9 melanoma cells treated for the indicated time period with DMSO (D), GDC-0941 (0941), GDC-0032 (0032), BYL719 (B), IPI145 (I) or BYL719+IPI145 (B+I, all at 5 μmol/L) were analyzed by immunoblotting.
PRAS-40 (Supplementary Fig. S2B). Moreover, PI3Kβ inhibition had only modest antiproliferative effects on M249 cells, suggesting that residual PI3Kβ-independent PI3K signaling was sufficient to sustain cell proliferation.

The lack of robust single-agent activity on BRAF/PTEN melanoma cells observed with PI3K selective inhibition led us to hypothesize that BRAF/PTEN human melanoma cells might require the combined activity of PI3Kα and PI3Kβ for sustained proliferation. To test this, we assessed cell proliferation of BRAF/PTEN melanoma cells treated with BYL719 in the presence or absence of a fixed concentration of GSK771 (Fig. 2C). Although PI3Kα inhibition had a modest inhibitory effect on melanoma cell proliferation, the addition of a PI3Kβ inhibitor did not dramatically enhance this effect, suggesting that BRAF/PTEN melanoma cells do not rely exclusively on the combined activity of PI3Kα and PI3Kβ for their proliferation.

To test whether BRAF/PTEN melanoma cells require PI3Kβ to promote PI3′-lipid signaling with downstream effects on cell proliferation, we used GDC-0032, a PI3Kβ-sparing class I PI3K inhibitor that inhibits PI3Kα, PI3Kδ, and PI3Kγ (29). Initially, we compared the antiproliferative activity of GDC-0032 to that of GDC-0941, a pan-class I PI3K inhibitor (30). Both GDC-0032 and GDC-0941 displayed equivalent GI50 values in all BRAF/PTEN melanoma cells tested (Fig. 2D and Supplementary Fig. S2C). Furthermore, treatment with GDC-0032 elicited a dose-dependent reduction in pAKT and its downstream effectors with modestly enhanced potency compared with GDC-0941 (Fig. 2E and Supplementary Fig. S2D). Taken together, these data indicate that PI3Kβ contributes little or nothing to PI3′-lipid signaling or proliferation of BRAF/PTEN melanoma cells.

To confirm that BRAF/PTEN melanoma cells rely on the combined activity of PI3Kα plus PI3Kδ and/or PI3Kγ for sustained proliferation, we investigated whether the effects of GDC-0032 could be mimicked by combined use of PI3Kα (BYL719) and PI3Kδ/γ (IPI145) selective inhibitors (31). Treatment of BRAF/PTEN melanomas in vivo with GDC-0032, either alone or in combination with GDC-0973, to that end, BRAF/PTEN melanoma was initiated in adult Tyr:CreER;Braf<sup>†</sup>F<sup>8</sup>R<sup>lox/lox</sup> mice and, 7 weeks after initiation, mice were randomized to receive vehicle, GDC-0973, GDC-0032, or combination therapy, with melanoma size measured weekly (Fig. 3C). As with BRAF/PI3K<sup>CA110</sup> melanomas treated with BYL719, inhibition of PI3K signaling with GDC-0032 had largely cytostatic effects on BRAF/PTEN melanomas (Fig. 3C and D). In contrast, MEK1/2 inhibition with GDC-0973 elicited substantial regression of BRAF/PTEN melanomas, which was significantly enhanced by combined treatment with GDC-0032 (Fig. 3C and D).

Although the majority of patients with BRAF-mutated melanoma experience initial tumor regression in response to BRAF pathway–targeted therapies, the durability of response is limited by the onset of drug-resistant disease (6). Therefore, we wished to test whether inhibition of class I PI3K isoform(s) would influence the development of resistance to inhibitors that target BRAF signaling. We initially tested this question using vehicle-treated BRAF/PTEN melanoma-bearing mice enrolled in the study described in Fig. 3C. When these mice (n = 8) were near to end stage, they were randomly reassigned to receive extended treatment with
Figure 3. A PI3Kβ-sparing inhibitor enhances the effects of MEK1/2 inhibition on both BRAF<sup>V600E</sup>/PTEN<sup>Null</sup> human melanoma cells and autochthonous mouse melanomas. A, lysates of WM793, 1205Lu, or WM9 melanoma cells treated for 6 hours with DMSO, GDC-0973 (1 μmol/L, pink), GDC-0032 (5 μmol/L, blue), or GDC-0973 plus GDC-0032 (0973+0032) were analyzed by immunoblotting. B, WM793, 1205Lu, or WM9 melanoma cells were cultured in the presence of GDC-0032 (5 μmol/L, blue), GDC-0973 (1 μmol/L, pink), or GDC-0973 plus GDC-0032 (green) with cells fixed and stained with Crystal Violet every 24 hours for 72 hours. Crystal Violet staining was quantified as described above. Error bars represent SEM. Asterisks indicate significant difference between combination drug treatment and single-agent drug treatment (two-way ANOVA, ***P < 0.0005; ****P < 0.0001). C, BRAF<sup>V600E</sup>/PTEN<sup>Null</sup> melanomas were initiated in suitably manipulated adult Tyr::CreER;Braf<sup>CA</sup>; Pten<sup>lox/lox</sup> mice. Following randomization, mice were treated with vehicle, single-agent, or combination GDC-0032 (22.5 mg/kg) and GDC-0973 (4.5 mg/kg, every day). Melanoma growth or regression was measured weekly with digital calipers over the course of 37 days of continuous drug treatment. Mice received GDC-0032 (as single agent or combination therapy) on a twice-a-day regimen for the first 12 days of treatment, but due to apparent toxicity, mice were dosed once a day starting on day 13. Tumor sizes are displayed as the average percentage change in tumor size from the start of treatment, with error bars indicating SEM. Asterisks indicate significant difference between combination drug treatment and single-agent drug treatment (two-way ANOVA, P < 0.0001). D, a waterfall plot of the best tumor response for each of the 32 mice that received vehicle versus drug treatment in C. The percentage change in tumor size from the start of treatment is shown on the y-axis. Negative values indicate tumor shrinkage. The asterisks indicate significant difference between combination drug treatment and GDC-0032 (blue) or GDC-0973 (pink; unpaired t test, *, P < 0.05; ****, P < 0.0001).
either GDC-0973 monotherapy or combined GDC-0973 plus
GDC-0032. These mice received a reduced dose of GDC-0973
(2 mg/kg) and a full dose of GDC-0032 (22.5 mg/kg) to
minimize the toxicity of full-dose combination therapy. As
expected, mice in both treatment groups experienced initial
tumor regression, which was superior with combined treat-
ment compared with GDC-0973 monotherapy (Fig. 4A and B).
However, over the course of 113 days of treatment, all of the
mice receiving GDC-0973 monotherapy developed drug-
resistant disease, defined as tumor regrowth ≥100% of the
tumor volume at the initiation of therapy. In contrast, none
of the mice receiving combination therapy developed drug-
resistant disease. To further investigate the role of PI3K path-
way activity in promoting resistance to GDC-0973 treatment,
when the first mouse receiving GDC-0973 monotherapy
reached end stage, the tumor was resected, fragmented,
and implanted into a cohort (n = 8) of immunocompro-
mised mice. Immediately following implantation, these mice
received GDC-0973 monotherapy treatment. As expected, the
transplanted tumor displayed resistance to GDC-0973 and
grew progressively over 50 days. At this time, half of the mice
were randomly reassigned to receive GDC-0973 plus GDC-
0032 combination therapy, whereas the rest continued to
receive GDC-0973 monotherapy (Fig. 4C). The mice receiving
GDC-0973 plus GDC-0032 combination therapy experienced
potent tumor cytostasis, suggesting that PI3K pathway activ-
ity is necessary for the sustained growth of a melanoma that
has developed resistance to a MEK1/2 inhibitor.

To further validate the ability of PI3K inhibitors to forestall
the onset of resistance to targeted blockade of BRAFV600E
signaling, BRAFV600E/PIK3CAH1047R/H1047R melanoma was ini-
tiated in 13 adult Tyr::CreER;BrafT172A;Pik3caH1047R/− mice and, 61
days after initiation, mice were randomized to receive GDC-
0973 (2 mg/kg) monotherapy or GDC-0973 plus BYL719 (50
mg/kg, twice a day) combination therapy. As observed with
BRAFV600E/PTENNull-driven melanomas, mice in both treat-
ment groups experienced initial tumor regression, which was superior with combined treatment compared with GDC-0973
monotherapy (Fig. 4D and E). However, over the course of 106
days, 6 of 7 mice receiving GDC-0973 monotherapy developed
drug-resistant disease. In contrast, none of the mice receiving
combination therapy developed drug resistance.

DISCUSSION

At the initiation of these studies, the high rate of PTEN
silencing compared with mutational activation of PIK3CA
suggested that PTEN might exert PI3-lipid phosphatase-
dependent tumor-suppressor functions to restrain pro-
gression of BRAF-mutated melanoma (14). We previously
noted that, although heterozygous mutational activation of
Pik3caH124 was sufficient to promote melanoma progres-
sion, BRAFV600E/PIK3CAH1047R melanomas grew significantly
more slowly than did BRAFV600E/PTENNull melanomas
(11). However, BRAFV600E-driven melanomas homozygous
for PIK3CAH1047R expression grew even more rapidly than
BRAFV600E/PTENNull melanomas. Although we cannot for-
formally exclude a role for PI3-lipid phosphatase-independent
PTEN tumor-suppressor functions to restrain progression of
BRAF-mutated melanoma, there is no compelling rationale
to invoke such mechanisms. Importantly, the correlation
between PI3K pathway activation and melanoma growth
rate indicates that PI3K catalytic isoforms are relevant drug
targets in the treatment of BRAFV600E/PTENNull melanoma.

The “oncogene addiction” hypothesis posits that, despite a high
burden of genetic damage, tumors remain dependent on the sus-
tained activity of one or a small number of oncogenes for mainte-
nance of the malignant phenotype (33). As a corollary, inhibition
of the oncogene(s) to which a tumor is addicted can elicit profound
tumor regression (34). Accordingly, our model of BRAFV600E/
PIK3CAH1047R melanoma is driven by two oncogene activation
events, one of which, BRAFV600E, drives tumor initiation, and
the other of which, PIK3CAH1047R, promotes melanoma progres-
sion (22). Potent inhibitors of either BRAFV600E or PIK3CAH1047R
allowed us to characterize to which of these two oncoproteins
BRAFV600E/PIK3CAH1047R melanomas are most addicted in vitro.
Although inhibition of BRAFV600E elicited profound melanoma
regression, selective inhibition of PIK3CAH1047R elicited largely
cytostatic effects: Only 2 of 8 mice displayed a ≥30% reduc-
tion in melanoma size in response to BYL719 monotherapy,
and that response was largely transient (23). These results may
illustrate a fundamental difference between the effects of onco-
genic BRAFV600E and PIK3CAH1047R on the sustained survival of
melanoma cells, carrying potential clinical implications.

Previous studies of PTEN-deficient tumor cells have indi-
cated that PI3Kβ is an essential contributor to PI3-lipid
signaling and aberrant cell proliferation (24, 25). However,
our work suggests that PI3Kβ, either alone or in combina-
tion with PI3Kα, does not contribute to PI3-lipid signaling
or to the proliferation of human or mouse BRAFV600E/
PTENNull melanoma cells. Although PI3Kβ inhibitors mod-
estly attenuated pAKT in M249 cells, this did not translate
into suppression of cell proliferation. Furthermore, a PI3Kβ-
sparing inhibitor and a combination of agents that inhibits
PI3Kα, PI3Kβ, and PI3Kδ had potent inhibitory effects on
PI3-lipid signaling and proliferation of BRAFV600E/PTENNull
melanoma cells. Interestingly, SK-MEL-239 melanoma cells,
which express BRAFV600E and normal PTEN, also displayed
resistance to PI3Kβ inhibition and equivalent sensitivity to
can pan class I (GDC-0941) or PI3Kβ-sparing PI3K inhibition
(GDC-0032), demonstrating no role for PI3Kβ in these cells. It
is tempting to speculate that PI3Kβ may play a role in the
proliferation of melanomas in which RAC1 is mutated or ampli-
fied, as PI3Kβ is a direct target of activated RAC1-GTP (1, 35).

Despite the potent biochemical and antiproliferative effects
of the PI3Kβ-sparing inhibitor GDC-0032 in vitro, this agent
elicited largely cytostatic effects in our BRAFV600E/PTENNull
GEM melanoma model and showed modest, but significant,
cooperation with MEK1/2 inhibition to promote melanoma
regression. Perplexingly, although both human and mouse
cancer genetics indicate an important role for PI3K signaling
during disease progression, the limited activity of PI3K inhibitors
in solid tumor clinical trials does not correlate with PI3K
pathway activation (36). This may be due to a role for PI3K
signaling predominantly in promoting cell-cycle progression
and not for suppression of apoptosis. However, more promis-
ingly, treatment with GDC-0032 forestalled the onset of
resistance to a MEK1/2 inhibitor (GDC-0973) in our GEM
model of BRAFV600E/PTENNull melanoma. Importantly, upon
serial transplantation, MEK1/2 inhibitor–resistant melanomas
retained sensitivity to combined GDC-0973 plus GDC-0032 treatment, highlighting the importance of PI3K pathway signaling in maintenance of the MEK1/2 inhibitor-resistant phenotype. Further emphasizing the importance of PI3K signaling in MEK1/2 inhibitor resistance, the emergence of GDC-0973-resistant melanomas was forestalled by combined treatment with BYL719 in our BRAF^{V600E}/PIK3CA^{H1047R} GEM melanoma model. Because a major limitation in single-agent treatment of BRAF-mutated melanoma is the onset of drug-resistant disease, the observation that PI3K inhibition enhances the depth
and durability of response to BRAFV600E pathway–targeted inhibition may illuminate an arena in which PI3K inhibitors will offer substantial clinical benefit.

METHODS

Cell Culture and Drug Treatments

Human melanoma cell lines WM793, WM9, and 1205Lu were kindly provided by the well-curated cell line repository 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). Human melanoma cell lines M249 and M233 were kindly provided by Dr. Antoni Ribas (University of California, Los Angeles (UCLA), Los Angeles, CA) and authenticated by genomic sequencing as previously described (37). The human melanoma cell line SK-MEL-239 was kindly provided by Dr. David B. Solit (Memorial Sloan Kettering Cancer Center, New York, NY), authenticated by genomic sequencing as previously described (38). Mouse melanoma cell lines B10C, BPC, and BPC-5C were established as described previously and authenticated by PCR and immunoblot analyses (11). Efficient generation of melanoma cell lines from our various GEM models requires the silencing of the Cldn2a locus encoding INK4A and ARF. Mouse or human melanoma cell lines were cultured as described previously (11). Pathway-targeted pharmacologic agents were obtained from various colleagues in the public or private sector or from commercial sources (see Supplementary Table S1 for provenance).

Proliferation and Growth Assays

Melanoma cell proliferation was assessed over 72 hours by seeding 5 × 104 cells in 12-well dishes. Cells were treated with the various pharmacologic agents as described with viable cells enumerated using a Countess cell counter (Invitrogen). In addition, melanoma cells were seeded and treated with pharmacologic agents as described for 72 hours, at which time viable cells were stained with crystal violet and quantified by solubilization in 33% acetic acid with A562 absorbance assessed. GiO assays were performed by seeding 8.0 × 103 cells in a 96-well plate and treating cells with pharmacologic agents as described for 72 hours, at which time viable cells were stained with Crystal Violet and quantified by solubilization in 33% (v/v) acetic acid with A562 absorbance assessed. At least three independent experiments, performed in biologic triplicate, were completed for all 72-hour assays. Long-term colony formation assays were performed by plating 500 to 2,000 cells in a 10-cm dish for 6 to 11 days in the absence or presence of various agents with cell colonies fixed and stained with Crystal Violet.

Immunoblot Analysis

Cell lysates were generated for analysis of 50-μg aliquots by immunoblotting as described previously (20). Membranes were stained with primary antibodies with antigen–antibody complexes 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 Fc). Immunoblot data were analyzed using Image Studio v2.0 software (LI-COR Biosciences).

Experimental Animals

The University of California, San Francisco (UCSF; San Francisco, CA) Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures. Tyr::CreER, BrafV600E, Pten103/103, or Pkhd1L1−/−/− mice, maintained on an outbred background, were intercrossed to generate experimental mice which were genotyped as previously described (11, 12). Melanocyte-specific Cre activity was induced in adult mice by topical application of 1.5 μL of 5 mmol/L 4-hydroxymitoxifen (4-HT, 70% Z-isomer, in 100% ethanol; Sigma-Aldrich) to shaved back skin. Animals were euthanized based on a body-conditioning score (39) or when tumor volume reached 22 cm3, whichever occurred first. At necropsy, tissue was snap-frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer using the Tissue Lyser II (Qiangen) for immunoblotting as described previously (11).

Treatment of Mice with Pathway-Targeted Inhibitors

Melanoma-bearing mice were divided among treatment arms to give an equal distribution of tumor volume and gender when the mean tumor volume of the cohort exceeded 500 mm3. Mice were assigned to receive LGX818 (30 mg/kg, every day) or BYL719 (50 mg/kg, twice a day) formulated in 0.5% (v/v) carboxymethylcellulose/0.5% (v/v) Tween-80 (Sigma-Aldrich) or GDC-0973 (2.0 or 4.5 mg/kg, once a day) or GDC-0032 (22.5 mg/kg, every day) formulated in 0.5% (v/v) methylcellulose/0.2% (v/v) Tween-80 (Sigma-Aldrich) and administered via oral gavage 6 days per week. Melanoma growth was measured weekly using digital calipers with relative tumor volume (RTV) estimated using the ellipsoid volume formula as described previously (11).

Statistical Analysis

All quantitative data are represented as mean ± SEM. GraphPad Prism 6 statistical software was used to determine P values for the proliferation graphs by performing two-way ANOVA analysis and t tests as indicated.

Disclosure of Potential Conflicts of Interest

M. McMahon reports receiving commercial research grants from Novartis and Plexxicon. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M.M. Deuker, M. McMahon Development of methodology: V. Marsh Durban, M. McMahon Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Deuker, V. Marsh Durban, W.A. Phillips Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.M. Deuker, M. McMahon Writing, review, and/or revision of the manuscript: M.M. Deuker, V. Marsh Durban, M. McMahon Study supervision: M. McMahon

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REFERENCES
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