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

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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 BRAFV600E→MEK1/2→ERK1/2 mitogen-activated protein (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 phosphatidylinositide 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 deployment 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 Tyr;CreER;Braf mice that were homozygous for the Ptennull allele or either heterozygous or homozygous for the conditional Cre-activated Pkcdnull allele (Pkcδ hereafter) (Fig. 1A). As shown previously (11), BRAFV600E/PTENnull melanomas grew more rapidly than BRAFV600E/PIK3CAH1047R melanomas arising in heterozygous Pkcdnull mice (Fig. 1A). However, BRAFV600E/PIK3CAH1047R melanomas arising in homozygous Pkcdnull 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/Cdkn2anull (B10C) or BRAFV600E/PIK3CAH1047R/Cdkn2anull (BPC) melanomas grew more rapidly in vitro than did a cell line derived from a BRAFV600E/PIK3CAH1047R/Cdkn2anull (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**

![Graph showing tumor volume over time](image)

**B**

![Graph showing relative cell number over time](image)

**C**

![Images of cell cultures](image)

**D**

![Graph showing relative absorbance over time](image)

**E**

![Images of western blots](image)

**F**

![Graph showing relative absorbance over time](image)

**G**

![Images of western blots](image)

**H**

![Graph showing tumor volume over time](image)

**I**

![Graph showing maximum tumor volume change](image)
cell lines with pharmacologic inhibitors of PI3K (Supplementary Table S1). B1C 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). Indeed, there was a greater than 10-fold difference between the concentration of BYL719 required for 50% inhibition of proliferation (GL50) of BPC versus B1C cells (Fig. 1D). In addition, the B1C melanoma cell line derived from homozygous Pkdcα<sup>−/−</sup> mice displayed similar sensitivity to BYL719 as did the B1C cells (Supplementary Fig. S1A and S1B). Thus, BRAF<sup>600E</sup> /PIK3CA<sup>H1047R</sup> melanoma cells display the predicted genotype–drug response phenotype relationship. In contrast, BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> 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 B1C melanoma cells treated with BYL719 (5 μmol/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 B1C cells displayed only a partial and transient inhibition of pAKT with almost no effect on pPRAS40 or p4E-BP1.

Because BRAF<sup>600E</sup> and PI3K signal cooperatively through mTOR to regulate melanoma cell proliferation (20), we investigated whether PI3Kα inhibition would enhance the effects of BRAF<sup>600E</sup> inhibition in BPC or B1C melanoma cells. Although single-agent BRAF<sup>600E</sup> (LX818; 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 BRAF<sup>600E</sup> and PIK3CA<sup>H1047R</sup> signaling elicited a more robust inhibition of these phosphorylation events (Fig. 1G). Similar observations were made in the independently derived BP<sub>C</sub> melanoma cell line (Supplementary Fig. S1C). In contrast, whereas BRAF<sup>600E</sup> inhibition (LX818) potently suppressed B1C cell proliferation, the addition of BYL719 did not significantly enhance the antiproliferative effects of BRAF<sup>600E</sup> inhibition at any time point (Fig. 1F). In B1C cells, LX818 inhibited pERK but had little effect on pRPS6 or p4E-BP1 (Fig. 1G). Although treatment of B1C cells with BYL719 elicited a modest decrease in pAKT, there was no effect on pPRAS40, pRPS6, or p4E-BP1. Most importantly, combined treatment of B1C cells with LX818 plus BYL719 displayed no cooperative effects on pPRAS40, pRPS6, or p4E-BP1. Together, these data demonstrate that inhibition of PI3Kα enhanced the effects of BRAF<sup>600E</sup> inhibition in BRAF<sup>600E</sup>/PIK3CA<sup>H1047R</sup> but not in BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> melanoma cells.

The antiproliferative activity of PI3Kα selective inhibition on BRAF<sup>600E</sup>/PIK3CA<sup>H1047R</sup> cells in vitro prompted us to design a preclinical trial in mice to test the ability of BYL719, either alone or in combination with LX818, to elicit regression of autochthonous BRAF<sup>600E</sup>/PIK3CA<sup>H1047R</sup> melanomas. To that end, BRAF<sup>600E</sup>/PIK3CA<sup>H1047R</sup> melanomas were initiated on the back skin of adult Tyr::CreER;Braf<sup>600E</sup>/Pik3ca<sup>−/−</sup> mice. In this scenario, melanomas are elicited by the cooperative action of two dominantly acting oncogenes: BRAF<sup>600E</sup> and PIK3CA<sup>H1047R</sup> (11, 22). Seven weeks after initiation, mice were randomized to receive vehicle, LX818, BYL719, or combined LGX818 plus BYL719 treatment, with melanoma size measured weekly. Pharmacodynamic analysis of pAKT inhibition in BRAF<sup>600E</sup>/PIK3CA<sup>H1047R</sup> 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. 1I; 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 with single-agent LGX818 therapy (Fig. 1I).

Analysis of glioblastoma, breast cancer, or prostate cancer models suggests that PI3'-kinase-β (PI3Kβ) function (24). Consequently, we tested the effects of PI3Kβ selective inhibition on the proliferation of human BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> tumors (24, 25). Consequently, we tested the effects of PI3Kβ selective inhibition on the proliferation of human BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> 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 μmol/L), GSK771 failed to reach the GL50 of BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> human melanoma cells (Fig. 2A and Supplementary Fig. S2A). Furthermore, even at 5 μmol/L, GSK771 elicited only a minor reduction of pAKT in BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> 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 μmol/L KIN193 modestly suppressed pAKT in some human-derived BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> melanoma cells (Fig. 2B and Supplementary Fig. S2B), the ability of 5 μmol/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. B1C 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 BRAF<sup>600E</sup>/PTEN<sup>−/−</sup> melanoma cells, this inhibition did not result in reduced phosphorylation of
**Figure 2.** BRAF^V600E/PTEN^null 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 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. C, lysates of WM9 melanoma cells, treated for 72 hours with DMSO (D), GDC-0941 or GDC-0032, with drug treatment applied in a serial 2-fold dilution series from 10 μmol/L to 31.25 μmol/L as indicated by gradient, were analyzed by immunoblotting. 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 (0941), or GDC-0032, with drug treatment applied in a serial 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 PI3K-independent PI3K signaling was sufficient to sustain cell proliferation.

The lack of robust single-agent activity on BRAFV600E/PTENNull melanoma cells suggested that PI3K selective inhibition led us to hypothesize that BRAFV600E/PTENNull human melanoma cells might require the combined activity of PI3Kα and PI3Kβ for sustained proliferation. To test this, we assessed cell proliferation of BRAFV600E/PTENNull 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 that effect, suggesting that BRAFV600E/PTENNull melanoma cells do not rely exclusively on the combined activity of PI3Kα and PI3Kβ for their proliferation.

To test whether BRAFV600E/PTENNull 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 BRAFV600E/PTENNull 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 BRAFV600E/PTENNull melanoma cells.

To confirm that BRAFV600E/PTENNull 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 PI3Kβγ (IPI145) selective inhibitors (31). Treatment of BRAFV600E/PTENNull WM793, 1205Lu, or WM9 human melanoma cells with single-agent BYL719 or IPI145 failed to reach the GI50 even at 10 μmol/L (Fig. 2F). However, when the cells were treated with a fixed concentration of IPI145 (2.5 μmol/L) in the presence of BYL719, the combination elicited a more robust antiproliferative response similar to the effects of GDC-0032 (Fig. 2F). Furthermore, although single-agent treatment of WM9 cells with either BYL719 or IPI145 elicited only a modest reduction in pAKT with little or no effect on downstream pathway components, the combination of BYL719 plus IPI145 elicited a complete and sustained inhibition of pAKT that mirrored the effects of GDC-0032 (Fig. 2G). Similar results were obtained with 1205Lu BRAFV600E/PTENNull melanoma cells (Supplementary Fig. S2E).

Together, these results suggest that PI3Kβ activity is dispensable for the proliferation and PI3K pathway activation of BRAFV600E/PTENNull melanoma cells and that these cells instead rely upon the combined activities of PI3Kα and PI3Kδ and/or PI3Kγ.

To determine whether a PI3Kβ-sparing PI3K inhibitor might augment the effects of BRAFV600E pathway–targeted inhibition, we treated BRAFV600E/PTENNull human or mouse melanoma cells with GDC-0973 (an inhibitor of MEK1/2), GDC-0032, or GDC-0973 plus GDC-0032 (Fig. 3A and Supplementary Fig. S3A; ref. 32). Importantly, the combined use of these agents elicited robust suppression of pAKT and pERK, as well as more robust suppression of pRPS6 and p4EB-P1 than that achieved with either single agent alone. In addition, whereas single-agent GDC-0973 or GDC-0032 suppressed BRAFV600E/PTENNull human or mouse melanoma cell proliferation, combined treatment elicited a significant reduction in proliferation compared with either single agent alone (Fig. 3B and Supplementary Fig. S3B).

To investigate the potential role of PI3Kβ in BRAF-mutated melanoma cells in which there is no documented genetic alteration in components of PI3K signaling, we treated SK-MEL-239 human melanoma cells (BRAFV600E/PTENNull) with inhibitors of PI3Kα, and characterized the antiproliferative response (Supplementary Fig. S4A). Although we observed modestly enhanced potency for KIN193 as compared with GSK771, the GI50 for both PI3Kβ inhibitors on SK-MEL-239 melanoma cells was >10 μmol/L. Although SK-MEL-239 cells exhibit low basal levels of pAKT, inhibition of PI3Kβ did not suppress activation of downstream pathway components pPRAS40, pRPS6, or p4E-BP1 (Supplementary Fig. S4B). Moreover, both GDC-0032 and GDC-0941 displayed equivalent GI50 values in SK-MEL-239 cells (Supplementary Fig. S4C). The combined use of GDC-0032 plus GDC-0973 elicited robust suppression of pAKT and pERK, as well as an even more robust suppression of pRPS6 and p4E-BP1 than that achieved with either single agent (Supplementary Fig. S4D). Finally, whereas single-agent GDC-0973 or GDC-0032 suppressed SK-MEL-239 cell proliferation, combined treatment elicited a significant reduction in proliferation compared with either single agent alone (Supplementary Fig. S4E).

Collectively these results indicate that, in at least one BRAFV600E/PTENNull human melanoma cell line, PI3Kβ activity is dispensable for proliferation, and that these cells rely upon the combined activities of PI3Kα and PI3Kδ and/or PI3Kγ.

In vitro activity of GDC-0032 against BRAFV600E/PTENNull melanoma cells prompted us to conduct a preclinical trial to test the ability of GDC-0032 to elicit regression of BRAFV600E/PTENNull melanomas in vivo, either alone or in combination with GDC-0973. To that end, BRAFV600E/PTENNull melanoma was initiated in adult Tyr::CreER;BrafV600Emtm 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 BRAFV600E/PI3KCA1101R;Pten−/− melanomas treated with BYL719, inhibition of PI3K signaling with GDC-0032 had largely cytostatic effects on BRAFV600E/PTENNull melanomas (Fig. 3C and D). In contrast, MEK1/2 inhibition with GDC-0973 elicited substantial regression of BRAFV600E/PTENNull 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 BRAFV600E 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 BRAFV600E signaling. We initially tested this question using vehicle-treated BRAFV600E/PTENNull 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/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, 0973), GDC-0032 (5 μmol/L, 0032), 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/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 treatment 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 pathway 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 immunocompromised 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 activity 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 initiated in 13 adult Tyr::CreER;BrafV600E/PTEN−/− 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/PTEN−/−–driven melanomas, mice in both treatment 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-independent tumor-suppressor functions to restrain progression of BRAF-mutated melanoma (14). We previously noted that, although heterozygous mutational activation of Phik3ca was sufficient to promote melanoma progression, BRAFV600E/PIK3CAH1047R melanomas grew significantly more slowly than did BRAFV600E/PTEN−/− melanomas (11). However, BRAFV600E–driven melanomas homozygous for PIK3CAH1047R expression grew even more rapidly than BRAFV600E/PTEN−/− melanomas. Although we cannot 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/PTEN−/− melanoma.

The “oncogene addiction” hypothesis posits that, despite a high burden of genetic damage, tumors remain dependent on the sustained activity of one or a small number of oncogenes for maintenance 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 oncogenic activation events, one of which, BRAFV600E, drives tumor initiation, and the other of which, PIK3CAH1047R, promotes melanoma progression (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 vivo. Although inhibition of BRAFV600E elicited profound melanoma regression, selective inhibition of PIK3CAH1047R elicited largely cytostatic effects: Only 2 of 8 mice displayed a ≥30% reduction 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 oncogenic BRAFV600E and PIK3CAH1047R on the sustained survival of melanoma cells, carrying potential clinical implications.

Previous studies of PTEN-deficient tumor cells have indicated 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 combination with PI3Kα, does not contribute to PI3′ lipid signaling or to the proliferation of human or mouse BRAFV600E/PTEN−/− melanoma cells. Although PI3Kβ inhibitors moderately 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, PI3Kγ, and PI3Kδ had potent inhibitory effects on PI3′ lipid signaling and proliferation of BRAFV600E/PTEN−/− melanoma cells. Interestingly, SK-MEL-239 melanoma cells, which express BRAFV600E and normal PTEN, also displayed resistance to PI3Kβ inhibition and equivalent sensitivity to pan class 1 (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 amplified, 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/PTEN−/− 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 in 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 promisingly, treatment with GDC-0032 forestalled the onset of resistance to a MEK1/2 inhibitor (GDC-0973) in our GEM model of BRAFV600E/PTEN−/− melanoma. Importantly, upon serial transplantation, MEK1/2 inhibitor–resistant melanomas
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Figure 4. PI3K inhibition forestalls the development of MEK1/2 inhibitor resistance in two different GEM models of melanoma. A, after 2 weeks of vehicle treatment, BRAF V600E/PTENNull melanoma-bearing mice from the experiment described in Fig. 3C were crossed over to receive either GDC-0973 (2 mg/kg) or GDC-0973 (2 mg/kg) plus GDC-0032 (22.5 mg/kg). Tumors were measured weekly with digital calipers with tumor size displayed as the average percentage change in tumor size from the initiation of drug treatment, with error bars indicating SEM. B, a spider plot of the individual tumor response for each of the 8 mice treated in E. Horizontal dotted line indicates progressive disease when tumor volume was ≥100% of tumor volume before drug treatment. Tumor sizes are displayed as the average percentage change in tumor size from the crossover point. C, fragments of a single GDC-0973–resistant tumor from A were implanted into 8 immunocompromised mice and allowed to grow into measurable tumors over 50 days with daily administration of GDC-0973 (2 mg/kg). At that time, mice were randomized to receive either combination GDC-0973 (2 mg/kg) plus GDC-0032 (22.5 mg/kg; green) or continuation of single-agent GDC-0973 (2 mg/kg; pink). Tumor sizes were measured weekly and plotted as described previously. Tumor sizes are displayed as the average percentage change in tumor size from the start of treatment. D, BRAF V600E/PIK3CA H1047R melanomas were initiated in 13 adult Tyr::CreER;BrafCA;Pik3ca lat/lat mice and 8 weeks later they were randomized by tumor size and sex for treatment with GDC-0973 (2 mg/kg, once a day; n = 7) or GDC-0973 plus BYL719 (50 mg/kg, twice a day; n = 6). Melanoma growth or regression was measured weekly over the course of 106 days of drug treatment. Tumor sizes were measured weekly and plotted as described previously, with error bars indicating SEM. E, a spider plot of the individual tumor response for each of the 13 mice treated in D. The horizontal dotted line indicates progressive disease when tumor volume was ≥100% of tumor volume prior drug treatment. Tumor sizes are displayed as the average percentage change in tumor size from the start of treatment.

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 BRAF\(^{V600E}\) 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 from 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 BP\(^{2}\)C 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 Ink4a 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 \(\times 10^{3}\) 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. GI\(_{50}\) assays were performed by seeding 8.0 \(\times 10^{4}\) cells in 12-well dishes. Cells were treated with the various pharmacologic agents as described with viable cells enumerated 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, Braf\(^{CA}\), Pten\(^{fl/fl}\)\(\Delta\textrm{EX}^{4.5}\) or Pkfc\(^{KI}\)\(\Delta\textrm{EX}^{1078}\) 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-hydroxytamoxifen (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 ≥2 cm\(^3\), whichever occurred first. At necropsy, tissue was snap-frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer using the Tissue Lyser II (Qiagen) 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 mm\(^3\). Mice were assigned to receive LGX818 (30 mg/kg, every day) or BYL719 (50 mg/kg, twice a day) formulated in 0.5% (w/v) carboxymethylcellulose/0.2% (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% (w/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

**Acknowledgments**

The authors thank all of the members of the McMahon laboratory for advice and guidance on this project, with special thanks to Jillian Silva for stimulating discussions on melanoma cell signaling and immunoblotting. The authors also thank Adil Daud (UCSF) for invigorating discussions on melanoma therapy, Meenhard Herlyn (Wistar Institute) and Antoni Ribas (UCLA) for melanoma cell lines, and Marcus Rosenberg (Yale School of Medicine, New Haven, CT) for Tyr::CreER mice. The authors thank colleagues for providing access to the following compounds and information on their use: Emmanuelle Di Tomaso, Janet Lyle, and Darrin Stuart (Novartis) for BYL719 and LGX818, and Leisa Johnson, Deepak Sampath, and Lori Freedman (Genentech) for GDC-0941, GDC-0032, and GDC-0973. The authors also thank Byron Hann and the Helen Diller Family Comprehensive Cancer Center’s Preclinical Therapeutics Core for advice and the Laboratory Animal Resource Center for animal husbandry.

**Grant Support**

This research was supported by grants from the National Health and Medical Research Council of Australia (to W.A. Phillips) and the National Cancer Institute (CA176839; to M. McMahon), the
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Melanoma Research Alliance (to M. McMahon), and the National Comprehensive Cancer Network (to M. McMahon).

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Received August 5, 2014; revised November 24, 2014; accepted November 25, 2014; published OnlineFirst December 3, 2014.

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


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Access the most recent version of this article at:
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