Phenformin enhances the therapeutic benefit of BRAFV600E inhibition in melanoma

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Biganuines, such as the diabetes therapeutics metformin and phenformin, have demonstrated antitumor activity both in vitro and in vivo. The energy-sensing AMP-activated protein kinase (AMPK) is known to be a major cellular target of biguanides. Based on our discovery of cross-talk between the AMPK and v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) signaling pathways, we investigated the antitumor effects of combining phenformin with a BRAF inhibitor PLX4720 on the proliferation of BRAF-mutated melanoma cells in vitro and on BRAF-driven tumor growth in vivo. Cotreatment of BRAF-mutated melanoma cell lines with phenformin and PLX4720 resulted in synergistic inhibition of cell viability, compared with the effects of the single agent alone. Moreover, treatment with phenformin significantly delayed the development of resistance to PLX4720 in cultured melanoma cells.

Biochemical analyses showed that phenformin and PLX4720 exerted cooperative effects on inhibiting mTOR signaling and inducing apoptosis. Noticeably, phenformin selectively targeted subpopulations of cells expressing JARID1B, a marker for slow cycling melanoma cells, whereas PLX4720 selectively targeted JARID1B-negative cells. Finally, in contrast to their use as single agents, the combination of phenformin and PLX4720 induced tumor regression in both nude mice bearing melanoma xenografts and in a genetically engineered BRAFV600E/PTEN−/−-driven mouse model of melanoma. These results strongly suggest that significant therapeutic advantage may be achieved by combining AMPK activators such as phenformin with BRAF inhibitors for the treatment of melanoma.

Significance

Inhibitors of BRAF protein kinase, such as Vemurafenib and Dabrafenib, have shown remarkable antitumor activity in patients with BRAF mutant melanoma. However, most of the patients developed drug resistance during the course of treatment, leading to resumed tumor growth. This drug resistance challenge underscores the need to improve on current BRAF-targeted therapy. In this study, we have shown that phenformin, a biguanide used for treating type 2 diabetes, enhances the antitumor activities of BRAF inhibitors in both cultured melanoma cells and a genetically engineered BRAFV600E-driven mouse model of melanoma. Our preclinical findings suggest that combining phenformin with a BRAF inhibitor may be a more effective treatment than a single-agent BRAF inhibitor for treating patients with melanoma whose tumor harbor BRAF mutations.


Conflict of interest statement: L.C.C. owns equity in, receives compensation from, and serves on the Board of Directors and Scientific Advisory Board of Agios Pharmaceuticals. Agios Pharmaceuticals is identifying metabolic pathways of cancer cells and developing drugs to inhibit such enzymes to disrupt tumor cell growth and survival.

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combination to suppress the growth of the JARID1B-positive subset of tumor cells, which are resistant to single agent therapy.

**Results**

To examine the effects of metformin and phenformin on cell viability as single agents, we performed MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] cell assays by using several BRAF mutated melanoma cell lines. Metformin had limited effect on reducing cell viability in these cell lines, when used at concentrations of up to 3 mM (Fig. 1A). In contrast, phenformin was much more potent, with an estimated IC<sub>50</sub> in the range of 0.5–1 mM (Fig. 1B). As expected, the effect of phenformin depended, at least partially, on AMPK, because shRNA knockdown of AMPKα1 in A375 melanoma cells (Fig. S1A) attenuated the reduction in cell viability in response to phenformin (Fig. 1C). A possible explanation for the observed difference in response seen here between these two biguanides is that metformin depends more on a family of organic cation transporters (OCT) for uptake into cells than phenformin (8, 12). Analysis of OCT2 protein expression levels in a panel of melanoma cell lines and MDA-MB-468 breast cancer cells, a metformin-sensitive breast cancer cell line, showed that melanoma cells express much lower levels of OCT2 than MDA-MB-468 cells (Fig. 1D). Knockdown of OCT2 by shRNA in MDA-MB-468 cells decreased the sensitivity of these cells to metformin (Fig. S1B). Conversely, overexpression of OCT2 in Sk-Mel-28 cells (Fig. S1C) increased the sensitivity of these cells to metformin (Fig. 1E), supporting the concept that OCT2 expression is a determinant of metformin sensitivity. These results together demonstrate that phenformin is much more potent than metformin in decreasing melanoma cell viability.

Based on these findings, we focused on phenformin as the biguanide AMPK activator of choice to examine the effects of its combination with BRAFi on melanoma cell viability. As shown in Fig. 1 F and G, cotreatment of Colo829 and WM115 melanoma cells with 1 mM phenformin and PLX4720 BRAFi led to a more pronounced decrease in cell viability compared with treatment with PLX4720 alone. In addition, a combination index of ~0.3 and 0.5, respectively, as calculated by the CalcuSyn software, was found for these cells. The combination index is a measurement of the combined drug interaction and is defined as <1 for synergism and >1 for antagonism (13). Similar synergistic effects were found for several other melanoma cell lines harboring the BRAF V600E mutation (Fig. 1H).

Based on the synergistic effect of combining phenformin and PLX4720 BRAFi, we hypothesized that combining phenformin with BRAFi may also influence the emergence of acquired resistance to BRAFi in melanoma cells. We first assessed this possibility in colony formation assays in vitro. Mel1617 cells were seeded at a low density in the presence of PLX4720, either alone or together with metformin or phenformin, and were then analyzed for the growth of resistant colonies from single cells. As shown in Fig. 2A, treatment with PLX4720 plus phenformin (but not metformin) significantly reduced the number of colonies formed compared with treatment with PLX4720 alone. As a second approach to address this question, we adapted a well-established in vitro assay for the development of drug resistance (14), in which A375 cells were chronically treated with stepwise increased concentration of PLX4720 alone (A375-BR, BRAFi-resistant) or PLX4720 together with fixed 0.3 mM of phenformin (A375-BPR, BRAFi- and phenformin-resistant). Cells were selected at each step until they resumed the normal growth kinetics of the untreated parental line before moving to the next step. After 7 wk of treatment, cells from both treatment groups were collected at each step until they resumed the normal growth kinetics of the untreated parental line before moving to the next step.
indicate that cotreatment with phenformin and PLX4720 suppresses the emergence of PLX4720 resistance in melanoma cells with BRAF V600E mutation.

To explore the molecular mechanisms underlying the synergistic effects of phenformin and PLX4720, we performed Western blot analyses to evaluate the activities of relevant signaling pathways in melanoma cells treated with PLX4720 and phenformin in combination compared with treatment with each drug individually. These analyses revealed that PLX4720 alone inhibited ERK activity dramatically, and cotreatment of phenformin with PLX4720 led to further reduction of pERK levels. In addition, combining phenformin and PLX4720 cooperatively activates AMPK (Fig. 3A). Because both AMPK and BRAF kinases are capable of regulating the mTOR signaling pathway (10), we further examined the effects of the phenformin and BRAFi combination on mTOR-dependent phosphorylation of S6. As shown in Fig. 3B, using either drug alone resulted in only modest inhibition of pS6 phosphorylation levels. However, when applied in combination, they essentially ablated activation of pS6. These results support the presence of a strong synergistic effect on mTOR inhibition for the phenformin/BRAFi combination.

We next used annexin V staining assays to further explore the mechanism underlying the synergistic effect of the combination. Although PLX4720 modestly induced apoptosis in Colo829 cells, phenformin alone did not. However, the combination of phenformin with PLX4720 greatly enhances the apoptotic activity of PLX4720 (Fig. 3B). Previously, metformin has been shown to selectively target cancer stem cell populations of several breast cancer cell lines (15, 16). Although it is not yet well established whether melanoma cancer stem cells exist, a slow-cycling subpopulation of melanoma cells, marked with expression of JARID1B, a H3K4 demethylase, has been described (17). Expression of JARID1B was shown to be critical for long-term maintenance of melanoma tumor growth (17). To examine the effect of phenformin/PLX4720 on JARID1B-positive and JARID1B-negative cells separately, we performed FACS analyses on WM115
cells exposed to phenformin, PLX4720, or both in combination. Treatment with PLX4720 alone for 3 d led to a decrease in the number of JARID1B-negative cells and an increase of JARID1B-positive cells (Fig. 3 C and E). In contrast, after 3 d of treatment with phenformin alone, the number of JARID1B-positive cells, but not that of JARID1B-negative cells decreased significantly compared with the control treatment group (Fig. 3 C and E). Importantly, shRNA knockdown of AMPKα1 increased the percentage of JARID1B-positive cells and abolished the inhibitory effect of phenformin on JARID1B expression (Fig. 3G), suggesting that AMPK is critical for the regulation of JARID1B expression by phenformin. Similar effects on JARID1B-positive and JARID1B-negative populations were also observed in BP01 cells (Fig. 3D), a mouse melanoma cell line derived from a genetically engineered BRAFV600E/PTENnull-driven mouse model (18). Interestingly, we detected increased JARID1B-positive cell populations in the BRAF-resistant A375 cells (A375-BR), compared with the parental BRAF-resistant Me1617 cells (Fig. S2). These results are consistent with the short-term effect of BRAFi on increasing the JARID1B-positive cell population (Fig. 3 C and D).

Importantly, combination treatment with PLX4720 and phenformin reduced the numbers of both JARID1B-positive and JARID1B-negative cells (Fig. 3 C–E). Overall, these findings indicate that PLX4720 preferentially targets the JARID1B-negative cells, whereas phenformin plus PLX4720 suppresses growth and survival of both JARID1B-positive and JARID1B-negative cells, most likely by suppressing the ability of the JARID1B-negative cells to switch to the more resistant JARID1B-positive subtype.

Finally, we investigated the effects of the phenformin/PLX4720 combination on BRAF-driven melanoma tumor growth in vivo by using both xenograft and GEM models. Colo829 and A375 melanoma cells were grown as xenograft tumors in nude mice to assess their sensitivities to various treatment options. Once tumor volumes reached between 80 and 100 mm3, animals were randomly assigned to four groups that were administered vehicle, PLX4720, phenformin, or PLX4720/phenformin combination by oral gavage twice per day. As shown in Fig. 4A and Fig. S3, although tumors in the vehicle-treated animals progressed steadily over the 2-wk course analyzed, those tumors treated with phenformin or PLX4720, respectively, showed slight and significant inhibition of tumor growth, but no evidence of tumor regression. In contrast, animals treated with the combination of PLX4720 and phenformin showed significant reduction in tumor size. We next examined the efficacy of the phenformin/PLX4720 combination on a genetically modified, BRAFV600E/PTENnull-driven melanoma mouse model (Tyr::CreER; BrafCA/+; Pten−/−) (18). This mouse model allows the simultaneous conditional conversion of a BrafCA allele to the active BrafV600E allele and deletion of the Pten allele in a melanocyte-specific fashion by administration of 4-hydroxotamoxifen (4-HT). Upon 4-HT–induced Cre expression, these mice develop melanoma with a high penetrance and a short latency (~3 wk) (18). For the GEM model, analyses similar to those conducted for the xenograft model were used to determine the effect of these drugs on tumor growth. We again observed significant tumor regression with treatment of the phenformin/PLX4720 combination in these mice (Fig. 4B). PLX4720 alone significantly reduced the rate of tumor progression, whereas phenformin alone only modestly attenuated tumor growth in these mice (Fig. 4B). Immuno histochemical analyses of these tumors indicated that the phenformin/PLX4720 combination dramatically induced apoptotic cell death and attenuated tumor cell proliferation in vivo (Fig. 4C). In addition, this combination strongly inhibited the pS6 levels in the tumors. Importantly, we also observed that the residual tumor from mice treated with PLX4720 exhibited enhanced expression of JARID1B, whereas the tumor from mice treated with phenformin or the phenformin/PLX4720 combination exhibited reduced expression of JARID1B (Fig. 4C). These results confirmed findings observed in melanoma cells in vitro (Fig. 3). Noticeably, we did not observe significant differences among different treatment groups in body weight, levels of glucose, IGF1, or insulin in blood plasma (Fig. S4), suggesting that the effect of the combination is unlikely to
be mediated indirectly through potential changes in whole-body metabolism. In summary, these data from both xenograft and BRAFV600E/PTEN−/−-driven GEM models demonstrate that the combination of the BRAF inhibitor PLX4720 and phenformin induces significant tumor shrinkage and has stronger antitumor activities than PLX4720 alone in vivo.

**Discussion**

In this study, we have demonstrated that the combination of phenformin and the BRAF inhibitor PLX4720 offers a therapeutical advantage against BRAF mutant melanoma over either agent alone in both cell culture and animal models. Metformin, an analog of phenformin, is widely used as a first-line therapy for type 2 diabetes. Recent epidemiological studies have found that the subset of patients with type 2 diabetes who were treated with metformin had lower cancer risk and lower cancer-related mortality rates compared with patients treated with other therapies (9). Moreover, both metformin and phenformin have antitumor activities in various xenograft, carcinogen-induced, and genetically modified mouse models, raising strong interest in repurposing these drugs for cancer therapy (9). Consistent with previous reports in other models (19, 20, 24), we found that phenformin is much more potent than metformin in suppressing tumor growth, apparently because metformin requires an organic cation transporter to enter tumor cells while phenformin does not. Although metformin was used in combination with BRAF inhibitors in melanoma cells in an in vitro study, the IC50 values for metformin were found to be in the ranges of 10−30 mM (21).

This range of doses appears to be much higher than what can be achieved in humans with oral administration of metformin (22). In our in vivo studies, we found that phenformin by itself possesses weak activity against BRAF V600E melanoma tumors in both xenograft and GEM melanoma models. We have also examined the effect of metformin in these mouse models (Fig. S5) and did not observe any significant effect of metformin compared with the vehicle treatment. Metformin monotherapy was also recently shown to be ineffective in A375 melanoma xenografts in nude mice (15). However, in another recent study, metformin alone was found to promote the growth of A375 xenografts in nude mice, possibly through ERK-dependent up-regulation of VEGF-A (19). The reason for the discrepancy among these studies regarding the effect of metformin on BRAFV600E+ driven melanoma tumor growth remains to be resolved.

Understanding mechanisms underlying the antitumor activities of metformin and phenformin remains an active research topic. AMPK-dependent inhibition of mTOR signaling and protein synthesis has been proposed as a major mechanism supporting their antiproliferative effects (10). However, it has been suggested that metformin and phenformin may also inhibit mTOR signaling in an AMPK-independent manner (23). More recently, phenformin was shown to induce apoptosis in LKB1-deficient nonsmall cell lung cancer cells independent of AMPK activation (24). In addition to these cancer cell-autonomous mechanisms, it is possible that the systemic metabolic effects of metformin and phenformin, such as lowering circulating insulin/IGF1 levels, may also play a role (9). These systemic effects of metformin and phenformin have been recently observed in some mouse model studies, such as one using a tobacco carcinogen-induced lung cancer mouse model (25), but not in others, including a study using the Pten−/− spontaneous lymphomamouse model (20) and the LKB1−/− NSCLC model (24). It is therefore possible that different mouse cancer models or the approaches of drug administration may affect systemic metabolic responses. Our data from the GEM melanoma model are in agreement with the second group of studies. Moreover, using the shRNA knockdown approach, we concluded that at least part of sensitivity to phenformin in melanoma cells is mediated by AMPK activation in the tumor cells. Future investigations should focus on further exploring both the tumor cell-intrinsic and extrinsic effects of phenformin in melanoma.

Our analyses have revealed several potential mechanisms that might be involved in the synergistic effects of phenformin and BRAFi. We have demonstrated that phenformin and BRAFi exert cooperative activities in inhibiting mTOR signaling and inducing apoptosis in both BRAF V600E-mutant melanoma cells in vitro and tumors in vivo. Moreover, BRAFi appears to target the JARID1B-negative population of melanoma cells, which represents the majority of cells in the tumors, and spares the JARID1B-positive slow-growing cycling population. Conversely, phenformin suppresses the JARID1B-positive population, either by inhibiting their growth or by suppressing the switch from the JARID1B-negative to JARID1B-positive phenotype. Although additional studies are needed to understand how phenformin suppresses this population of cells, it is clear that combining phenformin with PLX4720 reduces both cell populations and results in tumor regression. Previously, metformin was shown to specifically target the cancer stem cell population of breast cancer cells by inhibiting the inflammatory response associated with cell transformation (15, 16). A combination of metformin with doxorubicin resulted in tumor mass reduction and delayed tumor relapse in xenograft tumor models (15, 16). Intriguingly, two recent reports have shown that treatment with a BRAFi in Braf mutant melanoma enhanced oxidative phosphorylation capacity through up-regulation of PGC1α, a master transcription regulator for mitochondria biogenesis and function (26, 27). Because treatment of BRAFi appeared to render certain melanoma cells more addicted to oxidative phosphorylation, it is possible that BRAFi-treated melanoma cells would be more sensitive to phenformin, an inhibitor of mitochondrial oxidative phosphorylation (28), which may contribute to the synergistic effects of the phenformin/BRAFi combination. Future studies on the relationship among AMPK, JARID1B, and mitochondrial energy metabolism may reveal additional insight into the mechanisms underlying the synergistic effects of phenformin and BRAFi against melanoma.

BRAF-selective kinase inhibitors have shown great clinical benefits in malignant melanoma with BRAF V600E mutations in the initial phase of treatment. However, most of the responsive melanoma tumors treated with BRAF inhibitors developed resistance during the course of treatment, leading to resumed tumor growth. Recent studies have shown that reactivated ERK signaling due to amplification or mutation of proteins in the RAS-RAF-MEK-ERK pathway (i.e., NRAS, COT1, MEK1/2, and BRAF splicing variants) play a major role in acquired resistance to BRAFi (29). In addition, hyperactivation of the RTK (receptor tyrosine kinase)-PI3K-Akt survival pathway has also been identified as an alternative mechanism (29). Combination therapy strategies using BRAFi together with MEK inhibitors or the PI3K-Akt pathway inhibitors have been proposed to overcome the drug resistance and side effects associated with BRAFi single agent therapy. We demonstrated here that cotreatment of phenformin with BRAFi delayed the development of acquired resistance to BRAFi in melanoma cells in vitro. Moreover, our data suggest that JARID1B-positive, slow cycling cells could be involved in the development of acquired resistance to BRAFi. The effect of phenformin on limiting the switch to these slow cycling cells may make combination with phenformin more advantageous than other combinatorial therapy strategies. Consistent with this notion, we found that treatment of WM115 cells with the MEK inhibitor GSK1120212, similar to PLX4720 BRAFi, only reduced the number of JARID1B-negative cells, but not JARID1B-positive cells (Fig. S6).

The main serious adverse effect of both metformin and phenformin in diabetic patients is lactate acidosis, which is predominantly found in those with impaired renal function. However, phenformin has a higher incidence rate of lactate acidosis.
than metformin (~64 versus ~3 cases per 100,000 patient years) (30). Because of this adverse effect, phenformin was discontinued for use treatment of type 2 diabetes in the United States in the late 1970s, whereas it is still being used in some other countries. Multiple clinical trials are underway to explore the utility of metformin for cancer therapy. In addition to metformin, the adoption of phenformin, a more potent biguanide, for cancer treatment is also worthy of consideration, because in comparison with many commonly used cancer chemotherapy and adjuvant therapies, it possesses relatively lower toxicity and might be more acceptable for treatment of cancer than diabetes. In summary, our preclinical findings presented here demonstrate that the combination of phenformin and BRAFi for treating melanoma with BRAF V600E mutations, including in the adjuvant therapy setting, warrant future clinical evaluation.

Materials and Methods

Flow Cytometric Analysis. Apoptotic cells were detected by using BD FITC Annexin V Apoptosis Detection Kit I according to the manufacturer’s protocol and analyzed by BD FACScalibur. For JARID1B expression FACs analysis, cells were fixed in 1% formalin in PBS for 20 min, followed by incubation with 90% methanol for 30 min at −20 °C. After wash with 1% PBS in PBS, anti-JARID1B antibody (for human cells) or anti–PLU-1 (for mouse cells) was added to the cells and incubated for 30 min at room temperature. After wash, the cells were stained with Alexa Fluor 647 conjugated anti-rabbit IgG. The samples were analyzed by BD LSRII Flow Cytometer. All flow cytometry data were analyzed with FlowJo software.

Animal Studies. All animal experiments were performed by following Columbia University’s Institutional Animal Care and Use Committee guidelines. For xenograft models, 6-wk-old female athymic mice (NCr nu/nu) were purchased from Taconic Farms or Charles River. Animals were allowed a 1-wk adaptation period before tumor cell injection. A375 (2.5 × 10⁶) or Colo205 (2 × 10⁶) cells in 0.2 mL of basal culture medium were injected s.c. into the right lateral flank. For the genetically engineered mouse model, 6-8-wk-old Tyr::CreER; BrafCA/+; Ptenfl/fl mice were topically administrated 4-hydroxytamoxifen (70% Z-isomer; Sigma H6278) in ethanol to induce tumor formation. Treatment began when the tumor volume reached between 80 and 100 mm³. Tumor volumes were calculated from caliper measurements by using the following ellipsoid formula: \( V = \frac{4}{3} \pi \times r_1 \times r_2 \times r_3 \), where \( V \) represents the large diameter of the tumor, and \( r_1 \) represents the small diameter. Animals were randomly assigned to four groups that were administered vehicle (10% [vol/vol] DMSO in 1% [vol/vol] carboxymethylcellulose), PLX4720, Phenformin, or PLX4720/ Phenformin combination (same dose as used in the single-agent groups) by intravenous and oral gavage twice per day for the duration of the experiment. Mice were weighed daily, and drug doses were adjusted accordingly. Levels of insulin and insulin-like growth factor-1 (IGF-I) in plasma were measured by using the insulin ELISA Kit (Millipore) and the IGF-I Quantikine ELISA Kit (R&D Systems), according to manufacturers’ protocol. Plasma glucose levels were measured by using OneTouch Ultra (LifeScan).

Immunohistochemistry Analysis. Harvested mouse tissues were fixed in 10% neutral buffered formalin or in 70% ethanol and embedded in paraffin. Formalin fixed tissues were used for Ki67 and pAMPK staining, and ethanol fixed tissues were used for pERK, pS6, and TUNEL staining. The slides were deparaffinized by using HistoChoice clearing reagent (Amresco) and then rehydrated with water. Antigen retrieval for formalin fixed tissue sections was performed by heating slides in a pressure cooker for 10 min in citrate antigen retrieval solution. After wash with PBS, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS for 10 min at room temperature. For Ki67, slides were blocked with 5% normal goat serum in 0.3 M glycine and 0.25% Triton X-100 in PBS overnight at 4 °C and then incubated with anti-Ki67 antibody for 90 min, followed by incubation with biotinylated anti-rabbit IgG for 30 min (Vector Laboratories). For TUNEL staining, slides were treated with Proteinase K in PBS for 10 min at room temperature, followed by endogenous peroxidase quenching. The slides were then incubated with TdT reaction buffer for 10 min, followed by an incubation with terminal deoxynucleotidyl transferase reaction mix for 1 h at 37 °C and rinse with stop buffer for 10 min. All slides were then incubated with avidin-biotin peroxidase complex for 30 min, and the signals were visualized by using DAB Substrate Kit (Vector Laboratories). The tissue sections were counterstained with Gill’s hematoxylin Q5 and mounted with VectaMount after dehydration.

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Supporting Information

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SI Materials and Methods

Materials. Anti-Oct2 (SLC22A2) antibody was purchased from Sigma-Aldrich (HPA008567). Anti–phospho-AMPK (Thr172), anti-AMPK, anti–phospho-ERK1/2 (Thr202/Tyr204), anti-ERK, anti–phospho-S6 (Ser240/244), and anti-S6 antibodies were purchased from Cell Signaling Technology. Anti-AMPKα1 and anti-AMPKα2 antibodies were purchased from Millipore. Anti-JARID1B (22260002) and anti–PLU-1 (sc-67035) antibodies were purchased from Novus Biologicals and SantaCruz Biotechnology, respectively. Alexa Fluor 647 conjugated anti-rabbit IgG was purchased from Molecular Probes. pBabe-FLAG-OCT2 was generated by PCR-based subcloning from pCDNA3-OCT2 obtained from Kathleen Giacomini (University of California, San Francisco). pLKO-shAMPKα1 was obtained from Sigma. Metformin and phenformin were obtained from Sigma or Toronto Research Chemicals. PLX4720 used in Fig. 4A was obtained from Plexxikon. All of the other PLX4720 used in this work and GSK1120212 were purchased from ChemieTek.

Cell Culture. All melanoma cell lines used in this study contain BRAF V600E mutations, except otherwise indicated. BP01 mouse melanoma cells were derived from tumor induced from Tyr::CreER; Braf<sup>CA/+</sup>; Pten<sup>lox/lox</sup> mice. SK-Mel-28, WM115, A375, Colo829, UACC257, MeWo, and 501Mel cells were cultured in RPMI 1640 containing 10% (vol/vol) FBS and penicillin/streptomycin (P/S). Mel1617, 451Lu, and BP01 cells were maintained in DMEM containing 10% FBS and P/S. A375-BR Braf inhibitor resistance cells were maintained in completed media with 0.1 μM PLX4720. Mel1617 and Mel1617-BR cells were kindly provided by Jessie Villanueva and Meenhard Herlyn (Wistar, Philadelphia). For the cell viability analysis, cells were seeded onto a 96-well plates and drug treatment was started the following day. After a 3-d incubation, the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed according to the manufacturer’s protocol (Promega). For the colony formation assay, cells were seeded in 60-mm plates in triplicate at a low density of 6,400 cells per plate. The cells were stained with crystal violet after 15 d of treatment. For the drug resistance development assay, A375 cells were chronically treated with stepwise increased concentration of PLX4720 alone (A375-BR, BRAFi-resistant) or PLX4720 together with fixed 0.3 mM phenformin (A375-BPR, BRAFi- and phenformin-resistant). Cells were selected at each step until they resumed the same normal growth kinetics as the untreated parental line before moving to the next step. Throughout the 6 wk of treatment, the PLX4720 concentration was increased from 0.1 μM to 0.6 μM for the A375-BR group of cells, whereas the cells in A375-BPR did not achieve parental growth with the starting concentration of 0.1 μM PLX4720. At week 7, the sensitivity of cells to BRAFi was assessed by using MTS-based cell viability assays for both groups.

Statistical Analysis and Data Processing. Statistical analyses were performed by using Prism 6 (GraphPad Software). All values are expressed as mean ± SEM and the number (n) of samples used was as indicated. The statistical significance of differences between control and experimental groups was determined by Student’s t test with P values <0.05 considered significant. Two-way ANOVA with the Bonferroni post hoc test was performed on the tumor growth experiments. Combination index (CI) was calculated by CompuSyn software by using the method of Chou and Talalay.
Fig. S1. Expression of OCT2 is a key determinant of metformin sensitivity in cells. (A) Knockdown of AMPKα1 expression in A375 cells. The levels of AMPKα1 and AMPKα2 were analyzed by Western blot for A375 cells stably expressing pLKO shAMPKα1 or a scramble control. (B) Knockdown of OCT2 by shRNA in MDA-MD-468 cells decreased the sensitivity of these cells to metformin. MDA-MD-468 cells stably expressing OCT2 shRNA or the pLKO vector control were treated with phenformin for 3 d before MTS assays were performed. Cell lysates were analyzed by Western blot with the indicated antibodies. (C) Expression of FLAG-OCT2 in SK-Mel-28 stable cells. Lysates were analyzed by Western blot with the indicated antibodies.
Fig. S2. Increased JARID1B+ populations of cells in Mel1617 BRAFi-resistant (BR) cells compared with the parental Mel1617 cells. Mel1617 and Mel1617-BR cells were stained with anti-JARID1B antibodies before being subjected for FACS analysis.

Fig. S3. Combination treatment of phenformin and PLX4720 lead to tumor regression in the A375 xenograft mouse model. Nude mice bearing A375 xenograft tumors were treated with vehicle, PLX4720 (5 mg/kg), phenformin (100 mg/kg) or the combination of PLX4720 and phenformin twice per day when tumor volume reached between 80 and 100 mm³. The two-way ANOVA test was performed to compare between the PLX4720 group vs. the combination group. *P < 0.05.
Fig. S4. Combination treatment of phenformin and PLX4720 does not significantly affect the whole body metabolism in Tyr::CreER; BRAFCA/+; PTENlox/lox mice. (A) Changes of body weight for Tyr::CreER; BRAFCA/+; PTENlox/lox mice in various treatment groups were shown. (B–D) Levels of blood plasma glucose (B), insulin (C) and IGF1 (D) for Tyr::CreER; BRAFCA/+; PTENlox/lox mice in various treatment groups are shown. No significant difference was found in Student’s t tests in any of these measurements.

Fig. S5. Metformin does not promote melanoma tumor growth in the A375 xenograft model or the BRAFV600E/PTENnull GEM model. (A) Nude mice bearing A375 xenograft tumors were treated with vehicle or metformin in drinking water (300 mg/kg) starting the day before cell induction. Tumor size was determined daily and plotted. No significant difference was found. (B) Tyr::CreER; BRAFCA/+; PTENlox/lox mice were treated with treated with vehicle or metformin through oral gavage (350 mg/kg) when tumor volume reached between 80 and 100 mm³. Tumor size was determined daily and plotted. No significant difference was found by the two-way ANOVA test.
Fig. S6. GSK1120212 MEK inhibitor specifically targets JARID1B− but not JARID1B+ populations of WM115 cells. WM115 cells were treated with DMSO or 0.1 μM GSK1120212 for 3 d before, and the numbers of JARID1B− and JARID1B+ cells were analyzed by FACS and plotted. Data from three independent experiments were represented as mean ± SEM. The Student t test was performed to compare between the control or parental vs. treated group.