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Selective RAF inhibitor impairs ERK1/2 phosphorylation and growth in mutant NRAS, vemurafenib-resistant melanoma cells

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SUMMARY

The RAF inhibitor vemurafenib achieves remarkable clinical responses in mutant BRAF melanoma patients. However, vemurafenib is burdened by acquired drug resistance and by the side effects associated with its paradoxical activation of the ERK1/2 pathway in wild-type BRAF cells. This paradoxical effect has driven the development of a new class of RAF inhibitors. Here, we tested one of these selective, non-paradox-inducing RAF inhibitors termed paradox-breaker-04 (PB04) or PLX7904. Consistent with its design, PB04 is able to efficiently inhibit activation of ERK1/2 in mutant BRAF melanoma cells but does not hyperactivate ERK1/2 in mutant RAS-expressing cells. Importantly, PB04 inhibited ERK1/2 phosphorylation in mutant BRAF melanoma cells with acquired resistance to vemurafenib/PLX4720 that is mediated by a secondary mutation in NRAS. Consistent with ERK1/2 re-activation driving the re-acquisition of malignant properties, PB04 promoted apoptosis and inhibited entry into S phase and anchorage-independent growth in mutant N-RAS mediated vemurafenib-resistant cells. These data indicate that paradox-breaker RAF inhibitors may be clinically effective as a second line option in a cohort of acquired vemurafenib-resistant patients.

Keywords

BRAF; NRAS; paradox breaker; vemurafenib

INTRODUCTION

Advances in the treatment options for metastatic melanoma led to the FDA-approval in 2011 of two targeted therapies: ipilimumab and vemurafenib. The latter of these agents is a RAF inhibitor that selectively inhibits mutant BRAF signaling in V600 mutant BRAF-harboring cells. BRAF is a serine-threonine kinase that is mutated in 40–50% of melanomas; the most frequent alteration is a valine to glutamic acid substitution within the activation loop, V600E (Wellbrock et al., 2004). The V600E form of BRAF constitutively signals through the MAPK/ERK kinases 1 and 2 (MEK1/2) to extracellular signal-regulated kinase (ERK) 1/2 (Conner et al., 2003; Wan et al., 2004). V600E BRAF is a driver mutation in melanoma (Bollag et al., 2010; Tuveson et al., 2003) and targeting it with vemurafenib leads to tumor shrinkage in the majority of mutant BRAF melanomas and increases patient progression-free survival and overall survival (Bollag et al., 2010; Flaherty et al., 2010; Sosman et al., 2012).

Despite this success, there are two main limitations of vemurafenib. Firstly, RAF inhibitors exhibit paradoxical effects on the ERK1/2 pathway; thus, in cells with high RAS activity vemurafenib activates MEK1/2-ERK1/2 signaling (Halaban et al., 2010; Heidorn et al., 2010; Kaplan et al., 2011; Poulikakos et al., 2010). Paradoxical activation of MEK-ERK1/2 is mediated by activation of CRAF, and likely underlies the high frequency (25%) of keratoacanthoma (KA), cutaneous squamous cell carcinoma (cuSCC) of the KA type, or well-differentiated cuSCC lesions that develop in vemurafenib-treated patients (Su et al., 2012). It has also been linked to the formation of adenomas and in at least one patient to leukemia (Callahan et al., 2012; Chapman et al., 2012). The second limitation is drug resistance. Approximately 10–15% of patients are intrinsically resistant and no tumor shrinkage is achieved with vemurafenib. Other patients (~50%) show tumor shrinkage greater than 30% but ultimately the disease progresses with a median of 6.3 months for progression-free survival. The mechanisms underlying this acquired resistance to vemurafenib have been actively studied and an overarching mode is reactivation of the MEK1/2-ERK1/2 pathway (Aplin et al., 2011). Reactivation may be initiated through a secondary mutation in NRAS, expression of splice variant of V600E BRAF, enhanced expression of the alternative MAP3K, COT1/Tpl2, or downstream through secondary mutation in MEK1/2 (Johannessen et al., 2010; Nazarian et al., 2010; Poulikakos et al., 2011). Of these mechanisms, secondary mutations in NRAS are frequent and have been reported independently by multiple groups. Lo et al., described one melanoma patient with disease progression while on vemurafenib treatment at multiple sites, two of which harbored activating mutations in NRAS (Nazarian et al., 2010). Additionally, Poulikakos and colleagues identified NRAS mutations in 4 of 19 samples following progression on vemurafenib (Poulikakos et al., 2011).

A new class of RAF inhibitors is being developed to potentially inhibit mutant BRAF unencumbered by the effects of paradoxical activation. These paradox breaker (PB) inhibitors are expected to elicit a narrower spectrum of side effects; thus, permitting higher dosing. Here, we investigated the PB inhibitor PB04/PLX7904 in melanoma cells harboring either BRAF or NRAS mutations. Given the similarities in the mechanisms of RAF inhibitor paradoxical effects and mutant NRAS-mediated by-pass of RAF inhibitor blockade of the ERK1/2 pathway during acquired resistance, we also tested PB04 in vemurafenib-resistant cells harboring both BRAF and NRAS mutations.

RESULTS

PB04 potentially blocks ERK1/2 activation in mutant BRAF melanoma cells but does not induce hyperactivation of ERK1/2 in mutant NRAS cells

The selective RAF inhibitor PB04 (also known as PLX7904) is designed to avoid the paradoxical effects of RAF inhibitors. Initially, we compared the dose response of PB04 with that of PLX4720 (the tool compound for vemurafenib) in mutant BRAF WM793 melanoma cells. Similar to PLX4720, PB04 potentially blocked phosphorylation of MEK1/2 and ERK1/2 at high nmol to low μ mol doses (Fig. 1A). We expanded our analysis of PB04 to an additional 5 mutant BRAF harboring melanoma cells. This panel includes mutant BRAF cells that are PTEN deficient (WM115 and WM9) or PTEN and Rb deficient (SK-MEL207). Phosphorylation of ERK1/2 was rapidly inhibited by PB04 in all cell lines (Fig. 1B) and the inhibition was durable as indicated by the 16 h treatment (Fig. 1C). RAF inhibitors hyperactivate the ERK1/2 pathway in wild-type BRAF cells harboring RAS mutations (Halaban et al., 2010; Heidorn et al., 2010; Kaplan et al., 2011; Poulikakos et al., 2010). We have previously reported paradoxical activation of MEK and ERK1/2 by PLX4032/PLX4720 in the mutant NRAS melanoma lines: SbCl2, WM1346, WM1366 and WM1361A (Kaplan *et al.*, 2011). Similar effects were observed in SK-MEL 2 cells (Fig. 1D). Next, we analyzed the effects of PB04 in these melanoma cells harboring NRAS

mutations. Consistent with paradox breaker design, PB04 did not enhance or only modestly enhanced the phosphorylation of ERK1/2 after either 1 h (Fig. 1E) or 16 h (Fig. 1F) of treatment in the panel of 5 mutant NRAS cells lines. Together, these data show that PB04 potently inhibits phosphorylation of ERK1/2 in mutant BRAF melanoma cells without eliciting paradoxical activation in wild-type BRAF, mutant NRAS melanoma cells.

PB04 does not paradoxically activate MEK-ERK1/2 signaling in mutant HRAS-expressing immortalized keratinocytes or squamous carcinoma cells

Paradoxical activation of the ERK1/2 pathway by vemurafenib in keratinocyte cells has been linked to the formation of cuSCC/KA lesions (Arnault et al., 2011; Su et al., 2012). Since this effect was associated with mutations in HRAS in 46% of cases, we tested the effects of PB04 in keratinocyte lineage cell lines that harbor HRAS mutations. We utilized two cells lines: human immortalized keratinocytes HaCaTs that ectopically express HRAS G12V and mouse squamous cell carcinoma cells PAM 212 that endogenously express HRAS G12V (Yuspa et al., 1980). In both HaCaT-HRAS G12V and PAM 212 cells, PLX4720 treatment enhanced phosphorylation of MEK and ERK1/2 (Fig. 2A & B). By contrast, PB04 treatment led to a slight reduction in the levels of phospho-MEK and phospho-ERK1/2. These data indicate that PB04 does not elicit paradoxical activation in keratinocyte cells harboring mutant HRAS.

PB04 elicits similar short-term responses compared to vemurafenib

Vemurafenib/PLX4720 treatment induces short-term responses that may modulate the initial apoptotic and proliferative response to mutant BRAF inhibition. For example, the stemness transcription factor FOXD3 is up-regulated following inhibition of the BRAF-MEK signaling in mutant BRAF melanoma cells (Abel and Aplin, 2010) and provides adaptive resistance to vemurafenib/PLX4720 (Basile et al., 2012). Other examples of resistance mechanisms to vemurafenib include up-regulation of PDGFR- β or IGF-1R genomic amplification/enhanced expression of mutant BRAF, up-regulation of COT1 and activation of AKT signaling (Das Thakur et al., 2013; Johannessen et al., 2010; Nazarian et al., 2010; Paraiso et al., 2011; Shao and Aplin, 2010; Shi et al., 2012; Villanueva et al., 2010). We determined the effect of PB RAF inhibitors on the aforementioned mechanisms in three mutant BRAF melanoma lines: 1205Lu, WM115 and A375 cells. No up-regulation of FOXD3 was observed after 1 h treatment with PB04 (data not shown); however, FOXD3 was detected in all 3 lines after 16 h of PB04 treatment (Fig. 3). PDGFR- β expression was up-regulated in all three melanoma cell lines following PB04 treatment (Fig. 3), similar to findings with PLX4720 in these cell lines (data not shown). However, no changes in the levels of BRAF and phospho-AKT were detected and IGF-1R expression decreased with PB04 treatment (Fig. 3). COT1 expression was not detected in these cells lines (data not shown). Overall, these data show that PB04 acts similarly to other RAF inhibitors in terms of leading to up-regulation of FOXD3 and PDGFR- β .

PB04 inhibits ERK1/2 activation on mutant BRAF/NRAS co-expressing melanoma cells

Next, we analyzed PB04 in the context of acquired resistance to vemurafenib that was recently FDA approved for the treatment of mutant BRAF melanoma patients. Re-activation of the ERK1/2 signaling pathway is a major mode of acquired resistance to vemurafenib and is frequently mediated by acquired mutations in NRAS and/or selection of a small population of cells co-expressing mutant BRAF and mutant NRAS (Nazarian et al., 2010; Poulikakos et al., 2011). In other studies, we generated a subset of BRAF^{V600E} WM793 melanoma cells with acquired resistance to PLX4720 (the tool compound for vemurafenib) that co-expressed NRAS^{Q61K} and BRAF^{V600E} (Kaplan et al., 2012). In these cells (hereafter termed WM793-Res NRAS^{Q61K}), ERK1/2 reactivation is dependent on NRAS and is regulated by both BRAF and CRAF in manner similar to the mechanism of paradoxical

activation of the pathway by vemurafenib in cells with high RAS activity (Kaplan et al., 2012). We tested the effect of PB04 in two sub-lines of WM793-Res NRAS^{Q61K} (#5 and #12). Both sub-lines exhibited a high degree of resistance to PLX4720-mediated inhibition of phosphorylated ERK1/2 (Fig. 4A). By contrast, 1 h treatment with PB04 effectively blocked activation of ERK1/2 at doses equal to/above 1 μ M. Similar effects of PB04 were observed after 6 and 24 h of drug treatment in both sub-lines (Fig. 4B). To further test the effects of PB04 in PLX4720-resistant cells, we utilized WM793 cells that were engineered to ectopically express NRAS^{Q61K}. In contrast to parental WM793 cells, PLX4720 does not inhibit phosphorylation of ERK1/2 in WM793 cells expressing exogenous NRAS^{Q61K} cells (Fig. 4C). PB04, however, more effectively inhibited activation of ERK1/2 in NRAS^{Q61K}-expressing WM793 cells compared to PLX4720 (Fig. 4D). We note that the level of resistance to PLX4720 appeared to decrease with continued passaging of these cells probably due to reduced expression of ectopic mutant NRAS. Nevertheless, these overall data show that PB04 inhibits ERK1/2 phosphorylation in mutant NRAS-mediated PLX4720-resistant cells.

Enhanced apoptosis and inhibition of anchorage-independent growth in PB04 treated vemurafenib-resistant cells

To examine the effect of PB04-mediated ERK1/2 pathway inhibition in PLX4720-resistant cell lines, we analyzed apoptosis by annexin V staining. We have previously shown that WM793-Res NRAS^{Q61K} cells are resistant to apoptosis induced by PLX4720 (Kaplan et al., 2012). PB04 effectively enhanced annexin V staining in parental WM793 cells as well as in the resistant sub-lines in 2D conditions (Fig. 5A). Cutaneous metastases reside in type I collagen-rich dermal microenvironment. To assay the effects of PB04 in dermal mimetic conditions, we performed apoptosis assays in 3D collagen. Parental WM793 cells are more susceptible to BRAF inhibition in 3D collagen compared to 2D cell culture conditions (Boisvert-Adamo and Aplin, 2006). Surprisingly, WM793-Res NRAS^{Q61K} cells showed high levels of annexin V staining in the absence of any inhibitors (Fig. 5B). This effect may be related to loss of fitness in the absence of RAF inhibitor (Fig. 5B). Notably, PB04 treatment further enhanced levels of apoptosis in both WM793-Res NRAS^{Q61K} cell lines compared to DMSO controls (Fig. 5B).

Anchorage-independent growth in soft agar is often used as an *in vitro* measure of tumorigenicity. A375 cells readily form colonies in soft agar and we have shown that that A375-NRAS^{Q61K} cells are resistant to PLX4720, whereas A375-NRAS^{WT} cells are sensitive in colony formation assays (Kaplan et al., 2012). Notably, both A375-NRAS^{Q61K} and A375-NRAS^{WT} cells were sensitive to PB04 treatment, as measured by decreased colony number (Fig. 5C). PB04 treatment increased levels of annexin V staining in both A375-NRAS^{WT} and A375-NRAS^{Q61K} cells (Fig. 5D). Since the level of apoptosis induced by PB04 was noticeably lower in A375 cells compared to WM793 cells, we analyzed effects on entry into S phase. PB04 significantly inhibited the incorporation of the thymidine analog EdU in both A375 NRAS^{WT} and A375 NRAS^{Q61K}, although expression of NRAS^{Q61K} provided a partial degree of resistance to PB04 in these assays (Fig. 5E). Together, these data show that PB04 is effective at inhibiting the growth of vemurafenib/PLX4720-resistant cells.

DISCUSSION

RAF inhibitors are the new first-line therapy for V600 BRAF melanoma and form the building blocks for further improvements to achieve more durable responses with reduced side effects. One approach is to develop a new generation of RAF inhibitors that do not elicit the paradoxical activation of MEK-ERK1/2 signaling in wild-type BRAF cells (Halaban et al., 2010; Heidorn et al., 2010; Kaplan et al., 2011; Poulikakos et al., 2010). Theoretically,

this would enable enhanced tolerability and, in turn, increased drug dosage. This approach has led to the generation of a series of drugs known as paradox breakers. In this study, we analyzed the ability of one of these inhibitors, PB04.

Initially, we show that PB04 is an efficient inhibitor of ERK1/2 activation in a panel of mutant BRAF melanoma cells but does not hyperactivate ERK1/2 in the mutant NRAS melanoma cells. These data are consistent with the paradox breaker design of this RAF inhibitor. The development of PB inhibitors represents a major advance in the field given that most of clinical grade RAF inhibitors to date elicit ERK1/2 hyperactivation *in vitro* and the formation of cuSCC/KA *in vivo*. Indeed, a second clinical grade RAF inhibitor, dabrafenib, effectively inhibits mutant BRAF signaling, elicits strong clinical responses in patients but also induces the formation of cuSCC/KA (Falchook et al., 2012). In mutant NRAS cells, which utilize CRAF to activate ERK1/2 (Dumaz et al., 2006), PB04 did not effectively inhibit pathway activation. This is likely due to the 21-fold higher IC₅₀ of PB04 towards CRAF compared to BRAF^{V600E}, as measured by *in vitro* kinase assays. Similar to vemurafenib/PLX4720, PB04 led to an up-regulation of FOXD3. Since FOXD3 may be associated with an adaptive response to RAF inhibitors (Abel and Aplin, 2010; Basile et al., 2012), a similar primary/intrinsic resistance profile may be associated with paradox breakers as with vemurafenib.

In the phase 2 and 3 trials with vemurafenib, approximately 50% of V600 BRAF melanoma patients responded with at least 30% tumor shrinkage (Chapman et al., 2011; Sosman et al., 2012). As with targeted therapies in other tumor types, the benefit provided by vemurafenib was limited in time and many of initial responders ultimately developed progressive disease. This acquired resistance to vemurafenib is frequently associated with re-activation of the ERK1/2 pathway that is mediated by secondary mutations in NRAS or MEK1 and the expression of BRAF splice variants (Nazarian et al., 2010; Poulikakos et al., 2011; Wagle et al., 2011). We studied whether PB04 could inhibit activation of ERK1/2 in the setting of mutant NRAS-mediated resistance to vemurafenib. Vemurafenib and PLX4720 are not able to efficiently inhibit phospho-ERK1/2 in BRAF^{V600E} melanoma cells with an acquired endogenous NRAS^{Q61K} allele or co-expressing ectopic NRAS^{Q61K} (data within and (Kaplan et al., 2012)). However in these systems, PB04 was able to efficiently inhibit phosphorylation of ERK1/2, induce apoptosis and inhibit anchorage-independent growth. Activation of ERK1/2 in the NRAS^{Q61K}/BRAF^{V600E} melanoma cells is dependent upon both BRAF and CRAF (Kaplan et al., 2012); thus, the inhibitory effect of PB04 in this system is likely due to inhibition of BRAF^{V600E} activity and the lack of transactivation of CRAF. While secondary mutations in NRAS are frequent in acquired resistance to RAF inhibitors in melanoma, multiple other mechanisms of resistance exist. Further studies will determine the effect of PB inhibitors on other resistance mechanisms especially those that involve altered dimerization properties of RAFs.

The main potential use of selective, paradox breaker RAF inhibitors in BRAF^{V600} melanoma patients is as a first line therapy. Theoretically, increased dosing of PB drugs should lead to effective inhibition of the ERK1/2 pathway with a decreased incidence of associated cuSCC/KA. In this setting, it is likely that the frequency of secondary mutations in NRAS leading to acquired resistance will be reduced. Our studies indicate a second potential use is in vemurafenib-treated patients who show disease progression that is associated with a secondary mutation in NRAS. Such patients may benefit from a switch in their RAF inhibitor treatment from vemurafenib to a paradox breaker. While ongoing trials are testing the combinatorial approaches such as RAF inhibitors (dabrafenib, GSK2118436) plus MEK inhibitors (trametinib, GSK1120212) [Clinical Trials.gov ID: NCT01072175 and NCT01619774 and (Flaherty et al., 2012)], it is likely that the numbers of drugs being combined will increase. For example, there is already considerable interest in combining

RAF or MEK inhibitors with drugs targeting the PI3K-AKT-mTOR pathway. Since combining drugs often produces new, unanticipated toxicities and side effects, the use of a single drug to effectively inhibit the ERK1/2 pathway without eliciting paradoxical effects may be more tolerable in these combinatorial regimens.

MATERIALS AND METHODS

Inhibitors

PB04 and PLX4720 were kindly provided by Dr. Gideon Bollag (Plexxikon Inc., Berkeley, CA). AZD6244 was purchased from Selleck Chemicals LLC (Houston, TX).

Cell lines

Melanoma cells were cultured, as previously described (Hu and Aplin, 2010). WM793, WM115, WM9, WM278, WM1346, WM1366, WM1361A, Sbcl2 and 1205Lu were cultured in MCDB 153 containing 20% Leibovitz L-15 medium, 2% FBS, 5 µg/ml insulin and penicillin/streptomycin. SK-MEL 24, SKMEL-2, and SK-MEL 207 cells were cultured in RPMI with 10% FBS. A375 cells were cultured in DMEM with 10% FBS. The BRAF and NRAS genotypes were verified by Sanger sequencing. A375-NRAS^{WT}, and the PLX4720-resistant cell lines, A375-NRAS^{Q61K} and 1205TR-NRAS^{Q61K} have been previously described (Kaplan et al., 2012). HaCaT and PAM212 cells were grown in DMEM with 10%FBS and in in RPMI 1640 and 10%FBS, respectively.

Generation of resistant cell lines

WM793 cells were cultured in the aforementioned MCDB 153 medium in the presence of 5 µM PLX4720 for four weeks. Medium containing PLX4720 was replenished every two d. Resistant sublines were generated by seeding cells at low density and allowing isolated colonies to form. These colonies were picked and cells expanded in the continued presence of PLX4720 (Kaplan et al., 2012).

Western blotting

Western blotting was performed, as previously described (Hu and Aplin, 2010), and quantitated using a Versadoc Imaging system (BioRad, Hercules, CA). Primary antibodies utilized were: phospho-MEK (S217/S221), phospho-ERK1/2 (Thr202/Tyr204, #4377), and PDGFRβ (#3169) from Cell Signaling Technology, Beverly, MA., MEK (sc-219), ERK1/2 (sc-094), BRAF (sc-5284), IGF-1Rβ (sc-713), HRAS (sc-520) and NRAS (sc-519) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, FOXD3 (Poly6317) from BioLegend, San Diego, CA, and β-actin (A5316) from Sigma Aldrich, Inc., St Louis MO.

Three-dimensional (3-D) collagen gels

Collagen gels were cast by mixing the following on ice: MEM (Lonza, Inc. Walkersville, MD), 2 mM L-glutamine, 2 % FBS, 0.15 % sodium bicarbonate and 0.8 mg/ml bovine type-I collagen (Organogenesis Inc., Canton, MA). Cells were seeded in 2 ml collagen gels and incubated at 37°C for 30 min. After polymerization, the collagen gel lattice was overlaid with 1.5 ml medium and inhibitor at 37°C for 48 hrs. 3-D collagen gels were dissolved in 1 mg/ml collagenase (Sigma-Aldrich, Inc.) solution to release cells.

Apoptosis assays

Apoptosis assay was done as previously described (Shao and Aplin, 2010). Cells were washed once in PBS and resuspended in 100 µl binding buffer (10 mM HEPES, 0.14 M sodium chloride, 2.5 mM calcium chloride), stained with 5 µl Annexin V-APC (BD Biosciences, Franklin Lakes, NJ) for 15 min and finally an additional 400 µl binding buffer

was added. Apoptosis was analyzed by flow cytometry on the FACSCalibur (BD Biosciences). Data were analyzed using Flowjo software (Three Star, Inc. Ashland, OR).

EdU (5-ethynyl-2'-deoxyuridine) incorporation assays

A375 NRAS^{WT} and A375 NRAS^{Q61K} cells were treated with PB04 for 48 h before the addition of 10 μ M EdU for another 16 hrs. Cells were then processed using the Click-iTTM EdU Alexa Fluor 647 Flow Cytometry Assay kit (Invitrogen, Carlsbad, CA) for flow cytometry analysis.

Soft agar assays

Cells (3×10^3 cells/mL) were grown in 0.2% soft agar, as previously described (Abel and Aplin, 2010). Cells were grown for 7 d, replacing the medium every 3 d. Three random fields per chamber were acquired using NikonTM Eclipse Ti inverted microscope (Nikon, Tokyo, Japan) with NIS-Elements AR 3.00 software (Nikon).

Statistical analysis

Statistical analysis of the data was performed using an unpaired student *t*-test assuming unequal variance.

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ABBREVIATIONS

cuSCC	cutaneous squamous cell carcinoma
ERK	extracellular signal-regulated kinase
KA	keratoacanthoma
MAPK	mitogen-activated kinase
MEK1/2	MAPK/ERK kinases 1 and 2

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SIGNIFICANCE

We provide studies on a new class of RAF inhibitors to inhibit the ERK1/2 pathway in mutant BRAF melanoma cells. RAF inhibitors that do not elicit the paradoxical activation of the ERK1/2 pathway in wild-type BRAF cells have clear utility as a first-line therapy that permits increased dosing with a decreased incidence of associated cutaneous squamous cell lesions. Furthermore, our studies indicate that they may be useful as a second-line option in vemurafenib-treated patients who show disease progression that is associated with a secondary mutation in NRAS.

Figure 1. PB04 inhibition of ERK1/2 in a panel of melanoma cell lines

(A) WM793 cells were treated with PLX4720 or PB04 at different concentrations (0, 0.05, 0.1, 1, 5 μM) for 24 h. Cells were lysed and analyzed by Western blotting with phospho-MEK1/2, total MEK1/2, phospho-ERK1/2 and total ERK1/2 antibodies. (B) A panel of mutant BRAF melanoma cell lines were treated with PB04 at different concentrations (0, 0.5, 1, 5 μM) for one h. Cells were lysed and analyzed by Western blotting with phospho-ERK1/2 and total ERK1/2, and actin antibodies. (C) As in B, except with a 16 h treatment of PB04. (D) Mutant NRAS cells, SK-MEL 2 cells were treated with PLX4032 at different concentrations (0, 0.5, 1, 5 μM) for one or sixteen h. Cells were lysed and analyzed by Western blotting with phospho-ERK1/2 and total ERK1/2, and actin antibodies. (E) Mutant NRAS melanoma cell lines were treated with PB04 at different concentrations (0, 0.5, 1, 5 μM) for one h. Cells were lysed and analyzed by Western blotting with phospho-ERK1/2 and total ERK1/2, and actin antibodies. (F) As in D, except with a 16 h treatment of PB04. For all phospho-ERK1/2 and phospho-MEK blots, normalized quantitation is indicated.

Figure 2. PB04 effects in keratinocyte/squamous cell carcinoma lines harboring mutant HRAS (A) HaCaT HRAS G12V cells and (B) PAM 212 cells were treated with PLX4720 or PB04 at different concentrations (0, 0.05, 0.1, 1, and 5 μ M) for 1 h. The levels of phospho-MEK1/2, total MEK1/2, phospho-ERK1/2 and total ERK1/2 were assessed by Western blotting.

Figure 3. Up-regulation of FOXD3 in mutant BRAF melanoma cells treated with PB04
 1205Lu, WM115, and A375 cells were treated with PB04 at different concentrations (0, 0.5, 1, 5 μ M) for 16 h, as indicated. Cells were lysed and analyzed by Western blotting with FOXD3, phospho-ERK1/2, total ERK1/2, PDGFR, IGF-1R, phospho-AKT, total AKT, BRAF, and actin antibodies. Fold changes in FOXD3, PDGFR and IGF-1R are indicated.

C

D

Figure 4. PB04 inhibits ERK1/2 in PLX470-resistant cell lines

(A) WM793R NRAS Q61K cell sub-lines (#5 and #12) were treated with PLX4720 or PB04 at different concentrations (0, 0.05, 0.1, 1, 5 μ M) for 24 h. Cells were lysed and analyzed by Western blotting with phospho-ERK1/2 and total ERK1/2 antibodies. (B) WM793R NRAS Q61K clone #5 and clone #12 cells were treated with DMSO, 1 μ M PLX4720 (PLX), and 1 μ M PB04 for 1, 6, or 24 h. Cells were lysed and analyzed by Western blotting with phospho-ERK1/2 and total ERK1/2 antibodies. (C) WM793 and WM793-NRAS Q61K cells were treated with PLX4720 at different concentrations (0, 0.1, 1, 5, and 10 μ M) for 24 h. Cells lysates were analyzed, as above. (D) As in C, except cells were treated with PB04 (0.1 – 5 μ M) and PLX4720 (5 μ M), as indicated. Normalized quantitation is shown for phospho-ERK1/2 blots.

A

B

Figure 5. PB04 treatment promotes apoptosis and inhibits anchorage-independent growth of vemurafenib resistant cells

WM793 and WM793R NRAS Q61K clone #5 and clone #12 cells were plated in (A) 2D culture dishes or (B) seeded in 3D collagen gels and treated with 1 μM PB04 or DMSO (control) for 48 h. (C) A375 NRAS^{WT} and A375 NRAS^{Q61K} cells were plated in 0.2% bacto-agar and treated with DMSO or PB04 (1 μM for 7 d). Random fields per wells were acquired using NIS-Elements software from Nikon. The number of colonies was counted and the size of the colonies was measured. Results represent the mean ± STD of 3 independent experiments. (D) A375 NRAS^{WT} and A375 NRAS^{Q61K} cells were treated with PB04 for 48 h. After 48 h, collected cells were analyzed by annexin V-APC staining and flow cytometry analysis. Quantitation of data from three independent experiments was represented by the mean percentage of cells staining positive for annexin V-APC. (E) Similar to (D), except that cells were treated with PB04 for 48 h in the presence of EdU for 16 h. Cells were collected and analyzed by EdU incorporation proliferation assay. Assays are completed in triplicate.