**Preclinical Development**

**Epithelial Tissue Hyperplasia Induced by the RAF Inhibitor PF-04880594 Is Attenuated by a Clinically Well-Tolerated Dose of the MEK Inhibitor PD-0325901**

Vince R. Torti¹, Donald Wojciechowicz², Wenyue Hu¹, Annette John-Baptiste¹, Winston Evering¹, Gabriel Troche², Lisa M. Marroquin¹, Tod Smeal², Shinji Yamazaki³, Cynthia L. Palmer⁴, Leigh Ann Burns-Naas¹, and Shubha Bagrodia²

**Abstract**

Clinical trials of selective RAF inhibitors in patients with melanoma tumors harboring activated BRAFV600E have produced very promising results, and a RAF inhibitor has been approved for treatment of advanced melanoma. However, about a third of patients developed resectable skin tumors during the course of trials. This is likely related to observations that RAF inhibitors activate extracellular signal–regulated kinase (ERK) signaling, stimulate proliferation, and induce epithelial hyperplasia in preclinical models. Because these findings raise safety concerns about RAF inhibitor development, we further investigated the underlying mechanisms. We showed that the RAF inhibitor PF-04880594 induces ERK phosphorylation and RAF dimerization in those epithelial tissues that undergo hyperplasia. Hyperplasia and ERK hyperphosphorylation are prevented by treatment with the mitogen-activated protein/extra-cellular signal–regulated kinase (MEK) inhibitor PD-0325901 at exposures that extrapolate to clinically well-tolerated doses. To facilitate mechanistic and toxicologic studies, we developed a three-dimensional cell culture model of epithelial layering that recapitulated the RAF inhibitor–induced hyperplasia and reversal by MEK inhibitor in vitro. We also showed that PF-04880594 stimulates production of the inflammatory cytokine interleukin 8 in HL-60 cells, suggesting a possible mechanism for the skin flushing observed in dogs. The complete inhibition of hyperplasia by MEK inhibitor in epithelial tissues does not seem to reduce RAF inhibitor efficacy and, in fact, allows doubling of the PF-04880594 dose without toxicity usually associated with such doses. These findings indicated that combination treatment with MEK inhibitors might greatly increase the safety and therapeutic index of RAF inhibitors for the treatment of melanoma and other cancers. *Mol Cancer Ther; 11(10); 2274–83.* ©2012 AACR.

**Introduction**

The BRAF protein kinase plays a central role in promoting normal cell proliferation and survival as a component of the RAS/RAF/MEK/ERK signaling pathway. Mutations in the BRAF gene that activate BRAF kinase activity independently of RAS activation have been observed in approximately 50% of malignant melanomas (1–3). BRAF has therefore attracted much attention as a potential target of inhibitor therapies in melanoma and other tumors in which BRAF is mutated. Indeed, the selective RAF inhibitor vemurafenib (PLX4032) was recently approved by the U.S. Food and Drug Administration for treatment of metastatic melanomas in patients with BRAFV600E mutation. In a pivotal phase III trial of 675 patients with previously untreated metastatic melanoma, 74% had a reduction in risk of progression or death with vemurafenib compared with the reference drug, dacarbazine (4). Mean progression-free survival with vemurafenib was 5.3 months, compared with 1.6 months for the dacarbazine cohort, and response rates of 48% with vemurafenib and 5% with dacarbazine were observed. Another selective RAF inhibitor, GSK2118436, has recently entered phase III trials after showing similar effectiveness in melanoma patients, and other compounds are in early clinical development.

Despite the remarkable clinical effectiveness of vemurafenib and other RAF inhibitors, there remain 2 key...
Improving Tolerance to RAF Inhibitors

Concerns over long-term efficacy and safety. First, clinical development of vemurafenib revealed that a large number of patients with BRAF V600E mutation had primary or acquired resistance to the RAF inhibitor. Acquired resistance does not seem to develop as a result of mutation of "gatekeeper" residues in BRAF itself, as occurs in resistance to BCR-ABL and EGFR inhibitors (5), but from activation of other proteins in the same or parallel signaling pathways (6). Two of the most common resistance mechanisms involve mutation in the NRAS gene and upregulation of PDGFRβ expression (7). Second, a high percentage of patients in clinical trials of vemurafenib and GSK2118436 developed skin tumors, particularly keratoacanthoma and squamous cell carcinoma. This is regarded as an acceptable risk for treatment of advanced melanoma because in all cases, the tumors were resected and did not progress to metastases. Nonetheless, the possibility of RAF inhibitor-induced development of tumors in internal organs less amenable to observation remains a major concern, and understanding the causative mechanism has been a priority.

The most likely explanation for development of skin tumors during RAF inhibitor treatment comes from studies showing that RAF inhibitors cause activation of the extracellular signal-regulated kinase (ERK) pathway in cells with wild-type BRAF (8–11). Pathway activation involves formation of homo- or heterodimers between BRAF and CRAF, in which an inhibitor-bound protomer transactivates its dimeric partner in a RAS-GTP-dependent manner (12, 13). In keeping with this model, ectopic expression of kinase-defective BRAF also causes ERK pathway activation in tumor cells with wild-type BRAF (14). Stimulation of ERK activity, cell proliferation, and tumor growth in vivo was observed in response to RAF inhibitor treatment in preclinical models with both mutant and wild-type RAS genes (11).

It is possible that development of squamous cell carcinoma in RAF inhibitor–treated patients depends on pre-existing mutations or it is also possible that tumorigenic genetic changes accumulate as a secondary consequence of hyperplasia. Indeed, Carnahan and colleagues (8) showed increased cell proliferation and hyperplasia in mouse stomach epithelial tissue after treatment with the RAF inhibitor C-19 that was prevented by cotreatment with the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor PD-0325901. In a follow up study, Wisler and colleagues (15) showed hyperplasia in response to a series of RAF inhibitors in multiple rat epithelial tissues. Moreover, severe hyperplasia in the bladder transitional epithelium was found to be associated with development of a very rare carcinoma in one rat (15). Clearly this will need to be reproduced, but it does raise the possibility of development of nonskin neoplasias in melanoma patients receiving RAF inhibitor therapy.

PD-0325901, a highly potent, second-generation selective MEK1/2 inhibitor that inhibits phosphorylation of ERK1/2 and proliferation in BRAF-mutant tumor cells (16). PD-0325901 showed promising efficacy in early clinical development, but trials were discontinued after phase II because of unacceptable musculoskeletal, neurologic, and ocular toxicities at doses greater than 15 mg dosed twice daily intermittently (17–19). The potent and selective RAF inhibitors PF-04880594 (20) and WYE-130600 (21), which inhibit both wild-type and mutant BRAF and CRAF, have shown tumor growth inhibition in BRAF-mutant melanoma xenograft models. However, during the course of preclinical studies, we observed hyperplasia in response to both inhibitors. Because the 2 inhibitors are structurally unrelated, this indicated that the effect was mechanism specific. Indeed, we had observed activation of ERK in cell lines with mutant RAS or activated growth factor receptor signaling (unpublished observations). On the basis of these observations, we investigated whether RAF inhibitor–induced hyperplasia was associated with ERK activation and BRAF-CRAF dimerization in hyperplastic tissues and, most importantly, whether hyperplasia could be prevented by doses of MEK inhibitor that would extrapolate to clinically safe doses. To facilitate further studies, we also developed a three-dimensional (3D) cell culture model that recapitulated the RAF and MEK inhibitor effects on epithelial hyperplasia in vitro. We were also able to show that RAF inhibitors induce expression of the inflammatory cytokine interleukin 8 (IL-8), providing a possible explanation of some of the observed preclinical effects of RAF inhibitor treatment.

Materials and Methods

Modulation of ERK phosphorylation in mice treated with the RAF inhibitor (PF-04880594) and the MEK inhibitor (PD-0325901)

Nude mice were treated with either 10 mg/kg of PF-04880594 alone, 0.5 mg/kg of PD-0325901 alone, or in combination. Chemical structures of the 2 drugs are provided (Fig. 1). Animals were administered 3 doses over 2 days: an am and pm dose on day 1 and then an am dose on day 2 approximately 2 hours before the animals were necropsied and the tissues harvested. A selective panel of tissues, including esophagus, skin, tongue, and urinary bladder, were collected and flash frozen in liquid nitrogen. Samples were powdered with a mortar and pestle and homogenized in NP40 lysis buffer (25 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, and 2% NP-40, pH 7.4), with 10× Halt protease and phosphatase inhibitors (Thermo Scientific). Lysate was centrifuged at 12,000 × g for 10 minutes at 4°C and the supernatant was collected. Protein concentration was determined using the bicinchoninic acid protein assay (Thermo Scientific).

Immunoprecipitation and immunoblotting

Lysates (500 μg of protein) were incubated with 2 μg of anti-BRAF antibody (sc-166; Santa Cruz Biotechnology) for 1 hour at 4°C while rocking, followed by an additional 30 minutes incubation with 40 μL of resuspended A/G Plus agarose (Santa Cruz Biotechnology). Samples were spun down at 200 × g for 1 minute and washed with 500 μL
of NP40 buffer 3 times. Then 35 μL of lithium dodecyl sulfate sample loading buffer with reducing agent was added. Samples were boiled at 95°C to 100°C for 5 minutes and then spun at 2,000 g for 1 minute and supernatant was collected. Immunoprecipitates or protein homogenates were electrophoretically separated with a NuPAGE 4% to 12% Bis-Tris gel (Invitrogen) and transferred to a 0.45-μm nitrocellulose membrane. After blocking in fluorescent blocking buffer (Rockland Immunochemicals) for 1 hour at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. Primary antibodies included anti-phospho-MAPK (ERK1/2; M 8159; Sigma Chemical Co.), anti-MAPK (ERK 1/2; 9102; Cell Signaling), anti-CRAF (610152; BD Biosciences), and anti-BRAF (sc-5284; Santa Cruz Biotechnology). ERK control extracts (9194; Cell Signaling) and Caco-2 whole-cell extract were used as negative and positive controls for p-ERK and BRAF/CRAF measurement. Membranes were washed, probed with secondary infrared antibodies (donkey anti-rabbit IgG or rabbit anti-mouse IgG; Rockland Immunochemicals) for 1 hour at room temperature, and washed again. After the final wash, membranes were visualized on the Odyssey infrared scanner (Li-Cor Biosciences).

Microscopic pathology of tissues from PF-04880594 and PD-0325901–treated nude mice

Nude mice (6–8 weeks old) were treated with either PF-04880594 10, 20, or 40 mg/kg twice daily or PD-0325901 at 1 mg/kg and/or at 0.1, 0.3, 0.5, 1.0, or 2.5 mg/kg twice daily in combination with PF-04880594 as indicated in Table 1. Treatment occurred daily for 3 weeks, after which all surviving mice were euthanized and necropsied. The esophagus, tongue, footpad, urinary bladder, and dorsal skin were excised and preserved in 10% neutral buffered formalin (VWR). Following fixation, all tissues were embedded in paraffin blocks, sectioned, mounted on slides, and stained with hematoxylin and eosin (H&E). Slides were evaluated for the incidence and severity of a hyperplastic response.

All animal studies were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. In addition, all animal experiments were carried out in accordance with the principles described in the guide for care and use of laboratory animals by the NIH (22) and approved by the Institution Animal Care and Use Committee associated with the facility in which the laboratory animals were housed.

Reconstructed human epidermis 3D cell culture and treatment

The in vitro reconstructed human epidermis (RHE; Skinethic Laboratories) consists of normal human keratinocytes cultured on an inert polycarbonate filter at the air–liquid interface in the proprietary growth culture medium. These cells have been tested and authenticated. These cells express major differentiation markers that include (i) filaggrin and involucrin in granular layers, (ii) transglutaminase I and keratin 10 in suprabasal cell layers, and (iii) loricrin in upper granular cell layers, and (iv) the basement membrane markers, type IV collagen, integrin α6, integrin β4, antigen BP, laminin 1, and laminin V. The cells were tested for identity via immunohistochemistry by Skinethic Laboratories for each batch before shipping. Cells were treated with dimethyl sulfoxide (DMSO) or RAF inhibitor PF-04880594 at 62.5 nmol/L and MEK inhibitor PD-0325901 at 5 nmol/L separately or in combination in the basal chamber for 2 days. Duplicate samples from each treatment were fixed overnight in 10% formalin and subjected to histologic evaluation using H&E staining. A second set of samples with the same treatments was flash frozen in liquid nitrogen for protein extraction. The phosphorylated and total forms of ERK were evaluated using immunoblotting, as described.

Induction of IL-8 release from HL-60 cells by PF-04880594 and attenuation of release by PD-0325901

HL-60 cells were procured from the American Type Culture Collection and stored for 5 years. Cells were tested for authenticity by differentiating with phorbol myristate acetate (PMA), as has been previously reported (23). On an intermittent basis, PMA was added to HL-60
cells in RPMI-1640 at a concentration of 100 ng/mL, and the cells were observed for morphologic signs of differentiation to authenticate their identity. Secreted IL-8 was determined using a standard ELISA-based assay (SA Biosciences/Qiagen). 3/C2 106 HL-60 cells were incubated with 100 nmol/L PF-04880594 or 100 nmol/L MEK inhibitor or both for 48 hours in RPMI media containing 10% FBS. Control cells were treated with DMSO (0.25% final). After 48 hours, the culture media was separated from the cells by centrifugation at 200/C2 188 g for 5 minutes. The culture supernatant was stored at 4/C14 C and assayed the following day. The cells were washed once in cold PBS and lysed in a buffer as described above. Protein determination was carried out using the BCA protein assay (Pierce).

Results

The RAF inhibitor PF-04880594 induces ERK phosphorylation and BRAF-CRAF dimerization in multiple epithelial tissues

Hatzivasiliou and colleagues (13) showed elevation of p-ERK, by immunohistochemistry, in skin sections from mice treated with RAF inhibitor. To extend this observation, we examined p-ERK levels in target tissues from mice dosed with PF-04880594 alone or in combination with PD-0325901. As shown in Fig. 2A, tissue (urinary bladder, tongue, skin, and esophagus) homogenates from mice dosed with PF-04880594 at 10 mg/kg for 2 days showed higher levels of p-ERK compared with tissue homogenates from animals treated with vehicle. The induction of ERK phosphorylation by PF-04880594 treatment was attenuated by cotreatment with 0.5 mg/kg PD-0325901. A similar induction of p-ERK was seen in tissues taken from dogs dosed with the RAF inhibitor WYE-130600 (data not shown).

It has been shown that the activation of ERK in tumor cells with wild-type BRAF involves the formation of homo- and heterodimers between BRAF and CRAF isoforms (12, 13). To determine whether dimerization might be responsible for the observed ERK in normal mouse tissues, we immunoprecipitated BRAF from tissues homogenate lysates and immunoblots were probed with anti-CRAF antibodies. As shown in Fig. 2B, CRAF was detectable in BRAF immunprecipitates from skin and esophageal lysates from animals that were treated with PF-04880594 alone or in combination with MEK inhibitor, but not in animals treated with vehicle or MEK inhibitor alone. These results are entirely consistent with the proposed model of RAF inhibitor transactivation of RAF isoforms via inhibitor-induced dimerization. The MEK inhibitor, although blocking ERK activation, would not be expected to inhibit dimer formation.

Induction of epithelial hyperplasia by PF-04880594 is prevented by low doses of the MEK inhibitor PD-0325901

Wisler and colleagues showed hyperplasia in multiple stratified squamous and transitional epithelial tissues after treatment of rats with a series of RAF inhibitors for 7 days (15). In agreement, we observed a similar level of hyperplasia in squamous epithelial layers from esophagus, tongue, urinary bladder, and footpad in mice treated for 21 days with the RAF inhibitor PF-04880594 at 10, 20, and 40 mg/kg (Fig. 3 and Table 1). Carnahan and colleagues (8) have previously shown that RAF

Table 1. Severity and incidence of hyperplastic lesions in mice treated with BRAF inhibitor (PF-04880594) and MEK inhibitor (PD-0325901)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Esophagus</th>
<th>Tongue</th>
<th>Feet</th>
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<tr>
<td>PD-0325901</td>
<td>PF-04880594</td>
<td>4/6 (1,2)</td>
<td>4/6 (1)</td>
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<tr>
<td>1</td>
<td>0</td>
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<tr>
<td>13</td>
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*Tissues positive for hyperplasia are denoted as a/b (c), in which a = the number of hyperplasia positive mice, b = the total number of mice examined per group, and c = the severity. Severity of lesions is described as 1, minimal, 2, mild, 3, moderate, and 4, severe.

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inhibitor–induced hyperplasia in nonglandular stomach epithelium was blocked by the MEK inhibitor PD-0325901 at 3 mg/kg. To test the effectiveness of PD-0325901 in preventing RAF inhibitor–induced hyperplasia, we used a dose range of PD-0325901 (0.1–1 mg/kg) that, on the basis of pharmacokinetic and toxicologic considerations, extrapolated to a dose range that was well tolerated in clinical trials (Supplementary Fig. S1). At 1 mg/kg, PD-0325901 blocked hyperplasia in response to 10 mg/kg PF-04880594 (Fig. 3). Titration of the PD-0325901 dose revealed that it was effective at ratios up to at least 1:33 at all doses of PF-04880594 (Table 1).

PF-04880594 at 10 mg/kg was well tolerated in these studies; however, mice treated with 20 and 40 mg/kg PF-04880594 were euthanized at week 1 and 2, respectively, because of moribundity associated with toxicity. Postdose observations included up to a 9% body weight loss over 6 days (Supplementary Fig. S2) and noticeable hypoactivity. Mice in the 10 mg/kg RAF inhibitor dose group were beginning to show signs of RAF inhibitor–induced toxicity by the end of the study just before scheduled euthanasia after 3 weeks of dosing. However, mice in RAF inhibitor–treated groups with up to 40 mg/kg PF-04880594 treatment that also received PD-0325901 treatment survived the treatment period and showed lesser weight loss to no weight loss (Supplementary Fig. S2).

A 3D human epidermal model recapitulates PF-04880594–induced hyperplasia in vitro

To further evaluate the clinical relevance of the hyperproliferative response to RAF inhibitors, we used a 3D culture model of RHE cells (Skinethic), which bears histologic similarity to human epidermal layers. We observed growth and development of all squamous epithelial cell layers (stratum geminativum, stratum spinosum, stratum granulosum, and stratum corneum) across all groups (Fig. 4A). There was a thick layer of viable cells that was 4 to 6 cells deep and covered by a cornified layer (stratum corneum) that was approximately half the total thickness of the sample (Fig. 4A). After the RHE cell model was treated with PF-04880594 at 62.5 nmol/L for 2 days, marked necrosis was evident, characterized by the presence of ghost cells in a layer superficial to the viable cells, with cell debris scattered at the ghost cell–viable cell interface and within the viable cell layer (Fig. 4B). The ghost cell layer was considerably thicker than the viable cell layer below it, approximately 6 to 7 cells deep and consisted approximately 50% to 60% of the thickness of the culture, which were approximately 10 to 14 cells deep, compared with 4 to 6 cells deep in untreated cells (compare Fig. 4A with B). Cells treated with PF-04880594 and PD-0325901 in combination closely resembled untreated cells, with a viable cell layer 4 to 6 cells

Figure 2. RAF inhibitor (PF-04880594) induces ERK phosphorylation and BRAF/CRAF dimerization in epithelial tissues. Tissue homogenates from urinary bladder, tongue, skin, and esophagus were made from mice treated with PF-04880594 at 10 mg/kg or PD-0325901 at 0.5 mg/kg separately or in combination for 2 days. A, levels of phosphorylated and total ERK were evaluated using immunoblotting and the band intensity was digitally quantified (Odyssey 2.1 software). The levels of phosphorylated ERK were normalized against total ERK and expressed as a fold change over vehicle control. Jurkat whole cells extract treated with U0126 at 10 μmol/L for 1 hour were used as negative control, and Jurkat cells treated with TPA at 200 nmol/L for 10 minutes served as positive control for ERK phosphorylation. B, the dimerization of BRAF/CRAF was evaluated in the esophagus and skin samples via immunoprecipitation using BRAF–specific antibody, followed by immunoblotting using CRAF–specific antibody. The total level of CRAF was also assessed in the same membrane as the loading control. Caco-2 whole-cell extract was used as positive control.
deep covered by a cornified layer that was approximately 40% to 50% of the total thickness of the culture with minimal single cell necrosis and no detectable ghost cells. The level of p-ERK and total ERK were evaluated in RHE cells treated with PF-04880594 or PD-0325901 separately or in combination (Fig. 4D). Consistent with observations in mouse epithelial tissues, the level of p-ERK was significantly induced by RAF inhibitor treatment in RHE cells. This increase in p-ERK was attenuated by cotreatment with PD-0325901, at doses comparable with the in vivo exposure level in mice.

RAF inhibitors induce IL-8 release in vitro

After relatively short periods of exposure (1–3 days), mice treated with PF-04880594 and dogs treated with WYE-130600 were seen to experience flushing of the skin (Supplementary Fig. S3). We speculated that this effect might be because of production and release of proinflammatory factors in response to RAF inhibitor–induced ERK pathway activation. To address this possibility, HL-60 cells were treated with the RAF inhibitor WYE-130600 (300 nmol/L for 48 hours) and analyzed the tissue culture supernatant for the presence of inflammatory cytokines. The only cytokine detected at elevated levels after inhibitor treatment was IL-8 (data not shown). Interestingly, exposure of HL-60 cells to WYE-130600 did not increase proliferation, but markers of differentiation were detected, consistent with previous reports of constitutive ERK phosphorylation inducing differentiation of HL-60 cells (ref. 18; data not shown). To confirm these findings and establish the role of ERK activation, we treated HL-60 cells with PF-04880594 (100 nmol/L), with or without PD-0325901 (100 nmol/L for 48 hours) and measured IL-8 levels in tissue culture media. As shown in Fig. 5, PF-04880594 caused ERK activation and stimulation of IL-8 release by more than 5-fold, both of which were blocked by PD-0325901 treatment.

Discussion

Previous studies have shown that different RAF inhibitors activate the ERK pathway in cells harboring wild-type BRAF via a mechanism involving BRAF/CRAF dimerization and transactivation of the inhibitor-free pro- tomer in a RAS-GTP–dependent manner (12, 13). It has also been shown that a series of RAF inhibitors induce hyperplasia in multiple epithelial tissues and that hyperplasia in the nonglandular stomach epithelium can be prevented by coadministration of a MEK inhibitor,
suggesting ERK activation as a contributing factor (8). We have now extended these sets of studies and characterized the underlying biology by showing that treatment of mice with the RAF inhibitor PF-04880594 results in CRAF-BRAF dimerization, ERK activation, and hyperplasia in the same epithelial tissues. Most importantly, we have shown that hyperplasia induced by the RAF inhibitor PF-04880594 can be prevented by relatively low, clinically relevant doses of the MEK inhibitor PD-0325901 and that this translates to human epithelial tissue model.

The RAF inhibitors vemurafenib and GSK2118436 showed excellent effectiveness in clinical trials of advanced melanoma in patients with BRAFV600E mutations (4, 24, 25). For instance, vemurafenib had a 48% response rate, with 5.3 months progression-free survival (1.8 months for the dacarbazine comparator). However, 15% to 30% of patients treated with these RAF inhibitors developed skin tumors diagnosed as keratoacanthomas and squamous cell carcinomas, as did patients treated with less selective RAF inhibitors such as sorafenib (24, 26, 27). These tumors were successfully resected and did not metastasize; however, there remain concerns about the possibility of patients developing undetected skin tumors or tumors at sites less amenable to detection and resection. The hyperplasia associated with ERK activation in epithelial tissues, including skin, reported here and in previous studies (8, 13, 15) lend strong support to the idea that skin tumor formation in patients is linked to RAF inhibitor–induced ERK activation. Tumor formation may be secondary to ERK-mediated hyperproliferation, by promoting accumulation of oncogenic mutations, or it may require the presence of preexisting mutations, such as sunlight-induced RAS or p53 mutations. In either case, it will be important to minimize this unwanted effect of RAF inhibitors; although keratoacanthomas generally spontaneously regress and do not metastasize, squamous cell carcinomas do have metastatic potential if undetected. Moreover, the ERK pathway is ubiquitous and, as shown here and elsewhere (15), hyperplasia occurs in multiple epithelial tissues in response to RAF inhibitors, raising the possibility of tumor formation at extracutaneous sites.

Our finding that treatment of HL-60 cells with PF-04880594 increases IL-8 release raises further concerns, as IL-8 promotes angiogenesis, stimulates proliferation...
and survival, and facilitates cancer cell migration (28). It is also possible that IL-8 may mediate the acute redness, or flushing, in the skin observed in dogs after RAF inhibitor treatment on areas where the fur was thin and the skin was visible (areas surrounding the muzzle, nose, and eyelids). This observation was noted in dogs administered with structurally distinct RAF inhibitors, suggesting the effect is target mediated. Interestingly, production of IL-8, which plays a role in the extravasation of melanoma cells by mediating ICAM-1 interaction with β2 integrin, was shown to be decreased following inhibition of BRAFV600E (29). Moreover, elevated plasma levels of IL-8 in mice bearing BRAFV600E melanoma xenografts were decreased by RAF inhibitor treatment (30). Our observation is consistent with these findings and, taken together, these findings suggest that reduction of IL-8 production may be important for prevention of metastasis in melanoma by RAF inhibitors and minimizing tumorigenic risk in nontarget tissues by MEK inhibitors.

The MEK inhibitor PD-0325901 was found to effectively block PF-04880594–induced hyperplasia and ERK activation in epithelial tissues and IL-8 release from cells in vitro. These results suggest a means of reducing the risk of tumorigenesis in nontarget tumor tissues with wild-type BRAF in patients treated with RAF inhibitors. Moreover, we found that the coadministration of PD-0325901 allowed elevation of the PF-04880594 dose by 2-fold without the toxicity usually observed at such doses. This indicates that combination with a MEK inhibitor will both increase RAF inhibitor safety and potentially increase efficacy by allowing dose escalation. A key point to be raised here is that the effective systemic exposures of PD-0325901 (MEK inhibitor) used in these studies extrapolate to exposures that were shown to be well tolerated in phase I and II clinical trials (17, 18). Specifically, the doses of PD-0325901 used in this study (0.1–1 mg/kg) have been calculated to correlate with clinical exposures resulting from oral twice-daily doses in the 4 to 10 mg range. In those trials, higher exposures of PD-0325901 were found to have unacceptable toxicity, particularly neurotoxicity and retinal vein occlusion. Unfortunately, these higher exposures were needed to achieve efficacy and thus led to the discontinuation of the clinical trials (17–19). When used at the lower levels shown here, the MEK inhibitor (PD-0325901) is highly effective at inhibiting RAF inhibitor-induced ERK activation and would be expected to be well-tolerated clinically.

The reconstructed human epidermis 3D cell model described here will be useful for future mechanistic and screening studies. First, rodent and human skin have significant differences in architecture and reconstructed human skin models can be used to study melanoma progression and invasion (31) and may be useful as a predictor of drug efficacy. Second, as shown here, the model can be used as a measure of adverse effects, in this case skin hyperplasia. Finally, the model may prove most useful in screening future drug candidates, particularly those impacting the ERK pathway, for their potential to cause epidermal hyperplasia.

A major challenge facing the clinical application of RAF inhibitors as antitumor agents (aside from the squamous cell carcinoma side effect) lies in the development of drug resistance. Most patients treated with vemurafenib acquired resistance within 8 to 12 months after initiation of treatment. In the case of other kinase-targeted therapies, such as those targeting BCR-ABL, KIT, or EGFR, resistance arises as a result of acquisition of "gatekeeper" mutations in the target kinase (5). However, so far analogous mutations in BRAFV600E have not been detected in tumors from patients receiving RAF inhibitors. Nazarian...
and colleagues (7) analyzed 12 matched pairs of drug-sensitive and drug-resistant tumors and discovered activating mutations in NRAS or activation of PDGFRβ in half of the samples, suggesting that acquisition of mutations that bypass BRAF to activate ERK (NRAS/PDGFRβ) or activate parallel pathways (PDGFRβ) may cause RAF inhibitor resistance. Combination treatment with a MEK inhibitor would most likely prevent tumor cells with the former category of mutation, including NRAS mutation, from gaining a proliferative advantage and therefore protect against resistance development. Early clinical studies in which both RAF and MEK inhibitors were administered seem to validate the predicted advantages of this combination and has recently been published (32).

It is not clear whether a MEK inhibitor would protect against PDGFR-mediated resistance, as there is the potential for signaling through multiple pathways, including ERK. Indeed, Shi and colleagues (33) have shown that a combination of RAF inhibitor with PI3K/mTOR inhibitor confers sensitivity to PDGFR-β-positive, vemurafenib-resistant melanoma cell lines. Also a recent report describes the presence of an activating mutation in MEK1 in a resistant tumor (34). The extent to which MEK inhibitors protect against RAF inhibitor–acquired resistance will no doubt become clearer with more extensive genotyping of drug-resistant tumors.

Disclosure of Potential conflicts of Interest
All authors are either former or current employees of Pfizer Inc.

References


Authors’ Contributions


Development of methodology: V.R. Torti, D. Wojciechowicz, W. Hu

Animals/cell lines/data (provided animals, acquired and managed patients, provided facilities, etc.): V.R. Torti, D. Wojciechowicz, W. Hu, G. Troche, I.D. Marroquin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.R. Torti, D. Wojciechowicz, W. Hu, G. Troche

Writing, review, and/or revision of the manuscript: V.R. Torti, D. Wojciechowicz, W. Hu, A. John-Baptiste, W. Evering, T. Smeal, S. Yamazaki, L. A. Burns-Naas, S. Bagrodia

Administrative, technical, or material support: V.R. Torti, D. Wojciechowicz, W. Hu, G. Troche

Study supervision: V.R. Torti, D. Wojciechowicz, T. Smeal, S. Bagrodia

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