MEK Inhibitor PD-0325901 Overcomes Resistance to PI3K/mTOR Inhibitor PF-5212384 and Potentiates Antitumor Effects in Human Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: Head and neck squamous cell carcinomas exhibit variable sensitivity to inhibitors of the PI3K/mTOR pathway, an important target of genomic alterations in this cancer type. The mitogen-activated protein kinase kinase (MEK)/ERK/activator protein 1 (AP-1) and nuclear factor-kB (NF-kB) pathways are also frequently co-activated, but their roles in resistance mechanisms to PI3K/mTOR inhibitors and as therapeutic targets in head and neck squamous cell carcinoma (HNSCC) are not well defined.

Experimental Design: We determined the IC50s of dual PI3K/mTOR inhibitor PF-05212384 (PF-384) by XTT assays in 14 HNSCC lines with PI3K/Akt/mTOR cascade alterations. In two resistant models, we further characterized the molecular, cellular, and in vivo attributes and effects of combining PF-384 with MEK inhibitor PD-0325901 (PD-901).

Results: PF-384 IC50 varied between 0.75 and 133 nmol/L in 14 HNSCC lines with overexpression or mutations of PIK3CA, and sensitivity correlated with increased phospho-AKT(T308/S473). In resistant UMSCC-1 and -46 models, PF-384 increased G0/G1-phase accumulation but weakly induced sub-G0 cell death. PF-384 inhibited direct targets of PI3K/mTOR, but incompletely attenuated co-activated ERK and UMSCC-1 xenograft growth in vivo. PD-901 strongly inhibited MEK/ERK targets, and the combination of PF-384 and PD-901 inhibited downstream NF-kB and AP-1 transactivation, and IL8 and VEGF production in vitro. PD-901 potently inhibited tumor growth alone and with PF384, enhanced antiproliferative, apoptotic, and anti-angiogenesis activity in vivo.

Conclusions: PI3K/mTOR inhibitor PF-384 exhibits variable activity in a panel of HNSCC cell lines with differing PIK3CA expression and mutation status. MEK inhibitor PD-901 overcomes resistance and enhances antitumor effects observed with PF-384 in vivo. Clin Cancer Res; 21(17); 3946–56. ©2015 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and standard treatment with surgery or chemoradiation has significant morbidity, with 5-year survival of <50% (1). Molecularly targeted inhibitors for HNSCC gained interest following identification of agents targeting EGFR.

However, when used either as single agents or in combination with radiation, these studies provided evidence of clinical activity in only ~10% to 15% of patients (2). Cumulative observations from mechanistic studies in HNSCC lines and proteomic and genomic studies in tumors suggest that multiple signaling molecules and pathways contribute to pathogenesis and a rationale for combination-targeted therapy in HNSCC.

We identified several growth factor and inflammatory cytokine receptor tyrosine kinases (RTKs) and signal pathways forming a signal network that can promote growth and survival of HNSCC, and lead to resistance to EGFR and other targeted inhibitors. EGFR (3, 4), hepatocyte growth factor receptor (c-MET; refs. 5 and 6), and tumor necrosis factor receptor (TNFR; ref. 7) are among the co-activators of the PI3K/Akt/mTOR and mitogen-activated protein kinase kinase (MEK)/ERK signal cascades, which induce transcription factors nuclear factor-kB (NF-kB) and activator protein 1 (AP-1), respectively (4, 8, 9). Supporting the importance of co-activation of these pathways, first-generation PI3K and MEK antagonists inhibited EGFR, HGF, or TNF-induced NF-kB, AP-1, target angiogenesis factor gene expression, and cell growth in vitro (4, 6, 8). Clinical trials with EGFR inhibitors or proteasome inhibition of NF-kB showed limited inhibition of PI3K–AKT–NF-kB or MEK–ERK signaling and clinical activity in vivo (3, 10).
Translational Relevance

The PI3K/AKT/mTOR and MEK/ERK pathways are frequently altered cascades in head and neck cancers that promote co-activation of oncogenic transcription factors NF-kB and AP-1, tumorigenesis, and resistance against anticancer therapies. In this study, we show that dual PI3K/mTOR inhibitor PF-384 exhibits nanomolar range activity in a panel of 14 HNSCC lines with differing PIK3CA expression and mutation status, with sensitivity correlating with phospho-AKT(T308/S473). Although PF-384 inhibited the PI3K/mTOR pathway, it incompletely attenuated ERK, AP-1, IL8, and VEGF in less sensitive lines, implying a potential resistance mechanism. MEK inhibitor PD-901 potently suppressed MEK/ERK pathways and displayed strong antiproliferative, apoptotic, anti-angiogenesis, and antitumor activity in xenografts in vivo. Our data suggest that MEK/ERK and PI3K/mTOR inhibition could be a more effective strategy to target this co-activated signal and transcriptional network, tumorigenesis, and resistance in HNSCC with various alterations activating PI3K or MEK.

Recent analysis by The Cancer Genome Atlas (TCGA) Network has identified genetic drivers that support PI3K and MEK as key common signal cascades and potential therapeutic targets for head and neck cancer (11). Overall, TCGA data shows >60% of HNSCC tumors harbor genomic alterations among various RTKs, PIK3CA, or HRAS, which converge on PI3K and MEK pathways. Among human papilloma virus negative (HPV−) HNSCC, ~30% display mutations or amplification of the PI3K catalytic subunit PIK3CA, whereas ~30% have activating alterations distributed among EGFR, ERBB2, FGFRs, EPHA2, IGF, and HRAS, which can potentially activate both PI3K and MEK pathways. In HPV+ HNSCC, nearly 60% of tumors harbor PIK3CA mutations and/or amplification.

Proteomic studies indicate levels of active phosphorylated AKT are highest in HPV+ HNSCC (12). In recent preclinical studies with a dual PI3K–mTOR inhibitor PF-502, we observed antitumor activity in a subset of human HPV+ HNSCC xenograft models, which overexpress PIK3CA protein (13). Independent reports using other PI3K or PI3K/mTOR inhibitors suggested that HPV− or HPV+ HNSCC with PIK3CA mutations may exhibit greatest sensitivity (14–16). However, the potential basis for varying sensitivity and role for PI3K/mTOR and MEK inhibitors among the major subset of tumors with amplification and overexpression of PIK3CA has not yet been fully explored.

To examine the role of PI3K–mTOR and MEK inhibition in HNSCC, we selected two compounds to test in our models. PF-384(PKI-587/PF-5212384) and PD-901(PD-0325901/PF-0192513) are selective small molecule inhibitors of PI3K–mTOR and MEK, respectively, in early-phase clinical trials, including one joint combination trial (NCT01347866). PF-384 is a reversible, ATP-competitive dual inhibitor of PI3K, PI3Kγ, and mTOR (17). PD-901 is a second-generation, highly potent, and specific non-ATP competitive inhibitor of MEK.

Here, we examined sensitivity to PI3K/mTOR inhibitor PF-384 in an expanded panel of 14 HNSCC lines, including nine with a defined range of PIK3CA protein expression (13), and two with known H1047R activating mutations (14). We observed that sensitivity correlated with increased phospho-AKT(T308/S473). Conversely, the relative resistance to PF-384 correlated with lower phospho-AKT(T308/S473), but was not consistently dependent on expression or mutation of PIK3CA, implying a role for other pathway(s). Among two HPV− HNSCC lines with PIK3CA protein overexpression that exhibited relative resistance to PF-384 in vitro and in vivo, we observed potent inhibition of PI3K–mTOR-mediated AKT and S6 kinase, but not MEK-mediated ERK phosphorylation. MEK inhibitor PD-901 potently inhibited ERK, tumor growth, and overcame the resistance to PI3K–mTOR inhibitor in vivo. The inhibitors blocked transactivation of AP-1 and NF-kB transcription factors, target angiogenesis factor reporter genes and proteins in vitro, and inhibited proliferative and angiogenesis markers in vivo. Together, our findings provide a rationale for incorporating MEK inhibitors to overcome resistance to PI3K–mTOR inhibitors in HNSCC with co-activation of these pathways.

Materials and Methods

Cell lines and culture

A panel of unique genotyped UMSCC cell lines were obtained from Dr. T.E. Carey at the University of Michigan (Ann Arbor, MI; Supplementary Table S1), including nine previously shown to overexpress PIK3CA (p110α) protein (13). Genotyped HPV(+) UPCI, UDSCC-2, and 93VII lines were kindly provided by Drs. Thomas E. Carey with permission of originating scientists, and characteristics are as described (Supplementary Table S1). The origin of these HNSCC cell lines were authenticated by genotyping with nine markers, and preserved in frozen stocks that were used within 3 months of culture as cited in Supplementary Materials and Methods. Two additional HNSCC lines containing PIK3CA H1047R mutations (14) were generously provided by Dr. J.S. Gutkind of the National Institute of Dental and Craniofacial Research (Bethesda, MD).

Therapeutic agents

PF-5212384 (PF-384) and PD-0325901 (PD-901) were acquired through a Materials Transfer Agreement between Pfizer, Inc. and the National Institute on Deafness and Other Communication Disorders (NIDCD, Bethesda, MD, USA). Preparations for use are described in Supplementary Materials and Methods.

XTT cell proliferation assay

XTT was performed according to manufacturer's instructions (Roche #11465015001) and as described in Supplementary Materials and Methods. Half maximal inhibitory concentration (IC50) was determined 3 days after treatment using the nonlinear four-parameter regression function in GraphPad Prism.

Flow cytometric analysis of cell cycle

Flow cytometry was performed according to Cycletest Plus DNA Reagent Kit instructions (BD Biosciences #340242) and as described in Supplementary Materials and Methods. Data from 10,000 cells per treatment group and time point were analyzed using BD FACSDiva software (BD Biosciences).

Western blots

Specific antibodies, reagents, and methods are explained in Supplementary Materials and Methods. Densitometry was

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Transient siRNA transfection
SMARTpool ON-TARGETplus PIK3CA (#L-003018) or Non-Targeting pool (#D-001810) siRNA (Thermo Scientific) were complexed with Lipofectamine RNAiMAX transfection reagent (Invitrogen) in Opti-MEM reduced-serum media (Invitrogen) per manufacturer’s instructions. Specific reagent concentrations, plating densities and times are outlined in Supplementary Materials and Methods.

Real-time quantitative PCR
RNA isolation, cDNA synthesis, and amplification were performed using the kits, primers, probes, and thermal cycle listed in Supplementary Materials and Methods. Relative gene expression was normalized to 18S endogenous control.

Luciferase gene reporters
Reporters, reagents, and conditions are described thoroughly in Supplementary Materials and Methods. Data are normalized to cell density.

Enzyme-linked immunosorbent assay
Cytokines were measured using Quantikine IL8 (#D8000C) and VEGF (#DVE00) Immunoassay Kits from R&D Systems following Supplementary Materials and Methods, and normalized to cell number.

HNSCC xenograft studies
All animal studies were carried out under protocols approved by the Animal Care and Use Committee, and in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2011). Twenty gram, 4- to 6-week-old SCID/NCr-Balb/c mice were obtained from Frederick Cancer Research and Development Center (National Cancer Institute, Frederick, MD, USA) and housed in a pathogen-free facility. UMSCC-1 or -46 cells were injected subcutaneously in flanks and animals were treated as described in Supplementary Materials and Methods.

Immunohistochemical analysis of tumors
Immunohistochemical staining and quantification were performed using a previously described method (13). Specific reagents and antibodies are listed in Supplementary Materials and Methods.

Statistical analysis
In vitro experiments were performed in triplicate, with samples assayed in at least triplicate. Data are presented as the mean ± SD. Significance was determined using the Student t test and P values of <0.05 were considered statistically significant. For tumor growth analysis, significance was determined using the Student t test and P ≤ 0.05. For survival analysis, the Gehan–Breslow–Wilcoxon test was used and significance was set to 0.05 using the Bonferroni method.

Results
Dual PI3K/mTOR inhibitor PF-384 variably attenuates cell growth and survival of HNSCC lines in vitro and displays limited antitumor activity in less sensitive models in vivo
For initial screening for sensitivity and resistance to PI3K/mTOR inhibitor PF-384, we assembled a panel of 14 HNSCC lines (Supplementary Table S1), which includes HPV− lines previously demonstrated to overexpress PIK3CA protein (UMSCC-1, -6, -9, -11A, -11B, -22A, -22B, -38, -46; ref. 13), HPV− line UMSCC-47, and two HPV+ lines, which contain activating H1047R PIK3CA mutations (CAL-33 and Detroit-562) reported to exhibit enhanced sensitivity to PI3K/mTOR inhibitors (14). For ease of comparison, the XTT assay curves for 14 lines treated with concentrations of PF-384 between 1 nmol/L and 5 μmol/L in Fig. 1A are empirically separated into relatively high (0.75–10.2 nmol/L, left), intermediate (11.1–22.2 nmol/L, middle), or lower (48.5–133 nmol/L, right panel) drug IC50s. Overlap is observed between those with overexpression or mutations of PIK3CA. In an independent analysis comparing IC50s that included 11 HPV− and 8 HPV+ lines with PIK3CA amplification, HPV− lines were significantly more resistant to PF-384 when compared with HPV− lines (Supplementary Fig. S1, P = 0.021).

We selected two of the less sensitive HPV− lines UMSCC-1 and -46, with similar overexpression of PIK3CA protein (13), to further characterize the limiting effects of PF-384 on G–S–M cell-cycle phase and sub-G0 cell death using DNA fluorometry (Fig. 1B). Cells were treated at a concentration exceeding the IC50 of both cell lines (200 nmol/L) and harvested at 24, 48, and 72 hours. At the time points studied, UMSCC-1 cells showed augmented G0–G1, G1 accumulation, but a relatively small increase in sub-G0 DNA fragmentation. In UMSCC-46, modest increases in the G0–G1 fraction were noted with treatment at 24 to 48 hours, and a delayed increase in sub-G0 fraction was observed 48 to 72 hours after treatment. We next tested PF-384 as a single agent using a previously defined dosing schedule (17) in UMSCC-1 and 46 xenografts in vivo (Fig. 1C). UMSCC-1 showed a modest, but statistically significant reduction in tumor growth and prolonged survival. UMSCC-46 showed delayed tumor growth and prolonged survival, which did not reach statistical significance. Overall, tumor growth was minimally delayed and resumed growth parallel to controls during PF-384 treatment, and the difference in median survival was less than the 3-week course of treatment, consistent with relative resistance. Thus, in less sensitive HNSCC lines, PF-384 inhibited cell-cycle progression but exhibited limited cytotoxic activity in vitro and antitumor activity in vivo.

Relationship of PF-384 sensitivity and resistance to AKT phosphorylation, and effects on PI3K/Akt/mTOR, MEK/ERK, and IKK/NF-κB pathway signaling in resistant lines
As we previously quantified the constitutive expression of PIK3CA protein and phosphorylation of AKT(T308/S473) sites for PI3K–PKD1 and mTOR activation for a subset of this panel (13), we explored if sensitivity was associated with PIK3CA expression or AKT activation (Fig. 2A–C). Sensitivity (lower IC50) for PF-384 was significantly correlated with higher phosphorylation of AKT(T308/S473) (Fig. 2A and B), but not with PIK3CA overexpression (Fig. 2C), or H1047R mutation alone.
These observations suggested that sensitivity to PF-384 correlates with signal phosphorylation of AKT via PI3K/mTOR, whereas resistance may be associated with other pathway(s) in addition to PIK3CA expression or mutational status. As we had observed that HNSCC often display co-activation of PI3K/AKT/mTOR and MEK/ERK signaling (4, 6, 8), we examined their activation and effects of PF-384 by Western blot in UMSCC-1 (Fig. 2D) and UMSCC-46 cells (Supplementary Fig. S2). PF-384 induced near complete, sustained inhibition of mTORC2 target p-Akt(S473), PI3K/PDK1 target p-Akt(T308), and mTORC1 targets p-S6(S240/244), and p-4E-BP1(S65) through 48 hours, indicating the limited sensitivity was not because of inadequate target inhibition. However, MEK target p-ERK1/2(T202/Y204) activation was only partially inhibited by PF-384 treatment. Because PI3K/AKT/mTOR have also been reported to modulate NF-kB via either inhibitor of IκB kinase (IKK)-dependent p65 phosphorylation or cofactor-dependent transactivation (18–24), we first examined the effects of PF-384 on IKK-dependent phosphorylation of serine 536-p65 RELA subunit of NF-κB, without or with inducer TNFα (20 ng/mL; Supplementary Fig. S3A and S3B). PF-384 partially inhibited TNFα-induced p-p65(S536) at early time points, but not at later time points. We employed PIK3CA siRNA to examine specific effects on downstream molecules. Strong knockdown of PIK3CA mRNA and protein was confirmed by RT-PCR and Western blot (Supplementary Figs. S4A and S4B). Knockdown of PIK3CA decreased pAKT(S473) and pAKT(T308), but had minimal effects on IKK-dependent phospho- or total p65. Together,
these data indicate PF-384 potently inhibits direct PI3K/mTOR targets, but incompletely inhibits co-activated MEK–ERK and IKK-dependent NF-κB phosphorylation in HNSCC.

PF-384 inhibits transcription factor NF-κB and AP-1 reporter gene transactivation

PI3K/AKT/mTOR signaling has also been implicated in nuclear cofactor-mediated transactivation of transcription factors NF-κB and AP-1 (4, 21), which coregulate a variety of genes important in cancer cell proliferation, survival, inflammation, and angiogenesis (25). To explore this, we investigated how PF-384 affected the regulation of transcription factor NF-κB and AP-1 reporter activity, with or without TNFα stimulation in UMSSC-1 and -46 cells (Fig. 3A). The PI3K/mTOR inhibitor significantly suppressed basal and/or TNFα-induced NF-κB and AP-1 at growth inhibitory concentrations between 50 and 200 nmol/L. PIK3CA-specific siRNA knockdown alone partially inhibited NF-κB and AP-1 activities in both cell lines (Fig. 3B). These data indicate PI3K–mTOR inhibitor PF-384 significantly attenuates NF-κB and AP-1-mediated gene transactivation, and this is mediated at least in part by PIK3CA.

PF-384 modulates NF-κB/AP-1 and mTOR-regulated inflammatory and angiogenic cytokine expression

To further examine the effects of PI3K/mTOR inhibition on known NF-κB/AP-1 transcriptional and mTOR translational targets important in angiogenesis and tumorigenesis of HNSCC (4, 8, 26–29), we next assessed the effects of PF-384 (200 nmol/L) on expression of IL8 and VEGF angiogenesis factors as measured by ELISA, with or without TNFα (20 ng/mL) stimulus (Fig. 4A). PF-384 strongly blocked IL8 and VEGF expression. We further delineated effects of PF-384 and contribution of transcription factor activation for the IL8 promoter by performing IL8 luciferase reporter assays with mutated NF-κB, AP-1, and NF-IL6 (CEBPb) binding sites (Fig. 4B). PF384 partially inhibited TNFα-induced IL8 reporter activity in UMSSC1, and NF-κB binding site mutation most strongly reduced basal and TNFα-induced reporter activity in both cell lines. Together, these data support the potential of PI3K–mTOR inhibitor PF-384 to suppress expression of angiogenesis factor IL8 and VEGF, known targets of NF-κB–dependent transcription and/or mTOR-regulated protein translation.

Figure 2.
Relationship of PF-384 sensitivity and resistance to pAKT and effects on PI3K/mTOR and MEK/ERK pathways in HNSCC. PF384 IC50 shows a significant inverse correlation with (A) pAkt (T308) and (B) pAkt(S473), but not for (C) PIK3CA (p110α). Scatter plot of PF384 IC50 and basal protein intensity for pAKT(T308) and pAKT (S473). D. Western blot of the effects of PF-384 on PI3K/AKT/mTOR and MEK–ERK signaling in UMSSC-1 and -46 (Supplementary Fig. S2). Cells were treated with 200 nmol/L PF-384 or 0.01% DMSO diluent in media as a control for 6, 12, 24, and 48 hours, and midway to collection, TNFα (20 ng/ mL) as an inducer or control diluent was added. Western blots and quantification were performed as described in methods.
and angiogenesis factors IL8 and VEGF. These data suggest that PF-384 and PD-901 and their targets both modulate activity of transcription factors and targets implicated in cell growth, survival, angiogenesis, and tumorigenesis.

Effects of PF-384 and PD-901 on tumorigenesis in vivo

We tested the effects of PF-384 and PD-901 on tumor growth and survival in vivo individually and in combination in the UMSCC-1 xenograft model (Fig. 5D). Tumor bearing mice were divided into four groups, vehicle control, 10 mg/kg PF-384 every 4 days, 1.5 mg/kg PD-901 everyday, or the combination for a single 21-day cycle of treatment. Notably, PD-901 and the combination potently suppressed tumor growth in this PF-384-resistant model. Although all three drug-treated arms showed statistically significant delayed tumor growth (P < 0.05) until day 25, the PD-901, and combination groups remained significant until day 35. Compared with control, all three treatment arms also showed a statistically significant survival advantage, with the longest survival in the combination group. Tumor growth in PD-901 only and combination groups eventually caught up with other groups with similar survival by day 55. These observations are consistent with the effects of PD-901 and the combination being tumoostatic, with the implication that added cycles of treatment may be required to evaluate the durability of efficacy in clinical trials. In addition, mice treated with PF-384, but not PD901, showed lack of weight gain as compared with controls (Supplementary Fig. S5), consistent with PI3K–mTOR inhibitory effects on metabolism. No significant toxicities were observed in any treated groups.

To examine the effects of PF-384 and PD-901 treatment on PI3K/Akt/mTOR and MEK/ERK targets, proliferation (Ki-67), apoptosis (TUNEL), and vessel density (CD31), we performed immunostaining and determined histoscores for these markers (30) in UMSCC-1 tumors isolated from additional animals (Fig. 6A–C, Supplementary Fig. S6). PF-384–treated tumor sections showed significantly decreased staining of direct PI3K and mTOR targets p-Akt(S473), p-Akt(T308), and weaker inhibition of p-ERK1/2 and p-S6(240/244). PD-901–treated tumor sections showed reduced staining of p-ERK1/2 and lesser reduction of p-S6. Combination treatment most potently reduced staining of PI3K/mTOR targets and p-S6. Proliferation marker Ki-67 was reduced in all three conditions, with greatest inhibition in the combination group. Apoptosis (TUNEL) staining was significantly increased in all treatment groups, with greatest increase in PD-901 or combination groups. Vessel density (CD31) and proangiogenic factor IL8 was most significantly reduced with PD-901 and combination treatment. (Fig. 6C). Together, these findings demonstrate significant in vivo antitumor activity of PD-901 and its role in modulating MEK and corresponding pharmacodynamic markers in an HNSCC model exhibiting lower sensitivity to PI3K–mTOR inhibitor PF-384.

Discussion

Previously we showed that a subset of HPV− HNSCC models overexpressing PIK3CA protein were sensitive to an earlier generation dual PI3K–mTOR inhibitor, PF-502, in vitro, and in vivo (13). Subsequent studies of HNSCC panels with defined mutant lines suggest HPV− and HPV+ tumors with activating PI3KCA mutations may be more sensitive to other PI3K and PI3K–mTOR agents (14–16). Here we found that a panel that includes PIK3CA...

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overexpressing and H1047R PIK3CA mutant HPV- HNSCC lines, displays an overlapping range of sensitivity and resistance to dual PI3K–mTOR inhibitor PF-384 (Fig. 1), and relatively greater overall resistance when compared with HPV+ lines in vitro (Supplementary Fig. S1). Higher sensitivity (lower IC50s) among HPV- HNSCCs overexpressing PIK3CA protein was correlated with phospho-AKT(T308 and S473), consistent with activation by signaling via PI3K-PDK1 and mTOR, respectively (Fig. 2A and B). As AKT activation has been associated with sensitivity to PI3K or mTOR inhibitors in other types of tumors (31–36), p-AKT may warrant investigation as a selective and pharmacodynamic biomarker for PI3K–mTOR inhibitors in future clinical studies. By contrast, PF-384 sensitivity was not correlated with expression or mutation of PIK3CA alone (Figs. 2C and 1A). The inverse relationship between resistance (higher IC50) and lower p-AKT, suggested that co-activation of other pathway(s) in addition to PI3K–AKT signaling could contribute to differences in sensitivity observed.

In depth, study of two HPV- lines that displayed relative resistance in vitro also showed limited response to PF-384 in vivo, despite similar overexpression of PIK3CA protein (13). Limited response was not because of lack of target activity, as PF-384 potently blocked PI3K–AKT–mTOR signal and downstream functional NF-κB transactivation. However, it incompletely inhibited MEK–ERK signal phosphorylation, which we previously showed to be frequently co-activated in HNSCC (8). Targeting MEK with PD-901 alone potently inhibited ERK signaling and tumor growth, and overcame the resistance when given together with PF-384 in vivo. Inhibition of MEK with PI3K/mTOR further inhibited p-AKT, pS6 as well as p-ERK, cell proliferation, survival, and angiogenesis in vivo (Fig. 6). These observations support a model whereby co-activation and cross-talk between PI3K–mTOR and MEK–ERK contribute to cell proliferation, survival, target transcription factor activity, and expression of angiogenesis factors IL8 and VEGF, angiogenesis, and tumorigenesis (Supplementary Fig. S6).

It is well accepted that the RTK-RAS and -PI3K/Akt/mTOR axes promote cell survival, growth, and metabolism (Supplementary Fig. S7). EGFR, other RTKs, and RAS implicated in HNSCC by TCGA and functional studies can trigger phosphorylation and activation of PI3K, which leads to activation of AKT and eventually mTORC1 through negative inhibition of TSC1/2 and Rheb (10). MEK/ERK co-activation may enhance activation of mTOR and its downstream effectors, ribosomal protein S6 and translation repressor 4E-BP1, to integrate the function of ERK and Akt/mTOR signaling in transcription and translation in tumors (38).
data showing that addition of PD901 potentiated the inhibitory
effects of PF-384 on p-AKT (T308/S473) and p-S6 in tumors
in vivo (Fig. 6A) suggest that the mechanisms of cross-talk between MEK
–ERK and PI3K
–mTOR warrant further investigation in HNSCC.
Recent studies have emphasized that several HNSCC lines with
activating mutations of
PIK3CA
show greater sensitivity to various
PI3K or PI3K
–mTOR inhibitors (14
–16). In this study, we
explored mechanisms whereby HNSCC tumors evade molecular
therapies targeting these pathways in models with wild-type
PIK3CA
and
RAS
(H. Cheng, unpublished data), which represent
the majority of HNSCC tumors (11). The efficacy of combined
MEK and PI3K/mTOR treatment in other studies conducted on
adenocarcinoma of the colon (37, 39) and lung (40
–42) is
consistent with frequent alteration of
RAS
/RAF in those cancers.
Considering much of the literature regarding PI3K and MEK
combination therapy uses
RAS
or
PIK3CA
mutant models, the
mechanisms of monotherapy resistance and efficacy of combined
therapy targeting these pathways need to be better characterized in
HNSCC and cancers lacking
PIK3CA or RAS mutation, as
established in this study and another in colon cancer (43).

Although HPV
+ HNSCC exhibit a lower rate of
PIK3CA mutation, they exhibit frequent amplification of several other RTKs,
PIK3CA, and express higher levels of active AKT and its phos-
phorylated downstream targets (11, 12). Activation of AKT or ERK
has been shown to predict resistance to standard HNSCC thera-
pies such as cetuximab (44, 45) and radiation (46), which can be
augmented with PF-384 treatment (47). Studies in other cancer
types have shown higher basal p-Akt to modulate increased
response to PI3K inhibition (31, 32) or mTOR inhibition (33
–36), regardless of
PIK3CA mutational status, which corroborate
our findings in HNSCC. In addition, the effectiveness of com-
bined MEK and PI3K/mTOR inhibition in colorectal cancer
reduction is not dependent on
KRAS or
PIK3CA mutational status
(43). Thus, resistance to PI3K/mTOR inhibition through sus-
tained MEK/ERK signaling, and resistance to standard radio-
chemotherapies because of elevated p-AKT, can be overcome with
combined PI3K/mTOR and MEK inhibition in HPV
+ HNSCC
lacking
PIK3CA mutation but exhibiting
PIK3CA amplification and p-AKT activation. The function of MEK–ERK signaling in
HNSCC is likely to include tumor-microenvironment dependent
with chemotherapy (26). These factors are under the transcriptional control of both NF-κB and AP-1 transcription factors (4), and serve to collectively regulate angiogenesis and lymphangiogenesis (29). Both the PI3K and ERK pathways contribute to expression of IL8 and VEGF through input from NF-κB and AP-1, respectively (8). Although the in vitro capacity of PF-384 to inhibit PI3K/mTOR signaling and NF-κB transactivation was observed, xenografts were relatively insensitive to the drug. Incomplete inhibition of ERK, AP-1, and IL8 and angiogenesis were associated with treatment resistance, which was overcome by single agent or combination therapy with PD-901. The rationale for this combination is further supported by other recent studies on HNSCC biomarkers of resistance to PI3K inhibitors (15).

The mechanisms involved in PI3K/Akt/mTOR-mediated NF-κB activation, which is a well-described mediator of inflammation and oncogenesis, are complex. In this study, we establish the effects and contribution of PI3K/mTOR inhibition by PF-384 and PIK3CA siRNA on NF-κB reporter gene activation, confirming our earlier observation that PI3K inhibition blocks NF-κB transactivation (4). Further, E545K and H1047R activating mutations of PIK3CA prevalent in HNSCC have been directly implicated in upregulating NF-κB–dependent gene expression in other models (21). Although classical IKK signaling is a well-described mediator of NF-κB nuclear translocation and phosphorylation, we observed limited and transient inhibition of classical IKK-dependent RELA/p-p65(S536) phosphorylation by PF-384 or PIK3CA siRNA, suggesting the potent inhibition of NF-κB transactivation observed occurs as a result of dual PI3K/mTOR pathway restriction (co-activating mechanisms). Further downstream, Akt and mTOR have been reported to coregulate IKK–NF-κB transcriptional machinery (18–24). Akt mediates phosphorylation of nuclear cofactors such as IKKα and CBP/p300, which can bind and coregulate transcriptional activation of NF-κB, AP-1, and p53 transactivation (24, 48, 49). Signal-regulated interaction of CBP/p300 with prosurvival transcription factors such as NF-κB and AP-1 can promote their co-activation, and limit its availability and accessibility to p53. This is supported by the co-reduction of NF-κB and AP-1 transcriptional activation upon treatment with PF-384, and our previous finding that another dual PI3K/mTOR inhibitor, PF-502, could inversely enhance p53 expression and activity in these cell lines (13). In addition, MEK–ERK-induced AP-1 often binds and co-regulates many NF-κB–modulated genes. Thus, it is apparent that NF-κB and AP-1 co-activation can be triggered at any number of points along the RTK/RAS/PI3K/Akt/mTOR signaling route, underscoring the importance and rationale for inclusion of MEK and PI3K–mTOR inhibition in targeting HNSCC harboring co-activation of these signal pathways.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S. Mohan, R. Vander Broek, Z. Chen, C. Van Waes
Development of methodology: R. Vander Broek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Mohan, R. Vander Broek, S. Shah, D.F. Eytan, M.L. Pierce, S.G. Carlson, Z. Chen
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