PI3K/mTOR Inhibitor PF-04691502 Antitumor Activity Is Enhanced with Induction of Wild-Type TP53 in Human Xenograft and Murine Knockout Models of Head and Neck Cancer

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Abstract

Purpose: Phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway activation is often associated with altered expression or mutations of PIK3CA, TP53/p73, PTEN, and TGF-β receptors (TGFBR) in head and neck squamous cell carcinomas (HNSCC). However, little is known about how these alterations affect response to PI3K/mTOR-targeted agents.

Experimental Design: In this preclinical study, PI3K/Akt/mTOR signaling was characterized in nine HNSCC (UM-SCC) cell lines and human oral keratinocytes. We investigated the molecular and anticancer effects of dual PI3K/mTOR inhibitor PF-04691502 (PF-502) in UM-SCC expressing PIK3CA with decreased wild-type TP53, mutant TP53/C0/C0 mutant TGFBR2, and in HNSCC of a conditional Pten/Tgfbr1 double knockout mouse model displaying PI3K/Akt/mTOR activation.

Results: UM-SCC showed increased PIK3CA expression and Akt/mTOR activation, and PF-502 inhibited PI3K/mTORC1/2 targets. In human HNSCC expressing PIK3CA and decreased wtTP53 and p73, PF-502 reciprocally enhanced TP53/p73 expression and growth inhibition, which was partially reversible by p53 inhibitor pifithrin-α. Most UM-SCC with wtTP53 exhibited a lower IC50 than those with mtTP53 status. PF-502 blocked growth in G0–G1 and increased apoptotic sub-G0 DNA. PF-502 suppressed tumorigenesis and showed combinatorial activity with radiation in a wild-type TP53 UM-SCC xenograft model. PF-502 also significantly delayed HNSCC tumorigenesis and prolonged survival of Pten/Tgfbr1-deficient mice. Significant inhibition of p-Akt, p-4EBP1, p-S6, and Ki67, as well as increased p53 and TUNEL were observed in tumor specimens.

Conclusions: PI3K-mTOR inhibition can enhance TP53/p73 expression and significantly inhibit tumor growth alone or when combined with radiation in HNSCC with wild-type TP53. PIK3CA, TP53/p73, PTEN, and TGF-β alterations are potential modifiers of response and merit investigation in future clinical trials with PI3K-mTOR inhibitors.

Introduction

The phosphoinositide 3-kinase, viral-akt homolog, and mammalian target of rapamycin (PI3K/Akt/mTOR) kinases act as key regulators of a cascade of signal and effector molecules that are aberrantly activated in a broad variety of human cancers (1, 2). The PI3K/Akt/mTOR pathway is emerging as important in oncogenesis of head and neck squamous cell carcinomas (HNSCC), which arise from the upper aerodigestive tract (3, 4). We previously showed that aberrant PI3K-mediated Akt phosphorylation may be induced by EGF receptor (EGFR), hepatocyte growth factor receptor (c-MET), and their ligands, which are often overexpressed by HNSCC (5, 6). Genetic alterations in the PIK3CA gene encoding the key catalytic subunit of PI3K were also subsequently found to be prevalent, with amplification or mutations reported in approximately 30% to 55% of HNSCC tumors (7–9). Genetic alterations in the PIK3CA gene encoding the key catalytic subunit of PI3K were also subsequently found to be prevalent, with amplification or mutations reported in approximately 30% to 55% of HNSCC tumors (7–9). Genetic alterations in the PIK3CA gene encoding the key catalytic subunit of PI3K were also subsequently found to be prevalent, with amplification or mutations reported in approximately 30% to 55% of HNSCC tumors (7–9).
PTEN expression is decreased in ~60%, and mutated in ~7% of HNSCC (9–11). We recently detected increased phospho-Akt concurrent with decrease in PTEN and TGFBR1 expression in 8 of 20 (40%) human HNSCC (11). Conditional double knockout of Pten/Tgfbr1 enhanced development of HNSCCs with increased expression and phosphorylation of the EGFR/Akt/mTOR axis in mice (11, 12). Similarly, knockout of Tgfbr1 in combination with activating mutation of Kras also enhanced tumorigenesis of HNSCC with increased EGFR (13). In human HNSCC, we found that TGFBR2 may be inactivated infrequently by mutation, or repressed by mTOR (14). Mutation or inactivation of TP53 and/or inactivation of related family member p73 occurs in more than 80% of HNSCC (15–18). Altogether, widespread activation of PI3K/Akt/mTOR, and downstream mediators has been shown in more than 90% of human HNSCC, implicating it as a key oncogenic pathway overlapping loss of these tumor suppressors in pathogenesis of HNSCC (19–21).

Inhibitors of PI3K, Akt, or mTOR individually have shown antitumor activity in human and murine HNSCC models. We reported that a selective PI3K inhibitor strongly attenuated Akt phosphorylation, cell growth, and angiogenesis factor expression by HNSCC in vitro, indicating that PI3K is important in pathway activation and promotion of the malignant phenotype and as a potential therapeutic target (5, 6). An Akt inhibitor attenuated growth and migration of HNSCC lines in vitro, and tumorigenesis and metastasis in an orthotopic HNSCC model in vivo (22). Targeting mTOR by rapamycin or analogues was found to potentially inhibit tumorigenesis of human HNSCC tumor xenografts (21, 23, 24). Rapamycin also inhibited development of carcinogen-induced squamous cell carcinoma (SCC) of the oral cavity and skin (25,26), and HNSCC that develop in genetically engineered KrasG12D/p53−/−, Pten−/−, or Pten−/−/Tgfbr1−/− mice in vivo (27–29). In a pilot pharmacodynamic clinical study with rapalog temsirolimus for 3 weeks, evidence for tumor reduction was observed in 8 of 14 patients (30). In that study, p-S6 was strongly inhibited, but p-AKT was only weakly inhibited, suggesting that combined targeting of PI3K-mTORC1/2 warrants investigation in HNSCC.

Although the aforementioned studies indicate that PI3K/mTOR activation occurs via multiple mechanisms together with frequent alterations in tumor suppressor TP53 and other genes, little is yet known about how these alterations may be related or affect response to PI3K-mTOR-targeted agents. Interestingly, we observed decreased expression of wild-type (wt)TP53, p73, and its cofactor YAP in HNSCC tumors and cell lines displaying enhanced p-AKT, suggesting a potential inverse link between these alterations (16–18). Furthermore, an anti-inflammatory drug, quinacrine, that restored TP53 expression, growth arrest, and sensitivity to DNA-damaging therapy with cisplatin (16), was later reported to be an inhibitor of PI3K/Akt signaling (31). Quinacrine or an Akt inhibitor was also found to enhance nuclear expression and proapoptotic function of cofactor YAP, important in stabilization and proapoptotic function of p73 (ref. 18; R. Ehsanian, unpublished data). These findings suggest the hypothesis that oncogenic PI3K/AKT/mTOR activation may be linked to repression of TP53/p73 expression and function, and potentially reversible by inhibitors of PI3K/AKT/mTOR signaling in HNSCC. Such agents establishing re-expression of TP53/p73 could potentially enhance response in combination with DNA-damaging radiation or chemotherapeutic modalities, important in therapy of HNSCC.

Based on the evidence that amplification of PIK3CA, upstream growth factor receptors, or tumor suppressor inactivation may promote or complement PI3K/AKT/mTOR pathway activation in HNSCC, we explored the activity of a novel selective competitive kinase inhibitor of both class I PI3Ks and mTORs (C1 and C2), PF-04691502 (PF-502; Pfizer; refs. 32 and 33). The effects of PF-502 on PI3K/mTOR signaling, TP53/p73 expression, growth, apoptosis, and sensitivity to DNA-damaging radiation treatment were examined in human HNSCC cell lines; xenograft models with alterations in PI3KCA, TP53/p73, and TGFBR2; and our newly developed Pten/Tgfbr1 double knockout (2cKO) mouse model.

**Materials and Methods**

**Cell lines**

A panel of 9 HNSCC cell lines from the University of Michigan squamous cell carcinoma (UM-SCC) series was obtained from Dr. T.E. Carey (University of Michigan; ref. 34; Supplementary Table S1). The origin of these UM-SCC cell lines was authenticated by genotyping with 9 markers as listed and described in 2010 (34), and preserved in frozen stocks that were used within 3 months of culture. These...
HNSCC are well characterized for status of TP53 and TGFBR tumor suppressor alterations (Supplementary Tables S2 and S3; refs. 14, 16, and 17). The UM-SCC cells were cultured in minimal essential medium and 10% fetal calf serum. Human oral keratinocytes (HOK) obtained from oral gingival mucosa from Lonza were used as a control cell line.

**Reagents**

PF-04691502 was provided by Pfizer through a Materials Transfer Agreement with the National Institute of Deafness and Other Communication Disorders (NIDCD), and described previously (32, 33). The drug was resuspended to a stock solution of 10 mmol/L in dimethyl sulfoxide (DMSO) for *in vitro* experiments and 2.35 mmol/L in 0.5% methylcellulose (Sigma-Aldrich) to deliver 5 to 10 mg/kg for *in vivo* experiments. TP53 inhibitor Pifithrin (35) was from Sigma-Aldrich.

**Western blot**

Western blots were done as previously described (11, 16, 29), using whole cell lysates and antibodies as described in Supplementary Methods. The quantification of the protein expression detected by Western blots was done by protein densitometry with ImageJ 1.45 k software, and the calculation was done using a formula described in Supplementary Methods.

**MTT/XTT cell-density assay and cell-cycle analysis by DNA flow cytometry**

Cell density was evaluated using MTT or XTT assay as indicated, and cell-cycle effects were characterized by DNA flow cytometry as described previously (16, 18) and in Supplementary Methods.

**HNSCC xenograft and radiation regrowth delay tumor models**

All animal experiments were carried out under protocols approved by the Animal Care and Use Committee of the NIDCD or NCI, and were in compliance with the Guide for the Care and Use of Laboratory Animal Resource (1996) National Research Council. Four- to 6-week-old female BALB/c severe combined immunodeficient (SCID; for xenografts) or nu/nu mice (for radiation experiments) were obtained from Frederick Cancer Research and Development Center (National Cancer Institute) and housed in a specific pathogen-free animal facility. Mice were injected s.c. with 5 to 10 mg/kg of PF-502–treated human HNSCC xenograft and radiation regrowth delay tumor models.

**Tgfbr1/Pten cKO mouse model studies**

The Tgfbr1/Pten cKO mice were generated from crosses between Tgfbr1 cKO mice (K14-CreERT2; Tgfbr1fl/fl) and Ptenfl/fl mice, induced as recently reported (11), and treated as described in Supplementary Methods and Fig. S5.

**Immunohistochemical analyses of tumors**

Immunohistochemical staining (IHC) and quantifications of slides with tumor from control or PF-502–treated mice were done using a previously published method (11), and as described in Supplementary Methods.

**Statistics**

Statistical analyses used were as indicated in figure legends and Supplementary Methods.

**Results**

**Differential expression and molecular effects of dual PI3K/mTOR inhibitor PF-502 on PI3K/Akt/mTOR pathway components and TP53/p73 in HNSCC cell lines**

We examined the expression and phosphorylation of key PI3K/Akt/mTOR pathway proteins in a panel of 9 human HNSCC (UM-SCC) cell lines, which have been previously genotyped and also demonstrate alterations prevalent in HNSCC tumors, including EGFR and PI3K/Akt activation (5, 6), as well as inactivation or mutations of TP53/p73 and TGFBRs (refs. 14, 16–17; Supplementary Tables S1–S3). Expression and/or phosphorylation of several PI3K/Akt/mTOR pathway components were differentially increased in most of the UM-SCC lines when compared with control HOK (Fig. 1A) and normalized to β-actin and UM-SCC1. A marked increase in protein expression of the PI3K p110alpha catalytic subunit (PIK3CA), p-Akt (S473 and T308), p-S6 (S240/244), and p-4EBP1 (T37/46) was observed in two thirds of wtTP53 deficient (UM-SCC1–6) and most mutant (mt)TP53 (UM-SCC1A-46) lines. Thus, the majority of UM-SCC lines showed differentially increased expression and/or phosphorylation of several PI3K/Akt/mTOR pathway components, consistent with previous studies in HNSCC tumors (7, 8, 19).

To compare the molecular effects of the PI3K-mTOR inhibitor PF-502 in UM-SCC with different molecular alterations, UM-SCC lines 1 and 46 were selected for initial study (Fig. 1B). These lines showed increased expression of PIK3CA, p-Akt, and mTOR targets, but either express low levels of wtTP53 (UM-SCC1), or express mtTP53 and mtTGFBR2 tumor suppressor proteins (UM-SCC46), previously observed in HNSCC subsets (14, 16, 18, 36). Based on differing IC50s for UM-SCC1 and 46 of ~2 and 0.6 μmol/L, respectively (Fig. 2A and B), we selected an intermediate concentration of PF-502 of 1.25 μmol/L, which resulted in detectable inhibition of UM-SCC1 and was ~2× the IC50 for UM-SCC46. Treatment of both UM-SCC cell lines with PF-502 strongly inhibited PI3K and TORC2 targets p-Akt (T308/S473), and mTORC1 targets p-4EBP1 (S65/T37/46 residues) and pS6 (S240/244) by 6 hours postadministration, with sustained inhibition of p-S6 or p-4EBP1 (S65) observed up to 24 hours postadministration (Fig. 1B). However, greater than baseline phosphorylation was seen for PI3K/PDK1 target p-Akt (T308) and mTORC2 target p-Akt (S473) in UM-SCC1 by 12 to 24 hours, whereas earlier rebound for mTORC1 target p-4EBP1 (T37/46) was observed in UM-SCC46. PF-502 treatment had no inhibitory effect on total PI3K p110α or Akt expression, consistent with its action as an inhibitor of kinase activity.
As we have previously found evidence for repression of TP53 and p73 expression and tumor suppressor function in HNSCC (16, 17), we examined if PI3K/mTOR inhibition modulated expression of these proteins (Fig. 1B). Interestingly, PF-502 treatment induced an increase in expression of TP53 up to 50-fold in the UM-SCC1 line expressing minimal detectable levels of wtTP53, whereas a lesser fold increase in TP53 was seen in the UM-SCC46 line with already elevated basal levels of mtTP53 between 6 and 24 hours. A moderate increase in p73 was also observed in both UM-SCC1 and 46 after PF-502 treatment. To confirm these findings and control for experimental variability, protein loading, cell density, or cell line variation, we conducted additional experiments (Supplementary Fig. S1). Reproducible inhibition of selected PI3K/mTOR targets and a clearly inducible expression of TP53/p73 proteins were observed in replicate as well as independent experiments with time/density-matched controls for UM-SCC1 and 46 (Supplementary Fig. S1A–S1D). In addition, inhibition of selected PI3K/mTOR targets and induced expression of TP53/p73 proteins were observed in an independent wtTP53 line UM-SCC6 expressing detectable TP53 and p73 (Supplementary Fig. S1E). These findings indicate that dual PI3K/mTOR inhibitor PF-502 had similar inhibitory effects on PI3K/mTOR signaling, while enhancing expression of TP53 and p73 expression in multiple UM-SCC lines with wtTP53 or mtTP53.

**Dual PI3K/mTOR inhibitor PF-502 differentially modulates cell growth and survival of HNSCC in vitro**

UM-SCC1 and 46 cell lines treated with increasing PF-502 concentrations between 25 nmol/L to 10 µmol/L over a 5-day MTT assay exhibited a dose-dependent decrease in cell density (Fig. 2A and B). UM-SCC1 had a higher IC₅₀ value of ~2 µmol/L as compared with an IC₅₀ of ~0.6 µmol/L for UM-SCC46. As we observed PF-502–modulated TP53 and p73 expression in multiple UM-SCC lines with wtTP53 or mtTP53.
When compared using robust least squares regression analysis, the IC₅₀ levels were significantly higher for most mtTP53 relative to wtTP53 cell lines (P = 0.049; Supplementary Methods). As we observed PF-502–increased TP53 in UM-SCC1, we examined if the decrease in cell density was attributable in part to TP53 pretreatment with TP53 inhibitor, pifithrin-α (35), as observed previously with pifithrin and TP53 short hairpin RNA upon quinacrine-induced TP53 activation (16). PF-502 treatment alone decreased cell density of UM-SCC1 (Fig. 2D) and was associated with a change in cellular morphology (rounding, detachment; Supplementary Fig. S3). Although pifithrin alone had no appreciable effect on cell density, significantly higher cell density was observed when pifithrin was combined with PF-502, consistent with partial contribution of TP53 to growth inhibition (Fig. 2D and Supplementary Fig. S3).

To further evaluate the effects of PF-502 on cell cycle and death, DNA cyt fluorometric analysis of UM-SCC1 and 46 cells was compared after treatment with PF-502 at twice their established IC₅₀ values (Fig. 3). UM-SCC1 cells treated with PF-502 (4 μmol/L) exhibited increased G₀–G₁ accumulation over 24 to 48 hours, and sub-G₀ DNA over 24 to 72 hours, consistent with the G₀–G₁ growth arrest and cell death expected with the PI3K/Akt/mTOR signal inhibition, and increased TP53/p73 protein expression observed with PF-502 above. By contrast, treatment of UM-SCC 46 cells with 1.2 μmol/L PF-502 showed a lesser increase in G₀–G₁ and no further increase in the elevated sub-G₀ fraction observed with late growth. Because the differences in DNA fraction could be related to lower concentration used for UM-SCC46, we also compared the effects after treating both at the same concentration of ~IC₅₀ for UM-SCC1 (2 μmol/L). Under these conditions, PI3K/Akt/mTOR...
inhibition still had a differentially greater effect on G0–G1 cell-cycle arrest in UM-SCC1 than 46, and similar enhancement of sub-G0 DNA in both lines required a concentration equal to approximately 2 times the IC50 values (final concentration of 4 and 1.2 μmol/L, respectively). Cells were harvested for cell-cycle analysis at 24, 48, and 72 hours posttreatment. A, UM-SCC1 cells showed increased G0–G1 arrest and sub-G0 DNA fragmentation. B, UM-SCC46 cells showed limited increase in G0–G1 or sub-G0 fraction with PF-502 treatment compared with matched controls.

PF-502 differentially affects tumor growth and survival and delays tumor growth with radiation in a human HNSCC xenograft model with wtTP53

To evaluate the activity and tolerability of PF-502 in HNSCC in vivo, pilot dosage studies were done using the UM-SCC1 (wtTP53) and UM-SCC46 (mtTP53 and mtTGFBR2) as human HNSCC xenograft models. Tumor cells were implanted s.c. into the flank of SCID mice, and treatment was initiated beginning 14 days after implantation when tumors were palpable. PF-502 (5, 7.5, and 10 mg/kg) or control vehicle were given via oral gavage for 21 consecutive days (see schema; Supplementary Fig. S5). After establishing 10 mg/kg of PF-502 as the most effective tolerated dose (data not shown), we conducted a larger trial (n ≥ 12 per group) in UM-SCC1 and UM-SCC46 xenograft mice, and the effects on tumor growth and survival were
compared (Fig. 4A, B). Although UM-SCC1 showed more rapid tumor growth than UM-SCC46 in vivo, PF-502 had a greater inhibitory effect on the tumor growth in the wtTP53 UM-SCC1 xenograft mouse model than in UM-SCC46 with mtTP53 and mtTGFBR2 (Fig. 4A). At the completion of treatment on day 21, the difference in tumor volume versus control was highly statistically significant ($P < 0.0001$) in the UM-SCC1 model and associated with an improvement in median survival of $\sim$10 days, whereas UM-SCC46 xenograft mice did not show improved survival with PF-502 treatment. (C) The effects of control (0.5% methyl cellulose/PBS), PF-502 (10 mg/kg), 15 Gy radiation, and PF-502 plus radiation on the growth of UM-SCC1 xenografts. PF-502 was administered by oral gavage once daily for 5 days, and radiation treatment was delivered on the fourth day, 2 hours after PF-502 gavage (arrows). Error bars, SEM ($n = 5$ mice/group).

In both HNSCC xenograft mouse models, tumor growth resumed after daily PF-502 treatment was withdrawn on day 21. This growth rate was relatively consistent with that seen in vehicle-treated control mice. In the UM-SCC46 xenograft model, this tumor growth increase after treatment cessation led to equalization of tumor volume by about day 32, accounting for the lack of effect on survival. Regarding the drug toxicity, 10 mg/kg of PF-502 was well tolerated, with all mice having normal body conditioning scores of 3. Although weight increase was delayed in UM-SCC1 as compared with UM-SCC46 xenograft mice treated with PF-502, this approximated the difference in tumor volume and mass of $\sim$2.25 g between treated and control mice for days 18 to 21 (Supplementary Fig. 6A and B).

As PF-502–enhanced expression of wtTP53 and p73 in UM-SCC1, we examined if combining PF-502 with DNA-damaging therapy with radiation further delayed tumor regrowth. As shown in Fig. 4C, 5 daily doses of PF-502 or 15 Gy single-dose radiation inhibited tumor growth compared with control. The combination of PF-502 and radiation further delayed tumor growth. The times for tumors to reach 4 times the initially measured tumor volume relative to the control for PF-502 alone, 15 Gy, and PF-502 plus 15 Gy were $23.2$ ($P = 0.20$), $23.6$ ($P = 0.04$), and $29.4$ ($P = 0.02$) days, respectively.

PF-502 delays HNSCC tumor development and growth and improves survival in Pten/Tgfbr1 2cKO mice

Decreased PTEN and TGFBR1 has been detected in $\sim$40% of human HNSCC and implicated in PI3K/Akt/mTOR
activation and tumor development (10–12). As we recently established that increased PI3K-Akt-mTOR activation and HNSCC tumorigenesis with 100% penetrance is a consequence in Pten/Tgfbr1 2cKO mice (11), we examined the potential of PF-502 to inhibit development and growth of HNSCC in this defined transgenic mouse model. After a pilot dosage escalation study, PF-502, 10 mg/kg, for 3 weeks, was found to be well tolerated and without significant weight loss or toxicity for up to 6 months (data not shown). Treatment of larger groups of Pten/Tgfbr1 2cKO mice (n > 18) with PF-502 was then initiated 4 weeks after deletion of Pten and Tgfbr1 via tamoxifen-induced Cre expression and 2 weeks before full development of HNSCC, when ~50% of mice exhibited small visible lesions. 2cKO mice were treated with 10 mg/kg of PF-502 for 21 consecutive days by oral gavage. PF-502–treated 2cKO mice exhibited a significant decrease in development and growth of external head and neck and oral tongue tumors (Fig. 5A–D; P < 0.05), as compared with control mice. Delayed tumor development and growth were seen after treatment cessation, requiring ~20 days after treatment cessation before reaching similar tumor volumes as controls (Fig. 5D). This PF-502–mediated tumor growth delay in 2cKO mice corresponded to an improvement in median survival of >20 days, from a median survival of ~40 days in control mice to >60 days in PF-502–treated mice (Fig. 5E). PF-502 was well tolerated, without significant weight loss in treated versus control mice (Supplementary Fig. 6C).

PF-502 inhibited PI3K/Akt/mTOR and Ki-67 and increased TP53 and TUNEL staining in xenograft and 2cKO models in vivo

Effects of PF-502 treatment for 21 days on PI3K/Akt/mTOR, TP53/p73, and related proliferation (Ki67) and apoptosis (TUNEL) markers were examined by immunostaining in HNSCC tumors from UM-SCC1 and 46 xenografts and Pten/Tgfbr1 2cKO mice (Fig. 6A). Tumor immunostaining was quantified by histoscores (Fig. 6B). PF-502 treatment for 21 days significantly decreased PI3K/Akt/mTOR

Figure 5. PF-502 delays HNSCC development in Tgfbr1/Pten 2cKO mice. Treatment was initiated 4 weeks after 5-day tamoxifen administration to induce Cre expression and approximately 2 weeks before development of HNSCC. Tgfbr1/Pten 2cKO mice were treated with PF-502, 10 mg/kg, or 0.5% methylcellulose vehicle alone by oral gavage for 21 days. A, representative HNSCC in 4 control mice on day 21. B, representative PF-502–treated mice showed visibly reduced tumor burden on day 21. C, PF-502–treated mice had fewer tongue tumors as compared with control mice (30% vs. 55.6%, respectively) on day 21 despite similar initial tongue tumor incidence. Tongue tumors also visibly covered a smaller surface in PF-502 mice (9.8%) as compared with control mice (21.3%) on day 21 (P < 0.0001). D, 2cKO mice treated with PF-502 (n = 19) showed decreased tumor volume versus control vehicle (n = 18) during 21 days of PF-502 treatment. E, PF-502–treated 2cKO mice showed improved median survival of >60 days as compared with 40 days for control mice (median survival advantage of >20 days; n = 19 and 18, respectively).
pathway signaling as measured by decreased staining of pAkt (S473) and downstream p-4EBP1 (T37/46) and p-S6 (S240/244) in tumor specimens from all three models (Fig. 6). Conversely, TP53 was significantly increased in tumors from wtTP53 UM-SCC1 and mtTP53 UM-SCC46 xenografts but did not reach significance in 2cKO mice. p73 staining was slightly increased but also did not reach statistical significance in the three tumor models. There was a statistically significant decrease in proliferation (Ki67) and an increase in apoptosis (TUNEL) marker staining in tumor specimens from all three models, with greater relative increase in TP53 and TUNEL staining observed in UM-SCC1 with wtTP53. TP53 inducible cell-cycle–dependent kinase inhibitor p21Cip1 gene expression was increased in wtTP53 UM-SCC1 but not in mtTP53 UM-SCC46 tumors, providing further evidence for functional activation of TP53 (Supplementary Fig. S7).

Discussion

Here, we show that PI3K/mTOR antagonist PF-04691502 has antitumor activity in PI3KCA-overexpressing/TP53-deficient human and Pten/Tgfbr1-deficient murine head and neck cancer models, which reflect alterations found concurrently with PI3K/Akt/mTOR activation in human HNSCC subsets. Notably, in HNSCC from the subset in which we previously showed decreased wtTP53/p73 expression (16, 17), PF-502 inhibited PI3K/Akt/mTOR signaling and reciprocally enhanced TP53 and p73 expression, supporting a model whereby oncogenic activation of the PI3K/Akt/mTOR pathway can repress TP53 and p73 expression (Supplementary Fig. S8). PF-502 significantly delayed HNSCC tumorigenesis and prolonged survival of mice bearing wtTP53 UM-SCC1 xenografts, and in Pten/Tgfbr1-deficient mice, in which PI3K/Akt activation is a major driver of HNSCC (11, 12). More limited activity was
observed in human UM-SCC46 with PIK3CA overexpression. Akt/mTOR activation, but mutations of both TP53 and TGFBR2 (14, 16). Our findings comparing IC50 in a wider panel of UM-SCC in vitro suggest that TP53 status as well as other factors contribute to the sensitivity of UM-SCC to PF-502. Preliminary findings from the The Cancer Genome Atlas have detected amplifications or putative activating mutations in PIK3CA in ~30% of 279 HNSCC tumors, which overlap with wild-type and mutant TP53 subsets, and other less prevalent candidate gene alterations (36). Together, these observations underscore the importance of evaluating molecular and antitumor effects of PI3K/Akt/mTOR pathway targeted therapy in experimental models and clinical subsets with different genetic and molecular alterations of biologic relevance in HNSCC.

The greater than baseline increase in p-Akt (S473 and T308) and p-S6 (Fig. 1A; Supplementary Fig. S8), as often observed in human tumors (7, 19). Treatment with PF-502 blocked downstream targets of PI3K and mTORC2 (pAkt T308/S473, mTORC1 [p-S6(S240/244)], and p-4EBP1(Thr37/46), consistent with dual PI3K/mTORC1/2 inhibitor activity. However, the duration of blockade was limited for p-Akt (S473 and T308) and some other downstream components in UM-SCC1 and 46 by 12 to 24 hours, as was also observed using lower concentrations of PF-502 in other tumor types in vitro and in vivo (33). Reactivation of PI3K/Akt with mTOR inhibition has been reported to result from loss of feedback inhibitory loop involving p-S6 and IRS-1 (Supplementary Fig. S8; ref. 2). The greater than baseline increase in p-Akt (S473 and T308) by 24 hours for UM-SCC1 (Fig. 1B) could potentially explain the requirement for markedly higher concentrations of PF-502 to inhibit proliferation of UM-SCC1 relative to UM-SCC 46 in vitro (Fig. 2A and B). However, these p-Akt markers were similarly inhibited in all three tumor models in mice receiving 10 mg/kg every 24 hours in vivo, indicating that steady-state concentrations of PF-502 were adequate to inhibit p-Akt and p-S6 in vivo. A phase I clinical trial with daily PF-502 for recurrent cancers has recently been completed, and p-Akt as a candidate marker of PF-502 activity is planned (R. Millham; personal communication; Pfizer). Preliminary analysis of this phase I clinical trial provides evidence for clinical activity, with prolonged stable disease in 12 of 36 (33%) patients (R. Millham; personal communication; Pfizer), similar to tumoristatic effects we observed in UM-SCC1 and 2cKO models. These data also suggest that a subset of tumors are sensitive to clinically achievable concentrations, whereas alterations in others may attenuate the antitumor effects of PI3K/mTOR inhibition.

In this regard, the reciprocal enhancement in expression of TP53/p73 tumor suppressor proteins and the apparent relationship between TP53 status and IC50 for the majority of UM-SCC lines with blockade of the PI3K/Akt/mTOR pathway are important findings of this study. Increases in TP53 and p73 were detected in wtTP53 UM-SCC1 and UM-SCC 6 lines in vitro, and mtTP53 and p73 were also further increased in UM-SCC46. Pifithrin partially attenuated PF-502–induced growth inhibition in wtTP53 line UM-SCC1, consistent with contribution of TP53 to growth inhibition. We further show that UM-SCC1 with inducible wtTP53 exhibits greater G0–G1 growth arrest, and that concentrations ~3 × IC50 for UM-SCC46 with mtTP53 are required to induce a comparable increase in sub-G0 DNA (Fig. 3 and Supplementary Fig. S4). Furthermore, wtTP53 UM-SCC6 and 9 showed relatively higher baseline and PF-502 inducible sub-G0 fraction by 24 hours, whereas PF-502 did not induce increased sub-G0 DNA until 48 hours in UM-SCC38 with mtTP53/wtTGFBR2 (Supplementary Fig. S4, right panels). Together, these data suggest that TP53 status as well as other factors, such as PI3KCA mutations, contribute to the sensitivity of HNSCC to PI3K/mTOR inhibitors (37).

Similar effects of PF-502 associated with genotype were observed in vivo (Fig. 6). We found that UM-SCC1, which is wtTP53 genotype and exhibits greater differential induction of TP53 and growth arrest than mtTP53/TGFBR2 line UM-SCC46, shows a greater reduction in proliferation (Ki67) and induction of apoptosis (TUNEL), consistent with the higher sensitivity to tumor inhibition observed. Furthermore, PF-502 induced TP53 target and growth arrest gene p21 in UM-SCC1 tumors, which was not observed in mtTP53 line UM-SCC46 (Supplementary Fig. S7). These data support that wtTP53 genotype and function contribute to the difference observed. The capability of PI3K/mTOR inhibition to enhance TP53 expression and effects when combined with radiation has broader implications for combination with radiation or chemotherapies that enhance the TP53 DNA-damage response.

We previously showed that wtTP53 mRNA and protein expression is repressed in UM-SCC1 and a subset of HNSCC, and that the repression of TP53 mRNA, protein, and TP53-dependent growth arrest and cytotoxicity was reversible by treatment with quinacrine (16), but its target of action was unclear. Subsequent studies suggested that quinacrine enhances TP53 reexpression via an Akt-dependent mechanism (32). We also reported that Akt mediates phosphorylation and cytoplasmatic inactivation of p73 cofactor YAP in the same subset, which is important in p73 stabilization and transcription (18). Recently, mTOR inhibitor rapamycin was implicated in modulating p73 genes involved in mesenchymal differentiation and tumorigenesis in rhabdomyosarcoma (38). Our current results showing that a dual PI3K/mTOR inhibitor increases TP53, and to a lesser extent, p73 expression, further support the hypothesis that this pathway contributes to inactivation of TP53/p73 in this HNSCC subset.

Our 2cKO transgenic mouse model enabled evaluation of the effect of PF-502 on HNSCC with defined genetic alterations in PTEN and TGF-β signaling, which we have shown enhance PI3K/Akt activation (11). Treatment of the novel Tgfbr1/Phen 2cKO HNSCC mouse model with PF-502 inhibited p-Akt and p-S6 and significantly inhibited tumor development and prolonged survival. These effects were also
associated with an increase in TP53, marked decrease in proliferation marker Ki67, and increase in TUNEL apoptosis marker immunostaining in PF-502–treated 2cKO tumors. Clinical studies will be needed to determine how individual and combined TP53 and TGF-β pathway alterations affect sensitivity to PI3K/mTOR inhibitors. In addition, inclusion of HPV+ HNSCC, which also shows concurrent PIK3CA activation and TP53 inactivation (36, 37), and sensitivity to mTOR inhibitor rapamycin in preclinical models (39), is warranted.

Other inhibitors of the PI3K/Akt/mTOR pathway have shown varying levels of activity in HNSCC and lung SCC models associated with status of PI3K, PTEN, TP53, or p73/YAP1. In a recent study, lung SCC lines harboring receptor tyrosine kinase activation, PI3K mutation, or amplification, and PTEN loss were reported to be most sensitive to a PI3K inhibitor, GDC-0941. Furthermore, activity of this inhibitor was enhanced by combination with chemotherapy (40). PI3K inhibitor BMK120 and PI3K/mTOR inhibitor BEZ235 showed activity in lung SCC with wtTP53 (41). In contrast, BMK120 and BEZ235 showed more limited cytotoxicity alone in HNSCC line SCC25 (42), which we note was previously reported to lack TP53 resulting from deletion of 2 base pairs in codon 209 (43). BMK120 also lacked antiproliferative effect in cell lines without high PI3K p85-subunit expression (44). The dual PI3K/mTOR inhibitor, BEZ235, was recently reported to show preclinical activity in an HNSCC xenograft model with reduction in tumor volume (44). An Akt inhibitor was shown to reduce metastasis in an orthotopic model of wtTP53 HNSCC line SCC1 (22). We previously showed that an Akt inhibitor can enhance YAP in wtTP53 lines, an important cofactor for p73-mediated tumor suppression (18). However, the suppression of tumorigenesis but lack of complete regression observed with PI3K/Akt/mTOR inhibition by monotherapy with PF-502 or these agents suggests that combination with other therapies merits investigation.

As PF-502 induced TP53 and/or p73, and showed evidence of increased activity with radiation, combination of PI3K/mTOR inhibition with DNA-damaging therapies such as radiation or cisplatin active in HNSCC holds promise. Reduction of radiation toxicity by mTOR inhibitor rapamycin has been reported (45). Combination of PI3K/Akt/mTOR inhibition with other targeted agents could also potentially lead to improved antitumor effects with lower doses of each specific inhibitor, thereby limiting toxicity while improving efficacy (2). As we showed that MEK–ERK signaling is often coactivated with PI3K in HNSCC (5, 6), combined inhibition of these pathways could help improve on the tumoristatic effects observed with PI3K/mTOR inhibition alone.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Knockout Models of Head and Neck Cancer with Induction of Wild-Type TP53 in Human Xenograft and Murine PI3K/mTOR Inhibitor PF-04691502 Antitumor Activity Is Enhanced

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