Radiation Enhancement of Head and Neck Squamous Cell Carcinoma by the Dual PI3K/mTOR Inhibitor PF-05212384

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Translational Relevance:

Combined modality treatment of locoregional head and neck squamous cell carcinoma (HNSCC) has improved outcomes over the last two decades, but 5 year survival rates remain poor with significant morbidity. Thus, there is an urgent necessity to improve treatment outcomes without added toxicity. This study reports the evaluation of PF-05212384, a novel dual PI3K/mTOR inhibitor currently in phase I-II clinical trials, as a HNSCC radiation sensitizer. Selectively targeting the PI3K/mTOR axis significantly radiosensitized proliferative tumor cell lines, plateau phase tumor cells, and tumor xenografts, with only minimal normal fibroblast radiosensitization. These data suggest the PI3K/mTOR axis is a potential selective radiation target for malignant head and neck squamous cells and warrants assessment of PF-05212384 in clinical trials as a radiation modifier.
Abstract:

Purpose: Radiation remains a mainstay for the treatment of non-metastatic head and neck squamous cell carcinoma (HNSCC), a malignancy characterized by a high rate of PI3K/mTOR signaling axis activation. We investigated the ATP-competitive dual PI3K/mTOR inhibitor, PF-05212384, as a radiosensitizer in pre-clinical HNSCC models.

Experimental Design: Extent of radiation enhancement of two HNSCC cell lines (UMSCC1-wtP53, UMSCC46-mtP53) and normal human fibroblast (1522) was assessed by in vitro clonogenic assay with appropriate target inhibition verified by immunoblotting. Radiation induced DNA damage repair was evaluated by γH2AX western blots with mechanism of DNA-DSB repair abrogation investigated by cell cycle analysis, immunoblotting, and RT-PCR. PF-05212384 efficacy in vivo was assessed by UMSCC1 xenograft tumor regrowth delay, xenograft lysate immunoblotting, and tissue section immunohistochemistry.

Results: PF-05212384 effectively inhibited PI3K and mTOR resulting in significant radiosensitization of exponentially growing and plateau-phase cells with 24 hr treatment following irradiation, and variable radiation enhancement with 24 hr treatment prior to irradiation. Tumor cells radiosensitized to a greater extent than normal human fibroblasts. Post-irradiation PF-05212384 treatment delays γ-H2AX foci resolution. PF-05212384 24 hr exposure resulted in an evident G1/S phase block in p53 competent cells. Fractionated radiation plus IV PF-05212384 synergistically delayed nude-mice bearing UMSCC1 xenograft regrowth, with potential drug efficacy biomarkers identified, including pS6, pAkt, p4EBP1, and Ki67.

Conclusions: Taken together, our results of significant radiosensitization both in vitro and in vivo validates the PI3K/mTOR axis as a radiation modification target and PF-05212384 as a potential clinical radiation modifier of non-metastatic HNSCC.
Introduction:

Despite significant advances in the treatment of non-metastatic head and neck squamous cell carcinoma (HNSCC) the 5-year disease-specific survival rate remained poor at ~66% from 2002 to 2006 (1, 2). Although improved from ~55% a decade earlier, increased survival arose partially secondary to an epidemiological shift (↑HPV rate); thus, the necessity to improve HPV negative HNSCC treatment remains critical as ever. In an attempt to improve outcomes, research efforts have subsequently shifted to focus on rational based treatments via molecularly targeting oncogenic signaling pathways. In two landmark phase 3 clinical trials, the molecularly targeted EGFR inhibitor cetuximab, a chimeric monoclonal antibody, in combination with radiation or platinum-fluorouracil for the treatment of HNSCC demonstrated an absolute survival benefit of 10% and 20% respectively (3, 4). Although improving overall survival, given the near ubiquitous overexpression (≥90%) of EGFR in HNSCC the demonstrated benefit of cetuximab was lower than anticipated (5). The two aforementioned trials did, however, demonstrate the validity of molecularly targeted therapy for the treatment of HNSCC. Excluding early disease, radiation alone or in combination with other modalities plays a central role in the treatment of all anatomical sites of HNSCC (6). The pivotal role radiation plays in the treatment of HNSCC warrants pre-clinical investigation into identifying better novel molecularly targeted radiation modifiers.

The Cancer Genome Atlas (TCGA) and other studies have rapidly identified numerous cancer cell dysregulated signaling pathways (7-9). Among the identified HNSCC aberrations, the most prevalent of genetic and epigenetic alterations lie along the PI3K/Akt/mTOR pathway. The PI3K/Akt/mTOR axis is a central signaling pathway that plays a crucial role in metabolism, cell growth, apoptosis, survival, and differentiation (10). Genetic aberrations along this key axis can lead to persistent activation and consequently a malignant phenotype. Along with driving malignancy, the PI3K/Akt/mTOR signaling cascade has been implicated in the radiation response and radioresistance (11-14).
Molecularly targeted agents inhibiting PI3K and mTOR have been tested in pre-clinical models, and as expected demonstrated significant radiation enhancement (15-17). An orally available dual PI3K/mTOR inhibitor, PF-04691502, in preliminary studies has demonstrated HNSCC radiosensitization with single fraction radiation in vivo (17). Additionally, a dual PI3K/mTOR inhibitor, NVP-BEZ-235, has displayed radiosensitization in numerous pre-clinical studies (18-20). However, Burma et al. have eloquently demonstrated that the radiosensitizing effect propagated by NVP-BEZ-235 is in part secondary to catalytic inhibition of the ATM and DNA-PKc enzymes (21, 22). The ATM and DNA-PKc protein kinases are critical components of the DNA-DSB repair response and belong to the PI3K-like kinase (PIKK) family, along with PI3K, mTORC1, and mTORC2 (23, 24). DNA repair enzyme inhibition radiosensitization may be a viable approach in select anatomic sites, but normal tissue radiosensitization will likely limit this approach clinically for the vast majority of malignancies (25). Unlike quiescent normal tissue sites, a majority of the aerodigestive tract irradiated during the course of HNSCC treatment comprises rapidly dividing mucosal epithelium, adding credence to identifying selective radiation modifiers. The necessity to selectively target HNSCC versus normal tissue is reinforced by RTOG 0522, a randomized phase 3 clinical trial of radiation and cisplatin with or without cetuximab for locally advanced HNSCC. Disappointingly, survival was not improved when adding cetuximab, which may have been a consequence of radiotherapy interruptions (~27%) secondary to added toxicity (26).

We hypothesize that given PI3K/Akt/mTOR pathway’s oncogenic role in HNSCC, along with early PF-04691502 single radiation fraction data, that dual PI3K/mTOR inhibition by PF-05212384, an intravenous delivered compound currently in phase I-II clinical trials, would result in significant HNSCC radiosensitization, and to a greater extent than normal tissue (17, 27). To test this hypothesis, we performed clonogenic survival studies in HNSCC cell lines, including non-dividing cells (G1 enriched) and normal tissue. To identify the most appropriate clinical regimen, separate in vitro drug regimens prior to and after irradiation were studied. With significant radiation enhancement identified, we went on to validate appropriate target inhibition by in vitro western blotting, as well as identify potential radiation
enhancement mechanism by cell cycle analyses, DNA-DSB repair assay, and senescence pathway
directed RT-PCR genomic expression analysis. Additionally, the ability of PF-05212384 to
radiosensitize in vivo in combination with fractionated radiation was tested in a human head and neck
squamous cell carcinoma xenograft model. Associated xenograft harvests were additionally utilized to
assess in vivo target inhibition as well as potentially identify clinical biomarkers.

Materials and Methods:

Cell Culture and Drug Solutions

UMSCC1 wild type p53 (HNSCC) and UMSCC46 mutant type p53 (HNSCC) were kindly provided by
Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI) and were grown in MEM supplemented
with 10% Fetal Bovine Serum (FBS) and streptomycin/penicillin (17). FaDu (mutant type p53) was
purchased from American Type Culture Collection (ATCC) and maintained in DMEM supplemented
with 10% FBS and streptomycin/penicillin (28). Normal human lung fibroblasts (1522) were purchased
from the Coriell Institute for Medical Research and were grown in Nutrient Mixture F12 supplemented
with 20% FBS and streptomycin/penicillin. Cell culture maintenance and all in vitro work were
performed at atmospheric oxygen levels (20% O2; 5%CO2). All cell lines were authenticated within the
past six months by IDEXX Bioresearch, Columbia, MO using Cell Check 9 (9 allele marker STR (short
tandem repeat) profile and inter-species contamination test). For in vitro studies, PF-05212384 (Pfizer
and Selleck Chemicals) was dissolved in dimethyl sulfoxide and stored in aliquots of 10mM
concentration at -70°C. For xenograft tumor growth delay, PF-05212384 was dissolved in 5%
dextrose/.25% lactic acid at 1 mg/mL and raised to a pH of 3.3 with 1M NaOH.

Cell Survival Studies

Cells were plated (5x10^5) in 100 mm dishes and incubated overnight at 37°C. Exponentially growing cells
were subsequently exposed to 10µM PF-05212384 for 24 hr then irradiated (PF-05212384 removed
immediately following IR) or irradiated then immediately exposed to 10 µM PF-05212384 for 24 hr in
For separate experiments. For plateau phase clonogenic survival, UMSCC1 cells were grown to confluence with G1 phase enrichment verification by cell cycle analysis; confluent cells were then subsequently treated as described above. Following drug exposure and irradiation, cells were rinsed, trypsinized, counted and plated in triplicate for macroscopic colony formation and allowed to grow for 10-14 days at 37°C. Colonies were fixed and stained with methanol/crystal violet, and counted. After correcting for plating efficiency and PF-05212384 toxicity, survival data were plotted and fitted with the linear quadratic model according to Albright (29). The dose modification factor (DMF) for each clonogenic survival curve was calculated as the control (DMSO) radiation dose for 10% survival divided by radiation dose for 10% survival with PF-05212384 treatment (30). Dose modification factors are subsequently expressed as the mean and standard error of measurement (SEM) of multiple experiments.

**Immunoblotting**

For 24 hr PF-05212384 exposure then irradiation, exponentially growing cells were washed twice with 37°C fresh medium immediately following irradiation and collected as a function of time following irradiation. Total protein from cultured cells, excluding γH2AX, and xenograft tumor protein extraction was performed as previously described (30). Acid soluble histone proteins were extracted in 0.2mol/L sulfuric acid as previously described (31). Protein concentrations were determined with a DC-Protein Assay (Bio-Rad); samples were then aliquoted and stored at -70°C. Protein samples of equal amount (5-40 µg) were subjected to SDS PAGE on 4-20% Novex Tris-Glycine gels or NuPAGE 3-8% Tris-Acetate gels (Invitrogen). Proteins were then transferred to a nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). Nitrocellulose membranes were then incubated with primary antibody according to manufacturer recommended dilutions overnight with gentle agitation at 4°C followed by incubation with the appropriate secondary antibody (1:2,000) for 1 hr at room temperature. Protein bands were visualized by chemilluminescence (Thermo Scientific). To ascertain equal protein loading and transfer, antibody was stripped by ReBot Plus mild antibody stripping solution (Millipore) and membranes were probed with the appropriate loading control. Protein band image capture and quantification was performed with a
Fluor Chem HD2 imager (Alpha Innotech) coupled with image analyzer software. Density values for each band were normalized to the appropriate loading control and expressed as fold change compared to control condition. The following primary antibodies were used: pS6 (Cell Signaling #5364), S6 (Cell Signaling #2217), p4EBP1 (Cell Signaling #2855), 4EBP1 (Cell Signaling #9644), pAkt (Cell Signaling #9271), Akt (Cell Signaling #9272), pH2AX (Upstate #05636), H2A (Millipore #07146), pATM (Cell Signaling #5883), ATM (Cell Signaling #2873), pDNA-PKc (Cell Signaling #4215), DNA-PKc (Cell Signaling #4602), p21 (BD Pharmingen #554228), p27 (Epitomics #27471), p53 (Abcam ab32049), Actin (Millipore MAB1501R), Cyt C (Santa Cruz G2909), HSC70 (Santa Cruz J2212).

**Xenograft Studies**

All animal experiments were carried out in accordance with protocols approved by the National Cancer Institute’s Animal Care and Use Committee (ACUC). For radiation regrowth delay studies, UMSCC1 cells (1.0x10^6) were injected into the subcutaneous space of the right hind leg of athymic nude mice, 5-6 weeks of age, bred at the National Cancer Institute’s Animal Production Area (Frederick, MD). Individual mice were ear tagged and randomized on day 6 of tumor growth into the following 4 groups (8 mice/group): vehicle control, vehicle control + fractionated radiation, PF-05212384 control, and PF-05212384 + fractionated radiation. Fractionated radiation (3 Gy) was delivered daily for two full work weeks starting on Monday with a hiatus of two days following five fractions, with a total dose of 30Gy delivered. PF-05212384 (6.7mg/kg) was delivered via tail vein injection (<200 µL) 2 hr prior to Monday, Wednesday, and Friday’s fractionated radiation with an additive total of 40 mg/kg. Two to three additional mice per group were treated in parallel for tumor harvest. UMSCC1 tumor xenografts were harvested 2 hr following the third PF-05212384 injection and fifth 3 Gy fractionated radiation dose. Tumor samples were immediately divided with one piece flash frozen in liquid nitrogen for immunoblotting and the second embedded with OCT over dry ice followed by storage at 70°C. Individual mice tumor volume growth data were fitted with an exponential growth curve and days to
thrice starting tumor volume were determined. For control and treated groups, growth delay is expressed as mean time (days) to thrice starting tumor volume.

**Cell Cycle Analysis, RT-PCR, Immunohistochemistry, and Irradiation**

Cell Cycle Analysis, RT-PCR genomic expression analysis, immunohistochemistry, and irradiation technique followed standard experimental methodology (32, 33). A detailed breakdown can be found in the Supplementary Materials and Methods section.

**Statistical Analyses**

Statistically significant differences for immunofluorescence densitometry, immunohistochemistry total fluorescence and xenograft group growth differences were assessed using the Student’s t test in Microsoft Excel.

**Results**

**PF-05212384 sensitzes head and neck squamous cell carcinoma to radiation**

To determine the ability of PF-05212384 to radiosensitize HNSCC, pilot studies were performed indicating radiation enhancement both with pre-irradiation PF-05212384 (10µM) treatment and post-irradiation PF-05212384 (10µM) treatment protocols. Radiation enhancement was optimized with 24 hr drug exposure regardless of pre or post irradiation treatment protocols (Supplementary Figure S1A). Minimal toxicity was observed for the majority of cell lines treated with PF-05212384 alone (Table 1). Treatment with PF-05212384 24 hr prior to irradiation led to enhancement in radiosensitivity in UMSCC1 cells, with a DMF (±SEM) of 1.9±.07. UMSCC46 cancer cells and normal human fibroblasts (1522) displayed no pre-treatment radiation enhancement (Figure 1A-C). Post-IR PF-05212384 treatment radiation enhancement was observed in all cell lines studied, with PF-05212384 enhancing radiation to a greater extent in HNSCC cells than normal human fibroblasts (DMFs, UMSCC1 – 3.6 and UMSCC46 – 1.9 vs. 1522 – 1.4, Figure 1D-F). A third HNSCC line (FaDu), was exquisitely sensitive to PF-05212384
treatment alone, and displayed both pre and post IR PF-05212384 treatment radiation enhancement (Supplementary Figure S1D). All data described to this point applies to exponentially growing cells. Radiation enhancement, although reduced, was observed with both 24 hr pre-IR and 24 hr post-IR PF-05212384 treatment (DMFs of 1.6 and 2.2 respectively) of plateau phase UMSCC1 cells (Table 1, Supplementary Figure S1B-C). UMSCC1 confluence (plateau phase) was verified with G1 phase enrichment of ~70% in confluent cells vs. ~40% in exponentially growing cells (Table 1). Reduced radiation enhancement in plateau phase cells indicates some dependence on active cell cycling.

**PF-05212384 in the setting of radiation prolongs PI3K/mTOR signaling cascade knockdown**

To identify possible variable drug response contributing to differential radiation enhancement, we next investigated the downstream targets of the PI3K/mTOR cascade (phosphorylated-S6, phosphorylated-4EBP1, and phosphorylated-Akt) with both pre-IR PF-05212384 treatment and post-IR PF-05212384 treatment. Reduced inhibition of 4EBP1 phosphorylation (not pS6 alone) secondary to mTORC1 inhibitor resistance has previously been shown to correlate with limited radiation enhancement and drug response (34, 35). UMSCC1 and UMSCC46 cells were treated with PF-05212384 (10µM) for 24 hr then irradiation with immunoblots demonstrating a dramatic reduction in total p4EBP1 (with or without IR) >24 hr following irradiation; the same effect was observed with the mTORC1 target S6 (Figure 2A-B). PF-05212384 treatment following irradiation resulted in a reduction in mTORC1 target protein phosphorylation (with or without IR) with only 2 hr of treatment (Supplementary Figure S3A-B).

Additionally, treatment with PF-05212384 (10 µM) dramatically reduced mTORC2 phosphorylation of Akt (Figure 3C). Given the central role mTOR plays in integrating metabolic and growth signaling and its intricate interplay with the tumor suppressor p53 we assessed for cell cycle perturbation secondary to PI3K/mTOR inhibition (24 hr PF-05212384 exposure) with or without radiation (36). PF-05212384 exposure (24 hr) alone induced significant cell cycle G1 arrest, in FaDu (72% vs. 49% control), UMSCC1 (88% vs. 39% control) and 1522 (81% vs. 65% control). The increase in G1 was mirrored by a reduction in S-phase in these 3 cell lines. As has been seen with another dual PI3K/mTOR inhibitor, the
UMSCC46 cells had a much reduced G1 phase increase after PF-05212384 incubation (45% vs 32% control) and little reduction in S-phase (Table 1, Supplementary Figure S2A) (17). PF-05212384 treatment following irradiation (8 Gy) did not abrogate radiation induced G2/M arrest (data not shown), which corresponds well to Cyclin B1 down regulation both with irradiation and drug treatment (Supplementary Table S1). Similarly to the oral dual PI3K/mTOR inhibitor PF-04691502, dual PI3K/mTOR inhibition by PF-05212384 induced p53, p21, and p27 expression in UMSCC1 cells (Supplementary Figure S2) (17). PF-0521384 incubation did not significantly change p53, p21, or p27 in the p53 mutated UMSCC46 cells (Supplementary Figure S2) and this corresponded to the lack of G1 inhibition in the USMCC46 cells (Table 1).

**PF-05212384 significantly inhibits radiation-induced DNA-DSB repair with post-IR treatment**

We investigated the influence PF-05212384 has on radiation induced DNA damage by assessment of phosphorylated γH2AX induction and resolution. Immunoblots demonstrating PF-05212384 impact on phosphorylated γH2AX kinetics, in UMSCC1, are show in Figure 3A-C for both pre-IR and post-IR treatment protocols. As expected, relative phosphorylated γH2AX rapidly increased (0.5 hr) following cell irradiation, but with resolution of radiation induced DNA double strand breaks 24 hr following radiation in non-treatment controls. With post IR 24 hr treatment, PF-05212384 resulted in a persistent elevation of phosphorylated γH2AX significantly (P<.05) different from 24 hr radiation control (Figure 3A-B). Additionally, PF-05212384 24 hr exposure alone resulted in a significant (P<.05) reduction in total phosphorylated γH2AX versus control (Figure 3A-B). With post-IR treatment in UMSCC46, multiple experiments indicated phosphorylated γH2AX was significantly (P<.05) elevated in PF-05212384 + radiation (4 Gy) versus radiation (4 Gy) control at 6 hr post-IR (Supplementary Figure S4). Twenty four hour PF-05212384 treatment followed by irradiation, although appearing to impede DNA-DSB repair, did not result in a statistically significant difference in phosphorylated γH2AX resolution versus radiation control at 24 hours post-IR across multiple independent experiments (Figure 3C). As is suggested by PF-05212384 post-IR treatment γH2AX data, radiation modulation is at least in part...
secondary to mitigation of key components of the DNA-DSB repair pathway. ATM and DNA-PKc protein kinases belong to the PI3K-like kinase (PIKK) family, along with PI3K, mTORC1, and mTORC2 (21-23). Given the considerable homology between the catalytic sites of the previously listed protein kinases we performed immunoblots investigating ATM and DNA-PKc activation following IR with and without PF-05212384 treatment. Treatment with PF-05212384 following IR did not impact resulting DNA-PKc or ATM protein kinase activation (Supplementary Fig. S5A). Treatment with PF384 for 24 hr then IR followed by cellular incubation with drug free medium for 2 hr (sample then extracted) demonstrated a slight reduction in total phosphorylated DNA-PKc, but not phosphorylated ATM, with 7.5 Gy + PF-05212384 versus 7.5 Gy alone (Supplementary Fig. S5B). However, this data when normalized to total DNA-PKc for the given sample time point demonstrates minimal to no DNA-PKc catalytic site inhibition. Our assessment of total phosphorylated ATM and DNA-PKc with PF-05212384 treatment (± IR) demonstrates DNA-DSB repair inhibition although mitigated is likely not secondary to direct ATM or DNA-PKc catalytic site inhibition.

**IR plus PF-05212384 in vitro does not accelerate cellular senescence**

The interplay between the PI3K/mTOR signaling cascade and the two central regulators of cellular senescence, p53 and Rb, is intricate and yet to be fully understood (37, 38). Given the significant association we performed senescence pathway directed RT-PCR genomic expression analysis investigating accelerated senescence on the order of days as a potential mechanism of PF-05212384 reduced clonogenicity and impeded repair of radiation induced DNA-DSB following IR. Another valuable in vitro cellular senescence analysis, β-galactosidase staining, was considered, but provided no information as β-galactosidase staining of the HNSCC cell lines under investigation lead to cellular morphology changes, cell culture detachment, and cell death. Azad et al. demonstrated accelerated senescence of irradiated human cancer cells with NVP-BEZ-235 treatment, thought to be secondary to selective DNA-PKc inhibition (39). siRNA DNA-PKc knockdown provided distinct evidence of the role DNA-PKc inhibition plays in irradiated tumor cell accelerated senescence, but without studies
investigating siRNA and NVP-BEZ-235 co-treatment, dual PI3K/mTOR inhibition cannot be ruled out as a contributing factor. For our analysis, exponentially growing UMSSC1 cells were treated with 24 hour PF-05212384 (10 µM) or DMSO (0.1%) followed by irradiation (10 Gy) or mock irradiation. mRNA was collected 24 hours following irradiation for analysis with a cellular senescence microarray kit.

Additionally, one set of UMSSC1 cells were irradiated and treated with PF-05212384 as stated above, but 24 hours following irradiation cells were washed with 37°C medium and left to recover for 48 hr; mRNA was then extracted for senescence gene analysis. All 84 genes were compared to control untreated UMSSC1 cells with 77 giving measurable signal for unsupervised clustering analysis. The heat map for the drug and IR exposure is shown in Supplementary Figure S6. First, immediately after IR exposure only 3 transcripts were up-regulated (>2 fold ratio) and 4 were down-regulated (<0.5 ratio) compared to control untreated cells. In contrast, the PF-05212384 24 hr treated cells had 11 up-regulated and 44 down-regulated genes. The heat map clearly shows that PF-05212384 treatment dominated the gene response and the addition of IR did little to alter this response. Forty eight hours after PF-05212384 was washed away many of the 77 genes were beginning to return to control levels (ratio = 1) with 2 major cell cycle/damage response genes p53 and CDKN1C going from a ratio of 15.6 to 1.2 and 9.0 to 1.4 respectively (Supplementary Table S1). Secondly, two genes measured qualitatively changed in the same manner as the proteins measured (AKT1, p21). Finally, genes involved with DNA damage response were greatly reduced by PF-05212384 exposure and several were not recovered after wash out followed by additional 48 hr incubation. An interesting finding of the RT-PCR analysis is the significant up regulation of Fibronectin 1 (>100 fold-change; Supplementary Table S1). Experimental data indicates cellular adhesion to fibronectin activates PI3K (40). Additionally, Fibronectin 1 has been implicated as a biomarker of HNSCC radiosensitization (41). The increased genomic expression of Fibronectin 1 then may simply represent an attempt to overcome PI3K inhibition. Taken together, it re-emphasizes PI3K as a potential radiation modulation target in HNSCC.
PF-05212384 and fractionated IR combination delay UMSCC1 xenograft growth

Based on significant pre and post IR PF-05212384 treatment radiosensitization in vitro we next evaluated PF-05212384 with fractionated radiation (3 Gy) in vivo via nude mice bearing right flank UMSCC1 xenografts. A preliminary in vivo experiment demonstrated that fewer than three PF-05212384 (20mg/kg/week) injections per week resulted in no radiosensitization. Mice were irradiated (3Gy fraction) daily Monday-Friday, with a weekend hiatus, for two weeks. During the two week fractionated radiation course PF-05212384 (6.7 mg/kg) was delivered via tail vein injection Monday, Wednesday, and Friday two hours prior to irradiation. In the absence of radiation, PF-05212384 had minimal effect on UMSCC1 xenograft growth with time to thrice initial tumor volume of 3.1 (±.9) days (P=.04) versus control (Figure 4A). Days to thrice initial tumor volume relative to control for fractionated radiation and fractionated radiation + PF-05212384 was 7.1 (±1.4; p<.005) and 13.0 (±1.6; p<5.0×10^-6) days respectively. Fractionated radiation + PF-05212384 UMSCC1 xenograft regrowth was significantly prolonged (5.9 days) relative to fractionated radiation alone (p<.05). Combined treatment (fractionated radiation + PF-05212384) growth delay of 13.0 days demonstrates a synergistic effect as the sum of the individual treatments equates to 10.2 days.

Moderately reduced PI3K/mTOR inhibition with PF-05212384 and fractionated IR in UMSCC1 tumor xenografts

To assess the molecular inhibition of PI3K and mTOR in HNSCC tumor xenografts, we analyzed the downstream targets Akt, S6, and 4EBP1 on day 5 of treatment with immunoblotting, as well as immunohistochemistry of the mTORC1 phosphorylation target S6. The mTORC1 downstream targets 4EBP1 and S6 demonstrated moderately reduced phosphorylation, with no difference in mTORC1 inhibition between xenografts treated with drug alone and xenografts treated with drug plus fractionated radiation (Figure 4B). Inhibition of the PI3K/mTORC2 was verified by reduced phosphorylation of the protein kinase Akt. As with S6 and 4EBP1, xenografts treated with drug plus radiation displayed an
equivalent level of reduced pAkt as in the xenografts treated with drug alone. In addition to xenograft lysate immunoblotting, immunohistochemistry of xenograft tissue sections demonstrated reduced pS6 (green) fluorescence in drug treated xenografts, as shown in Figure 5A-B. Drug treated irradiated xenografts displayed a statistically significant (p<.05) reduction in pS6 fluorescence versus irradiated xenografts with no drug treatment. In addition to downstream target inhibition, cellular proliferation was significantly (p<.0005) reduced in tumor xenografts treated with drug versus no drug as indicated by Ki67 (green) immunohistochemistry shown in Figure 5C-D. There was no difference in Ki67 fluorescence between drug treatment alone xenografts and irradiated plus drug treatment xenografts. This is consistent with the cytostatic effect of PF-05212384 and the timeframe of UMSCC1 xenograft radiation regrowth delay, see Figure 4A.

Discussion:

The identification of viable clinical radiosensitizers necessitates extensive pre-clinical investigation into treatment schedule, efficacy, biomarker identification, and normal tissue toxicity (42). With the identification of the PI3K/mTOR axis as a potential radiation modulation target in HNSCC, we sought to investigate the dual PI3K/mTOR inhibitor PF-05212384 as a radiosensitizer through rigorous pre-clinical investigation. Through our use of multiple treatment schedules, we have found that dual PI3K/mTOR inhibition significantly radiosensitizes HNSCC tumor cells with PF-05212384 treatment following irradiation, with variable radiation enhancement following a pre-IR treatment regimen (Figure 1). Regardless of p53 status, dramatic radiosensitization was evident in all HNSCC cell lines studied with PF-05212384 treatment following irradiation. Importantly, radiosensitization occurring in normal fibroblasts was significantly reduced as compared to malignant cells (post-IR treatment DMF of 1.4 vs. ≥1.9 respectively). To ascertain efficacy, we show that PF-05212384 (IV) with fractionated radiation synergistically delays UMSCC1 xenograft regrowth (Figure 4A). Along with the key finding of UMSCC1 xenograft regrowth delay, multiple potential clinical pharmacodynamics effects on targets were identified, including pAkt, p4EBP1, pS6, and Ki67 (Figure 4B, Figure 5). Overall, the significant
targeted radiation modulation along with the extensive pre-clinical investigation presented here support clinical assessment of PF-05212384 as a radiosensitizer in the combined modality treatment of HNSCC.

An incentive for utilizing a molecular targeted agent in combination with radiation for the treatment of HNSCC is the potential for differentially abrogating tumor cell radiation response versus normal tissue. A number of PI3K, mTOR, and dual PI3K/mTOR inhibitors have been tested in hopes of achieving a differential radiation response. One such agent, the dual PI3K/mTOR inhibitor NVP-BEZ-235, radiosensitizes a number of tumor cell lines (18, 20-22). However, as stated earlier, a major component of this compound’s radiosensitization is secondary to off-target effects via catalytic inhibition of the key DNA repair enzymes DNA-PKc and ATM, which could hinder this drug’s use in combination with radiation near the rapidly proliferative oral and pharyngeal mucosa. Here we not only demonstrated PF-052123384 did not alter levels of activated DNA-PKc and ATM enzymes directly (Supplementary Figure S5), but by clonogenic assay normal fibroblasts were radiosensitized to a lesser extent as compared to tumor cells (Figure 1). Notably, inhibition of the PI3K/mTOR pathway has been noted to reduce cellular mitochondrial oxygen consumption preventing tumor hypoxia (43). In a clinical setting, reduced tumor hypoxia could result in an enhanced radiation response, but this mechanism could not account for reduced normal tissue radiation enhancement as compared to HNSCC in vitro, particularly the studies performed here, as all cell culture work comparing normal versus tumor cells was performed at constant atmospheric oxygen level on exponentially growing cells (i.e. equivalent free radical formation). Although not directly inhibiting DNA-DSB repair enzymes, targeting the PI3K/mTOR axis with PF-05212384 clearly abrogates DNA-DSB repair following ionizing radiation as evidenced by a statistically significant (p<.05) delay in phosphorylated γH2AX resolution with PF-05212384 24 hour treatment versus no treatment (Figure 3). The exact mechanism or mechanisms linking dual PI3K/mTOR inhibition to ionizing radiation induced DNA-DSB repair inhibition remains to be elucidated.

To further assess PF-05212384 mechanism in conjunction with IR, cellular senescence PCR array analysis was performed. As was expected, dual PI3K/mTOR inhibitor had significant effects on both cell
cycle active and cell cycle inhibition genes (Supplementary Table S1). All the cyclins (A, B, and D) were markedly down-regulated and several cyclin dependent kinase inhibitors were up-regulated (CDKN1A, CDKN1C, p53, and CDKN2B). The net effect on these genes was consistent with the cell cycle flow analysis showing a significant block in G1 and a loss in S, and G2 phase (Table 1). A potentially interesting finding was that PF-05212384 down-regulated several genes involved in DNA damage response (Table 1) and that this might be the mechanism of radiosensitization in the UMSCC1 cells. Additional experiments to determine if these genes are altered in UMSCC46 cells and normal fibroblasts are planned.

As has been reiterated throughout the literature and reinforced by clinical trials, an effective molecular targeted agent spares normal tissue while at the same time synergistically killing tumor cells in conjunction with radiation. An agent’s ability to radiosensitize tumor cells in vitro is most often assessed by survival of exponentially growing cells following drug plus IR exposure. However, an exponentially growing clonogenic cell population bathed in nutrient rich growth medium at best represents a fraction of the cellular system near vasculature within a three dimensional malignant tumor. A large fraction of the tumor, in contrast, lies a considerable distance from the nearest blood vessel. This tumor microenvironment is distinct from normal physiologic conditions and is characterized by a reduction in proliferative rate, extracellular pH, oxygen tension, and nutrients, with an increase in lactic acid and catabolites (44, 45). To appropriately model this distinct non-cycling cellular population, considerable efforts were expounded in the early 1970s identifying plateau-phase monolayer cultures as a system closely resembling a hypoxic microenvironment (46). Here we have demonstrated that PF-05212384 via dual PI3K/mTOR inhibition significantly radiosensitizes plateau-phase UMSCC1 tumor cells with pre-treatment and post-treatment DMFs of 1.6 and 2.2 respectively (Supplementary Figure S1B). Radiosensitization of the non-proliferative and proliferative HNSCC cellular populations in vitro in conjunction with the validating synergistic UMSCC1 xenograft regrowth delay in vivo strongly support PF-05212384 as an agent capable of radiosensitization in physiologic and non-physiologic tumor
microenvironments. These results using fractionated radiation, together with preliminary data using single large doses of radiation combined with PF-04691502, indicate that PI3K/mTOR inhibition may be a good target for clinical radiotherapy (17).

Experimental evidence has surfaced indicating PF-05212384 dual PI3K/mTOR inhibition rescues HNSCC from cetuximab resistance (47). The ability to re-sensitize tumor cells previously resistant to a known radiosensitizing agent indicates PF-05212384, a significant radiosensitizing agent solely by itself, could potentially be quite efficacious for the treatment of cetuximab resistant HNSCC. However, PF-05212384 as a rescue therapeutic necessitates the combination of multiple molecular targeted agents in combination with radiation. A phase II clinical trial, RTOG 0411, and a phase III clinical trial, RTOG 0522, have assessed the impact on survival with the addition of a molecular targeted agent, bevacizumab and cetuximab respectively, to concurrent chemoradiation. The addition of a molecular targeted agent in both clinical trials did not improve outcome. Although increased toxicity may have played a role as a consequence of treatment breaks, a criticism of these clinical trials is the lack of biomarker studies and the inability to assess appropriate target inhibition (42). In this study, several tissue proteins were identified from our UMSCC1 xenograft regrowth delay studies, including pAkt, p4EBP1, pS6, and Ki67, that could be evaluated as candidate biomarkers to guide future clinical trials investigating PF-05212384 as a radiosensitizing agent (Figure 4B, Figure 5). The identification of multiple potential surrogates is key as DNA-damaging agents, such as cisplatin, modulate components of the mTOR signaling pathway (48, 49).

That data presented here represent the most comprehensive pre-clinical evidence that true dual PI3K/mTOR inhibition by PF-05212384 radiosensitizes HNSCC, with limited normal fibroblast radiation enhancement. Prior to the initiation of clinical trials investigating PF-05212384 in the combined modality treatment of HNSCC a few key issues need to be addressed. Immunosuppressant mTOR inhibitors, such as everolimus, have a narrow therapeutic window with high concentrations resulting in serious adverse effects (50). Thus, it will be a necessity to carefully craft the PF-05212384 treatment schedule and dose when combined with radiation based both on extensive pre-clinical data and current in human Phase I-II
clinical trials. Additionally, although multiple potential biomarkers were identified, the most appropriate biomarker or permutation of biomarkers establishing clinically relevant outcomes remain to be clarified. In the context of cetuximab resistance, PF-05212384 action as a re-sensitizing agent may prove to be quite efficacious reinforcing appropriate schedule, dosing, and clinical biomarker identification. Finally, this study lays the foundation for further investigation into the PI3K/mTOR axis as a radiation modulation target in HNSCC and has identified the dual PI3K/mTOR inhibitor PF-05212384 as a potential clinical radiosensitizer.

References


7. The cancer genome atlas [homepage on the Internet]. [cited October 7th, 2014].


33. Alvarez ML, Done SC. SYBR(R) green and TaqMan(R) quantitative PCR arrays: Expression profile of genes relevant to a pathway or a disease state. Methods Mol Biol 2014;1182:321-59.


Table 1. Summary of dose modifying factors (DMFs), PF-05212384 (10µM) toxicity alone, and PF-05212384 impact on cell cycle distribution.

<table>
<thead>
<tr>
<th>24 Hr Pre-XRT Treat DMF (±SEM)</th>
<th>24 Hr Post-XRT Treat DMF (±SEM)</th>
<th>24 Hour PF-384 (10µM) Exposure (No IR) Surviving Fraction (±SEM)</th>
<th>G1(%)* (Control G1%)</th>
<th>S(%) (Control S%)</th>
<th>G2/M(%) (Control G2/M%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSCC1</td>
<td>1.9 (±.07)</td>
<td>3.6 (±.14)</td>
<td>.71 (±.06)</td>
<td>88% (39%)</td>
<td>6% (56%)</td>
</tr>
<tr>
<td>UMSCC46</td>
<td>1.0 (±.00)</td>
<td>1.9 (±.25)</td>
<td>.84 (±.09)</td>
<td>45% (32%)</td>
<td>24% (43%)</td>
</tr>
<tr>
<td>1522</td>
<td>1.0 (±.00)</td>
<td>1.4 (±.15)</td>
<td>.84 (±.06)</td>
<td>81% (65%)</td>
<td>3% (21%)</td>
</tr>
<tr>
<td>UMSCC1 (Plateau)</td>
<td>1.6 (±.05)</td>
<td>2.2 (±.55)</td>
<td>.62 (±.03)</td>
<td>87% (69%)</td>
<td>7% (26%)</td>
</tr>
</tbody>
</table>

*Cell cycle distribution plots are shown in Supplementary Figure S2. Cell cycle distribution data representative of multiple experiments.
Figure Legends

**Figure 1.** Radiation survival curves for cell lines treated with PF-05212384 (10µM, Dashed Curve) or with DMSO Control (0.1%, Solid Curve) for 24 hours prior to irradiation (Pre-IR Treatment), **A-C** UMSCC1, UMSCC46, and 1522, or for 24 hours following irradiation (Post-IR Treatment), **D-F** UMSCC1, UMSCC46, and 1522. Data representative of n = 2-3 independent experiments fitted using the linear quadratic model. Dose modifying factors (DMFs) and drug treatment toxicities are shown in Table 1.

**Figure 2.** The effects of PF-05212384 on the phosphorylation of the PI3K/mTOR downstream targets S6, 4EBP1, and Akt. **A-C** UMSCC1 and UMSCC46 HNSCC cells were treated with PF-05212384 (10µM) for 24 hours then irradiated (7.5 Gy); immediately following irradiation cells were washed twice with 37°C medium and were subsequently incubated with drug free medium for the given time frame. PF-05212384 effect on PI3K/mTOR targets with treatment following irradiation demonstrated in Supplementary Figure S3A-B.

**Figure 3.** The effects of PF-05212384 (10µM) on UMSCC1 radiation induced DNA damage repair. Western blot analysis of phosphorylated γH2AX from **A** UMSCC1 as a function of PF-05212384 (10µM) drug treatment time following irradiation (7.5 Gy) with associated **B** mean (±SEM) densitometry of three independent γH2AX post-IR PF-05212384 treatment westerns. Individual experiment densitometry results were normalized based on the corresponding lane H2A loading control. Characteristic western blot of γH2AX from **C** UMSCC1 as a function of time following irradiation (7.5 Gy) with 24 hour PF-05212384 (10µM) pre-IR treatment; DNA-DSB repair inhibition at 24 hours is not statistically significant across multiple independent experiments. With pre-IR treatment, cells washed twice with 37°C medium at time of irradiation and incubated with drug free medium for the given timeframe. Statistically significant difference indicated by Θ as calculated by student’s t-test.
**Figure 4.** Radiation modulation of UMSCC1 tumor xenografts by dual PI3K/mTOR inhibition via PF-05212384 treatment.  
**A)** Athymic nude mice bearing right flank UMSCC1 tumor xenografts were treated with fractionated radiation (daily 3 Gy fraction M-F x 2 weeks) with and without IV PF-05212384 (6.7mg/kg Mon, Wed, and Fri 2 hours prior to radiation fraction). Xenograft regrowth delay (±SEM) relative to the control arm for IV PF-05212384, fractionated radiation (30 Gy total), and IV PF-05212384 plus fractionated radiation were as follows: 3.1±0.9 days, 7.1±1.4 days, and 13.0±1.6 days respectively. Fractionated radiation plus PF-0521283 regrowth delay is significantly different from fractionated radiation alone regrowth delay (P<.05; student’s t-test).  
**B)** Immunoblots of the PI3K/mTOR downstream targets S6, Akt, and 4EBP1 from UMSCC1 xenografts harvested 2 hours following the 5th 3 Gy fraction and 3rd PF-05212384 injection. Each lane (column) represents one UMSCC1 xenograft harvest. Normalized protein densities for each band are in parenthesis below each row.

**Figure 5.** Representative immunohistochemistry images of UMSCC1 tumor xenograft tissue sections harvested on treatment day 5 (after 5 x 3 Gy fractions and 3 x 6.7 mg/kg PF-05212384 injections).  
**A,B)** pS6 (green fluorescence) immunostaining with relative pS6 fluorescence.  
**C,D)** Ki67 (green fluorescence) immunostaining with relative Ki67 fluorescence. Relative fluorescence (green fluorescence area / nuclear count) expressed as mean (±SEM) of 3 random fields per mouse for ≥2 mice per treatment group.  
\[\text{\textsuperscript{\textsuperscript{v}}/\textsuperscript{\textsuperscript{\textbeta}}} \] Fractionated radiation plus PF-05212384 statistically different from fractionated radiation alone (P<.05 for pS6; P<.0005 for Ki67).  
\[\text{\textsuperscript{\textsuperscript{\textbeta}}} \] Fractionated radiation plus PF-05212384 versus PF-05212384 treatment alone not statistically different (P>.05) for pS6 or Ki67 immunostaining. DAPI nuclear counterstain (blue fluorescence). Statistical analyses performed utilizing student’s t-test. Imaged at 20x magnification.
Figure 2

A

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Time Post Wash/IR

- 7.5 Gy
- PF-05212384

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<tr>
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Time Post Wash/IR

- 7.5 Gy
- PF-05212384

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C

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Time Post Wash/IR

- 7.5 Gy
- PF-05212384

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**Figure 3**

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**B**

- Graph showing density (relative to control) for various conditions over 6 and 24 hours post IR and wash/IR with PF-05212384.
- *p < .05*

**C**

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<th>24 (hr)</th>
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Figure 4

A

![Graph showing Tumor Volume (mm$^3$) vs. Time (Days) with different treatments and time points.]

Time (Days)

- 3 Gy
- 6.7mg/kg IV PF-05212384

B

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<tr>
<td>pAkt</td>
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<td>(.55)</td>
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<td>p4EBP1</td>
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<td>(1.0)</td>
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<tr>
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<td>Cytochrome C</td>
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*Relative to HSC70, †Relative to Cytochrome C

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Figure 5

Panel A: Immunofluorescence images of pS6 expression in control injection and IV PF-05212384 treated cells under Mock Irradiate and Fractionated IR conditions.

Panel B: Bar graph showing relative fluorescence of pS6 expression in control, PF-384, and Fractionated IR + PF-384 conditions.

Panel C: Immunofluorescence images of Ki67 expression in control injection and IV PF-05212384 treated cells under Mock Irradiate and Fractionated IR conditions.

Panel D: Bar graph showing relative fluorescence of Ki67 expression in control, Fractionated IR, PF-384, and Fractionated IR + PF-384 conditions.
Radiation Enhancement of Head and Neck Squamous Cell Carcinoma by the Dual PI3K/mTOR Inhibitor PF-05212384

Andrew Leiker, William G. DeGraff, Rajani Choudhuri, et al.

Carcinoma by the Dual PI3K/mTOR Inhibitor PF-05212384

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