Taselisib (GDC-0032), a Potent β-Sparing Small Molecule Inhibitor of PI3K, Radiosensitizes Head and Neck Squamous Carcinomas Containing Activating PIK3CA Alterations

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Abstract

Purpose: Activating PIK3CA genomic alterations are frequent in head and neck squamous cell carcinoma (HNSCC), and there is an association between phosphoinositide 3-kinase (PI3K) signaling and radioresistance. Hence, we investigated the therapeutic efficacy of inhibiting PI3K with GDC-0032, a PI3K inhibitor with potent activity against p110α, in combination with radiation in HNSCC.

Experimental Design: The efficacy of GDC-0032 was assessed in vitro in 26 HNSCC cell lines with crystal violet proliferation assays, and changes in PI3K signaling were measured by Western blot analysis. Cytotoxicity and radiosensitization were assessed with Annexin V staining via flow cytometry and clonogenic survival assays, respectively. DNA damage repair was assessed with immunofluorescence for γH2AX foci, and cell cycle analysis was performed with flow cytometry. In vivo efficacy of GDC-0032 and radiation was assessed in xenografts implanted into nude mice.

Results: GDC-0032 inhibited potently PI3K signaling and displayed greater antiproliferative activity in HNSCC cell lines with PIK3CA mutations or amplification, whereas cell lines with PTEN alterations were relatively resistant to its effects. Pretreatment with GDC-0032 radiosensitized PIK3CA-mutant HNSCC cells, enhanced radiation-induced apoptosis, impaired DNA damage repair, and prolonged G2-M arrest following irradiation. Furthermore, combined GDC-0032 and radiation was more effective than either treatment alone in vivo in subcutaneous xenograft models.

Conclusions: GDC-0032 has increased potency in HNSCC cell lines harboring PIK3CA-activating aberrations. Further, combined GDC-0032 and radiotherapy was more efficacious than either treatment alone in PIK3CA-altered HNSCC in vitro and in vivo. This strategy warrants further clinical investigation.

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Introduction

Radiotherapy is the primary local treatment used for head and neck squamous cell carcinomas (HNSCC) arising from many anatomic sites, including the oropharynx, hypopharynx, nasopharynx, and larynx. Concurrent administration of cisplatin-based chemotherapy has been shown to improve overall survival in patients with locally advanced HNSCC undergoing definitive radiation, primarily by decreasing locoregional relapse (1). However, concurrent chemotherapy also significantly increases toxicity during, and possibly after, radiotherapy (2–5). In addition to cisplatin, the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab has also been shown to improve overall survival and locoregional progression-free survival, without increasing acute or late radiation toxicity, when administered concurrently with radiation (6). Despite this success, EGFR inhibitor–based radiation regimens may be less effective than platinum-based chemoradiation (7). Therefore, novel agents that radiosensitize HNSCC in a tumor specific manner continue to be sought.

The most common oncogenic alteration in HNSCC is the aberrant activation of PI3K via mutations or amplification of PIK3CA, the gene encoding the α-isoeform of catalytic subunit of PI3K (p110α; refs. 8–12). According to The Cancer Genome Atlas (TCGA; ref. 13), activating PIK3CA alterations are present in 56% and 34% of HPV+ and HPV– HNSCCs, respectively. Activation of PI3K leads to synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the plasma membrane that, in turn, leads to the recruitment of the pleckstrin homology domain–containing proteins phosphoinositide dependent protein kinase-1 (PDK1) and AKT. PDK1 phosphorylates AKT at threonine 308 and activates AKT and downstream signaling elements, including mammalian target of rapamycin (mTOR) complex 1 (mTORC1), thereby promoting cell growth, proliferation, survival, and angiogenesis and regulating glucose metabolism (14).
or knockdown of We and others have demonstrated that inhibition of PI3K downstream signaling within minutes of treatment (15 of AKT, both at threonine 308 and serine 473, and activate damaging agents, including radiation, induce phosphorylation more, inhibition of PI3K 
izes breast cancer cells to PARP inhibition (19, 20). Further-

**Materials and Methods**

**Reagents**

GDC-0032 was provided by Genentech. For in vitro assays, all drugs were dissolved in dimethyl sulfoxide. For in vivo experi-
ments, GDC-0032 was dissolved in sterile water, 0.5% methyl-
cellulose, and 0.2% Tween-80.

**Cells and cell culture**

All HPV-negative cells were obtained directly from the American Type Culture Collection (Cal-33, FaDu, Detroit 562, SCC-4, SCC-9, SCC-15, and SCC-25), the European Collection of Cell Cultures via Sigma-Aldrich (BICR-16, BICR-18, BICR-22, and BICR-31), the Japanese Collection of Research Bioresources (HSC-2, HSC-3, and HSC-4), or the Korean Cell Line Bank (SNU-46, SNU-1076, SNU-1214, and YD-8), with the exception of LB-771, which was obtained from The Center for Molecular Therapeutics at Massachusetts General Hospital. The HPV-posi-
tive cell lines UD-SCC-2, UPCI-SCC-90, and 93-VU-147T were kind gifts from the Paul Harari lab at the University of Wisconsin (Madison, WI). UMSCC-104 was purchased from the lab of Thomas Carey at the University of Michigan (Ann Arbor, MI), and UPCI-SCC-154 was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All cell lines were maintained in humidified incubators at 37°C in Dulbecco’s modified Eagle’s medium/Ham’s F-12 1:1, with the exception of SNU-1076, SNU-46, SNU-1214, and YD-8, which were grown in RPMI-1640. Cell culture media were supplemented with 10% heat-
inactivated fetal calf serum, 2 mM l-glutamine, penicillin (20 U/mL), and streptomycin (20 μg/mL).

**Determination of PIK3CA mutation and copy number status**

PIK3CA mutation and amplification status information for each cell line was obtained from the Cancer Cell Line Encyclo-
pedia (www.broadinstitute.org/ccle; ref. 31), except for LB-771 and the six HPV-positive cell lines, which were sequenced using the MSK-IMPACT next-generation sequencing platform (32). Amplification was defined as greater than or equal to 4 copies of the PIK3CA gene.

**Proliferation assays**

Cells were seeded in replicates of 6 in 96-well plates with 5 to 5,000 cells/well overnight and then treated with GDC-0032. After 4 days, the media were removed and the cells were fixed with 4% glacial acetic acid for 30 minutes. Fixed cells were stained with 0.1% crystal violet and washed, and the absorbance was measured using a Biotek Synergy H1 plate reader.

**Clonogenic survival assays**

Cells were plated in 6-well plates in appropriate dilutions (37, 111, 333, 1,000, 3,000, or 9,000 cells/well) and allowed to attach overnight. Cells were then treated with DMSO or drug for 24 hours and irradiated at various dosing using a cesium irradiator. Drugs were left in following irradiation. Ten to 14 days after irradiation, cells were fixed with 4% glacial dehyde and stained with 0.1% crystal violet. Colonies with greater than 50 cells were counted. Surviving fractions were calculated by normalizing to the plating efficiency at 0 Gy for either control- or drug-treated plates.

**Western blot analysis**

Cells were washed twice in ice-cold phosphate-buffered saline, scraped from the plate, centrifuged, and then frozen at −20°C after the supernatant was removed. The cells were then
lysed in ice-cold radioimmunoprecipitation buffer supplemented with phosphatase inhibitor cocktails (Complete Mini and PhosphoStop, Roche), centrifuged, and the supernatant was removed for protein quantification (Pierce BCA Protein Assay Kit, Thermo Scientific). Twenty-five to 50 μg of protein was loaded into NuPAGE 4% to 12% bis–tris gels (Life Technologies) and resolved via electrophoresis, then transferred to Immobilon transfer membranes (Millipore). Membranes were blocked in 5% BSA in TBS-T for 1 hour prior to overnight incubation in primary antibody at 4°C, and incubated in either mouse or rabbit horseradish peroxidase–conjugated secondary antibodies (1:50,000; Amersham Biosciences) in 2% BSA in TBS-T for 1 hour. Membranes were imaged using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and images were captured using a GBOX camera system. Antibodies used are listed in Supplementary Table S1.

Immunofluorescence

Cells were seeded into 8-chamber culture slides (BD Falcon), fixed at each time point with 4% formaldehyde at room temperature, and then rinsed with PBS and stored in PBS at 4°C. Cells were permeabilized with 0.8% Triton X-100 and 0.2% BSA in PBS for 10 minutes on ice and then blocked with 1% BSA in TBS-T for 1 hour at room temperature, followed by incubation with pH2AX (1:500; Millipore) and 53BP1 (1:200; Bethyl) antibodies in 1% BSA in TBS-T for 2 hours at room temperature. The cells were then washed with TBS-T and incubated with Alexa 555 anti-rabbit and Alexa 488 anti-mouse secondary antibodies (both 1:1,000; Life Technologies) at room temperature. The cells were washed with TBS-T, rinsed in ddH2O, and coverslipped with ProLong with DAPI (Life Technologies). Slides were digitally scanned with a Panoramic Flash scanner (3DHistech) using 20X/0.8NA objective. The images were then analyzed using Metamorph software (Molecular Devices); briefly, nuclear regions were segmented using the DAPI channel, and the number of foci was counted using spot detection.

Annexin V staining

Cells were treated with either GDC-0032 or DMSO for 24 hours, irradiated or mock-irradiated, then trypsinized and harvested along with cells in the media after an additional 72 hours. Cells were resuspended in Annexin V buffer, stained with Annexin V–FITC and propidium iodide (PI) according to the manufacturer’s instructions (BD Biosciences), and analyzed for fluorescence with a Fortessa flow cytometer. Resulting data were analyzed using FlowJo software.

Cell-cycle analysis

Cells were collected by trypsinization, washed in ice-cold PBS, resuspended in 70% ethanol and stored at −20°C for at least 2 hours. Ethanol was then removed, cells were washed in wash buffer (1% BSA, 0.25% Triton X-100, and 2.5 mmol/L EDTA in PBS), then incubated in wash buffer containing 40 μg/mL PI (Sigma) and 100 μg/mL RNase A (Sigma) for 20 minutes. Cells were characterized for fluorescence with either a FACSCalibur or a Fortessa flow cytometer, and the resulting distributions were analyzed using FlowJo software.

In vivo xenograft studies

All in vivo studies were conducted according to the Memorial Sloan Kettering Research Animal Resource Center-approved protocols and Johns Hopkins University Animal Care and Use Committee approved protocols. Six-week-old Nuf/Nu mice were order from Harlan Laboratories. For cell line–derived xenograft studies, mice were injected bilaterally with $5 \times 10^6$ cells resuspended in 200 μL of culture media and Matrigel (BD Biosciences) mixed in a 1:1 ratio. After tumors reached approximately 100 to 200 cm³, mice were randomized into treatment arms with 8 to 10 tumors per group. GDC-0032 (5 mg/kg) was dissolved in a vehicle containing 0.5% methylcellullose with 0.2% Tween-80 and was administered via daily oral gavage. Tumors were irradiated using an X-RAD 320 X-ray system with appropriately sized lead shields.

For patient tumor–derived xenograft studies, mice were implanted with a tumor obtained from a patient with oropharynx squamous cell carcinoma. The tumor DNA was sequenced using CancerSelect-203 R platform (Personal Genome Diagnostics) and found to have a PIK3CAE542K mutation. After tumors reached approximately 100 to 250 cm³, mice were randomized into treatment arms with 5 to 6 tumors per group. GDC-0032 (5 mg/kg) was administered via daily oral gavage for 14 days. Tumors were irradiated 2 Gy daily on days 2 to 4 using a Small Animal Radiation Research Platform at the Johns Hopkins University. Tumor volumes were calculated as $(\pi/6) \times length \times width^2$.

Results

GDC-0032 is active in HNSCC cell lines harboring activating PIK3CA aberrations and wild-type PTEN

We tested the antiproliferative activity of GDC-0032 across a panel of 26 HNSCC cell lines, including the majority of commercially available HPV-positive HNSCC cell lines. As expected, there was a gradient of sensitivity in HNSCC cell lines. Cell lines harboring either mutations or amplification of PIK3CA tended to be sensitive to GDC-0032, with IC50 values in the nanomolar range (Fig. 1A). In contrast, 4 of the 6 most resistant cell lines to GDC-0032 had mutation or loss of PTEN, consistent with previous reports of PTEN aberrations leading to resistance to PI3K inhibitors through upregulation of PI3Kb signaling (33–35). The observation of a preferential antiproliferative effect of GDC-0032 in cells with activated PIK3CA has also been observed with other isotype-specific PI3K inhibitors in other tumor types, suggesting that this selectivity could be important in the clinic (36, 37). In contrast, neither PIK3CA nor PTEN status correlated with sensitivity to GDC-0941, a pan-PI3K inhibitor with similar potency against all class IA PI3K isoforms (Fig. 1B). Thus, although PIK3CA and PTEN status may help identify tumors sensitive to GDC-0032, this is not a property shared amongst all classes of PI3K inhibitors.

GDC-0032 induces apoptosis in cell lines with PIK3CA alterations

Next, we investigated the effects of GDC-0032 on downstream PI3K signaling in several genetic contexts. Treatment with GDC-0032 in Cal-33 cells (harboring a PIK3CAH1047R mutation) prevented phosphorylation of AKT and inhibited downstream mTOR targets, such as ribosomal protein S6 kinase (S6K), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP-1), and S6 (Fig. 2A). This translated into an induction of apoptosis, as assessed by cleavage of poly-ADP ribose polymerase (PARP).

Similar results were obtained in LB-771, a cell line containing amplification of PIK3CA (Supplementary Fig. S1A). In cell lines containing either PTEN homzygous deletion (UD-SCC-2) or
mutation (UPCI-SCC-90). GDC-0032 was appreciably less effective at downregulating AKT/mTOR signaling and inducing cell death (Fig. 2A and Supplementary Fig. S1B). This supports the notion that downregulation of PI3K signaling is necessary for the proapoptotic effects of GDC-0032 in HNSCC.

The dose of 100 nmol/L GDC-0032, which inhibits AKT/mTOR signaling in PIK3CA mutant cell lines but not in cells with loss or mutation of PTEN, was chosen for subsequent time-course experiments. This dose of GDC-0032 inhibited phosphorylation of AKT and downstream signaling in Cal-33 (PIK3CA mutant) and LB-771 (PIK3CA amplified) cells, but had little effect on PI3K signaling in UD-SCC-2 (PTEN homozygous deletion) or UPCI-SCC-90 cells (PTEN mutant; Fig. 2B), confirming that this is a concentration of GDC-0032 that inhibits PI3K but not PI3K-dependent signaling.

GDC-0032 radiosensitizes cells with PIK3CA mutation/amplification

Given that inhibition of PI3K signaling has been purported to affect expression of DNA damage repair (DDR) proteins (19, 20, 38) and alter DDR signaling in response to radiation (15, 17), we next sought to study the effect of GDC-0032 on HNSCC cell lines treated with radiation. In Cal-33 cells (PIK3CAH1047R), the combination of GDC-0032 and radiation resulted in both more apoptotic (Annexin V–positive, PI-negative) and nonapoptotic (Annexin V–positive, PI-negative) cell death than either treatment alone (Fig. 3A). GDC-0032 and radiation also slowed cell growth rates more than either treatment alone in PIK3CA-mutant cell lines Cal-33 and HSC-2, but had little effects in PTEN-altered cell lines UPCI-SCC-90 and UD-SCC-2 (Fig. 3B). The increased antitumor effects of combined radiation and GDC-0032 compared with either treatment alone were confirmed using Annexin staining in three additional cell lines bearing activating PIK3CA alterations (LB-771, SNU-1076, and HSC-2), whereas no significant additional activity was observed in cell lines with wild-type PIK3CA and/or inactivating PTEN alterations (HSC-3, UD-SCC-2, HSC-4, and FaDu; Fig. 3C). Similar results were seen with the structurally unrelated p110α inhibitors BYL719 (Supplementary Fig. S2) and A66 (Supplementary Fig. S3), suggesting that...
GDC-0032 induces cell death following radiation primarily through p110α inhibition, rather than inhibition of other PI3K isoforms or off target enzymes.

Inhibition of other downstream PI3K components using an allosteric AKT inhibitor MK-2206 or the allosteric mTORC1 inhibitor RAD001 also increased radiation-induced apoptosis, albeit to a smaller degree than GDC-0032 (Fig. 3D). Similar results were observed with the PIK3CA-mutated cell line HSC-2, although RAD001 did not enhance radiation-induced apoptosis in this cell line (Supplementary Fig. S4).

The gold standard for assessing radiosensitization is clonogenic survival. Using this assay, Cal-33 cells pretreated with GDC-0032 had significantly decreased cell survival following radiation (Fig. 3E). Similar radiosensitization was seen when LB-771 cells were treated with GDC-0032 prior to radiation (Supplementary Fig. S5). However, GDC-0032 had no effect on the radiation response of HSC-3, a PIK3CA wild-type cell line resistant to single-agent GDC-0032 (Fig. 3D).

GDC-0032 delays the resolution of DNA double-strand breaks following radiation

PI3K signaling is a key regulator of the DNA damage response (15, 17–21, 39). Therefore, we decided to study whether the GDC-0032–dependent radiosensitization was at least in part attributable to impaired DDR under a state of PI3Kα inhibition. We quantified the amount of DNA double-strand breaks (DSB), as assessed by γH2AX foci, with and without GDC-0032 pretreatment in Cal-33 cells. Cells pretreated with GDC-0032 had significantly more γH2AX foci at 24 and 48 hours after irradiation than control-treated cells (Fig. 4A). This increase in DNA

Figure 2.
GDC-0032 is a more potent inhibitor of downstream AKT and mTOR signaling in head and neck cancer cell lines containing PIK3CA-activating alterations than in cell lines containing PTEN alterations. A, increasing doses of GDC-0032 were administered in serial dilution in Cal-33 (PIK3CA H1047R) or UD-SCC-2 (PTEN deletion), and cells were harvested after 4 hours. B, time course following 100 nmol/L GDC-0032 in Cal-33, LB-771 (PIK3CA amplified), UPCI-SCC-90 (PTEN mutated), and UD-SCC-2.
Figure 3.
GDC-0032 enhances radiation-induced apoptosis and inhibits growth in head and neck cancer cell lines that are sensitive to its single-agent activity. A, Cal-33 (PI3KCA^H1047R) cells were treated with DMSO or 100 nmol/L GDC-0032 for 24 hours, irradiated with 4 Gy, then analyzed for Annexin V–FITC and PI staining 72 hours later. B, Cal-33, HSC-2 (PI3KCA^H1047R), UD-SCC-2 (homozygous PTEN deletion), and UPCI-SCC-90 (PTEN mutant) were treated with either DMSO, 100 nmol/L GDC-0032, 4 Gy of radiation, or both. Cell proliferation was measured by counting cell numbers on the indicated days with a hemocytometer. (Continued on the following page.)
damage upon combination of radiation and GDC-0032 was accompanied by increased formation of p53-binding protein 1 (53BP1) foci, a mediator of the DSB repair downstream of γH2AX (Fig. 4B; ref. 40), and induction of PARP cleavage (Supplementary Fig. S6A). Consistent results were also observed in LB-771 cells (Supplementary Fig. S6B), supporting the notion that GDC-0032 impairs DSB repair in these cells following radiation.

GDC-0032 enhances G2–M arrest following radiation

Ionizing radiation induces two molecularly distinct G2–M checkpoints (41). The first, known as the “early” G2–M checkpoint, consists of a transient ATM-dependent mitotic block affecting cells in late G2 occurring within minutes of irradiation and can be assessed by the proportion of cells with phosphorylation of histone H3 (HH3) after irradiation (41). The second, more prolonged and known as the "late" G2–M checkpoint, is independent of ATM and results in an accumulation of cells with 4N DNA content (41). It is well accepted that the duration of the G2–M checkpoint reflects the number of unrepaired DSBs (42). Therefore, based on our results we hypothesized that GDC-0032 may alter the DNA damage–induced cell-cycle arrest that occurs following irradiation.

As single agent, treatment with GDC-0032 resulted in a mild increase in the proportion of cells in G1 over 72 hours, with a concomitant decrease in the G2 phase of the cell cycle (Fig. 5A).

(Continued.) C, various cell lines with or without PIK3CA or PTEN alterations were treated with 100 nmol/L for 24 hours, then irradiated with 4 Gy, and analyzed 72 hours later. D, Cal-33 cells were treated with either DMSO, 100 nmol/L GDC-0032, 2 mmol/L MK-2206, 100 nmol/L RAD-001, or a combination of MK-2206 and RAD-001, then irradiated with 4 Gy after 24 hours. E, Cal-33 or HSC-3 (PIK3CA wild-type) were treated with 100 nmol/L GDC-0032 for 24 hours, then irradiated, then fixed and stained with crystal violet 10 days later. Surviving fractions were calculated based on the plating efficacy of the 0-Gy plate for DMSO or GDC-0032, respectively.
As expected, radiation induced both the early and late G2–M checkpoints, with a marked decrease in pHH3-positive cells (Supplementary Fig. S7) and an accumulation of cells with 4N DNA content (Fig. 5B). However, by 48 hours after irradiation, the cell-cycle profile was nearly back to baseline. When we examined the impact of PI3Kα inhibition in this setting, we observed that,
while treatment with GDC-0032 for 24 hours prior to irradiation did not significantly alter the cell-cycle profile of Cal-33 cells (Fig. 5B) or affect the early G2–M arrest as assessed by pH3-positive cells (Supplementary Fig. S7), the proportion of cells with 4N DNA content at 24 and 48 hours after irradiation was considerably higher with than in control-treated cells. This suggests that the late G2–M arrest induced by radiation is enhanced by pharmacologic inhibition of PI3Kα (Fig. 5B).

Because the late G2–M checkpoint is known to be ATM independent, we investigated whether the enhanced late G2–M checkpoint induced by GDC-0032 could be abrogated by either inhibition of other known regulators of the G2–M cell-cycle progression, such as Wee1 and ATR. We found that although both Wee1 inhibition with AZD-1775 and ATR inhibition with VE-821 abrogated the early G2–M checkpoint following irradiation as assessed by pH3 (Supplementary Figs. S8 and S9), only ATR inhibition with VE-821 reversed the late G2–M arrest induced by the combination of GDC-0032 and radiation (Fig. 5C and Supplementary Fig. S10). Taken together, these results indicate that GDC-0032 enhances the late G2–M checkpoint induced by irradiation in an ATR-dependent fashion, resulting in increased DNA damage overtime.

GDC-0032 enhances the antitumor effects of radiotherapy in vivo

In order to test the ability of GDC-0032 to inhibit PI3K signaling in vivo, we treated nude mice implanted with subcutaneous Cal-33 xenografts with 5 mg/kg of GDC-0032 and harvested the tumors after 2, 6, and 24 hours of treatment. As expected, treatment with GDC-0032 resulted in nearly complete abrogation of AKT and PRAS40 phosphorylation, as well as decreased phosphorylation of 4EBP-1 and S6, at 2 hours after drug administration (Fig. 6A). However, by 6 hours after oral gavage, a rebound in phosphorylation of all of these PI3K targets was detected, probably a reflection of the short half-life of the compound.

We next assessed the efficacy of combined PI3K inhibition and radiation. Mice received daily GDC-0032 throughout the experiment and 20 Gy in 5 daily fractions on days 2 to 6. Although treatment with either radiation or GDC-0032 alone resulted in tumor growth delay compared with vehicle-treated mice, only the combination of GDC-0032 and radiation resulted in durable tumor regressions (Fig. 6B). In fact, at the experiment endpoint (90 days of treatment), none of the tumors in the combined radiation and GDC-0032 arm had progressed in comparison to the start of the treatment. We also saw superior activity with transient administration of GDC-0032 during radiation in a HPV+ PKI3CA[ES42K] patient-derived xenograft model (Supplementary Fig. S11). These data also suggest that both mutations (ES42K in the helical domain and H1047R in the kinase domain) are functionally active in HNSCC.

Moreover, our in vivo findings suggest that even a transient inhibition of the PI3K/AKT/mTOR pathway (Fig. 6A) is sufficient to sensitize tumors to radiation, an observation with potential clinical implications.

Discussion

In this study, we determined that HNSCC cell lines containing activating PIK3CA alterations are significantly more sensitive to GDC-0032, a novel, potent inhibitor of PI3Kα, than cell lines without these alterations. This was consistent with previous studies of other PI3Kα inhibitors studied in breast cancer (36, 37, 43). Additionally, we found that PTEN alterations were associated with resistance to GDC-0032, with 4 of the 6 most resistant HSNCC cell lines to this agent containing PTEN alterations, also consistent with previous data from our group (33).

Combining GDC-0032 and radiotherapy resulted in greater cell death than either treatment alone in cells with intrinsic sensitivity to GDC-0032. This translated in profound in vivo antitumor activity of the combination of GDC-0032 and radiation in PIKCA-mutant tumors.

The mechanisms underlying the strong antitumor efficacy of combined radiotherapy and PI3K inhibition in HNSCC are almost certainly multifactorial given the diverse phenotypic outputs induced by both radiation and PI3K signaling, including modulation of growth, survival, metabolic activity, angiogenesis, and immune response. However, we speculate that a major contributor to the efficacy of this combination is impaired DDR as a result of PI3K inhibition. Supporting this, we found that GDC-0032 pretreatment delays the resolution of deleterious DNA lesions following radiotherapy and prolongs the accumulation of cells in G2–M following irradiation in an ATR-dependent manner. It should also be noted that we observed strong in vivo effects of GDC-0032 and radiation despite the fact that inhibition of PI3K signaling was relatively transient. Although further studies may be needed with respect to the optimal timing and sequencing of GDC-0032 and radiation, our findings suggest that a strong...
pulsatile PI3K pathway inhibition would be sufficient to achieve effective radiosensitization.

Combining PI3K pathway inhibitors and radiation may be a particularly promising therapeutic strategy in HPV+ HNSCC for both biologic and clinical reasons. PIK3CA mutations and amplifications are present in 22% to 37% and 20% to 25% of HPV+ tumors, respectively, an approximately 2- to 3-fold higher incidence over what is observed in HPV- tumors (11, 12).

Furthermore, although PIK3CA mutations are found in the kinase domain, the helical domain, and other non-helical spots throughout the gene in HPV- HNSCC, almost all PIK3CA mutations in HPV+ HNSCC occur within the helical domain of p110α at either E542 or E545, suggesting that these specific helical domain codon changes confer a critical function in the pathogenesis of this disease (13). The limited PIK3CA mutation spectrum in HPV+ HNSCC also will likely simplify patient selection for clinical trials, obviating the complexity introduced by multiple mutations of unknown biochemical and phenotypic significance. Importantly, HPV+ tumors have increased radiosensitivity both preclinically and clinically in comparison to HPV- HNSCC (44, 45), and several clinical trials are currently under way to investigate strategies to de-escalate therapy without compromising cure rates. Thus, given the high prevalence of activating PIK3CA alterations in HPV+ HNSCC and the need to improve the toxicity profile of radiotherapy (by eliminating concomitant administration of chemotherapy), combining PI3K inhibitors with radiotherapy in select HPV+ patients may be a promising strategy for future clinical investigations. In fact, we are currently enrolling patients with HNSCC in a early phase clinical trial combining cetuximab, p110α-inhibition, and radiation. In summary, we have demonstrated that specific PI3Kα inhibition is an effective strategy for HNSCC tumors harboring activating PIK3CA alterations in combination with radiotherapy. Given the importance of PI3K signaling both in the pathogenesis of HNSCC and in the response of cells to radiation, these types of strategies may represent an important step to reducing the toxicity of treatment by personalizing therapy while maintaining the excellent outcomes provided by cytotoxic chemoradiation.

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

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Alterations PIK3CA Containing Activating of PI3K, Radiosensitizes Head and Neck Squamous Carcinomas -Sparing Small Molecule Inhibitor β-Taselisib (GDC-0032), a Potent

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