

PI3Kinase based degradation of NRF2 signaling in chemo-radiation resistant HNSCC

Pedram Yadollahi

Baylor College of Medicine

Yang Li

Baylor College of Medicine

Kalil Saab

Baylor College of Medicine

Fonma Nkeseobong Essien

Baylor College of Medicine

Imelda Yebra

Baylor College of Medicine

Hussam Dayoub

Baylor College of Medicine

Rutulkumar Patel

Baylor College of Medicine

Stephen Y Lai

MD Anderson Division of Radiation Oncology: The University of Texas MD Anderson Cancer Center
Division of Radiation Oncology

Jeffrey N. Myers

The University of Texas MD Anderson Cancer Center Department of Thoracic Head and Neck Medical
Oncology

Heath D. Skinner

UPMC Hillman Cancer Center

Mitchell J. Frederick

Baylor College of Medicine

Vlad Sandulache

vlad.sandulache@bcm.edu

Baylor College of Medicine <https://orcid.org/0000-0002-9205-385X>

Research Article

Keywords: Head and neck squamous cell carcinoma (HNSCC), PI3K/mTOR inhibition, Gedatolisib, Nrf2, GSK3, chemo-radiosensitization

Posted Date: August 18th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10606918/v1>

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Abstract

Background.

Hyperactivation of NRF2 is an established factor contributing to chemoradiation resistance in head and neck squamous cell carcinoma (HNSCC). In tumors with *KEAP1* loss or mutations in the oncogenic *PIK3CA* pathway, NRF2 accumulates, enhancing antioxidant defenses that mitigate the cytotoxic effects of cisplatin and ionizing radiation. An alternative approach to directly targeting NRF2 exploits the PI3K/AKT/GSK3 axis, which regulates a KEAP1-independent pathway for NRF2 proteasomal degradation.

Methods.

We evaluated the effects of the dual PI3K/mTOR inhibitor gedatolisib on NRF2-signaling and phenotype in various human and murine HNSCC cell lines, as well as genetically engineered mouse models (GEMMs) with conditional *Keap1* deletion and orthotopic xenografts of the cisplatin-resistant HN30R8 line. Proliferative responses were tracked using real-time IncuCyte imaging and label-free holotomographic microscopy. NRF2 protein regulation was assessed via western blot, nuclear/cytoplasmic fractionation, and RT-qPCR. The role of GSK3 and the proteasome in the gedatolisib-induced reduction of NRF2 was confirmed through pharmacological rescue with GSK3 and proteasome inhibitors and further validated by siRNA knockdown of GSK3- α , GSK3- β , β -TrCP, and SPOP.

Results.

Gedatolisib inhibited AKT phosphorylation and decreased NRF2 protein levels in a dose- and time-dependent manner across all tested cell lines, including those derived from GEMMs with *Keap1* deletion. This reduction was post-translational, as *NFE2L2* mRNA was upregulated via FoxO transcription factor activation, while NRF2 protein was directed towards GSK3-dependent, β -TrCP/SPOP-mediated proteasomal degradation. Holotomographic live-cell imaging revealed that cells treated with gedatolisib primarily underwent necrotic cell death, which was accompanied by mitochondrial accumulation and growth arrest. Gedatolisib effectively suppressed tumor growth in both *Keap1* wild-type and *Keap1*-deleted GEMMs. In orthotopic HN30R8 cisplatin-resistant xenografts, gedatolisib was the primary driver of antitumor activity. Additionally, the combination of gedatolisib and radiation showed synergistic tumor suppression according to the multiplicative model in a replicated experiment.

Conclusions.

Gedatolisib effectively overcomes NRF2-driven resistance in HNSCC by engaging a degradation pathway that does not rely on KEAP1. The drug simultaneously activated NRF2 transcription through FoxO and

accelerated the degradation of NRF2 protein via the GSK3/ β -TrCP pathway, resulting in decreased NRF2 protein levels. These findings support the potential of gedatolisib as a chemoradiosensitizer in HNSCC clinical trials.

Background

The transcription factor NRF2 (encoded by *NFE2L2*) has emerged as a central regulator in chemoradiation resistance across multiple solid tumors ^[1, 2]. Under homeostatic conditions, KEAP1 targets NRF2 for degradation via the Cul3 E3 ligase complex ^[3]. Oxidative stress modifies KEAP1's reactive cysteine residues, disrupting the KEAP1-NRF2 interaction. As a result, NRF2 accumulates, translocates into the nucleus, and subsequently activate a transcriptional program that includes genes involved in glutathione synthesis, NADPH regeneration, xenobiotic metabolism, and drug efflux ^[4]. This pathway serves a protective function in normal tissues but gives cancer cells a survival advantage by neutralizing reactive oxygen species (ROS) induced by radiation and platinum-based chemotherapy ^[2, 5].

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with over 900,000 new cases diagnosed annually ^[6]. Standard treatment for locally advanced disease combines cisplatin-based chemotherapy with radiation therapy, but treatment failure remains a major challenge. In HNSCC, NRF2 hyperactivation occurs through several mechanisms, including somatic mutations in *KEAP1* or *NFE2L2*'s Neh2 domain that disrupt the KEAP1-NRF2 interaction directly ^[7]. Genetic alterations in *KEAP1* are present in approximately 17% of HNSCC cases and are linked to NRF2 activation, enhanced cancer stem cell properties, and resistance to chemotherapy ^[8]. Environmental carcinogens, including cigarette smoke and polycyclic aromatic hydrocarbons, upregulate NRF2 through direct oxidative stress ^[9, 10]. Cisplatin exposure itself promotes *KEAP1/NFE2L2* mutations and epigenetic reprogramming of the NRF2 axis, creating a cycle in which treatment drives the very resistance mechanism it seeks to overcome ^[11, 12]. This convergence of mechanisms poses a therapeutic challenge as NRF2 itself is difficult to target, and upstream pathway inhibition has not been systematically explored as a strategy for NRF2 degradation in HNSCC.

In addition to the canonical KEAP1/Cul3 pathway, NRF2 is regulated by a KEAP1-independent degradation route mediated by GSK3 and β -TrCP. GSK3 phosphorylates a conserved DSGIS motif in the Neh6 domain of NRF2, creating a phosphodegron recognized by β -TrCP, which recruits the SCF E3 ubiquitin ligase complex to polyubiquitinate and degrade NRF2 ^[13, 14]. Chowdhry et al. further demonstrated that the Neh6 domain contains a second β -TrCP recognition motif (DSAPGS) that is not regulated by GSK3 phosphorylation ^[15]. Under conditions of PI3K/AKT pathway activation, AKT phosphorylates and inactivates GSK3 at Ser21 (GSK3 α) and Ser9 (GSK3 β), stabilizing NRF2 through this alternative route ^[16]. This raises the possibility that pharmacological inhibition of PI3K could reactivate GSK3 and restore NRF2 degradation even in tumors with KEAP1 loss.

Gedatolisib (PF-05212384) is a potent dual inhibitor of all class I PI3K isoforms and mTORC1/mTORC2 that is currently under clinical evaluation^[17]. In the phase 3 VIKTORIA-1 trial, gedatolisib in combination with fulvestrant and palbociclib demonstrated significant progression-free survival benefit in HR-positive, HER2-negative advanced breast cancer, leading to acceptance of a New Drug Application by the FDA with Priority Review^[18]. Its mechanism of action positions it well to engage the GSK3/ β -TrCP axis by preventing AKT-mediated GSK3 inhibition. Previously, we identified PI3K as a targetable vulnerability in NRF2-hyperactivated cisplatin-resistant HNSCC and demonstrated gedatolisib-cisplatin synergy across multiple cell lines^[19]. Here, we present evidence that gedatolisib degrades NRF2 through a GSK3-dependent, proteasome-mediated mechanism and combines effectively with cisplatin and radiation across a broad panel of HNSCC models. We further report gedatolisib's effectiveness in a novel therapeutic model – an orthotopic tongue xenograft model of cisplatin-resistant HNSCC – designed to evaluate a targeted agent in combination with fractionated radiation.

Methods

Cell lines. HN30 (RRID:CVCL_5525), HN31 (RRID:CVCL_5526), PCI-15B (RRID:CVCL_C756), MSK-922 (RRID:CVCL_6989), SCC152 (RRID:CVCL_C058), SCC154 (RRID:CVCL_2230), FaDu (RRID:CVCL_1218), UM47, Detroit 562, MDA 1986 LN, and MDA 886 LN were maintained in complete DMEM supplemented with 10% fetal bovine serum (FBS), 1% vitamins, sodium pyruvate, penicillin-streptomycin, L-glutamine, and non-essential amino acids. The cisplatin-resistant derivatives HN30R8 and HN31P10 were maintained in cisplatin as previously described^[11, 19]. Mouse oral carcinoma cell lines MOC1 (RRID:CVCL_ZD32) and MOC2 (RRID:CVCL_ZD33) were maintained in IMDM and Ham's F12 at a 2:1 ratio, supplemented with 5% FBS, 1% penicillin-streptomycin, insulin, hydrocortisone, and epidermal growth factor. All cells were routinely passaged in medium containing 0.2% Myco-Zap (Lonza) and authenticated by Short Tandem Repeat (STR) profiling every 3 months. Cells were cultured at 37 degrees C in a humidified atmosphere with 5% CO₂. Genetically engineered mouse model (GEMM)-derived cell lines 582679C (*Keap1*^{+/+}), 582579E (*Keap1*^{fl/fl}), 600714F (*Keap1*^{fl/fl}), and 600898C (*Keap1*^{fl/fl}) were established from primary oral tumors arising in different GEMMs of HNSCC. *Keap1*^{+/+} cell line was derived from the K5TP (*Krt5*^{CreER-T2/+}; *Trp53*^{fl/fl}; *Rosa26*^{LSL-PIK3CA(H1047R)/LSL-PIK3CA(H1047R)}; *Keap1*^{+/+}) model, while *Keap1*^{fl/fl} cell lines were derived from K5TPK (*Krt5*^{CreER-T2/+}; *Trp53*^{fl/fl}; *Rosa26*^{LSL-PIK3CA(H1047R)/LSL-PIK3CA(H1047R)}; *Keap1*^{fl/fl}) model. These were generated by inducing primary oral tumors through injection of 4-hydroxytamoxifen (4-OHT) into the oral buccal mucosa, with or without benzo[a]pyrene (BAP)^[20]. These lines were maintained in complete DMEM supplemented with 10% FBS, 1% gentamicin-amphotericin B, and 0.2% MycoZap at 37°C with 5% CO₂.

Western blot. Cells were lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad), blocked with 5% skim milk in TBS-T, and

incubated overnight at 4 degrees C with primary antibodies (Supplementary Table S1). After washing, membranes were treated with HRP-conjugated secondary antibodies (Supplementary Table S1). Protein bands were visualized using enhanced chemiluminescence reagent (Bio-Rad) and imaged on a ChemiDoc system (Bio-Rad). β -actin served as the loading control. For nuclear and cytoplasmic fractionation experiments, α -tubulin and Histone H3 (or Lamin A/C) served as compartment-specific controls.

Drug combination assay and Bliss independence analysis. Cells were seeded in 96-well plates and treated in a checkerboard format with cisplatin (0 to 2 μ M for human lines, 0 to 4 μ M for murine lines) and gedatolisib (0 to 1000 nM). Viability was measured after 7 days by resazurin assay. Drug interaction was quantified using the Bliss independence model with delta Bliss synergy values and 90% bootstrapped confidence thresholds, classifying interactions as strong synergy (delta Bliss > 10), moderate (> 5), or weak (> 2) [19].

siRNA knockdown. Cells were transfected with ON-TARGETplus Non-targeting Control Pool (siNT, D-001810-10-20) or ON-TARGETplus siRNAs SMARTPool targeting GSK3- α (L-003009-00-0020), GSK3- β (L-003010-00-0020), β -TrCP (L-003463-00-0020), or SPOP (L-017919-00-0020), individually or in combination from Horizon, using standard lipofection protocols. Knockdown efficiency was confirmed by Western blot prior to downstream experiments. For combination experiments, transfected cells were treated with gedatolisib (100 nM) 48 hours after transfection and harvested at the indicated time points.

Quantitative RT-PCR. Total RNA was extracted and reverse transcribed using standard methods. Quantitative RT-PCR was performed for NFE2L2, GPX2, and NQO1, with expression normalized to housekeeping genes and presented as fold change relative to DMSO controls.

Orthotopic xenograft mouse models of oral cancer and therapy. All animal experiments were conducted under IACUC-approved protocols at Baylor College of Medicine. For the orthotopic model, mice received 5×10^4 HN30R8 cells injected into the oral tongue. Once tumors reached approximately 0.5 mm³, mice were randomly assigned to treatment groups using the NCI Randomization Tool. Gedatolisib was administered at 20 mg/kg via tail vein injection twice weekly. Cisplatin was administered at 4 mg/kg i.v. weekly. Tumor volume was measured every three days by an investigator blinded to treatment, using the formula $V = (L \times W^2)/2$. For the radiation combination study, gedatolisib (20 mg/kg) was administered prior to each radiation fraction. Radiation was delivered at 6 Gy per fraction over 4 consecutive days (total 24 Gy; Biological effective dose (BED)₁₀ = 38.4 Gy₁₀; EQD2 = 32 Gy).

Orthotopic syngeneic mouse models of oral cancer and therapy. Mice were injected with 2×10^5 MOC1 cells into the oral tongue. Once tumors reached approximately 0.5 mm³, mice were randomly assigned to treatment groups using the NCI Randomization Tool. Gedatolisib was administered at 20 mg/kg via intraperitoneal injection twice weekly and prior to each radiation fraction. Radiation was delivered at 6 Gy per fraction over 4 consecutive days (total 24 Gy; Biological effective dose (BED) at α/β ratio of 10 = 38.4 Gy; EQD2 = 32 Gy). Tumor volume was measured every three days by an investigator blinded to treatment, using the formula $V = (L \times W^2)/2$.

Genetically engineered mouse models. Two GEMMs of HNSCC were used to evaluate the efficacy of gedatolisib *in vivo*: (1) K5TP ($Krt5^{CreER-T2/+}; Trp53^{fl/fl}; Rosa26^{LSL-PIK3CA(H1047R)/LSL-PIK3CA(H1047R)}; Keap1^{+/+}$) and K5TPK ($Krt5^{CreER-T2/+}; Trp53^{fl/fl}; Rosa26^{LSL-PIK3CA(H1047R)/LSL-PIK3CA(H1047R)}; Keap1^{fl/fl}$). Tumors were induced by injection of 4-OHT with or without BAP into the oral buccal mucosa as we described in our prior publication (Please cite GEMM paper, PMID: 4074237). Mice were treated with gedatolisib (20 mg/kg i.p., twice weekly for 8 weeks) or vehicle. Time to tumor quadrupling served as the primary endpoint [20].

Statistical Analysis. All *in vivo* tumor growth studies were analyzed using a standardized analytical framework. For studies with two treatment groups (GEMM efficacy, cisplatin combination replicate), Welch's t-test (unequal variance assumption) was used with Mann-Whitney U as non-parametric confirmation. For studies with four treatment arms (cisplatin factorial, radiation factorial, MOC1), two-by-two factorial ANOVA (Type II) with Tukey HSD post-hoc testing was used to evaluate main effects, interactions, and all pairwise comparisons. Effect sizes were reported as Cohen's d for two-group comparisons and partial eta-squared for ANOVA models. Four primary endpoints were evaluated across all *in vivo* studies: (1) tumor volume at pre-specified timepoints; (2) area under the tumor growth curve (AUC), which integrates cumulative tumor burden over time; (3) time to tumor volume threshold (TTV), which measures how long tumors take to reach clinically relevant sizes; and (4) restricted mean survival time (RMST). All endpoints were analyzed on both raw and log2 scales. For animals sacrificed before a study endpoint, last observation carried forward (LOCF) imputation was applied, which provides a conservative estimate by freezing tumor growth at the sacrifice value. Analyses at timepoints with zero or minimal imputation were prioritized for primary conclusions.

Synergy between treatment modalities was assessed using the interaction term in factorial ANOVA models. The log2 (multiplicative) scale was considered the biologically appropriate model for assessing drug-radiation interactions, given the exponential nature of tumor cell growth, where independent mechanisms of cell kill combine multiplicatively rather than additively. For *in vitro* drug combination assays, Bliss independence analysis was performed with delta Bliss synergy values and 90% bootstrapped confidence intervals, classifying interactions as strong (delta Bliss > 10), moderate (> 5), or weak (> 2) [19]. For RT-qPCR data, ANOVA with post-hoc Dunnett's test was used for multi-group comparisons. All analyses were performed in GraphPad Prism and MATLAB.

Results

Gedatolisib has potent anti-tumor activity against both NRF2 wildtype and hyperactivated HNSCC genetically engineered mouse models (GEMMs). To examine gedatolisib efficacy in an immunocompetent setting and across different NRF2 activation states, we tested gedatolisib in two GEMM-derived models of HPV-negative HNSCC: K5TP ($Keap1^{+/+}$) and K5TPK ($Keap1^{fl/fl}$). Both models carry conditional Trp53 deletion and express oncogenic PIK3CA(H1047R), mirroring the molecular landscape of human HNSCC. K5TPK mice also have conditional Keap1 deletion, resulting in constitutive

NRF2 hyperactivation^[20]. *In vivo* anti-tumor efficacy of gedatolisib was analyzed using 4 different approaches (Supplementary Table S2), tumor volume at a fixed time point, area under the tumor volume growth curve (AUC), time to reach pre-specified tumor volumes (TTV) to better differentiate tumor growth rates, and time to tumor quintupling (e.g., Fig. 1). In the K5TP model (*Keap1* wild-type), gedatolisib produced dramatic tumor regression (Fig. 1; **Supplementary Fig. 1**). By day 14, control tumors averaged 170.6 mm³ while gedatolisib-treated tumors averaged 34.9 mm³ (**Supplementary Fig. 1, Supplementary Table S2**), a 4.9-fold difference ($p = 0.013$, Cohen's $d = 1.40$). Two of seven treated animals achieved complete regression to 0 mm³, sustained for 25 to 46 days before eventual regrowth. Time-to-volume analysis at 150 mm³ produced the largest effect size in the study: gedatolisib delayed threshold crossing by 68 days (17.9 vs. 85.8 days, $p = 3.52 \times 10^{-9}$, Cohen's $d = 7.27$). All 24 volume and AUC comparisons across four timepoints were significant (all $p < 0.013$), though all tumors eventually regrew, with treated animals reaching sacrifice criteria between day 88 and day 116 (Fig. 1C-D; **Supplementary Table S2**).

In the K5TPK model (*Keap1* deleted, NRF2 hyperactivated), the treatment effect was growth suppression. At day 14, control tumors averaged 128.8 mm³ compared to 50.8 mm³ for gedatolisib-treated tumors (**Supplementary Fig. 1, Supplementary Table S2**), a 2.5-fold difference ($p = 0.0008$, Cohen's $d = 1.88$), with cumulative AUC yielding the strongest time-anchored result ($p = 6.27 \times 10^{-5}$, Cohen's $d = 2.82$). TTV analysis showed that gedatolisib delayed progression to 175 mm³ by approximately 40 days (22.0 vs. 61.5 days, $p = 5.5 \times 10^{-5}$, Cohen's $d = 3.64$). All 16 volume and AUC comparisons were highly significant (all $p < 0.008$, all Cohen's $d > 1.4$). Individual treatment responses showed heterogeneity, with strong responders maintaining volumes below 60 mm³ at day 28 and weak responders reaching 112 to 137 mm³ (Fig. 1E-F; **Supplementary Table S2**). This pattern supports the hypothesis that NRF2 hyperactivation driven by *Keap1* loss partially buffers the cytotoxic effects of PI3K/mTOR inhibition. Gedatolisib retained significant antitumor activity even in the *Keap1*-deleted setting, indicating that its effects are not fully dependent on the canonical KEAP1-mediated NRF2 regulation pathway and that the KEAP1-independent GSK3/ β -TrCP degradation route remains operative.

Gedatolisib suppresses proliferation and induces necrotic cell death. Gedatolisib inhibited proliferation in a concentration-dependent manner across all tested HNSCC cell lines, with phase confluency suppression evident at concentrations as low as 20 to 40 nM in the most sensitive lines (**Supplementary Fig. 2A-G**). Label-free holotomographic imaging of HN30R8 cells over approximately 58 hours following 3 days of drug pretreatment showed that gedatolisib produced dose-dependent suppression of proliferation and progressive loss of viability. Cell death across all treated conditions was predominantly necrotic, with minimal apoptotic events detected (**Supplementary Fig. 3A-C**). Subcellular analysis revealed increased mitochondrial counts per cell in all gedatolisib-treated conditions, while lipid droplet dynamics varied by concentration (**Supplementary Fig. 3D-F**). High-content morphological profiling by Cell Painting Assay demonstrated dose- and time-dependent alterations in DNA content consistent with cell cycle arrest across HN31P10, PCI15B, HN30R8, and FaDu (**Supplementary Fig. 4A-B**). MitoTracker phenotypic profiling showed concentration-dependent mitochondrial changes, with the magnitude of phenotypic deviation from vehicle increasing at higher concentrations and longer treatment durations

(**Supplementary Fig. 4C; Supplementary Table S3-8**). Gedatolisib also induced substantial nuclear morphological changes in irradiated HN30R8 cells following irradiation (8Gy) (**Supplementary Fig. 5A-B; Supplementary Table S9**). Complete Nanolive imaging data for all metrics are provided in **Supplementary Table S10**. Given the variation in baseline NRF2, GPX2, and NQO1 protein levels across cell lines (**Supplementary Fig. 6A-B**) and their distinct phenotypic responses to gedatolisib, we next investigated the underlying molecular mechanism, comparing cell lines with intact KEAP1 to those with constitutive NRF2 hyperactivation.

Gedatolisib reduces NRF2 protein through PI3K/AKT inhibition without suppressing NFE2L2 transcription. Gedatolisib produced dose-dependent reduction in phosphorylated AKT (Ser473) across all cell lines (Fig. 2A), confirming on-target PI3K/AKT inhibition. Concurrent with p-AKT loss, NRF2 protein was reduced across the panel with variable magnitude and kinetics (Fig. 2A). NRF2 reduction was more pronounced at 24 and 48 hours in HN31P10, FaDu, PCI15B, and MOC1 (Fig. 2A), as well as in HN30R8 and murine cell lines 582679 C (*Keap1^{+/+}*), 582579 E (*Keap1^{fl/fl}*), and 600714F (*Keap1^{fl/fl}*) (**Supplementary Fig. 7A**), suggesting NRF2 downregulation follows AKT inhibition with a temporal delay. Radiation alone (8 Gy) increased NRF2 protein in most cell lines, consistent with ROS-mediated stabilization (Fig. 2B). Pretreatment with gedatolisib prevented this accumulation (Fig. 2B; **Supplementary Fig. 7B**) and similarly attenuated NRF2 induction by 5 μ M benzo[a]pyrene and 5% cigarette smoke extract (Fig. 2C). Nuclear/cytoplasmic fractionation confirmed that gedatolisib reduced nuclear NRF2 accumulation and prevented radiation-driven nuclear translocation in HN31 and FaDu cells (Fig. 2D).

To determine whether NRF2 protein reduction was driven by transcriptional suppression, RT-qPCR was performed for *NFE2L2* and its downstream targets *GPX2* and *NQO1* in FaDu and HN31P10. Rather than decreasing, *NFE2L2* mRNA was significantly upregulated in a dose- and time-dependent manner, while *GPX2* and *NQO1* mRNA did not change substantially (**Supplementary Fig. 7C**). This paradoxical transcriptional increase despite concurrent protein loss demonstrates that gedatolisib-mediated NRF2 reduction occurs through a post-translational mechanism, with degradation outpacing the compensatory increase in transcription. As FoxO proteins can regulate NRF2, we investigated whether gedatolisib effects might be driven through this mechanism. Gedatolisib increased FoxO1 and FoxO3a protein levels in MSK922, PCI15B and HN31P10 at 6 hours, consistent with loss of AKT-mediated phosphorylation and subsequent stabilization (Fig. 2E). Nuclear/cytoplasmic fractionation confirmed dose-dependent nuclear accumulation of both FoxO1 and FoxO3a in HN30R8 and HN30 (Fig. 2F), as well as HN31P10 and FaDu (**Supplementary Fig. 7D**). These findings suggest that gedatolisib suppresses basal and stress-induced NRF2 signaling through a post-translational mechanism downstream of PI3K/AKT.

Gedatolisib activates GSK3, promoting proteasome-dependent NRF2 degradation. Gedatolisib reduced inhibitory GSK3 phosphorylation in human and murine HNSCC lines (Fig. 3A, **Supplementary Fig. 8A**) in a site- and cell line-dependent manner (**Supplementary Fig. 8A**), with some lines showing preferential changes in p-GSK3- α (Ser21) and others in p-GSK3- β (Ser9). The overall pattern supports that gedatolisib shifts the balance toward GSK3 activation. Co-treatment with the GSK3 inhibitor (CHIR-99021; 5 or 10

μM) rescued NRF2 protein levels from gedatolisib-induced loss in both PCI15B and HN31P10, confirming that GSK3 activity is necessary for gedatolisib-mediated NRF2 degradation. Total PDK1 levels were monitored as a marker of on-target PI3K pathway inhibition (Fig. 3B) as we previously reported.[21] Treatment with the proteasome inhibitor MG132 (10 μM) similarly rescued NRF2 accumulation in HN30R8, HN30, FaDu, and PCI15B (Fig. 3C). When GSK3-α, GSK3-β, or both isoforms were knocked down (confirmed in **Supplementary Fig. 9A**) prior to gedatolisib treatment, the drug's ability to reduce NRF2 was attenuated, with the most complete rescue observed under dual GSK3 knockdown (Fig. 4A). These results confirm that gedatolisib-induced NRF2 loss requires GSK3 activity downstream of AKT inhibition. Knockdown of either β-TrCP or SPOP (confirmed in **Supplementary Fig. 9B**) partially rescued NRF2 from gedatolisib-induced degradation, and combined knockdown of both adaptors provided the most complete rescue across all three cell lines tested (Fig. 4B). These genetic data, together with the pharmacological rescue experiments, establish a pathway in which gedatolisib-mediated AKT inhibition activates GSK3, which phosphorylates NRF2 at the Neh6 domain, creating a phosphodegron recognized by β-TrCP and SPOP for ubiquitin-dependent proteasomal degradation. The redundancy between these two adaptors likely accounts for the partial rescues seen with single knockdowns and the more complete rescue only when both were co-depleted. A schematic of the complete proposed mechanism is shown in Fig. 4C.

NRF2 phosphorylation analysis confirmed that gedatolisib reduced phosphorylation at the stabilizing Ser40 site while the pro-degradation Ser344 site showed altered phosphorylation dynamics, consistent with the model (**Supplementary Fig. 10A**). PDK1 downregulation confirmed sustained PI3K pathway inhibition at 24 and 48 hours (**Supplementary Fig. 10B**), and subcellular fractionation showed redistribution of GSK3 pathway components following gedatolisib treatment (**Supplementary Fig. 10C**). Together, these results demonstrate that gedatolisib destabilizes NRF2 by activating the GSK3/β-TrCP/SPOP proteasomal degradation axis.

Gedatolisib combined with cisplatin in cisplatin-resistant HN30R8 xenografts. To evaluate gedatolisib in the setting of acquired cisplatin resistance, we established orthotopic xenografts using the cisplatin-resistant cell line HN30R8, which exhibits NRF2 hyperactivation through epigenetic and genetic reprogramming of the KEAP1/NRF2 axis^[11]. In the first experiment, mice were randomized into four treatment arms: vehicle control (n = 9), cisplatin alone (n = 9), gedatolisib alone (n = 9), or gedatolisib plus cisplatin (n = 9). Factorial ANOVA revealed that gedatolisib was the primary driver of antitumor efficacy, with a highly significant main effect at every timepoint analyzed ($p < 10^{-10}$ for AUC at day 21, partial eta-squared = 0.77). Restricted mean survival time analysis at day 54 confirmed that gedatolisib-containing arms survived approximately 11 to 17 days longer than non-gedatolisib arms (combination RMST 43.1 days vs. control 27.7 days, $p = 0.005$), while cisplatin alone provided no survival benefit (RMST 26.3 days) (**Supplementary Fig. 11A-D; Supplementary Table S11**). A second independent experiment comparing vehicle control (n = 10) to the gedatolisib plus cisplatin combination (n = 10) confirmed robust treatment efficacy. The combination group had significantly smaller tumors at every timepoint analyzed (all $p < 0.0002$, Cohen's d = 2.40 to 3.82). By day 25, combination-treated tumors averaged 13.4 mm³ versus 28.1 mm³ for controls. Time to reach 10 mm³ was delayed by 67% (20.1 vs.

12.0 days, $p = 0.0015$), and 60% of treated tumors never reached 20 mm³ through the end of the study. Notably, tumor volumes in the combination arm remained stable well beyond the cessation of treatment, suggesting durable growth suppression rather than transient cytostatic arrest (Fig. 5A-B; **Supplementary Table S12**). Although the *in vivo* factorial analysis demonstrated that gedatolisib alone accounted for the observed antitumor effect in the HN30R8 model, *in vitro* Bliss independence analysis across a broader panel of seven human HNSCC cell lines and two murine lines revealed concentration-dependent synergistic interactions between gedatolisib and cisplatin in several lines, with PCI15B and FaDu showing the strongest synergy (**Supplementary Fig. 11E-F**). The discrepancy between *in vitro* synergy and *in vivo* additivity in HN30R8 may reflect the degree of cisplatin resistance in this particular model, which was selected for its treatment-refractory phenotype.

Gedatolisib combined with hypofractionated radiation therapy (hypoRT) in HN30R8 xenografts. NRF2 hyperactivation has been mechanistically linked to radiotherapy resistance in HNSCC and was confirmed here in a pilot experiment of HN30 and HN30R8 (**Supplementary Fig. 12A-C; Supplementary Table S13**)^[20]. Clonogenic survival assays confirmed that 48-hour gedatolisib pretreatment radiosensitized HN31, PCI-15B, FaDu, and MOC1 cells in a dose-dependent manner (**Supplementary Fig. 13A-D**). To test whether gedatolisib could enhance radiation sensitivity, we combined gedatolisib with hypoRT (6 Gy × 4; 6 Gy per day for four consecutive days; 24 Gy total) in the HN30R8 xenograft model. Two independent experiments were conducted, each with four treatment arms: vehicle, gedatolisib alone, RT alone, or the combination. Gedatolisib was administered before each RT fraction. In the first experiment ($n = 9$ to 10 per arm), both gedatolisib and RT showed significant single-agent activity by factorial ANOVA (gedatolisib $p < 0.0001$; radiation $p < 0.00001$ at day 36). Tumor volume at day 36 showed 5 of 6 possible Tukey pairwise comparisons reaching significance (R -squared = 0.63), with the combination producing a mean tumor volume of 10.3 mm³ compared to 42.2 mm³ for controls, a 76% reduction. The combination was significantly better than RT alone ($p = 0.033$) and gedatolisib alone ($p = 0.014$). Radiation and gedatolisib alone produced similar tumor volumes ($p = 0.99$), indicating comparable single-agent activity. Cumulative AUC analysis at day 43 confirmed the same pattern, with the combination achieving 71% lower tumor burden than control (**Supplementary Fig. 14A-D; Supplementary Table S14**). The interaction term was not statistically significant, indicating an additive effect in this experiment.

Based on the profound effects of gedatolisib alone across all murine models, we deliberately shortened the exposure period and demonstrated in a second experiment ($n = 10$ per arm) that the combination effect was even more pronounced. At day 8, all 6 Tukey pairwise comparisons were significant (R -squared = 0.864), with every treatment group distinguishable from every other group. Combination-treated tumors averaged 0.7 mm³, representing a 39.1-fold reduction compared to controls (27.4 mm³). Gedatolisib alone reduced tumors 3.0-fold (9.2 mm³) and hypoRT alone 2.3-fold (11.9 mm³). Using the multiplicative (log2) model, the interaction term was significant at multiple timepoints (volume log2 at day 11: $p = 9.04 \times 10^{-6}$, partial eta-squared = 0.426), indicating synergistic interaction between gedatolisib and RT (Fig. 5C-F; **Supplementary Table S15**). Gedatolisib combined with hypoRT was also evaluated in the syngeneic MOC1 model, where gedatolisib was again the primary driver of antitumor efficacy. At day 22, gedatolisib-treated tumors averaged 4.6 mm³ compared to 29.6 mm³ for controls, a

6.4-fold reduction, and gedatolisib delayed time to reach 40 mm³ by 14.2 days compared to control (p = 0.010; **Supplementary Fig. 15A-D; Supplementary Table S16**). Collectively, these findings demonstrate that gedatolisib significantly enhances the therapeutic efficacy of hypo-RT by producing additive to synergistic effects in NRF2-hyperactivated HNSCC *in vitro* and *in vivo*.

Discussion

Gedatolisib, a dual PI3K/mTOR inhibitor, degrades NRF2 protein through a KEAP1-independent, GSK3-dependent mechanism and overcomes NRF2-driven treatment resistance in preclinical HNSCC models. This work connects three previously unlinked observations into a single therapeutic strategy; NRF2 hyperactivation drives chemo-radiation resistance in HNSCC [1, 5, 8], the PI3K/AKT pathway stabilizes NRF2 by maintaining GSK3 in an inactive state [13–15], and pharmacological PI3K inhibition can restore NRF2 degradation even in tumors where KEAP1 function is lost. Gedatolisib's recent FDA Priority Review for breast cancer [18] makes these findings immediately relevant to HNSCC.

The degradation mechanism operates through the GSK3/β-TrCP axis, the principal KEAP1-independent route of NRF2 turnover [13–15]. Gedatolisib relieves AKT-mediated inhibition of GSK3, allowing phosphorylation of the DSGIS motif within the NRF2 Neh6 domain, which generates a phosphodegron recognized by β-TrCP for SCF-mediated ubiquitination and proteasomal degradation [14, 15]. Our genetic data extend this model by identifying SPOP as a second E3 ligase adaptor operating in parallel with β-TrCP. This redundancy between adaptors has not been reported in NRF2 degradation, explaining why disrupting either adaptor alone was insufficient to fully rescue NRF2 levels. Gedatolisib treatment unexpectedly increased *NFE2L2* mRNA levels, likely mediated by FoxO1/FoxO3a nuclear translocation upon AKT inhibition [22]. This transcriptional compensation is consistent with the established role of FoxO transcription factors as direct AKT substrates that activate stress-response genes when released from cytoplasmic sequestration [22]. However, the net effect on NRF2 protein still favored degradation, indicating that post-translational destruction outpaces the compensatory transcriptional response. This has practical consequences for clinical development: *NFE2L2* mRNA levels alone could be a misleading pharmacodynamic biomarker, and protein-level assessment of NRF2 should be prioritized.

In vivo studies revealed a genotype-dependent therapeutic response that aligns with the proposed mechanism. *Keap1* wild-type tumors were more sensitive to gedatolisib than *Keap1*-deleted tumors, as expected, since constitutive NRF2 stabilization through *Keap1* loss creates a pool of NRF2 protein that the GSK3/β-TrCP axis cannot fully eliminate. This mirrors observations in NSCLC, where *KEAP1*-mutant tumors show reduced sensitivity to PI3K pathway inhibitors [23, 24]. Notably, gedatolisib still achieved meaningful tumor control in the *Keap1*-null background, suggesting the drug's antitumor activity extends beyond NRF2 degradation and likely involves direct suppression of PI3K/mTOR-dependent survival signaling [25, 26]. Combination data revealed distinct interaction profiles for cisplatin and radiation. Gedatolisib retained full antitumor activity in a cisplatin-resistant model where cisplatin itself contributed no detectable benefit. This finding is consistent with known mechanisms of acquired

cisplatin resistance in HNSCC, which include NRF2-driven glutathione upregulation and drug efflux pump induction that are not addressed by continued cisplatin exposure but can be circumvented by targeting the PI3K/AKT axis ^[11, 19, 27, 28]. In contrast, gedatolisib combined with radiation produced synergistic tumor suppression, supporting the hypothesis that NRF2 degradation restores radiation sensitivity by removing the antioxidant buffer that quenches radiation-induced ROS ^[20, 29]. Cells treated with gedatolisib died predominantly through necrosis with mitochondrial accumulation, consistent with metabolic catastrophe following dual PI3K/mTOR blockade, a pattern distinct from the apoptotic cell death typically induced by radiation alone.

The translational relevance of these findings is strengthened by the preclinical models used. This study is the first to evaluate a targeted agent combined with fractionated radiation in an orthotopic oral cavity HNSCC model. Nearly all prior preclinical chemoradiation studies in HNSCC, including the only previous evaluation of gedatolisib as a radiosensitizer, have used subcutaneous flank implantation ^[30, 31]. Flank models fail to recapitulate the oral cavity microenvironment, locoregional vasculature, and anatomical constraints that define head and neck irradiation in the clinical setting ^[32, 33]. The use of a cisplatin-resistant, NRF2-hyperactivated cell line mirrors the patient population most likely to benefit from this strategy, since NRF2 pathway activation and acquired platinum resistance are defining features of treatment-refractory HNSCC ^[11, 20, 34].

Several limitations warrant consideration. The radiation combination findings are based on a limited number of models, and broader validation across genetically diverse HNSCC systems is needed. The pharmacokinetic profile and normal tissue toxicity of gedatolisib combined with standard chemoradiation have not been characterized, and the therapeutic window in the head and neck region will require careful definition. Heterogeneity in GSK3 isoform dependence across cell lines suggests that not all NRF2-hyperactivated tumors will respond equally, and predictive biomarkers for patient selection will be essential for clinical development. Durability of NRF2 suppression under chronic drug exposure and potential acquired resistance mechanisms through alternative NRF2 stabilization pathways remain untested.

This study establishes gedatolisib as a pharmacological tool for degrading NRF2 through a KEAP1-independent mechanism and demonstrates its potential as a radiosensitizer in HNSCC. The identification of a dual regulatory circuit, where GSK3-driven protein degradation overcomes FoxO-driven transcriptional compensation, provides a mechanistic framework for understanding how PI3K inhibition controls NRF2 at the protein level. Given that gedatolisib is already in advanced clinical development, these findings support its evaluation in radiation-based treatment regimens for HNSCC patients whose tumors exhibit NRF2 hyperactivation or PI3K pathway dependency.

Conclusions

Our findings identify dual PI3K/mTOR inhibition as an effective strategy to suppress NRF2 signaling and overcome NRF2-driven therapeutic resistance in head and neck squamous cell carcinoma. We

demonstrate that gedatolisib promotes KEAP1-independent degradation of NRF2 through activation of the GSK3/ β -TrCP/SPOP proteasomal pathway, thereby reducing NRF2 protein levels despite compensatory transcriptional upregulation of *NFE2L2* mediated by FoxO. These results establish the PI3K/AKT/GSK3 axis as a critical regulator of NRF2 protein stability and a therapeutically actionable mechanism for targeting NRF2-addicted tumors. The antitumor activity of gedatolisib was consistently validated in multiple complementary preclinical models, including genetically engineered mouse models with conditional *Keap1* deletion, cisplatin-resistant orthotopic xenografts, and human HNSCC cell lines. Furthermore, the enhanced efficacy observed in combination with radiation supports the potential of gedatolisib to improve responses to standard-of-care chemoradiotherapy, particularly in tumors characterized by NRF2 hyperactivation.

Taken together, this study provides mechanistic and preclinical evidence supporting the clinical evaluation of gedatolisib as a chemo-radiosensitizing agent for HNSCC. Given the frequent activation of NRF2 through KEAP1 loss or PI3K pathway alterations, our findings further suggest that molecular stratification based on NRF2/KEAP1/PI3K status may help identify patients most likely to benefit from PI3K/mTOR-targeted therapies in future biomarker-driven clinical trials.

Abbreviations

GEMMs

Genetically engineered mouse models

HNSCC

Head and neck squamous cell carcinoma

EA

Eve Analytics

LCA

Live Cytotoxicity Assay

SLDA

Smart Lipid Droplet Assay

SMtA

Smart Mitochondrial Assay

Declarations

Ethics approval and consent to participate.

All animal work performed in the current manuscript was in accordance with the rules of the Baylor College of Medicine Institutional Animal Care and Use Committee.

Consent for publication.

Not applicable

Competing interests.

The authors report no conflicts of interest relevant to the current work. V.C.S. is a consultant for, and equity holder in Femtovox Inc.

Funding.

This work was supported by the Sid Richardson Foundation, by the National Cancer Institute through U54CA274321, R01CA280980 and R01CA235620, by the National Institute of Dental and Craniofacial Research through R03DE034506 and by the Veterans Affairs Biomedical Laboratory Research and Development division (1I01BX006380). Imaging for this project was supported by the Integrated Microscopy Core at Baylor College of Medicine with funding from the NIH (P30 DK056338, P30 CA125123, S100D030414). This manuscript does not represent the policies, positions, views and opinions of the US Department of Veterans Affairs or the National Institutes of Health.

Authors' contributions.

Conceptualization: MJF, VCS, PY, SYL, RP. Methodology: PY, YL, RP, KS, FE, IY, MJF. Investigation: PY, YL, RP, HD, KS, FE, IY, SYL, JNM, HDS. Visualization: PY, YL, KS, FE, IY. Supervision: MJF, VCS, RP. Writing (original draft): PY, YL, RP, MJF, VCS. Writing (review and editing): PY, YL, RP, MJF, SYL, VCS.

Acknowledgements.

The authors gratefully acknowledge Celcuity Inc. for providing gedatolisib for this study. The authors also thank the Integrated Microscopy Core Services, particularly Michael A. Mancini, Adam Thomas Szafran, Devon Zachariah Suske, and Michael James Bolt, for their technical assistance with image acquisition and processing.

Availability of data and materials.

Data generated in this study are available within the article, its supplementary files, and supplementary data tables.

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