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Targeting TORC1/2 Enhances Sensitivity to EGFR Inhibitors in Head and Neck Cancer Preclinical Models[1]

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is characterized by overexpression of the epidermal growth factor receptor (EGFR) where treatments targeting EGFR have met with limited clinical success. Elucidation of the key downstream pathways that remain activated in the setting of EGFR blockade may reveal new therapeutic targets. The present study was undertaken to test the hypothesis that inhibition of the mammalian target of rapamycin (mTOR) complex would enhance the effects of EGFR blockade in HNSCC preclinical models. Treatment of HNSCC cell lines with the newly developed TORC1/TORC2 inhibitor OSI-027/ASP4876 resulted in dose-dependent inhibition of proliferation with abrogation of phosphorylation of known downstream targets including phospho-AKT (Ser473), phospho–4E-BP1, phospho-p70s6K, and phospho-PRAS40. Furthermore, combined treatment with OSI-027 and erlotinib resulted in enhanced biochemical effects and synergistic growth inhibition *in vitro*. Treatment of mice bearing HNSCC xenografts with a combination of the Food and Drug Administration (FDA)-approved EGFR inhibitor cetuximab and OSI-027 demonstrated a significant reduction of tumor volumes compared with either treatment alone. These findings suggest that TORC1/TORC2 inhibition in conjunction with EGFR blockade represents a plausible therapeutic strategy for HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide. Despite innovations in surgery, chemotherapy, and radiation delivery, survival rates have not significantly improved in decades. Cumulative evidence to date implicates increased expression of the epidermal growth factor receptor (EGFR) as a prognostic biomarker in HNSCC [1–3]. Successful targeting of EGFR in preclinical HNSCC models led to the rapid clinical development of EGFR inhibitors in this cancer, culminating in the FDA approval of the EGFR-targeted monoclonal antibody, cetuximab, in 2006. However, despite widespread expression of EGFR in HNSCC tumors, administration of EGFR targeting agents alone (either monoclonal antibodies or tyrosine kinase inhibitors) have been associated with only modest clinical responses to date [4–6]. While the mechanisms of *de novo* or acquired resistance to EGFR inhibitors remain incompletely understood, co-activation of alternative intracellular signaling pathways may contribute to tumor growth in the setting of EGFR blockade. We and others previously reported that persistent signaling through G protein–coupled receptors contributes to oncogenic signaling despite EGFR inhibition [7,8]. Targeting key intermediates that participate in the cross talk between G protein–coupled receptor and EGFR leads to additive or synergistic enhancement of tumor growth inhibition in preclinical cancer models [8,9]. Others have demonstrated that compensatory signaling of parallel oncogenic pathways may limit the sensitivity of cancer cells to single agents [10,11].

Abbreviations: HNSCC, head and neck squamous cell carcinoma; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; 4E-BP1, eukaryotic initiation factor 4E binding protein 1

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Mammalian target of rapamycin (mTOR) is activated by both EGFR-dependent and EGFR-independent pathways, including G protein–coupled receptors, and has been implicated as a potential therapeutic target in several solid tumors including HNSCC [9,12,13]. The mTOR complex is comprised of two components, TORC1 and TORC2. TORC1 includes the regulatory associated protein of mTOR (Raptor), mammalian LST8/G protein β-subunit–like protein (mLST8/GβL), and the more recently identified partners PRAS40 and DEPTOR [14–16]. The most well-characterized targets of TORC1 include p70 s6 kinase 1 (p70s6K) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which both serve as readouts for TORC1 activity [17,18]. TORC2 includes the rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress–activated protein kinase interacting protein 1 (mSIN1) [19,20]. TORC2 phosphorylates the serine/threonine protein kinase AKT/PKB at the serine residue S473 [21]. Phosphorylation of this serine stimulates AKT phosphorylation at a threonine T308 residue by PDK1 leading to full AKT activation [21]. TORC2 mediates TORC1 activation through AKT and TSC1/2 [22], while TORC1 regulates TORC2 through the inhibitory effects of p70s6K on Rictor [23].

Rapalogs, including rapamycin (sirolimus) and RAD001, are mTOR inhibitors that are under active clinical investigation in several cancers, including HNSCC. Both rapamycin and RAD001 bind to the cytosolic FKBP12 protein, leading to engagement of the TORC1 complex and attenuation of downstream TORC1 signaling [24]. However, inhibition of this complex results in attenuation of the S6K-IRS1–negative feedback loop, which leads to stabilization of IRS1 and stimulates phosphoinositide 3-kinase (PI3K) signaling. This triggers an increase in AKT phosphorylation, activating AKT-mediated cell survival while inhibiting p70s6K-mediated cell proliferation [10]. Thus, inhibition of TORC1 pathways with concomitant activation of TORC2 pathways represents a potential limitation of rapalogs.

OSI-027 (also known as A7486) is an orally available mTOR inhibitor that competitively binds to the adenosine triphosphate (ATP)-binding domain of mTOR, inhibiting both mTOR complexes. This dual TORC1/TORC2 inhibitory function of OSI-027 presents several advantages compared with rapalogs by decreasing signaling through TORC1-mediated mechanisms. OSI-027 is currently undergoing early phase clinical testing. The present study was undertaken to investigate the activity of OSI-027 alone or in combination with EGFR inhibitors in HNSCC preclinical models. Our results suggest that both TORC1 and TORC2 signaling are inhibited by OSI-027 in a dose-dependent fashion in a panel of HNSCC cell lines. We also show that combined treatment with OSI-027 and erlotinib result in significant decreases in TORC1 and TORC2 signaling and synergistic inhibition of *in vitro* proliferation compared to either drug alone. Furthermore, the combination of OSI-027 and cetuximab demonstrate significantly improved antitumor efficacy compared with either treatment alone in an HNSCC xenograft model. These cumulative findings suggest that OSI-027 may be used in combination with EGFR inhibitors to improve treatment responses.

Materials and Methods

Cell Lines, Tumors, and Reagents

Human HNSCC cell lines included UMSCC1 [25], a kind gift from Dr Thomas Carey (University of Michigan, Ann Arbor, MI), Cal 33 from Dr Gerard Milano (Centre Antoine Lacassagne, Nice, France) [26], 686LN from Dr Georgia Chen (Emory University, Atlanta, GA), and FaDu from Dr Jeffrey N. Myers (The University of Texas MD Anderson Cancer Center, Houston, TX) [27]. UPCI-15B, Cal 33, and UMSCC1 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS (Invitrogen). FaDu cells were maintained in DMEM with 10% FBS and 1% nonessential amino acids. 686LN cell lines were maintained in DMEM/F12 with 10% FBS. All cells were incubated at 37°C in the presence of 5% CO_2. All cell lines were validated through genotyping with AmpF/STR Identifiler PCR Amplification Kit (Applied Biosystems, Carlsbad, CA). OSI-027 (also known as A7486) and erlotinib were provided by OSI-Pharmaceuticals, Inc (Farmingdale, NY; now Astellas Pharma, Deerfield, IL).

Cytotoxicity Assays

One day prior to treatment, UMSCC1, Cal 33, and FaDu cells were plated at a density of 2×10^4 cells/ml in 96-well plates. Cells were treated with half-log concentrations of OSI-027 from 0.3 nM to 10 μM, rapamycin from 0.3 μM to 30 μM, and erlotinib from 0.1 μM to 8 μM. After 3 days, cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Variable-slope sigmoidal curves were plotted and EC_{50} values were computed through nonlinear regression analysis in GraphPad Prism software (GraphPad Software Inc, La Jolla, CA).

Immunoblot Analysis

HNSCC cells UMSCC1 and Cal 33 were plated at a density of 1×10^6 cells/dish in 100-mm petri dishes 1 day prior to treatment. Cells were treated with 0.3, 0.5, 0.75, 1.0, or 1.25 μM OSI-027. DMSO controls were included. For combined treatment with OSI-027 and erlotinib, UMSCC1 cells were treated with 0.5 μM OSI-027, 3 μM erlotinib, or both. Cal 33 cells were treated with 0.5 μM OSI-027, 4.0 μM erlotinib, or both. DMSO controls were included. After 24 hours, cells were lysed as described previously [28]. Protein levels were determined using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of protein was resolved on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes using the semidry transfer machine (Bio-Rad Laboratories). Membranes were blocked with Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, NE) for 1 hour at room temperature and incubated with primary antibodies in 5% BSA in Tris-buffered saline with Tween 20 (TBS-T) solution overnight at 4°C. After washing with TBS-T three times, membranes were incubated for 1 hour with IRDye 800CW goat anti-rabbit or IRDye 680LT goat anti-mouse secondary antibody (Li-COR Biosciences) diluted 1:5000 in 5% BSA in TBS-T. Membranes were rinsed with TBS-T three times and scanned with the Odyssey Infrared Imaging System (Li-COR Biosciences). Densitometry was performed with ImageJ software (National Institutes of Health, Bethesda, MD). The following monoclonal antibodies from Cell Signaling Technology (Danvers, MA) were used at a dilution of 1:1000 in 5% BSA in TBS-T: phospho-p70s6K (Thr389) (rabbit, No. 9205); p70s6K (rabbit, No. 9202); phospho-AKT (Ser473) (mouse, No. 4051S); AKT (rabbit, No. 9272); phospho-PRAS40 (Thr246) (rabbit, No. 2997); PRAS40 (rabbit, No. 2691); phospho–4E-BP1 (Thr37/46) (rabbit, No. 2855); 4E-BP1 (rabbit, No. 9452). β-Tubulin rabbit antibody (AbCam, Cambridge, United Kingdom) diluted 1:5000 was used as a loading control.

Synergy Studies

The method of Chou and Talalay [29] was used to determine levels of interaction between OSI-027 and erlotinib. Combination index (CI) values were determined using Calcusyn software (Biosoft, Cambridge, United Kingdom) at 50% inhibition of cell growth. Synergy was indicated by a CI of <1, additivity by a CI equal to 1, and antagonism by a CI of >1.

Clonogenic Studies

Cal 33 or UMSCC1 cells were plated the day before treatment at 5000 cells per well in a six-well plate. The next day, cells were treated with the EC_{50} values of each drug for each cell line. Cal 33 cells were treated either with 3 μM erlotinib, 0.5 μM OSI-027, or a combination of both drugs. A DMSO control was included. UMSCC1 cells were treated with 4 μM erlotinib, 0.5 μM OSI-027, or a combination of both drugs. A DMSO control was also included. Cells were allowed to form colonies for 5 days. These cells were then washed in phosphate-buffered saline and stained in crystal violet. Colonies of cells containing 50 cells or greater were counted. Experiments were repeated three times for each cell line.

In Vivo *Studies*

Female athymic nude mice, 5 to 6 weeks old, were purchased from Harlan (Indianapolis, IN) and maintained in a pathogen-free facility. Animal care was in compliance with the University of Pittsburgh's Institutional Animal Care and Use Committee. Mice were inoculated subcutaneously with a 100-μl suspension of 5×10^5 cells in Hanks' balanced salt solution subcutaneously in the left flank. Tumor-bearing mice were randomized to receive 0.02 mg of cetuximab (n = 10) i.p. twice a week, OSI-027 through oral gavage daily at 50 mg/kg daily (n = 10), a combination of cetuximab plus OSI-027 (n = 10), or vehicle alone (trappsol 20% through oral gavage and saline i.p.; n = 9). Tumors were measured with a vernier caliper thrice per week and the volume was calculated through use of the equation volume = length × $(\text{width})^2/2$. Tumors were harvested at the end of the study and fixed in 10% formalin before paraffin embedding. Tumor sections were assessed for Ki67 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemical staining by a pathologist (L.W.) blinded to the treatment groups. The percent positive Ki67 staining and the average number of TUNEL-positive cells were determined for each tumor.

Statistical Analysis

In vitro and *in vivo* data were analyzed using nonparametric Kruskal-Wallis exact test with Cytel Studio 9.

Results

OSI-027 Inhibits HNSCC Growth In Vitro *in a Dose-dependent Manner*

Rapalogs (including rapamycin) have demonstrated limited cellular cytotoxicity in HNSCC cell lines with reported EC_{50} values of 5, 12, and 20 μM for the HNSCC cell lines SCC61, SQ20B, and HEP2, respectively [30]. We performed EC_{50} determinations for rapamycin in a panel of HNSCC cell lines and found similar ranges with EC_{50} values ranging from 6 to 20 μM across a panel of five HNSCC cell lines (Figure 1*A*). To determine the antiproliferative effects of the selective TORC1/2 inhibitor OSI-027 in a panel of HNSCC cell

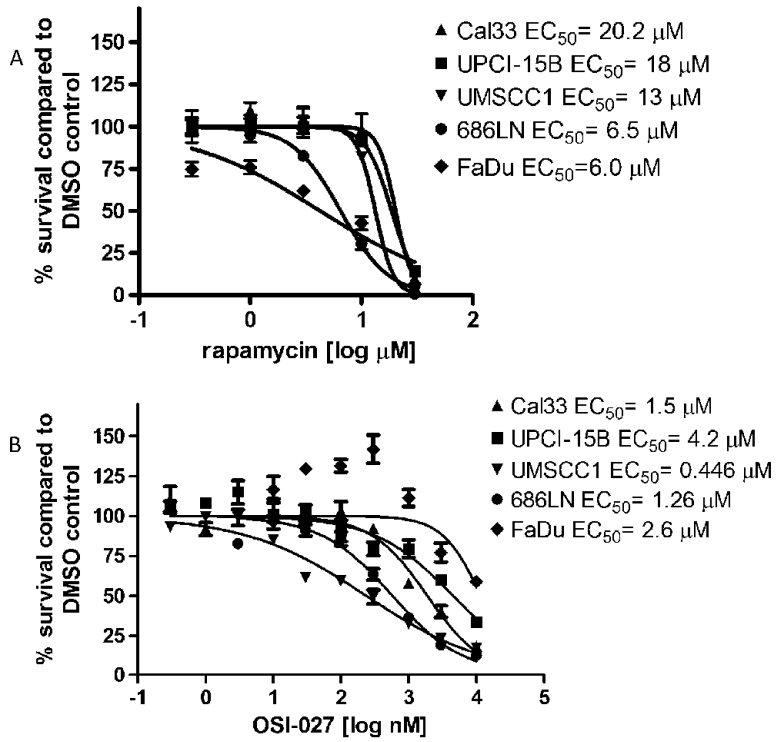


Figure 1. HNSCC cells are more sensitive to growth inhibition by OSI-027 than rapamycin. Representative HNSCC cell lines (PCI-15B, Cal 33, UMSCC1, 686LN, and FaDu) were plated at a density of 5000 cells in 100 μl in each well of a 96-well plate. Cells were treated for 3 days in triplicate with either (A) rapamycin or (B) OSI-027 followed by CellTiter-Glo Assay. For each cell line, EC_{50} values were obtained three times with similar results.

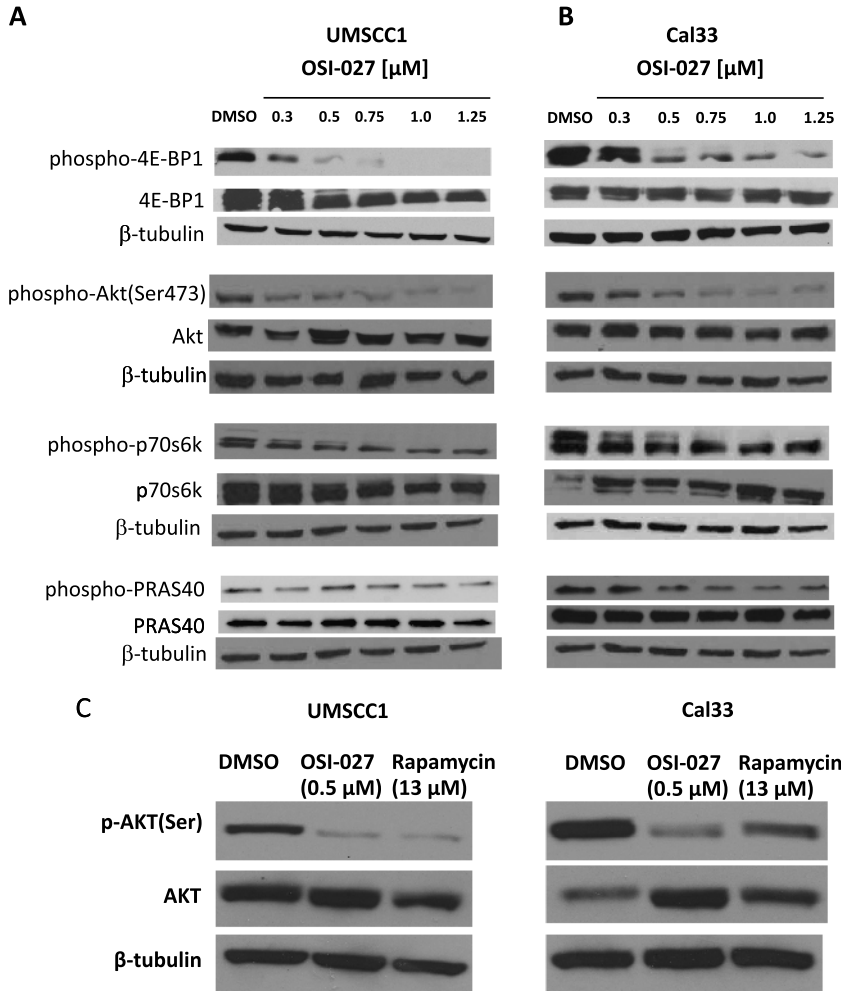


Figure 2. OSI-027 inhibits TORC1 and TORC2 downstream targets in a dose-dependent manner in HNSCC cell lines. HNSCC cells (A) UMSCC1 and (B) Cal 33 were treated for 24 hours with increasing concentrations of OSI-027 (0.3, 0.5, 0.75, 1, and 1.25 μM) or DMSO as a control. Western blot analysis was then performed for phosphorylated and total levels of p70s6K, AKT (Ser473), PRAS40, and 4EBP1. β-Tubulin was included as a loading control. (C) HNSCC cells (UMSCC1 and Cal 33) were treated with EC_{50} values of OSI-027 (0.5 μM for UMSCC1 and Cal 33) or rapamycin (13 μM for UMSCC1 and 20 μM for Cal 33). DMSO was used as a vehicle control. Cells were treated for 24 hours followed by Western blot analysis for phospho-AKT (Ser473) and total AKT. β-Tubulin was included as a loading control. The experiment was repeated three times with similar results.

lines compared to rapamycin, we treated the same cells with increasing concentrations of OSI-027 followed by assessments of cell growth 72 hours after the addition of the agent. As shown in Figure 1*B*, OSI-027 exhibited reproducible dose-dependent cytotoxicity with EC_{50}s in the nanomolar to low micromolar range. These values are consistent with the concentrations reported in leukemic and other solid tumor cell lines [31,32] and suggest that OSI-027 inhibits HNSCC cell line proliferation, more potently than rapamycin.

OSI-027 Inhibits TORC1 and TORC2 Downstream Targets in a Dose-dependent Manner in HNSCC Cell Lines

Previous studies of mTOR inhibition in HNSCC models have generally used rapalogs such as rapamycin or RAD-001 [33–37]. OSI-027, a novel inhibitor of the ATP-binding pocket, has not been studied in HNSCC. To evaluate the potency of OSI-027 in HNSCC preclinical models, we treated representative HNSCC cells (Cal 33 and UMSCC1) with a range of concentrations of OSI-027 (0.3–1.25 μM) for 24 hours followed by biochemical assessment of expression of total and phosphorylated forms of TORC1 and TORC2 substrates. As shown in Figure 2, treatment of HNSCC cells with increasing concentrations of OSI-027 was associated with decreased phosphorylation of downstream TORC1 and TORC2 signaling proteins, indicating mitigation of mTOR signaling by this compound. To determine if OSI-027 more potently decreased TORC1/2 signaling in HNSCC compared with rapalog treatment, we assessed activation of phospho-AKT (Ser473) in UMSCC1 and Cal 33 cells treated with EC_{50} concentrations of OSI-027 or rapamycin. As shown in Figure 2*C*, OSI-027 reduced expression of phospho-AKT (Ser473) at much lower concentrations (0.5 μM) compared to rapamycin (13 μM).

Combined Treatment with OSI-027 and Erlotinib Results in Synergistic Inhibition of HNSCC Cell Survival

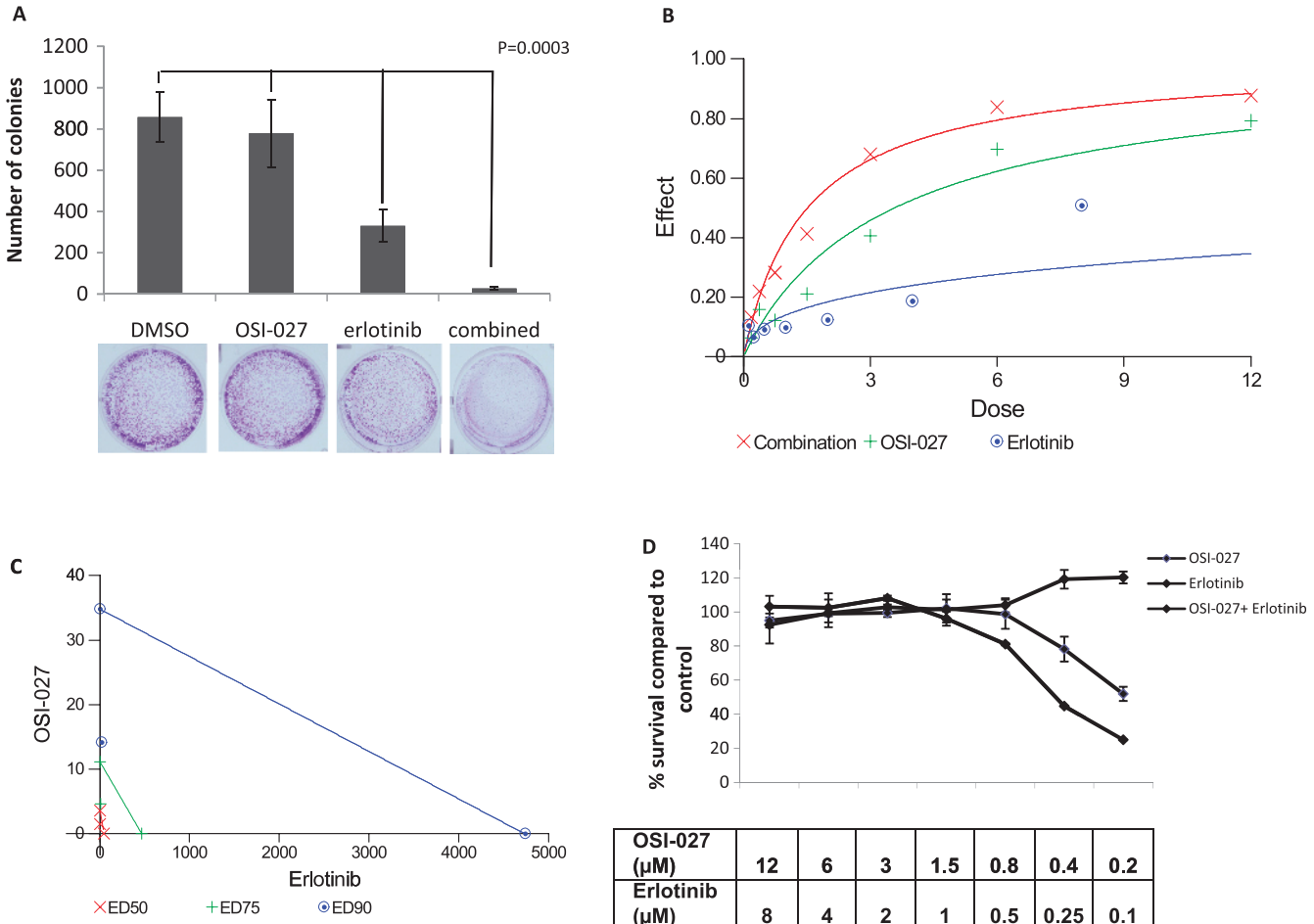
Targeting two pathways that both contribute to cell survival can lead to additive or synergistic growth inhibition. Rapamycin has been shown to synergize with erlotinib in a variety of solid tumors [10]. We have shown that the combination of RAD001, a TORC1 inhibitor, in combination with erlotinib resulted in enhanced decreases in cell survival, but these effects were only additive [38]. To determine the anti-

survival effects of combined TORC1/2 and EGFR inhibition, we performed clonogenic assays using OSI-027 and erlotinib. Representative HNSCC cells (UMSCC1 and Cal 33) were treated with 0.5 μM OSI-027 or erlotinib (3 μM for UMSCC1 and 4 μM for Cal 33) or a combination of OSI-027 plus erlotinib for 5 days followed by assessment of colony formation. As shown in Figure 3, *A* and *E*, inhibition of both EGFR and TORC1/2 pathways demonstrated a significantly reduced number of colonies compared with either agent alone (Cal 33, $P = 0.0004$; UMSCC1, $P = 0.0003$).

To further investigate the enhanced antiproliferative effects of OSI-027 and erlotinib in HNSCC cell lines, we treated UMSCC1 and Cal 33 cells with decreasing half-log concentrations of OSI-027, erlotinib, or a combination of the two drugs. CellTiter-Glo Assay was then performed and the data were analyzed by the method of Chou and Talalay. As shown in Figure 3, *B* and *F*, dose-response graphs show an enhanced effect. We also demonstrate that this effect was synergistic, as isobolograms show combination points well into the synergistic range for ED90, ED75, and ED50 inhibition (Figure 3, *C* and *G*). Additionally, at each combination of OSI-027 and erlotinib, CI values for each data point were less than 1.0 over a range of drug concentrations that included the EC_{50}s of both drugs (Figure 3, *D* and *H*).

Combined Treatment with OSI-027 and Erlotinib Enhances Inhibition of TORC1 and TORC2 Downstream Targets in HNSCC Cell Lines

Rapalogs have been combined with EGFR inhibitors in solid tumor cell lines and xenografts and have been shown to enhance the antitumor effects of EGFR inhibitors such as erlotinib [10]. However, the combined effect of OSI-027 and EGFR inhibitors has not previously been studied in HNSCC. Allosteric mTORC1 inhibitors combined with EGFR inhibitors resulted in enhanced down-regulation of TORC1 markers. However, this combination was shown to increase AKT phosphorylation [10,39]. To determine the biochemical consequences of targeting both TORC1 and TORC2 in conjunction with EGFR in HNSCC cells, representative cell lines (UMSCC1 and Cal 33) were treated with half of the EC_{50} concentrations of OSI-027



OSI-027 (μM)	12	6	3	1.5	0.8	0.4	0.2
Erlotinib (μM)	8	4	2	1	0.5	0.25	0.1
CI	0.16	0.13	0.27	0.74	0.92	0.76	0.98

Figure 3. Combined treatment with OSI-027 and erlotinib results in synergistic inhibition of cell survival. HNSCC cells (A–D) UMSCC1 and (E–H) Cal 33 were plated in 96-well plates at a density of 1000 cells per well in four wells of a six-well plate in DMEM + 10% FBS. The next day, cells were treated with 0.5 μM OSI-027, erlotinib (3 μM for UMSCC1 cells and 4 μM for Cal 33 cells), or a combination of both drugs at these concentrations for 5 days. Differences in treatment groups were tested through the Kruskal-Wallis exact test (Statxact software, Version 7). Dose-effect curves and isobolograms demonstrate synergistic effects of combined treatment. Synergy was determined using the CI values of Chou and Talalay with values less than 1 indicating synergy. The experiment was repeated at least three times with similar results.

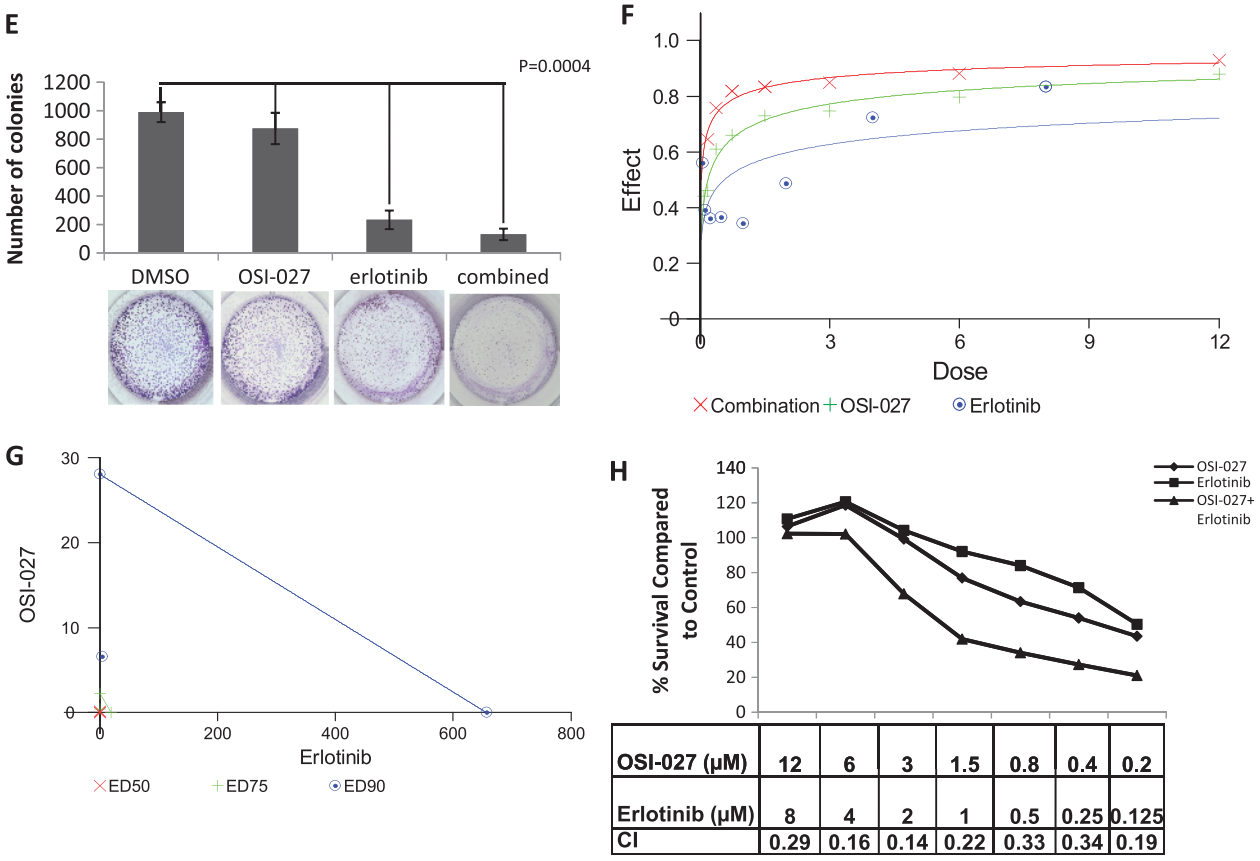


Figure 3. (*continued*).

(0.5 μM) and/or erlotinib (3 μM for UMSCC1 and 4 μM for Cal 33) for 24 hours followed by immunoblot analysis. As shown in Figure 4, *A* and *B*, combined inhibition of EGFR and TORC1/2 resulted in a significant decrease in the activation of both TORC1 and TORC2 substrates when compared to either treatment alone or DMSO control (UMSCC1: $P = 0.0014$ for phospho-AKT (Ser473), $P = 0.00006$ for phospho-p70s6K, and $P = 0.003$ for phospho-PRAS40; Cal 33: $P = 0.006$ for phosho-AKT (Ser473), $P = 0.014$ for phospho-p70s6K, and $P = 0.038$ for phospho-PRAS40).

The Combination of OSI-027 and Cetuximab Significantly Enhances Antitumor Effects in HNSCC In Vivo

OSI-027 was recently reported to demonstrate superior inhibition of tumor growth in breast and colon cancer models compared with rapamycin [31]. Others have shown a decrease in tumor burden when mice bearing human tumor xenografts were treated with a combination of rapamycin and erlotinib [10]. Treatment of HNSCC xenografts with rapalogs in combination with EGFR inhibition has demonstrated only modest enhanced effects [38,40]. Given the superior antiproliferative and biochemical effects of TORC1/TORC2 inhibition in combination with EGFR blockade *in vitro*, we next assessed the *in vivo* effects using cetuximab, which is FDA approved for the treatment of HNSCC. Mice were inoculated with a representative HNSCC cell line, Cal 33, randomized to one of four treatment groups including control ($n = 9$), OSI-027 alone (50 mg/kg daily by oral gavage, $n = 10$), cetuximab alone (0.02 mg twice/week, i.p., $n = 10$), or a combination of both OSI-027 and cetuximab using the same concentrations and dosing schedules ($n = 10$). As shown in Figure 5*A*, starting on day 9, tumor volumes were significantly decreased in the group receiving both OSI-027 plus cetuximab combined treatment compared to either treatment alone or vehicle control ($P = 0.001$). In addition, we examined Ki67 labeling and TUNEL staining in the xenografts and found that Ki67 labeling was reduced by treatment with cetuximab alone or combined cetuximab plus OSI-027 compared with vehicle control or OSI-027 alone (Figure 5*B*). TUNEL staining was decreased in all treatment groups (compared with vehicle control) to a similar degree (Figure 5*C*).

Discussion

EGFR is upregulated in HNSCC where expression levels generally correlate with decreased survival. Despite extensive evidence of the antitumor effects of EGFR targeting in HNSCC preclinical models, EGFR inhibition to date has been associated with limited clinical benefit. One plausible mechanism for the modest activity of EGFR inhibitors is the compensatory activation of alternative signaling pathways in the setting of EGFR blockade [10]. It has been demonstrated in a variety of cancers that EGFR inhibition results in increased AKT phosphorylation with subsequent mTOR activation [40,41]. In fact, while mTOR itself is infrequently mutated in most cancers, hyperactivation of mTOR can be detected in many tumors or cell lines, including those characterized by mutations of PI3K or AKT or loss of PTEN [42–44]. Targeting mTOR in conjunction with EGFR represents a rational approach to abrogate increased activation through the PI3K-AKT-mTOR pathway.

The results of the present study demonstrated that all HNSCC cell lines tested were sensitive to the antiproliferative effects of TORC1/TORC2 inhibition using OSI-027 with EC_{50} values ranging from

0.45 to 4.2 μM. In addition, growth inhibition was accompanied by decreased activation of downstream biochemical markers of TORC1 and TORC2 in a dose-response manner, demonstrating that not only does OSI-027 decrease growth but also does so in a manner that is consistent with its putative mechanism of action. Combined treatment of HNSCC cells with OSI-027 and erlotinib further decreased TORC1 and TORC2 activation when compared to either treatment alone. Using the method of Chou and Talalay, combinations of OSI-027 and erlotinib resulted in synergistic inhibition of HNSCC cell proliferation. *In vivo* studies using the FDA-approved EGFR targeting agent cetuximab demonstrated enhanced antitumor effects, suggesting that EGFR inhibition in combination with TORC1/TORC2 blockade is a plausible therapeutic strategy for the treatment of HNSCC.

In leukemic cell lines, TORC1/TORC2 blockade demonstrated improved antitumor effects compared with TORC1 inhibition using rapamycin [41]. While rapamycin was effective at a growth inhibitory concentration (GI) of 6.5 nM, it reached a plateau efficacy at 60% [41]. The combined TORC1/2 inhibitor PP242 suppressed growth by 90%, still maintaining a GI in the low nanomolar range in leukemic cells (12 nM) [41]. In the same study, similar results were observed for the combined PI3K-TORC1/2 inhibitor PI-103 (GI of 86 nM). These findings suggest that leukemic cell lines are more sensitive to the effects of dual TORC1/2 compared with TORC1 inhibition alone. In another study, the TORC1/2 inhibitor Ku-0063794 showed similar efficacy to PP242 with a GI in the nanomolar range in a leukemic cell line [45]. Vakana et al. reported that, in Ph+ leukemias, OSI-027 inhibited proliferation in both imatinib-sensitive and imatinib-resistant cells [46]. In the only prior study of OSI-027 in solid tumor models (but not HNSCC), IC_{50} values ranged from 0.5 to 4.5 μM [31]. We found similar EC_{50} values for all five HNSCC cell lines tested (0.45–4.2 μM). In the present study, we found that OSI-027 potently inhibited HNSCC cell proliferation at EC_{50} ranges far below those previously reported for single-arm TORC inhibitors such as rapamycin [30]. Our own investigation into the efficacy of rapamycin corroborates the ability of OSI-027 to inhibit growth at lower rapamycin concentrations ranging from 6.5 to 20 μM in HNSCC. These results indicate that dual targeting of TORC1 and TORC2 may be more efficacious in solid tumors such as HNSCC, compared with TORC1 inhibition alone. The biochemical effects of combined TORC1/2 inhibition have been reported to consist of dose-dependent decreases

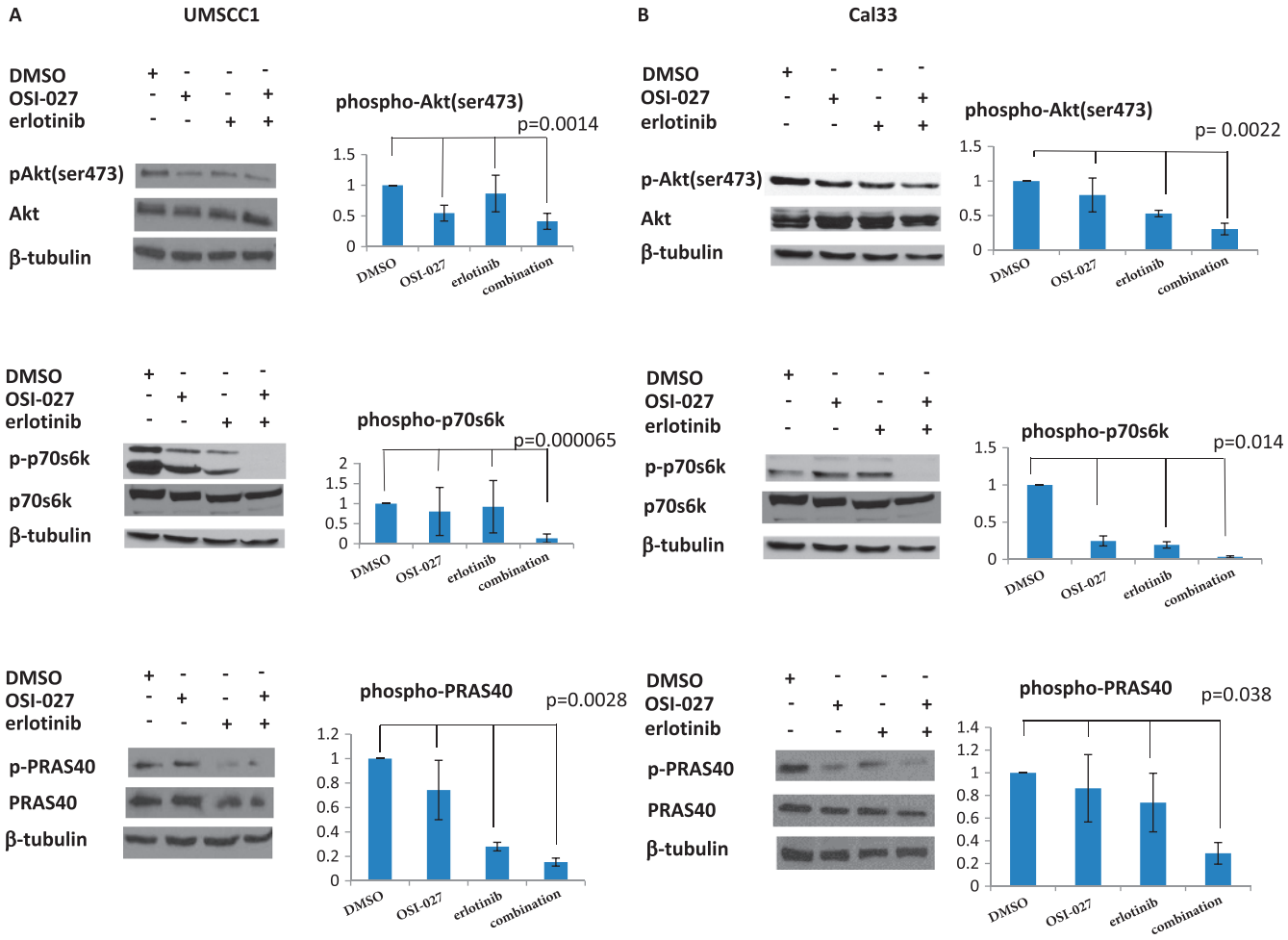


Figure 4. Combined treatment with OSI-027 and erlotinib enhances inhibition of TORC1 and TORC2 downstream targets in HNSCC cell lines. HNSCC cells (A) UMSCC1 and (B) Cal 33 were treated with (0.5 μM for UMSCC1 and Cal 33), erlotinib (3 μM for UMSCC1 and 4 μM for Cal 33), or a combination of the two drugs at these concentrations. DMSO was used as a control. Cells were treated for 24 hours followed by Western blot analysis for known TORC1 and TORC2 substrates. Cells were probed for phospho-AKT (Ser473) and total AKT, phospho-PRAS40 and total PRAS40, phospho-p70s6K and total p70s6K, and phospho–4E-BP1 and total 4E-BP1. Differences in treatment groups were tested through the Kruskal-Wallis exact test (Statxact software, Version 7). β-Tubulin was included as a loading control. Experiments were repeated three times with similar results.

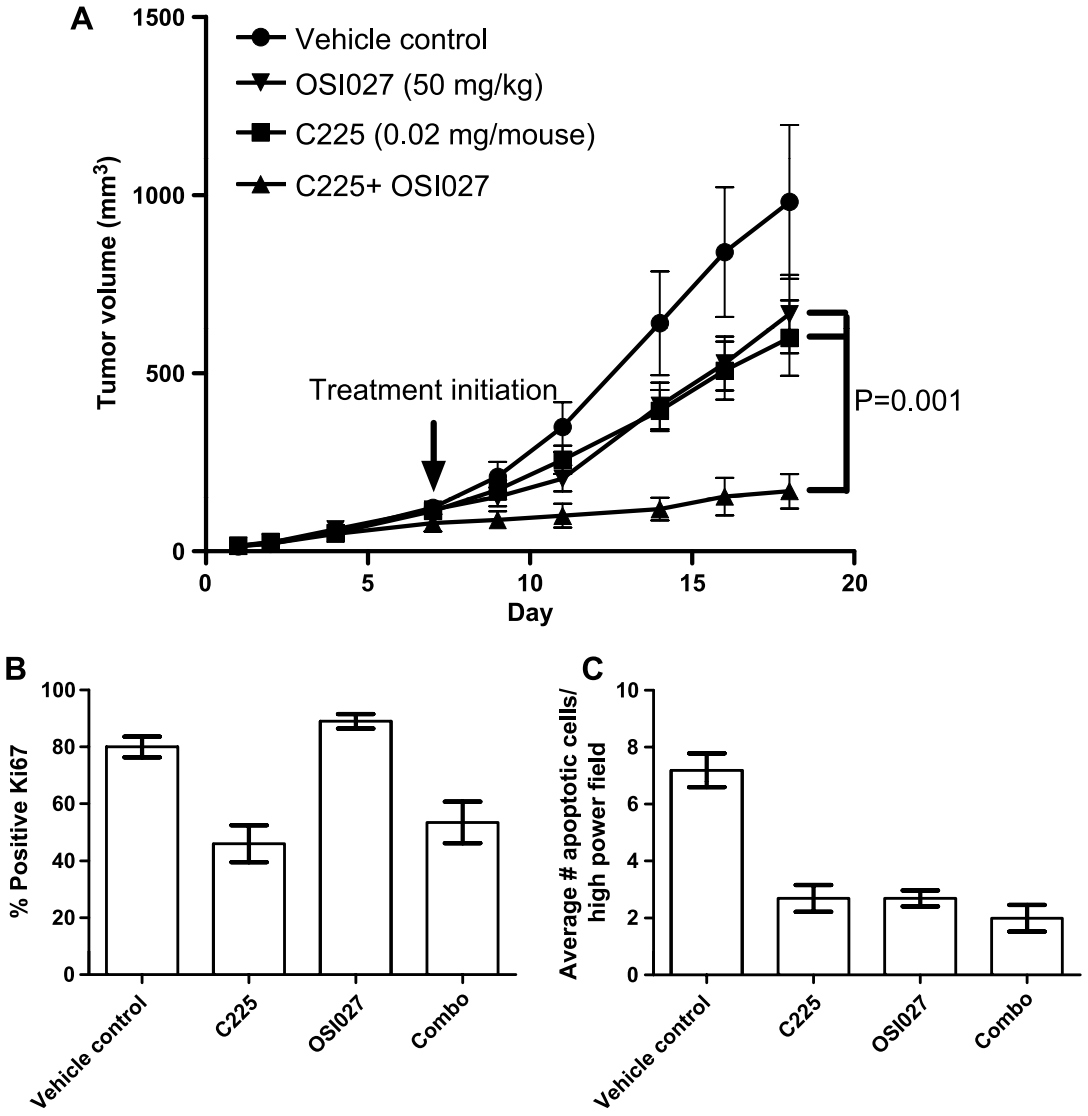


Figure 5. Combination of OSI-027 and cetuximab results in enhanced antitumor effects *in vivo*. Athymic nude mice were inoculated subcutaneously with 5×10^5 Cal 33 cells. After tumors were palpable, 39 mice were randomized into four groups and treated with either vehicle ($n = 9$), OSI-027 ($n = 10$; dose of 50 mg/kg daily, by oral gavage), cetuximab ($n = 10$; dose of 0.02 mg twice per week, i.p.), or a combination of cetuximab and OSI-027 ($n = 10$; above dosages). Tumor volumes were measured three times a week for 14 days. (A) Combined treatment with OSI-027 and C225 significantly reduced tumor growth compared to single agents alone ($P = 0.001$). Differences in treatment groups were tested through the Kruskal-Wallis exact test (Statxact software, Version 7). Xenograft tumors were assessed for (B) percent Ki67-positive cells and (C) average number of TUNEL-positive cells per high power field.

in the expression of downstream markers of TORC1/TORC2 with complete inhibition at the highest concentrations. Treatment of leukemic cells with 1 μM PP242 nearly completely abrogated expression of the mTOR downstream markers pAKT, phospho-p70, and phospho-mTOR (s2481) [47]. Similarly, others have shown decreased activation of both TORC1/2 markers in murine embryonic fibroblasts (MEFs) using a combined inhibitor, Torin1 [48]. Kinase assays to evaluate inhibition of both mTORC1 and mTORC2 reveal that, in the presence of Ku-0063794, levels of phosphorylated p70s6K, a marker for TORC1, decreased in a dose-dependent fashion [45]. Similar results were obtained for Ku-0063794 and AKT (s473), a marker for TORC2 [45]. In the few reports to date describing OSI-027, treatment of leukemic cell lines with OSI-027 was associated with decreased expression of markers for TORC1 and TORC2 activation [49]. Other studies report that in primary AML leukemic blasts, cells treated with OSI-027 showed a decrease in activation of TORC1 and TORC2 markers, including S6K and AKT (Ser473), respectively [32]. A variety of leukemic cell lines demonstrated decreased activation of mTORC1/2 markers in cells treated with OSI-027 [50]. In that study, OSI-027, unlike rapamycin, also decreased expression of 4E-BP-1 [50].

In another report assessing OSI-027 in solid tumor models, complete knockdown of both TORC1 and TORC2 markers was only observed at the highest concentration tested (10 μM) [31]. In the present study, we demonstrate that OSI-027 targets both mTORC1 and mTORC2 in HNSCC cell lines in conjunction with a dose-dependent decrease in activation of both TORC1 (levels of phospho-p70s6K) and TORC2 [levels of 4E-BP1, AKT (Ser473), and PRAS40]. Our findings suggest that combined inhibition of TORC1/TORC2 may be efficacious in solid tumors through predicted biochemical mechanisms at achievable concentrations *in vivo*.

Previous studies have found enhanced effects of combining TORC inhibitors with an EGFR inhibitor in preclinical cancer models. Combinations of rapamycin and either imatinib or dasatinib resulted in enhanced (and even synergistic) decreases in mTOR activation models of acute leukemia harboring the Philadelphia chromosome [10,41]. In addition, combinations of TORC1/TORC2 inhibition using PP242 and either imatinib or dasatinib resulted in further decreases in TORC1/2 activation in Ph(+) leukemic cells compared to either agent alone or the combination of rapamycin and imatinib [41].

In contrast to hematopoietic malignancies, EGFR is an established therapeutic target in several solid tumors, including HNSCC. Despite ubiquitous expression of EGFR in HNSCC, EGFR targeting to date has resulted in limited clinical benefit unless combined with cytotoxic chemotherapy and/or radiation [51]. There are no reports examining the therapeutic mechanisms of EGFR inhibition in combination with TORC1/2 blockade in any cancer, including a cancer where EGFR is an established therapeutic target, such as in HNSCC. The results of the present study show, for the first time, that a combination of erlotinib and OSI-027 results in synergistic antiproliferative effects *in vitro* and that combined inhibition of EGFR and TORC1/TORC2 *in vivo* using the clinical inhibitor cetuximab results in significantly enhanced antitumor effects. One of the limitations of this data is that treatment was initiated when tumors were at a median volume of 48 mm^3. In general, *in vivo* therapeutic agents are less effective when initiated in the setting of larger tumor volumes given the limited vascular supply in subcutaneous xenografts. Further, HNSCC tumor volumes in control mice approach the maximal limits permitted by the Institutional Animal Care and Use Committee making it difficult to acquire multiple measures of comparative tumor volumes. Another limitation of the study is that long-term effects of over 30 days of therapy to study mechanisms of resistance could not be assessed because of the short duration of the study. To address this limitation, we performed a similar *in vivo* therapeutic study using HNSCC cell line FaDu, where tumor-bearing mice were treated with OSI-027 and/or C225 for 14 days with additional observation and measurements through 33 days. However, tumor growth was inhibited by the combination throughout the dosing period, so assessment of mechanisms of resistance would not be supported by these studies (data not shown).

Levels of activated TORC1 markers were significantly decreased in HNSCC cells treated with erlotinib plus OSI-027 compared to either drug alone. Levels of TORC2 markers were similarly decreased to a greater extent in cells treated with both inhibitors (Figure 4). Clonogenic studies reveal a significant difference ($P < 0.05$) in the number of colonies after 5 days of treatment in the combination-treated group when compared to single agent(s) alone (Figure 3, *A* and *E*). A prior study investigating the antitumor effects of any tyrosine kinase inhibitor (TKI) with a TORC1/TORC2 inhibitor *in vitro* demonstrated synergistic growth inhibition in leukemic cells treated with a combination of PP242 in combination with imatinib or dasatinib compared with either agent alone [41]. In contrast to our findings, synergistic effects were much more pronounced when either TKI was combined with rapamycin [41].

Yu et al. recently reported a phosphoproteomic analysis of mTOR downstream signaling networks where mTOR was found to influence its upstream targets by mediating the activities of receptor-tyrosine kinases. Growth factor receptor–bound protein (grb10) showed decreased phosphorylation when cells were treated with rapamycin, revealing a novel downstream partner of mTORC1. Furthermore, it was determined that grb10 is directly phosphorylated by mTORC1 and is not phosphorylated by any upstream molecules when mTOR is activated [52]. Other phosphoproteomic studies following treatment with Torin1 in human embryonic kidney–293 cells that overexpress mTORC1 also showed that grb10 contributed to mTOR signaling [53].

The present study is one of the first to examine the effects of OSI-027 in combination with EGFR targeting strategies in tumor models where EGFR inhibition is an FDA-approved therapeutic strategy. We hypothesized that because EGFR and mTOR signaling contribute to HNSCC cell survival through both interacting and independent pathways, combined inhibition may improve treatment responses. Our findings that OSI-027 synergizes with erlotinib to decrease HNSCC survival *in vitro* and enhances the effects of cetuximab *in vivo* suggests that there is a rational basis for further research of TORC1/2 inhibitors such as OSI-027 for the treatment of solid tumors such as HNSCC.

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