Comparison of radiosensitizing effects of the mammalian target of rapamycin inhibitor CCI-779 to cisplatin in experimental models of head and neck squamous cell carcinoma

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Abstract

To determine if the mammalian target of rapamycin (mTOR) inhibitor CCI-779 can sensitize head and neck squamous cell carcinoma (HNSCC) to radiotherapy (XRT) and compare the radiosensitizing effects to cisplatin with its known considerable toxicity. Radiosensitizing effects of CCI-779 were assayed on HNSCC cell lines in vitro. CCI-779 (5 mg/kg), cisplatin (1 mg/kg), and XRT (2 Gy) alone and in combination were evaluated for antitumor activity in mice bearing FaDu and SCC40 xenografts. Effects of CCI-779 on radiation-induced activation of the Akt/mTOR pathway were analyzed. Although CCI-779 did not sensitize HNSCC cells to ionizing radiation in vitro, combination of CCI-779 and XRT significantly augmented the in vivo tumor growth-inhibitory effects of XRT and CCI-779 (P < 0.05). In addition, CCI-779 + XRT suppressed tumor growth more effectively than cisplatin + XRT (P < 0.05). CCI-779 + XRT significantly improved survival compared with XRT alone in both cisplatin-sensitive FaDu (P < 0.01) and cisplatin-resistant SCC40 (P < 0.05) xenograft mice. There were no additional benefits of adding cisplatin to CCI-779 + XRT. CCI-779 significantly attenuated irradiation-induced up-regulation of the mTOR pathway, increased apoptosis and displayed potent antiangiogenic activity in FaDu xenografts that was further enhanced by its combination with XRT (P < 0.05), which may explain the mechanism of its selective radiosensitizing effects in vivo and not in vitro. Antitumor activity of XRT was enhanced when combined with CCI-779 in HNSCC xenograft model. CCI-779 + XRT showed antitumor activity superior to conventional chemoradiotherapy with cisplatin. These results pave the way for clinical trials using molecular targeted therapy with CCI-779 in combination with XRT for HNSCC treatment. [Mol Cancer Ther 2009;8(8):2255–65]

Introduction

Head and neck cancer is one of the six most frequent cancers worldwide (1). Squamous cell carcinoma of the head and neck (HNSCC) remains a challenge because of the high locoregional recurrence rates, ~50% in advanced-stage disease (2, 3). The combination of organ preservation cisplatin-based chemoradiotherapy is now used as standard treatment (4). However, the incidence of severe acute adverse effects was higher in the group receiving cisplatin-based chemoradiotherapy than in the radiotherapy (XRT) group alone (77% versus 34%; ref. 4). Cisplatin displays considerable toxicity causing significant morbidity as a result of dysphagia, prolonged use of percutaneous endoscopic gastrostomy tube, and tracheostomy tubes (5). Additionally, many patients acquire resistance to cisplatin during therapy, suggesting a need for other treatment strategies (6).

The use of molecularly targeted agents in combination with XRT is a promising strategy. Cetuximab, an epidermal growth factor receptor monoclonal antibody, has been approved by the Food and Drug Administration for the treatment of HNSCC patients. The addition of cetuximab to XRT is beneficial in a small fraction of HNSCC patients (10-15%), and some patients acquire cetuximab resistance (7–9). The presence of mutant epidermal growth factor receptor (EGFRvIII) detected in 42% of HNSCC tumors might contribute to the limited clinical response. EGFRvIII-overexpressing cells and xenografts were more resistant to cetuximab treatment compared with cells and xenografts overexpressing wild-type epidermal growth factor receptor (10). Moreover, in models of non–small cell lung cancer and colon cancer, persistent activation of the phosphatidylinositol

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3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway was associated with resistance to anti-epidermal growth factor receptor drugs, including cetuximab (11, 12). HNSCC is characterized by persistent activation of the Akt/mTOR pathway that leads to phosphorylation of p70 S6 kinase and 4E-binding protein 1 (4E-BP1; ref. 13). The antiapoptotic role of Akt/mTOR accounts not only for its transforming potential but also for the resistance of cancer cells to the action of chemotherapeutic agents and ionizing radiation (14).

The PI3K/Akt/mTOR kinase pathway is a central regulator of cell metabolism, proliferation, and survival and is up-regulated in many tumors, including HNSCC (15–17). Although XRT initiates cytitotic cellular mechanisms through the induction of DNA damage and activation of the proapoptotic ASK1/JNK pathway, ionizing radiation also activates the antiapoptotic PI3K/Akt/mTOR pathway. For example, ionizing radiation activates mTOR signaling in breast cancer cells (18) and T lymphocytes (19). Rapamycin analogues, such as CCI-779 and RAD001, effectively inhibit signal transduction downstream of mTOR, attenuating cap-dependent translation of many pro-oncogenic and prosurvival factors, suggesting that rapamycin analogues can be potentially used as radiosensitizing agents.

mTOR inhibitors are well tolerated by patients with mild, manageable, and reversible toxicities (20–23). The radiosensitizing effects of mTOR inhibitors have been shown in breast and prostate cancer cell models (24, 25) and in glioma xenografts in mice (26). Radiosensitizing properties of mTOR inhibitors in HNSCC have not been elucidated. Moreover, antitumoral effects of mTOR inhibitors combined with radiation therapy have not been compared with conventional cisplatin-based chemoradiotherapy in any other organ system.

The goal of this study is to determine if the mTOR inhibitor CCI-779 can sensitize HNSCC to XRT in both in vitro and in vivo models and to compare the radiosensitizing effects of CCI-779 to cisplatin. In addition, the effects of radiation on proapoptotic and antiapoptotic pathways in HNSCC are elucidated.

Materials and Methods

Cell Lines

The HNSCC cell line FaDu (derived from a hypopharyngeal SCC) was obtained from the American Type Culture Collection. SCC40 (tongue cancer) and SCC66 (established from locally advanced primary cancer of the floor of mouth) were kindly provided by Dr. Susanne Gollin and PCI-15a (pyriform sinus cancer) was provided by Dr. Theresa L. Whiteside (both from the University of Pittsburgh Graduate School of Public Health). All cell lines were maintained in MEM (Sigma-Aldrich, except CCI-779, which was provided by Wyeth-Ayerst Research).

Radiation Cell Survival Clonogenic Assay

Radiation sensitivities of the HNSCC cell lines were determined by measuring colony formation after cells were exposed to ionizing radiation. Exponentially growing cells were pretreated with vehicle (DMSO) or CCI-779 at concentrations of 10 or 100 ng/mL or cisplatin at concentrations of 0.5 or 1.5 μmol/L for 24 h. The cells were then washed, suspended at a density of 2 × 10^6/mL, and irradiated with escalating doses of radiation from 0 to 8 Gy using a 137Cs γ-ray source (J.L. Shepard and Associates). After irradiation, cells were serially diluted and resuspended in fresh growth medium without CCI-779 or cisplatin. Cells were seeded in triplicate at various densities dependent on plating efficiency in 60 mm culture dishes to achieve ∼50 to 100 colonies per dish. The colonies were allowed to grow for 14 days and stained with gentian violet (1% gentian violet, 70% ethanol, 5% formaldehyde), and colonies, defined as >50 cells, were counted. The relative surviving fraction was determined by dividing the plating efficiency of the irradiated cells by the plating efficiency of the unirradiated control in at least three independent experiments. A Student’s t test was used to compare differences in clonogenic survival between treatment groups.

Proliferation and Vascular Endothelial Growth Factor ELISA Assay

The cytotoxic effect of cisplatin treatment on FaDu and SCC40 cells was evaluated using the CellTitre 96 AQueous cell proliferation assay according to the manufacturer’s instructions (Promega). Sensitivities of the cell lines to cisplatin were expressed as IC50 values. The growth-inhibitory effect of CCI-779 on HNSCC in vitro was tested in our laboratory previously (27).

The effects of CCI-779 treatment and ionizing irradiation on vascular endothelial growth factor (VEGF) expression in FaDu and SCC40 cells was determined with the ELISA commercial kit (R&D Systems) as described previously (27).

S.c. HNSCC Xenograft Model

In the xenograft model, BALB/c nu/nu mice (Harlan) were injected s.c. with 1 × 10^6 FaDu or SCC40 cells. These two cell lines were chosen because of their relative differences in sensitivity to cisplatin. Animals were housed in a barrier facility and maintained on a normal diet ad libitum. All studies were conducted in compliance with the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee guidelines.

Tumor volume [(length × width²) / 2] was determined with a digital caliper. The flow diagram of the in vivo studies is shown in Supplementary Fig. S1. To evaluate the early effects of CCI-779 and radiation therapy on the Akt/mTOR pathway and proapoptotic factors, a group of mice were injected with FaDu cells. When tumors reached ∼300 to 600 mm³, mice were divided randomly into several groups with three to four mice per group and treated either with vehicle, 5 mg/kg CCI-779, targeted radiation at
a single 2 Gy fraction, or combined CCI-779 and radiation. Tumors were harvested at 0, 1, 2, and 6 h after treatment, snap frozen in liquid nitrogen, and protein was extracted at a later time.

To determine prolonged effects of CCI-779 and XRT on the mTOR pathway, six mice from each group of the FaDu xenograft experiment described below were randomly selected to be sacrificed after 3 weeks of treatment and before the survival arm of the study to assay tumors for the mechanisms of antitumor action of different treatment modalities.

Antitumor activities of CCI-779, cisplatin, and XRT alone and in combination were evaluated in mice injected s.c. with FaDu or SCC40 cells. When tumors reached ~40 mm³ (defined as day 0), mice with established xenografts were stratified by tumor volume and randomized into experimental groups as follows: (a) control, vehicle only; (b) 5 mg/kg CCI-779 i.p. five times a week; (c) 1 mg/kg cisplatin i.p. twice a week before irradiation; (d) CCI-779 + cisplatin; (e) XRT at 2 Gy fractions twice a week for 3 weeks; (f) cisplatin + XRT; (g) CCI-779 + XRT; and (h) CCI-779 + cisplatin + XRT. CCI-779 was prepared in 4% ethanol, 5.2% Tween 80, and 5.2% polyethylene glycol 400 and administered i.p. The dose of 5 mg/kg CCI-779 was chosen, as it was the lowest dose tested in nude mice in our previous study (27). Cisplatin was prepared in sterile 0.9% saline, stored at 4°C, and protected from light until use. The dose of 1 mg/kg of cisplatin i.p. was chosen as a well-tolerated dose with significant antitumor efficacy against human oral squamous carcinoma xenografts in nude mice (28). Maximum tolerated dose of cisplatin for nude mice is ~2.9 mg/kg (28). Both CCI-779 and cisplatin were injected 1 h before XRT. The radiation groups received targeted irradiation delivered by a SL1 Plus linear accelerator with 6 MV photons (Elekta) at 2 Gy fractions. Each mouse was immobilized in a plastic tube during XRT. A sheet of 1-cm-thick bolus (MedTec) was overlaid on animals covering the entire irradiated area to ensure that tumors receive a maximum dose of 6 MV photon. All mice were treated for 3 weeks with 11 to 12 mice per group for the FaDu xenograft experiment and 5 to 6 mice per group for the SCC40 xenograft experiment. Tumors were measured twice a week. Xenograft tumor volumes were compared statistically on day 12 (FaDu) or day 17 (SCC40), the last day when all mice were still alive. In both experiments, some mice in the control and cisplatin alone groups were sacrificed due to severe tumor burden before the 3-week treatment was completed. The Kruskal-Wallis one-way ANOVA test was used to determine significant differences among the treatment groups. When an overall significant difference was indicated by the test, pairwise comparisons among the treatment groups were done using the Wilcoxon rank-sum test to determine which pairwise difference contributed to the overall significant difference.

As a surrogate marker of toxicity, body weight was measured two to three times a week for the duration of the experiment without any differences observed between treated and control animals (data not shown).

### Immunohistochemical Assessment of Intratumoral Microvessel Density with CD31

FaDu xenograft tumors from the mice treated for 3 weeks were fixed overnight with 10% phosphate-buffered formalin, rinsed twice with 50% ethanol, and embedded in paraffin. Tissue sections (5 μm) were deparaffinized and stained with CD31 antibody to determine microvessel counts. CD31 (PECAM-1) antibody was obtained from Santa Cruz Biotechnology and used at 1:300 dilution. Using low-power magnification, the regions containing the most intense area of vascularization were chosen for counting in each of the tumors. Individual microvessels were counted using a ×400 field (×40 objective lens and ×10 ocular lens). Any stained endothelial cells that were clearly separate in appearance were counted as individual vessels. Six random fields within the areas of intense vascularization were viewed and counted at ×400. Results were expressed as the average number of microvessels per field. To evaluate the differences between treatment groups, one-way ANOVA and Tukey’s multiple comparison tests were done.

### Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

Apoptosis was assayed by detecting terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) in paraffin sections using a commercially available kit (In situ Cell Death Detection Kit; Roche) and done according to the manufacturer's instructions. Slides were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). TUNEL labeling was observed using a Zeiss Axioplan fluorescence microscope (Carl Zeiss). Images were acquired using Axiovision software and an Axiocam MRm digital camera (Zeiss). The number of TUNEL-positive nuclei and total number of nuclei (4′,6-diamidino-2-phenylindole positive) per field of view were counted automatically after the threshold was set manually and this procedure was repeated in at least five fields from nonneoplastic regions of each tumor section. Percent of TUNEL-positive nuclei was counted separately for each image and averaged for the sample.

### Survival Study

In the survival arm of the xenograft study, treatment was discontinued after 3 weeks and mice were followed until one of the following end points were reached: xenograft volume of 2,000 mm³, mice lost >15% of their body weight, or maximum time point of 60 days after the treatment initiation. Kaplan-Meier curves in combination with log-rank test were used for survival analysis.

### Western Blot Analysis of Treated Cells and Tumors

Protein was extracted directly from cells and tumor tissues from the established tumor model (~5 mg) using lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄; Cell Signaling] containing 1× protease inhibitor cocktail (Roche Molecular Biochemicals). Western blot analysis was done according to previously published laboratory protocol (16). The following
antibodies were used: anti–4E-BP1, anti–S6 ribosomal protein, anti–phospho–S6 ribosomal protein (Ser235/Ser236), anti–phospho–4E-BP1 (Ser65), anti–Akt, anti–phospho–Akt (Ser473), anti–Bad, anti–phospho–Bad (Ser136), anti–β-actin, and anti–poly(ADP-ribose) polymerase (PARP). All antibodies were obtained from Cell Signaling, except PARP antibody obtained from Santa Cruz Biotechnology.

Results

mTOR Inhibition Does Not Radiosensitize HNSCC Cell Lines In vitro

To determine whether CCI-779 sensitizes HNSCC cell lines to radiation therapy, clonogenic assays were done (Fig. 1). In vehicle-treated samples, the surviving fractions were 0.68 ± 0.06, 0.64 ± 0.12, 0.75 ± 0.07, and 0.46 ± 0.05 after 2 Gy of irradiation for FaDu, SCC40, PCI 15a, and SCC66, respectively. There was no significant change in the surviving fractions of cells in the presence of CCI-779 at all doses tested compared with controls for all HNSCC cell lines (Fig. 1; Supplementary Fig. S2). The results show that neither 10 nor 100 ng/mL CCI-779 had any significant effects on the sensitivity of the HNSCC cell lines to radiation. CCI-779 treatment also had no significant effect on plating efficiency in all three HNSCC cell lines.

We also tested the radiosensitizing effects of cisplatin on FaDu, SCC40, and PCI 15a cell lines (Fig. 1). Cisplatin treatment had significant radiosensitizing effects on FaDu (P < 0.05 for 0.5 μmol/L cisplatin at 4 and 8 Gy and for 1.5 μmol/L cisplatin at exposure to all levels of radiation tested). Cisplatin treatment decreased plating efficiency of FaDu by 41.6 ± 14.4% and 96.9 ± 1.3% at 0.5 and 1.5 μmol/L, respectively. In the PCI 15a cell line, only a higher dose of cisplatin (1.5 μmol/L) had significant radiosensitizing effects. Cisplatin treatment decreased plating efficiency of PCI 15a cells by 14.6 ± 1.9% and 76.5 ± 2.6% at doses of 0.5 and 1.5 μmol/L, respectively. On the contrary, cisplatin did not sensitize SCC40 cells to radiation and affected plating efficiency of SCC40 at 1.5 μmol/L but not 0.5 μmol/L with a decrease of plating efficiency of only 37.9 ± 7.8% at 1.5 μmol/L. The results of the proliferation assay confirmed that FaDu is more sensitive to cisplatin with an IC50 of 3.9 ± 0.4 μmol/L, whereas SCC40 is a relatively cisplatin-resistant cell line with an IC50 of 7.6 ± 0.8 μmol/L (P < 0.01). Based on their differential sensitivities to cisplatin, these two cell lines were selected for the in vivo studies.

mTOR Inhibition Enhances XRT Efficacy in HNSCC Xenografts and Improves Survival

The effects of 3-week CCI-779 treatment on the sensitivity of FaDu (cisplatin-sensitive) and SCC40 (cisplatin-resistant) xenografts to radiation were compared with cisplatin (Table 1). On day 0, when mice were stratified into experimental groups, tumor volumes averaged ~40 mm3 and were not significantly different between groups. Tumor growth curves for FaDu and SCC40 xenografts are shown in Fig. 2A and B. CCI-779 treatment alone, even at the low dose of 5 mg/kg, displayed remarkable inhibition of tumor growth comparable with the effect of XRT alone in both HNSCC xenografts. The addition of CCI-779 to radiation therapy further enhanced radiation-induced tumor regression (P < 0.05 versus CCI-779 or XRT alone). CCI-779 + XRT suppressed xenograft growth significantly more than cisplatin + XRT for both cisplatin-sensitive FaDu and cisplatin-resistant SCC40 cell lines (P < 0.05). Notably, the addition of cisplatin to CCI-779 + XRT had no additional benefit compared with CCI-779 + XRT.

The Kaplan-Meier survival curves for the FaDu xenograft survival study are shown in Fig. 2C. The median survival time of the control group was only 19 days (Table 2). CCI-779 significantly extended survival time compared with control (35 days; P < 0.05), which was comparable with...
pared with control. However, all CCI-779–relatively cisplatin-resistant SCC40 xenograft tumors failed to significantly improve survival of mice bearing the tumor (median survival time of 38 days) compared with the control, which is greater than the sum (16 + 18 = 34) of single-modality treatments, suggesting a synergistic effect of combined treatment on survival in FaDu xenograft mice. Mice bearing the relatively cisplatin-sensitive FaDu xenografts treated with the combination of CCI-779 + XRT had a median survival time of 49 days, which represented an improved survival compared with XRT alone that, in turn, was as effective only as conventional chemoradiotherapy, which prolonged the median survival time by 18 days compared with the control group. Notably, CCI-779 alone prolonged the median survival time by 37 days compared with control and radiation therapy, which prolonged the median survival time by 16 days compared with control.

**Effects of Ionizing Radiation and CCI-779 on Proapoptotic and Prosurvival Factors in HNSCC Cells and Tumors**

The early effects of a single treatment of ionizing radiation (2 Gy) and CCI-779 (5 mg/kg in vivo or 24 h treatment of CCI-779 at 10 ng/mL in vitro) on the Akt/mTOR pathway were analyzed. Western blot analyses of tumors from irradiated FaDu xenografts showed that ionizing radiation activates antiapoptotic, prosurvival Akt/mTOR signaling. Although Akt phosphorylation at Ser473 was up-regulated after irradiation, phospho-Akt (Thr308) expression did not change (Fig. 3A). Western blot analysis of irradiated FaDu and SCC40 cells revealed that, in contrast to the in vivo data, radiation had no effect on Akt phosphorylation in vitro (Fig. 3B). We tested the effect of ionizing radiation (2 Gy) and CCI-779 treatment on downstream targets of mTOR, 4E-BP1, phospho-S6 ribosomal protein, and apoptotic markers. CCI-779 treatment effectively inhibited phosphorylation of S6 ribosomal protein at Ser235/Ser236 (a physiologic downstream target of p70 S6 kinase; Fig. 3B and C) and 4E-BP1 in HNSCC cell cultures and FaDu xenograft tumors as evidenced by a shift of the more phosphorylated S6 isoform after Western blot analysis of total and nonphosphorylated S6 isoform. In contrast, CCI-779 treatment significantly inhibited VEGF secretion in the medium when compared with the control or irradiated samples (Fig. 3B). CCI-779 treatment alone significantly inhibited expression of phospho-Bad (Ser136) in FaDu or irradiated samples (Fig. 3B). CCI-779 treatment effectively inhibited phosphorylation of Bad, which facilitates apoptosis. We found that PARP cleavage, a crucial early marker of apoptosis, was up-regulated in FaDu xenograft tumors at 6 h after irradiation (increased from 6% in control to 29% at 6 h after irradiation; Fig. 3C). CCI-779 treatment alone significantly inhibited expression of phospho-Bad (Ser136) in FaDu or irradiated samples (Fig. 3B). CCI-779 treatment effectively inhibited phosphorylation of Bad, which facilitates apoptosis. We found that PARP cleavage, a crucial early marker of apoptosis, was up-regulated in FaDu xenograft tumors at 6 h after irradiation (increased from 6% in control to 29% at 6 h after irradiation; Fig. 3C). CCI-779 treatment alone significantly inhibited expression of phospho-Bad (Ser136) in FaDu or irradiated samples (Fig. 3B). CCI-779 treatment effectively inhibited phosphorylation of Bad, which facilitates apoptosis. We found that PARP cleavage, a crucial early marker of apoptosis, was up-regulated in FaDu xenograft tumors at 6 h after irradiation (increased from 6% in control to 29% at 6 h after irradiation; Fig. 3C).
increased the percentage of cleaved PARP to 21% and substantially augmented radiation-induced cleavage of PARP to 40%, which also occurred at an earlier time point, that is, 2 h after irradiation (Fig. 3C), suggesting that the drug can facilitate apoptotic cell death caused by radiation in tumor tissues and therefore serve as a radiosensitizing agent.

CCI-779 treatment up-regulated Akt phosphorylation at Ser473 in both in vitro (Fig. 3B) and in vivo (Fig. 3C and D) settings, indicating that, in HNSCC experimental models, mTOR inhibition is disrupting the negative feedback loop that suppresses PI3K/Akt signaling when S6K is phosphorylated. The changes in Akt (Thr308) phosphorylation after CCI-779 were not significant (Fig. 3B and C). Although ionizing radiation treatment did not up-regulate Akt phosphorylation in our in vitro studies (Fig. 3B), it caused an increase in phospho-Akt (Ser473) expression in xenograft tissue as early as 2 h after irradiation (Fig. 3A, C, and D). Moreover, after 3 weeks of treatment, all three therapeutic agents (cisplatin, CCI-779, and XRT and their combinations) caused an increase of phospho-Akt expression (both sites) in the xenograft tumor tissues when compared with control (Fig. 3D). Importantly, CCI-779 treatment did not further exacerbate cisplatin- and XRT-induced up-regulation of Akt phosphorylation at Ser473 and Thr308.

The effects of prolonged 3-week treatment with CCI-779, cisplatin, and XRT on mTOR signaling were examined in the FaDu xenograft model. Although we did not observe radiation-induced up-regulation of 4E-BP1 and S6 ribosomal protein at early time points after irradiation (Fig. 3C), 3 weeks of XRT (6 fractions of 2 Gy) resulted in a 2-fold increased expression of pS6 and promoted phosphorylation of 4E-BP1 as evidenced by a shift from less phosphorylated to more phosphorylated isoforms (Fig. 3D).
contrast, CCI-779 decreased pS6 and caused a shift of 4E-BP1 phosphorylated bands to the unphosphorylated and less phosphorylated isoforms and significantly attenuated radiation-induced up-regulation of these mTOR downstream factors \( (P < 0.05) \).

### Effect of CCI-779 on Vascularization and Apoptosis of the FaDu Xenografts

The absence of radiation sensitizing effects noted in the in vitro group but not in the in vivo groups where CCI-779 augmented the tumor growth-inhibitory effects of XRT led us to evaluate the effects of CCI-779 on tumor-stromal interactions. To examine these interactions, we assessed the effects of CCI-779 on angiogenesis by counting the number of blood vessels in CD31-stained sections of xenograft tumors following 3 weeks of treatment. At \( \times400 \) magnification, the average counts per field were as follows: 30.4 ± 5.8 (mean ± SD) blood vessels for control tumors; 26.3 ± 4.7 for radiation alone, and 25.2 ± 1.4 for chemoradiotherapy with cisplatin (Fig. 4). XRT and chemoradiotherapy with cisplatin did not reduce the number of vessels significantly. Treatment with CCI-779 alone significantly reduced the intratumoral microvessel density compared with control (17.6 ± 2.3 vessels per field; \( P < 0.001 \) versus XRT alone). The combination of CCI-779 with XRT even further enhanced the antiangiogenic effects of CCI-779 (10.8 ± 2.3 vessels per field; \( P < 0.001 \) versus XRT alone; \( P < 0.05 \) versus CCI-779 alone).

Apoptosis was assessed by TUNEL staining in xenograft tumor tissues at early times after irradiation (2 h) and CCI-779 treatment (24 h) and following 3 weeks of treatment. Xenograft tumor tissues from vehicle-treated mice in the early effects study had 1.52 ± 0.27% (mean ± SE) of the apoptotic TUNEL-positive nuclei per field. CCI-779 (24 h), XRT (2 h), and CCI-779 + XRT treatment significantly increased the percentage of apoptotic nuclei to 3.48 ± 0.09%, 3.41 ± 0.21%, and 3.65 ± 0.33% correspondingly \( (P < 0.05 \) versus control). Three weeks of treatment further increased the apoptosis in the FaDu xenograft tissues (Fig. 4, bottom). The average percentage of apoptotic nuclei per field were as follows: 2.16 ± 0.45% for control tumors, 4.41 ± 0.57% for CCI-779 alone, 6.48 ± 0.46% for XRT, and 9.3 ± 0.27% for chemoradiotherapy with cisplatin (Fig. 4). The combination of CCI-779 and XRT augmented the percentage of apoptotic nuclei to 11.34 ± 0.92% \( (P < 0.001 \) versus XRT alone or CCI-779 alone; difference versus cisplatin + XRT is not significant). Representative photomicrographs of TUNEL-stained tumor sections from each treatment group are shown in Fig. 4.

### Discussion

The growth-inhibitory properties of mTOR inhibitors on various types of cancers have been well studied (29–32). We showed that CCI-779 is an effective agent against HNSCC in both in vitro and in vivo studies (27). The combination of mTOR inhibitors with cytotoxic treatments, such as radiation therapy or chemotherapy, might potentiate the antitumoral effects of mTOR inhibition (33). Hence, we wanted to elucidate the efficacy of using CCI-779 in combination with XRT in preclinical HNSCC models and in comparison with cisplatin. The efficacy of combining XRT with mTOR inhibitors have been studied in breast and prostate cancer cell models (24, 25) and murine glioma xenografts (26). However, the effects of CCI-779 and XRT have not been studied in HNSCC and have never been compared with cisplatin and XRT, which is a very commonly used radiosensitizer in several organ systems.

In radiation therapy, activation of cytotoxic cellular mechanisms, such as induction of DNA damage and up-regulation of proapoptotic ASK1/JNK pathway, in tumors is favorable. Additionally, irradiation further enhances the antiapoptotic PI3K/Akt/mTOR pathway, which is already up-regulated in many types of cancer, including HNSCC. In our experiments, Akt (at Ser\(^{473}\)) and mTOR were activated in FaDu xenografts after irradiation (Fig. 3A). Rapa-mycin analogues, such as CCI-779 and RAD001, effectively inhibit signal transduction downstream of mTOR, attenuating cap-dependent translation of many pro-oncogenic and prosurvival factors, which may explain their radiosensitizing effects.

We found that doses of CCI-779 that significantly inhibit mTOR signaling have a growth-inhibitory effect and importantly cause significant inhibition of VEGF production in the cells and did not sensitize HNSCC cell lines to radiation in vitro as evidenced by clonogenic assays. In
in contrast, our in vivo studies suggest that CCI-779 enhances radiation-induced cell death by decreasing 4E-BP1, S6, and Bad (Ser136) phosphorylation while promoting radiation-induced cleavage of PARP and ultimately apoptosis as evidenced by TUNEL staining of apoptotic nuclei. Based on these promising results, we hypothesize that, in combination with radiation, CCI-779 would be effective for treatment of HNSCC. This is not only the first study in HNSCC preclinical models but also the first study to compare cisplatin to CCI-779 as a radiosensitizer in any organ system. We found that compared with radiation therapy alone the combination of CCI-779 and XRT significantly inhibited tumor burden and extended survival of mice. In both xenograft models (cisplatin sensitive and cisplatin resistant), the combination of CCI-779 and XRT was significantly more effective in reducing tumor burden than conventional chemoradiotherapy with cisplatin. Although there was no significant difference in

Figure 3. Western blot analysis of the effects of ionizing radiation and CCI-779 on proapoptotic factors and antiapoptotic PI3K/Akt/mTOR signaling pathway in HNSCC cells and tumors. A to C, cells were treated with 10 ng/mL CCI-779 and mice were treated with 5 mg/kg of the drug 24 h before the time of irradiation. Cells and xenograft tumor tissues were harvested at 2 h after irradiation unless indicated otherwise. The extracts were separated by SDS-PAGE and various components of the AKT/mTOR pathway assayed by Western blot analysis as described in Materials and Methods. A, ionizing radiation activates antiapoptotic (phospho-Akt and phospho-mTOR) factors in FaDu xenograft tumors. B, CCI-779 treatment effectively inhibits signaling downstream of mTOR and VEGF production in vitro. C, CCI-779 treatment inhibits signaling downstream of mTOR in vivo and promotes radiation-induced cleavage of PARP in FaDu tumors. Band densities of phospho-Akt (Ser473; n = 4), phospho-Akt (Thr308; n = 4), and phospho-Bad (Ser136; n = 3) were quantitated using ImageQuant software and normalized to their respective total protein levels (total Akt or total Bad). Mean ± SE. *, P < 0.05 versus vehicle-treated control. No significant differences between groups were detected for phospho-Akt (Thr308) expression.

D, FaDu xenograft mice were treated with cisplatin, CCI-779, and XRT or their combinations for 3 wk (see details in Materials and Methods). Protein was extracted from the tissues and assayed by Western blot analysis. All therapeutic agents (cisplatin, CCI-779, and XRT and their combinations) caused an increase of phospho-Akt expression (both sites) in the xenograft tumor tissues when compared with control. The up-regulation of phospho-Akt (Ser473 and Thr308) by CCI-779 or CCI-779 + XRT in the 3 wk of treatment is no different from the up-regulation noted with radiation alone. Three weeks of XRT increased pS6 expression and induced a shift in phosphorylation of 4E-BP1 from the less phosphorylated "β" isoform and unphosphorylated "α" isoform to more phosphorylated "γ" and "δ" isoforms. CCI-779 treatment reversed radiation-induced phosphorylation of 4E-BP1 to some extent as well as decreased phosphorylation of S6 protein. Representative Western blots are shown. The intensity of the phospho-Akt (Ser473) bands was quantified in five independent sets of samples and the intensity of the phospho-S6 bands was quantified in six independent sets of samples. The intensities of phospho-S6 bands in CCI-779, cisplatin + CCI-779, CCI-779 + XRT, and cisplatin + CCI-779 + XRT groups were significantly different from those in XRT samples (P < 0.05).
survival of cisplatin-sensitive FaDu mice when compared with CCI-779, in the cisplatin-resistant SCC40 xenografts, CCI-779 and XRT combined significantly exceeded survival of mice treated with cisplatin and XRT. Also, there was no additional benefit when all three treatments (cisplatin, CCI-779, and XRT) were combined, which is important in designing clinical trials where novel therapeutics typically are added to standard of care that already cause significant toxicity. The potent antitumoral efficacy of CCI-779 as a single agent (>60% of tumor growth inhibition compared with control) precluded deciphering the exact mode of interaction (synergism, additive, and antagonism) between CCI-779 and XRT. Importantly, the addition of CCI-779 to XRT markedly enhanced the effects of radiation on tumor burden. Combined CCI-779 + XRT treatment inhibited tumor growth by >90% compared with control as well as more than doubled the survival benefit of mice compared with XRT alone in both xenograft models. Importantly, the effects of combined CCI-779 + XRT treatment on angiogenesis and apoptosis in xenograft tissues exceeded the sum of those by the drug or XRT alone.

Head and neck cancer is not the only type of cancer in which dissonant results are observed when examining the radiosensitizing properties of mTOR inhibitors in an in vitro versus in vivo model. For example, in a glioma model, there was no increased radiosensitivity with the mTOR inhibitor RAD001 treatment in vitro, whereas the drug significantly enhanced radiation-induced regression of glioma xenografts (26). Conversely, in breast cancer cell lines, radiation sensitization was observed with RAD001 treatment in vitro. In our study, Akt phosphorylation was affected differently by ionizing radiation in cell cultures compared with a xenograft model. Akt phosphorylation was not up-regulated after irradiation of FaDu and SCC40 cell cultures. It is known that ionizing radiation effectively induces phosphorylation of Akt at Ser473 in the vascular endothelium within minutes of irradiation (34), which may explain the observed increase of phospho-Akt (Ser473) levels in the FaDu xenografts that could most likely occur in tumor vascular endothelium. Radiation-induced activation of mTOR signaling was absent in glioma GL261 cells and mTOR inhibitors did not sensitize them to radiation (26) similarly to what we observed in the HNSCC model. Radiation-induced up-regulation of Akt/mTOR signaling was observed in breast cancer cells (24) and human umbilical vein endothelial cells (26). mTOR inhibition attenuated radiation-induced up-regulation of Akt/mTOR signaling and sensitized breast cancer and HUVEC cells to radiation in vitro (24, 26). Also, mTOR inhibition sensitized vascular endothelium to radiation injury in vivo (26). mTOR inhibition reduces microvesSEL density in tumor tissues by decreasing VEGF production (35–37). Our results show that CCI-779 significantly inhibited vascularization in the HNSCC xenografts. We have shown that treatment of HNSCC cells with CCI-779 decreases VEGF production that is consistent with antiangiogenic effects of the drug. Evidence suggests that the level of VEGF might not only affect angiogenesis and radiation resistance of endothelial cells but also may have an effect on radiation resistance of tumor cells, including HNSCC (38). Although CCI-779 treatment decreased VEGF production in HNSCC cells in vitro, it did not have an effect on radiation sensitivity, suggesting that a decrease in VEGF levels alone is not sufficient for radiation sensitization of HNSCC cells. We found that CCI-779 treatment up-regulated Akt phosphorylation (Ser473) in vitro and in vivo, indicating
that in HNSCC experimental models mTOR inhibition is disrupting the negative feedback loop that suppresses Akt phosphorylation (39, 40). However, the increase in phospho-Akt noted with CCI-779 and XRT was not significantly different from the increase in phospho-Akt noted with all other treatments, that is, cisplatin, XRT, and the combination. Additionally, this increase did not affect the potent antitumoral effects of CCI-779 or its radioenhancing effects.

In our study, CCI-779 showed potent antitumor activity against cisplatin-sensitive as well as cisplatin-resistant cell lines. Our data suggest that CCI-779 can be as effective as cisplatin in combination with XRT for the treatment of head and neck cancer. Because CCI-779 is a relatively well-tolerated drug with mainly manageable and reversible dermatologic side effects, it is a promising therapy that can potentially replace cisplatin with its known considerable toxicity. Because the PI3K/Akt/mTOR pathway in HNSCC is a critical regulator of multiple downstream effectors including angiogenesis, mitosis, and apoptosis, targeting this pathway during radiation therapy may be a key factor to blocking multiple other pathways enhancing the antitumoral action of XRT.

Disclosure of Potential Conflicts of Interest

C-A.O. Nathan: Wyeth-Ayerst funded clinical trial in Louisiana State University Health Sciences Center-Shreveport. No other potential conflicts of interest were disclosed.

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