Mammalian Target of Rapamycin Inhibitors as Possible Adjuvant Therapy for Microscopic Residual Disease in Head and Neck Squamous Cell Cancer

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Abstract

Molecular therapeutics identifies an aberration in tumors to select patients that benefit from molecular targeted therapy. Overexpression of eIF4E in histologically "tumor-free" surgical margins of head and neck squamous cell cancer (HNSCC) patients is an independent predictor of recurrence and is functionally activated through the Akt/mammalian target of rapamycin (mTOR) pathway. Although mTOR inhibitors are cytostatic agents, best used in combination therapy, we hypothesize that they can be used as long-term single agents in an HNSCC model of minimal residual disease (MRD). CCI-779, an mTOR inhibitor, arrested growth of a phosphatase and tensin homologue deleted on chromosome 10 (PTEN) abnormal HNSCC cell line FaDu, inhibiting phosphorylation of 4E-binding protein 1, resulting in increased association with eIF4E and inhibition of basic fibroblast growth factor and vascular endothelial growth factor. Fluorescence in situ hybridization detected PTEN abnormalities in 68% of patient tumors and 35% of tumor-free margins. CCI-779 inhibited growth of established tumors in nude mice. However, in the MRD model, there were significant differences in the tumor-free rate between the control (4%) and the treatment group (50%), and the median tumor-free time was 7 versus 18 days, respectively (P < 0.0001). In those animals that formed tumors, CCI-779 caused a significant decrease in the tumor volume. The Kaplan-Meier curve showed that CCI-779 significantly increased survival (P < 0.0001). The mTOR pathway was inhibited in peripheral blood mononuclear cells potential surrogate markers of response to therapy. Stable transfection of FaDu with luciferase allowed us to monitor the effects of CCI-779 with bioluminescence imaging in the MRD model. These results pave the way for a clinical trial using targeted molecular therapy with CCI-779 as a single agent for mTOR-activated residual cells. [Cancer Res 2007;67(5):2160–8]

Introduction

There has been no change in survival of head and neck squamous cell cancer (HNSCC) patients over the last few decades despite advances in treatment (1). One strategy to improve outcomes is to identify particular cohorts of patients who might more effectively respond to a given treatment. To this end, we have found that overexpression of the translation initiation factor eIF4E in histologically "tumor-free" surgical margins of HNSCC is an independent predictor of recurrence (2). The overexpression of eIF4E seems to be functionally activated in the tumor margins through the Akt/mammalian target of rapamycin (mTOR) pathway, suggesting the importance of this pathway in tumor cell survival and recurrence (3).

An important consequence of eIF4E overexpression is the increased translation of mRNAs with highly structured 5′-untranslated regions (UTR), many of which encode growth-promoting proteins directly related to tumorigenesis, such as c-myc, cyclin D1, ornithine decarboxylase, basic fibroblast growth factor (b-FGF), and vascular endothelial growth factor (VEGF; ref. 4). However, high levels of eIF4E alone are not capable of increasing translation of these proteins because eIF4E can be sequestered by binding to 4E-binding protein 1 (4E-BP1). Increased levels of functionally active eIF4E are achieved by activation of mTOR, which leads to the phosphorylation of 4E-BP1 releasing bound eIF4E, which can then stimulate cap-dependent translation especially of mRNAs with long 5′-UTRs (5). mTOR is an evolutionary conserved serine/threonine kinase that regulates cell growth and division by integrating signals from both nutrients and growth factors (6). Phosphorylation of 4E-BP1 can be inhibited by rapamycin through its action on mTOR, thus decreasing the available functionally active eIF4E (7, 8). Hence, it is attractive to hypothesize that mTOR inhibitors, such as rapamycin and its analogues, could be used as adjuvant long-term therapy in HNSCC patients that have activation of eIF4E in the margins leading to increased survival of this patient cohort.

Rapamycin analogues have been shown to cause a significant inhibition of tumor growth rather than tumor regression in experiments with tumor xenografts (9, 10). Hence, these drugs have been used in multiple clinical trials in combination therapy when treating established tumors (11). However, this class of compounds also represents an attractive single-agent long-term therapy for the inhibition of tumor regrowth following surgery in the patient cohort expressing high levels of eIF4E in otherwise histologically negative margins. Similar adjuvant therapy after initial tumor treatment has been used with both hormonal therapy and small-molecule inhibitors, such as imatinib, in a variety of cancers (12, 13). The mTOR inhibitor Sirolimus, which has been used to inhibit rejection in renal transplantation and to inhibit restenosis after coronary angioplasty, has a well-established safety profile that would allow for ready adaption as adjuvant therapy in HNSCC patients with a high risk of recurrence (14, 15).

Additionally, previous studies have shown that sensitivity to rapamycin is dependent on dysregulation of the tumor suppressor gene, phosphatase and tensin homologue deleted on chromosome 10.
(PTEN) in tumors (16). mTOR functions downstream of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and is activated in response to stimuli that activate the PI3K/Akt pathway. The PI3K pathway itself represents a major cell survival pathway and is often activated in cancer and contributes to cell cycle progression, to decrease apoptosis, and to increase metastatic capabilities of cancer cells (17). Normally, the phosphatase PTEN counters the PI3K activity and thus negatively regulates PI3K/Akt survival pathway (18). However, PTEN located on 10q23 is mutated in a wide range of human cancers with a frequency comparable with that of p53. Thus, dysregulation of PTEN could result in the activation of PI3K, which in turn would lead to activation of Akt/ mTOR, further supporting the use of the rapamycin class of inhibitors as adjuvant therapy in HNSCC patients.

Therefore, in investigating the efficacy of rapamycin analogues in preventing recurrence, we first determined whether PTEN ploidy was abnormal in HNSCC tumors and then tested whether the rapamycin analogue CCI-779 was capable of inhibiting growth of a PTEN abnormal HNSCC cell line in culture and in xenograft models through inactivation of mTOR. In the xenograft models, the activity of the mTOR pathway in peripheral blood mononuclear cells (PBMC) was monitored as a potential surrogate marker of tumor response. These studies present a novel approach for the potential use of a small-molecule inhibitor that specifically targets cells with a specific molecular aberration.

Materials and Methods

Cell line and generation of luciferase-expressing FaDu cells. The eIF4E-overexpressing HNSCC cell line FaDu derived from a hypopharyngeal SCC (obtained from American Type Culture Collection, Manassas, VA) was grown in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% bovine calf serum, nonessential amino acids (Life Technologies), and 100 units of penicillin with 100 μg streptomycin. Cells were grown in monolayers and maintained in humidified 5% CO₂ atmosphere at 37°C. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) except for CCI-779, which was provided by Wyeth-Ayerst Research (Philadelphia, PA).

To be able to image tumor cells in nude mice, FaDu cells were transfected with a luciferase-expressing vector (pMS110), a generous gift from Message Pharmaceuticals (Malvern, PA), using LipofectAMINE 2000 (Invitrogen). Transfected cells were selected with 500 μg/ml G418 and, individual clones were analyzed for luciferase expression by plating in a 96-well plate and, 24 h later, by lysing the cells and analyzing luciferase activity in a Victor III plate reader (Perkin-Elmer, Elm Grove, IL). FaDu9000 was the clone that expressed the highest level of active luciferase following transfection of pMS110.

Fluorescence in situ hybridization for PTEN in cell lines and paraffin-embedded tissue. Gene copy number was assayed by a multicolor fluorescence in situ hybridization (FISH). A commercial DNA probe (Vysis/Abbott, Des Plains, IL) 10q23/PTEN was used with a PTEN gene probe labeled with Spectrum Orange and a centromeric enumeration probe for chromosome 10 labeled with Spectrum Green. Cytosine preparations of FaDu cells were fixed in 3:1 methanol/acetic acid and processed for hybridization with the select probes. Target DNA and probe were codenatured, hybridized overnight, washed serially in decreasing concentrations of detergent wash solutions (sodium chloride and sodium citrate), and counterstained with 4,6-diamidino-2-phenylindole (DAPI). The slides were visualized using a Leica (Wetzlar, Germany) DMRB fluorescent microscope equipped with a rhodamine/FITC/DAPI triple band pass filter and imaged with a FISH imaging system (Cytovision, Applied Imaging, Inc., San Jose, CA). FISH analysis of paraffin-embedded tissue sections was carried out by the Molecular Pathology Laboratory using a well-established protocol (19). Approval was obtained from the Institutional Review Board.

Proliferation assay determining effects of CCI-779 on FaDu and FaDu9000. The effects of CCI-779 on the proliferation of FaDu and FaDu9000 cells were determined by plating exponentially growing cells in 96-well plates (3,000 per well) and medium with various concentrations of CCI-779 (0–10,000 ng/mL) for time points ranging from 0 to 96 h. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt/phenazine methosulfate (Promega Corp., Madison, WI) system using a previously established proliferation assay (20). The dose effects were calculated as a percentage of the cell growth obtained from vehicle-treated cells grown in the same culture plate for the same time point. In addition, the effects of 10 nmol/L rapamycin on luciferase activity in these cells were monitored using the Steady-Glow Luciferase kit (Promega) according to the manufacturer's instructions with detection of activity using the Lucy II (Rosys Anths, New Castle, DE) luminometer.

In vivo established tumor HNSCC xenograft models. In the established tumor model, RALB/c nu/nu mice (Charles River Laboratories, Wilmington, MA) were injected s.c. with 1 × 10⁶ FaDu cells. Animals were maintained in a barrier facility in accordance with Institutional Animal Care and Use Committee regulations. Tumor volume [(length × width²)/2] was determined with a digital caliper. On day 0, defined as average tumor volumes for the groups of approximately 60 to 90 mm³, animals were randomized into five groups of five mice each and treated with vehicle or CCI-779 prepared in 4% ethanol, 5.2% Tween 80, and 5.2% polyethylene glycol 400 (Sigma-Aldrich) at a daily ip dose of 0, 5, 10, 15, and 20 mg/kg for 3 weeks (3 days/wk). The doses were based on studies with CCI-779 on colon carcinoma cell lines (21). Tumor volumes were measured on days 7, 14, and 21 (three cycles). Because tumor volume was determined serially in each mouse, a two-way repeated measures ANOVA was done, and when the overall dose and time effects were significant, pairwise comparisons among the five dosage groups and the four time points were done using the Bonferroni post hoc test to control at 5% the overall level of significance.

As a surrogate marker of toxicity, body weight was measured weekly for the duration of the experiment at the time of tumor measurement without any differences observed between treated and control animals (data not shown).

Minimal residual disease model to measure the effects of CCI-779. This study was designed to evaluate the efficacy and potential therapeutic role of CCI-779 in a minimal residual disease (MRD) model that intends to mimic patients with molecular positive margins (22). Nude mice were anesthetized and incisions were made in the dorsal flanks and s.c. flaps were flaps with sharp dissection. Pipette dispensers were used to introduce the tumor cells (2 × 10⁶ FaDu cells) in 100 μL of culture medium into the flap, which was then sutured. Instead of allowing the tumor cells to form nodules, 72 h following tumor seeding, the animals were randomized into five groups of eight mice each and treated with vehicle or CCI-779 at daily ip doses of 5, 10, 15, and 20 mg/kg for 3 weeks as in the established tumor model and tumor volume was determined. In this manner, although no gross tumors were present, microscopic tumor cells were present within the surgical site mimicking the clinical dilemma of surgical excision of all gross tumor but leaving positive margins. Blood (500 μL) was obtained by orbital bleeding, and aliquots of PBMCs were collected using Histopaque-1083 at baseline and 72 h after treatment was completed to analyze the mTOR pathway (see below). The five groups were compared on proportion of mice that developed tumors and on average volume of tumor developed. Logistic regression analysis with tumor development as the dependent variable and group as the independent variable was used to determine significant effect of group on tumor development. When the effect of group on tumor development indicated an overall significant difference among the five groups, pairwise comparisons among the five groups were done using the Fisher exact test to determine which pairwise difference contributed to the overall significant difference. Because of the observed nonnormality of tumor volume in the drug dosage groups and significantly different variability among the groups, a nonparametric ANOVA, the Kruskal-Wallis test, was used to determine significant differences among the five groups. When an overall significant difference was indicated by the test, pairwise comparisons among the five groups were done using the Wilcoxon rank-sum test to determine which pairwise difference contributed to the overall significant difference.
The results of the initial experiment with the MRD model were used to determine the sample size for a definitive study with a single strength of CCI-779 (20 mg/kg) to determine if CCI-779 could prevent tumor formation in a model of MRD. The 20 mg/kg dose was chosen as there was a dose response in preventing tumor formation in the previous experiment. In the definitive study to confirm that CCI-779 had a significant effect on tumor development, MRD was established with 25 mice randomly assigned to a control group and 43 to receive CCI-779. The mice were sacrificed at day 21 to measure tumor volume with PBMCs also being obtained.

The Kaplan-Meier method and the log-rank test were used to estimate and compare the two groups on tumor-free rate and median tumor-free times.

**Survival study.** A survival study using the MRD model was undertaken to determine the time to form tumors of \( \geq 200 \) mm\(^3\) after cessation of CCI-779 treatment. In this experiment, 24 mice were treated with 20 mg/kg CCI-779 for 21 days and 16 control mice received vehicle alone. The control mice were sacrificed at day 21, as their tumors were already \( >200 \) mm\(^3\). The treated mice were followed until the tumors were \( >200 \) mm\(^3\) in size or up to 30 days after the drug was discontinued for those animals that did not develop tumors.

**In vivo imaging of tumor growth in a model of MRD by bioluminescence.** The MRD model was established with FaDu9000 cells with 10 animals subsequently receiving 20 mg/kg CCI-779 and 10 animals receiving vehicle alone. Bioluminescence imaging (BLI) was used to measure luciferase activity with \textit{In vivo} Imaging System (IVIS) technology (Xenogen, Alameda, CA) optimized for high sensitivity (23).

BLI was done twice weekly. Mice were anesthetized with ketamine (70 mg/kg i.p.) and xylazine (5 mg/kg i.p.) followed by the injection of luciferin (150 mg/kg i.p.; Xenogen) 10 min before imaging. Animals were then put in the light-protected chamber of the IVIS imaging system, and photons emitted were measured over 3 min. Regions of interest were drawn over the area of photon emission and quantified using the "Living Image" software. As luciferase-bearing cells proliferated \textit{in vivo}, occasional image saturation was encountered. This was overcome by shortening exposure time to reduce the number of photons collected.

**Western blot analysis of 4E-BP1, phospho-4E-BP1, phospho-S6 kinase, and b-FGF.** To determine if the observed effects of CCI-779 were a result of mTOR inhibition, the phosphorylation status of 4E-BP1 was analyzed in the cell lines, tumors, and PBMCs after treatment with CCI-779. Protein was extracted directly from cells and tumor samples from the established tumor model (\( \geq 5 \) mg) following treatment with CCI-779, and Western blot analysis was done according to previously published laboratory protocol (3). The following antibodies were used: rabbit polyclonal anti–4E-BP1 (1:500), rabbit anti-S6 kinase (1:100), rabbit anti–phospho-S6 kinase (Ser235/236; 1:100), rabbit anti–phospho-4E-BP1 (Ser65; 1:100), and rabbit anti–FGF-2 (1:100; all antibodies were obtained from Cell Signaling, Beverly, MA except anti–FGF-2 that was obtained from Santa Cruz Biotechnology, Santa Cruz, CA).

**VEGF ELISA assay with CCI-779 treatment on cell lines.** The translation of VEGF mRNA is dependent on eIF4E concentration. To determine if VEGF expression was inhibited by treatment with CCI-779, FaDu cells were seeded into six-well culture plates at a density of \( 4 \times 10^5 \) per well and incubated for 24 h in medium with 10% fetal bovine serum (FBS). The medium was then removed and replaced with fresh medium containing 10% FBS with and without 1 and 10 ng/mL CCI-779. Medium was removed at 30 min and 1, 2, 4, 6, 24, and 48 h after treatment with the drug and vehicle, centrifuged at 800 rpm for 5 min, and then stored at \(-80^\circ\mathrm{C}\). The VEGF protein concentration in the medium was determined by ELISA using a commercial kit (R&D Systems, Minneapolis, MN).

**Results**

**Analysis of PTEN ploidy by FISH in HNSCC.** As PTEN has been shown to influence sensitivity to rapamycin, we analyzed PTEN copy number by FISH in the HNSCC FaDu cell line and in the HNSCC tumors. In the FaDu cells, PTEN is aneuploid (hyper-diploid) cells with approximately three copies of the \( 10q23/\text{PTEN} \) locus identified by FISH analysis (Fig. 1A) because both the green centromeric marker and the orange \( 10q23/\text{PTEN} \) locus marker are increased. PTEN ploidy was examined by FISH in 22 HNSCC tumor samples.
samples and in 23 tumor-free margins, and the presence of deletions and/or aneuploidy was scored. Fifteen of 22 (68%) tumors showed abnormalities of PTEN with 8 tumors showing loss of one allele, 5 tumors with aneuploidy of chromosome 10, and 2 tumors containing cells either with deletions of PTEN and other cells with hyperploidy of the PTEN locus. The remaining seven tumors showed normal copy number of chromosome 10 and the 10q23/PTEN locus. Figure 1B is an example of a tumor with both deletions and aneuploidy of PTEN. Eight of the 23 (35%) margins showed deletions of PTEN. In addition, one margin with deletions also showed a gain of PTEN in some cells. Figure 1C is a histologically tumor-free margin with PTEN deletions present in the basal cell layer. Previously, overexpression of eIF4E in the basal cell layer has been shown to predict recurrence (2).

In vitro growth-inhibitory effects of CCI-779 on FaDu and FaDu9000. CCI-779 inhibited the growth of FaDu cells in a time-and dose-dependent manner at concentrations up to 100 ng/ml (Fig. 2A). At higher doses, there was no further growth inhibition. CCI-779 was found to have similar effects on the growth of FaDu9000 cells, indicating that transfection with the luciferase plasmid did not change the sensitivity of the cell line to CCI-779. Treatment of FaDu9000 cells with ~10 ng/mL rapamycin for 24 or 48 h did not alter luciferase activity (data not shown), showing lack of direct effect of rapamycin on the reporter gene.

Effects of CCI-779 on 4E-BP1 phosphorylation, eIF4E/4E-BP1 complex formation, and protein synthesis. Regulation of eIF4E activity is, in part, by phosphorylation of 4E-BP1 because phosphorylated 4E-BP1 does not bind eIF4E. CCI-779 treatment of cells should inhibit the phosphorylation of 4E-BP1 resulting in binding of 4E-BP1 to eIF4E. Western blot analysis of total 4E-BP1 showed that treatment with CCI-779 led to a concentration-dependent inhibition in the phosphorylation of 4E-BP1 with a decrease of the more phosphorylated ‘γ’ isoform and an increase of the less phosphorylated ‘β’ isoform and unphosphorylated ‘α’ isoform (Fig. 2B).

The sequestration of eIF4E by 4E-BP1 should result in an inhibition of protein synthesis, particularly the synthesis of proteins from mRNAs with highly structured 5'-UTRs. To test this, we assayed two important angiogenic factors, b-FGF and VEGF, both of which are encoded by mRNAs with highly structured 5'-UTRs. Two isoforms of b-FGF were noted due to alternative translation initiation from the CUG and AUG start codons. There was disappearance of the CUG isoform and decrease in the AUG isoform within 2 to 4 h of treatment with nearly complete loss of the AUG isoform by 48 h with 10 ng/ml CCI-779 (Fig. 2C). Similarly, 1 and 10 ng/ml treatment with CCI-779 significantly inhibited VEGF secretion in the medium over 48 h when compared with the control (Fig. 2D).

In vivo effects of CCI-779. The effect of CCI-779 was next tested in vivo on cell growth in the established xenograft tumor model (Table 1). In this model, there were highly significant (P < 0.0001) differences in average tumor size at days 7, 14, and 21 after initiation of CCI-779 treatment between the four treatment groups and the controls. There were no significant differences among the

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**Figure 2.** In vitro effects of CCI-779. A, growth-inhibitory effects of CCI-779 on FaDu cells showing a time- and dose-dependent inhibition. Bars, SD. B, Western blot analysis showing shift in phosphorylation from the more phosphorylated ‘γ’ isoform to the less phosphorylated ‘β’ isoform and unphosphorylated ‘α’ isoform after exposure to even 1 ng/mL of the drug, indicating that the antitumor effects are a result of mTOR inhibition. C, Western blot analysis showing a decrease in b-FGF expression with 10 ng/ml CCI-779. Two isoforms of FGF are noted due to alternative translation initiation from the CUG and AUG start codons. Note the disappearance of the CUG isoform and decrease in the AUG isoform at 2 h with complete disappearance of the AUG isoform by 48 h. D, ELISA showing that CCI-779 at 1 and 10 ng/mL causes significant inhibition of VEGF production in the medium of FaDu cells compared with the control. Bars, SD.
four drug groups. Analysis of the 4E-BP1/mTOR pathway in the established tumor model was undertaken after 21 days of treatment with CCI-779. Treatment with CCI-779 resulted in an inhibition in the phosphorylation of 4E-BP1 from the more phosphorylated ‘γ’ and ‘γ’ isoforms to the less phosphorylated ‘δ’ isoform and unphosphorylated ‘α’ isoform (Fig. 3A). mTOR activity also regulates a second pathway, S6K1, which functions as a kinase for the phosphorylation of the 40S ribosomal protein S6. CCI-779 resulted in a significant dose-dependent decrease in phospho-S6 in the established tumor model (Fig. 3B). These data strongly suggest that CCI-779 inhibits tumor growth in vivo through the mTOR pathway.

The effect of CCI-779 was next tested in the MRD model. With increasing doses of CCI-779, there was a significant decrease in the proportion of mice that developed tumors ($P = 0.025$, logistic regression analysis; Table 2). There was also an effect of the treatment on tumor volumes, which were decreased compared with control analyzed either by pairwise comparisons ($P = 0.003$) or by pooling all four treatment groups ($P = 0.0002$) with a decrease in tumor volume from an average volume of $360.3 \pm 118.6$ mm$^3$ in control mice to an average volume of $19.1 \pm 10.1$ mm$^3$ in the combined four treatment groups.

In a larger definitive study with the MRD model, tumor formation was measured at day 21 after treatment of 43 mice

### Table 1. Mean tumor volume by dosage group and by time point for the established tumor model (mean ± SE)

<table>
<thead>
<tr>
<th>CCI-779 dose</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.4 ± 18.6</td>
<td>623.8 ± 175.1</td>
<td>903.3 ± 169.6</td>
<td>2,200.3 ± 531.3</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>74.4 ± 26.9</td>
<td>88.8 ± 42.2</td>
<td>103.2 ± 51.0</td>
<td>106.4 ± 53.0</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>87.6 ± 27.7</td>
<td>167.8 ± 31.5</td>
<td>166.2 ± 19.4</td>
<td>203.4 ± 22.3</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>67.6 ± 34.1</td>
<td>59.6 ± 33.2</td>
<td>56.8 ± 31.0</td>
<td>57.2 ± 28.2</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>63.8 ± 50.9</td>
<td>87.0 ± 13.1</td>
<td>79.8 ± 8.5</td>
<td>90.4 ± 12.8</td>
</tr>
</tbody>
</table>

NOTE: The number of tumor values used was five except for days 14 and 21 of control, each of which was calculated with three tumor values.

Figure 3. In vivo effects of CCI-779 in an established tumor model. A, Western blots showing a shift in phosphorylation from the more phosphorylated ‘γ’ and ‘γ’ isoforms to the less phosphorylated ‘δ’ isoform and unphosphorylated ‘α’ isoform levels of 4E-BP1 after treatment with CCI-779 at all doses tested. B, Western blot analysis showing dose-dependent decrease in phospho-S6 after treatment with the drug, indicating inhibition of both downstream effects of mTOR.
with 20 mg/kg CCI-779 and compared with 25 control mice. There were again significant differences in the tumor-free rate between the control (4%) and the treatment group (50%). The median tumor-free time with 95% confidence intervals (95% CI) was 16 days (95% CI, 15–17 days) in the control group and 38 days (95% CI, 34–41 days) compared with the control group (4%) and the treatment group (50%). The median tumor-free time with 95% confidence intervals (95% CI) was 16 days (95% CI, 15–17 days) in the control group, whereas in the treatment group, it was 38 days (95% CI, 34–41 days).

In a “survival study” using the MRD model, CCI-779 (20 mg/kg) treatment was discontinued after 3 weeks and mice were followed until tumors reached an approximate volume of 200 mm³, at which time the animals were sacrificed (Fig. 4A). The Kaplan-Meier curve shows that CCI-779 significantly retarded the time to develop tumors of at least 200 mm³ (P < 0.0001). Although 50% of the treated mice formed tumors, when the size of these tumors was analyzed, the treatment was found to cause a significant decrease in the tumor volume (29.0 ± 32.8 mm³) compared with the control (162.5 ± 134.4 mm³; P < 0.0001).

In “vivo” BLI results in MRD model. The MRD model requires sacrifice of animals to monitor tumor growth. Because relatively small numbers of cells are implanted, large cohorts of animals are needed to monitor the effects of an intervention. Hence, BLI was used immediately after injection of FaDu9000 cells, modified to stably express luciferase, and then twice weekly in the control mice and mice treated with CCI-779 to measure the effects of CCI-779. An example of serial imaging is shown for both control and treated mouse (Fig. 4B) and shows both the similarity of cell numbers at the beginning of the experiment, the continued increase of bioluminescence with time, and the inhibition of luminescence by CCI-779. In the entire group, a significant difference was first seen by day 7 and persisted for the entire experiment with a 10-fold difference at day 21 (Fig. 4C).

### Table 2. Summary statistics for five drug groups on tumor development and average tumor volume (eight mice per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. with tumors (%)</th>
<th>P*</th>
<th>Mean ± SD1 of tumor volume</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 (87.5)</td>
<td>0.0257</td>
<td>360.3 ± 118.6</td>
<td>0.0037</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>6 (75.0)</td>
<td></td>
<td>157.7 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>4 (50.0)</td>
<td></td>
<td>245.1 ± 11.7</td>
<td></td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>3 (37.5)</td>
<td></td>
<td>133.3 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>1 (12.5)</td>
<td></td>
<td>35.03</td>
<td></td>
</tr>
</tbody>
</table>

*For effect of group on tumor development in logistic regression.

### Analysis of CCI-779 effects on the mTOR pathway in PBMCs of treated animals.

As an intermediate marker of the effect of CCI-779, phosphorylation of 4E-BP1 was examined in PBMCs from CCI-779–treated and control mice from the MRD model. Western blot analysis showed that each of the four doses of CCI-779 inhibited the phosphorylation of 4E-BP1 compared with control. CCI-779 also decreased phospho-S6 in the treated group (D1) compared with the control (C5; Fig. 4D).

### Discussion

A recent editorial suggests that clinical trials of targeted therapies should report not just the maximum tolerated dose but also the dose that reaches the target and produces the desired downstream effect (24). In addition, surrogate markers should be developed to facilitate monitoring of responses. Our “vivo” model meets these requirements, mimics the design of a clinical trial, and indicates that CCI-779 can potentially inhibit tumor formation in a model of MRD in HNSCC. The current study is the first to test a mTOR inhibitor in an MRD model and to show that PBMCs can serve as a surrogate marker of response.

It has also been suggested that to select patients whose cancers would most likely be amenable to treatment with mTOR inhibitors, tumors should be assayed for PTEN loss, Akt activation, and mTOR phosphorylation (11). We have identified previously a select population of patients whose tumor-free surgical margins have residual cells showing activation of the Akt/mTOR pathway who might potentially benefit from treatment with mTOR inhibitors (3). We now also report for the first time using FISH that about two thirds of HNSCC tumors have genomic alterations of PTEN alleles. Although the role of PTEN in HNSCC tumorigenesis is controversial, the loss of PTEN protein expression is an independent predictor of poor outcome in tongue cancers and also correlates with pathologic tumor stage in HNSCC (25, 26). The loss of PTEN is more likely associated with loss of heterozygosity. The gain in the copy of PTEN seen in some of our specimens may indicate the aneuploid nature of the tumor cells, indicating perhaps chromosomal instability that may cause tumorigenesis and the loss and/or gain of PTEN supports its role in HNSCC.

CCI-779 has shown antitumor activity against a broad range of human cancers both in tissue culture and in human tumor xenograft models (9, 21, 27). In this study, CCI-779 effectively abrogated the downstream effects of mTOR activation in an HNSCC cell line, decreasing expression of the translational effectors S6K1 and 4E-BP1 and resulting in decreased expression of bFGF and VEGF. Furthermore, in the xenograft models, CCI-779 inhibited this pathway in the tumor cells as well as in the PBMCs of treated animals. CCI-779 represents then a new targeted therapy for treatment of HNSCC because mTOR is activated in the majority of these tumors (3, 28).

The mTOR modulates two separate downstream pathways 4E-BP1 and the 40S ribosomal protein S6 kinase (p70 S6 kinase) that control the translation of specific subsets of mRNAs, including VEGF and FGF (29–31). The activation of mTOR leads to the phosphorylation of 4E-BP1, preventing it from binding to the cap-binding protein, eIF4E, which in turn leads to increased protein synthesis. Activation of eIF4E may be necessary although not sufficient for oncogenesis (4, 32–34). Overexpression of eIF4E in mouse B-cell lymphoma accelerates tumorigenesis and mimics the neoplastic effects of activated Akt in these cells (35). In HNSCC, breast, and bladder cancers eIF4E overexpression facilitates the
synthesis of b-FGF and VEGF, both potent angiogenic factors for tumor progression (36–38). We have shown previously that reduction of eIF4E with antisense RNA in human FaDu HNSCC and MDA-435 breast cancer cells suppressed the tumorigenic and angiogenic properties of these cells, concomitant with loss of FGF-2 synthesis (39, 40). Furthermore, rapamycin, which inhibits mTOR activation, has also been shown to have antiangiogenic properties by reducing the production of VEGF (41). In the present study, we show that in vitro and in vivo CCI-779 inhibition of growth of HNSCC is associated with inhibition of the downstream targets of mTOR, the ribosomal S6K1 and 4E-BP1, which could lead to the reduced production of VEGF and FGF-2. The critical role that this pathway plays in tumorigenesis is further supported by the observation that the primary effects of oncogenic Ras or Akt on gene expression are via induction of mRNA translation (42).

A key aspect of the current studies was to develop a preclinical model that would mimic the clinical situation of resection margins that had no residual cancer cells by histologic examination but still exhibited a molecular marker for cancer. The MRD seemed to be a reasonable preclinical model that could be made more useful by examining the response of cells longitudinally in the same animal. Using FaDu cells expressing luciferase did afford us this additional power and extends the usefulness of HNSCC to other tumor types (43, 44). The BLI results showed a significant decrease in photon emission in the CCI-779 treatment group compared with the control group. However, as expected with a cytostatic agent, tumor cells were still viable even during treatment. In the survival study, only 21% of the treated group did not form tumors after discontinuing the drug. Hence, patients with eIF4E overexpression in the surgical margins may require prolonged adjuvant therapy. The design of a clinical trial would involve the prolonged use of mTOR inhibitors. It is known that if tumor cells remain dormant and do not undergo the neovascularization required for tumor growth beyond a few millimeters that the tumor cells do not survive (45). Hence, prolonged treatment with mTOR inhibitors could potentially lead to ultimate death of tumor cells in the clinical setting of MRD. The intent of this study was to compare the effects of CCI-779 in a model of MRD and show increased survival compared with an established tumor model. Future studies are planned to vary timing of administration of mTOR inhibitors to

**Figure 4.** Effects of CCI-779 in the MRD model. A, Kaplan-Meier curves showing survival comparison between control and drug-treated mice. The curves indicate a significant difference in time to develop tumors at least 200 mm³ in volume for the two groups (P < 0.0001). B, Longitudinal growth of tumor cells using BLI of one mouse from each group. Top, control animal; bottom, animal treated with 20 mg/kg CCI-779. C, Graph showing luciferase activity for in vivo BLI. Results indicate significant differences in luciferase activity between the control and the treated group of mice. D, Western blot analysis of PBMCs as a surrogate marker of tumor response showing shift in the phosphorylated 'γ' isoform to the less phosphorylated 'α' isoform levels of 4E-BP1. A decrease in phospho-S6 is also seen in the treated animals, D1, compared with the control, C5.
determine the length of time needed to prevent tumor formation even when the drug is discontinued. Interestingly, preclinical and clinical studies indicate that intermittent administration of CCI-779 reduces its immunosuppressive properties while retaining its antitumor activity (46). Hence, in our preclinical model, we have used the intermittent dose scheduling.

Fortunately, both a low dose of CCI-779 is sufficient to inhibit the mTOR pathway, and the safety of the prolonged use of sirolimus has been shown in renal transplant patients (47). In other cancers, CCI-779 has shown strong antitumor potential and favorable pharmacological and toxicologic characteristics in early clinical trials with skin rash and mucositis being the prominent adverse effects (48, 49). In the survival study, there was an efficient block in tumor progression with this cell line during the 18 days after the treatment window, which indicates that long-term treatment with CCI-779 may lead to stable residual disease for as long as the treatment is continued. If our findings accurately predict human responses, then administration of CCI-779 would be continuous or long-term postresection. The long-term use of CCI-779 has been tested in a phase 3 randomized, three-arm study of temsirolimus as a single agent in the treatment of patients with advanced renal cell carcinoma with some patients receiving CCI-779 for as long as 4 years with an acceptable safety profile (50). The most frequently occurring adverse events were maculopapular rash, mucositis, asthena, and nausea, with the most frequently occurring grade 3 or 4 adverse events being hyperglycemia, hypophysphatemia, anemia, and hypertriglyceridemia.

A major challenge in clinical trials using signal transduction pathway inhibitors is to identify patients whose tumors have the appropriate molecular make up to respond to a specific inhibitor. A prospective trial has indicated that patients with molecular positive eIF4E margins are at high risk for relapse (2). Because standard adjuvant chemotherapy and radiotherapy have significant side effects and do not specifically target the eIF4E-positive cells, there is a need for a novel therapeutic approach targeting this pathway. mTOR and the associated signaling pathway represent an attractive target for adjuvant treatment in the cohort of eIF4E-positive margin patients. Rapamycin analogues cause significant tumor growth inhibition rather than tumor regression when tested in vivo experiments with tumor xenografts. In the present study, the results with the MRD model suggest that possible long-term administration of the rapamycin analogue, CCI-779, holds promise as a single agent in the treatment of patients with molecular positive eIF4E margins.

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