CCI-779 Inhibits Rhabdomyosarcoma Xenograft Growth by an Antiangiogenic Mechanism Linked to the Targeting of mTOR/Hif-1α/VEGF Signaling

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Abstract
Angiogenesis is one of the critical steps in tumor growth and metastasis. The goal of this study was to evaluate whether the antitumor activity of CCI-779 is related to antiangiogenic effects in vivo in tumors of mice bearing human rhabdomyosarcoma (RMS) xenografts. We now demonstrate that CCI-779 rapidly inhibits mTOR activity, as indicated by S6 reduction and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) phosphorylation in two xenograft models of RMS within 24 hours of treatment. Treatment with a single 20-mg/kg dose of CCI-779 suppressed S6 phosphorylation for more than 72 hours and 4E-BP1 phosphorylation for more than 96 hours. Based on these data, an intermittent treatment schedule (every 3 days for 30 days) was chosen and displayed a significant suppression of both tumor growth and mTOR signaling. Western blot analysis and immunohistochemical studies demonstrated that the antitumor activity of CCI-779 was associated with antiangiogenesis, as indicated by impaired levels of hypoxia-inducible factor-1α (Hif-1α) and vascular endothelial growth factor (VEGF) protein expression and by decreased microvessel density in Rh30 and RD xenografts. Together, these data suggest that CCI-779 inhibits human RMS xenograft growth by an antiangiogenic mechanism associated with the targeting of mTOR/Hif-1α/VEGF signaling.

Keywords: CCI-779, mTOR, Hif-1α, VEGF, rhabdomyosarcoma xenografts.

Introduction
Rhabdomyosarcoma (RMS) is the most common extracranial solid tumor in childhood that is related to myogenic lineage and represents approximately 60% of soft-tissue sarcomas in children [1–3]. The clinical nature of RMS involves rapid tumor progression with invasion of local tissues. Primary metastatic sites include the lungs, liver, bones, bone marrow, lymph nodes, and others. Concerted efforts to understand the clinical, pathological, and biologic factors of pediatric RMS have led to significant progress in the treatment of RMS [1–5]. However, nearly 30% of children with RMS experience progressive disease or relapse, and 50% to 95% of these patients die of progressive disease [6,7]. Patients with detectable metastases, patients not responding to therapy, and patients with disease relapse have a significantly poorer prognosis. Our studies have demonstrated that insulin-like growth factor II (IGF-II) is overexpressed in RMS and that IGF-II–mediated cell survival is associated with activation of the mammalian target of rapamycin (mTOR) pathway [8–10].
mTOR, a downstream mediator in the phosphatidylinositol-3 kinase/Akt (protein kinase B) signaling pathway, receives input from multiple signals (including growth factors such as insulin or IGFs, and nutrients such as amino acids or glucose) to regulate protein synthesis through two main pathways—ribosomal S6 kinases (S6K) 1 and 2, and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1)—that play fundamental roles in ribosome biogenesis and cap-dependent translation, respectively [11,12]. The mechanisms by which these signals impinge on mTOR have been highlighted by recent findings of the tuberous complex (TSC) and the small G-protein Rheb (Ras homolog enriched in brain) [13–16]. TSC1/2 inhibits the mTOR/S6K/4E-BP1 signaling pathway by stimulating the guanosine triphosphate hydrolysis of Rheb and Rheb functions between TSC2 and mTOR [17,18]. The S6K1 pathway controls the synthesis of proteins, such as IGF-II and ribosomal proteins, whereas the 4E-BP1 pathway controls many proteins involved in cell cycle regulation.

Rapamycin is a natural compound produced by bacteria that is currently undergoing clinical and preclinical evaluations as an immunosuppressant and anticancer agent [19,20]. Both immunosuppressive and anticancer properties of rapamycin are attributed to the inhibition of the mTOR signaling pathway.
which controls mRNA translation and cell proliferation. Rapamycin forms a complex with the immunophilin prolyl isomerase FK binding protein complex (FKBP-12) that binds with high affinity to mTOR. This interaction inhibits mTOR kinase activity and subsequently decreases the phosphorylation and activation of S6K1 and 4E-BP1. However, rapamycin has limited potential as an anticancer agent due to its poor water solubility and stability in solution. Recently, a rapamycin ester analogue, cell cycle inhibitor 779 (CCI-779; Wyeth-Ayerst, Philadelphia, PA), has been developed for intravenous use containing 10% fetal bovine serum, L-glutamine (2 mM), and 5% CO2 in a humidified incubator.

To provide a further preclinical rationale for the development of mTOR pathway inhibitors in patients, we initiated the current study, which evaluates the effects and mechanisms of CCI-779 against human RMS growth in murine xenograft models. Our data confirm that CCI-779 is effective in vivo against RMS and demonstrate that the effect of CCI-779 on the inhibition of primary tumor growth and on the induction of tumor cell apoptosis is associated with the suppression of the mTOR/Hif-1α/vascular endothelial growth factor (VEGF) pathway.

Materials and Methods

Cell Lines

Human RMS cell lines Rh30 and RD have been described previously [10]. These cells were maintained in RPMI 1640 containing 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C in 5% CO2 in a humidified incubator.

Antibodies and Reagents

Antibodies to phospho-S6 (Ser235/236), S6, phospho-4E-BP1 (Thr70), 4E-BP1, and caspase-3 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antiactin antibody was from Abcam, Inc. (Cambridge, MA). Rabbit anti–Hif-1α antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit anti–VEGF (A-20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CCI-779 was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD) and from Wyeth Laboratories (Philadelphia, PA).

In Vivo Tumor Model

Animal studies were performed in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee. Female 4- to 6-week-old female beige SCID mice were purchased from Charles River Laboratories (Wilmington, MA). Two million cells of each cell line (Rh30 and RD) were injected orthotopically into the gastrocnemius muscle in the left hind leg; after 3 weeks, mice were randomized to the control group or the CCI-779 treatment group. CCI-779 was prepared in 50 mg/ml 100% EtOH. On the day of injection, the drug was diluted in 5% Tween-80 and 5% polyethylene glycol 400 (Sigma, St. Louis, MO) up to a final concentration (20 mg/kg).

In an initial in vivo experiment, the CCI-779 or vehicle solution was administered intraperitoneally everyday for 5 days, followed by 2 days without the drug, and then followed by one additional injection (a total of six injections).

In the second in vivo experiment, to evaluate the pharmacodynamic effects of CCI-779 on mTOR target inhibition in vivo, a single treatment of CCI-779 (20 mg/kg) was given to mice bearing Rh30 xenografts.

In the third in vivo experiment, based on the results from the initial and the second experiments, mice were treated intraperitoneally with CCI-779 at 20 mg/kg every 3 days for 30 days.

Tumor growth was measured every 3 days with calipers, and tumor volume was calculated by the formula: V (mm3) = 0.5ab2, where a is the longest tumor axis and b is the shortest tumor axis. All mice were sacrificed by asphyxiation with CO2, and tumors were excised and snap frozen at −80°C until analysis.

Western Blot Analysis

Minced tumor pieces were sonicated in 1 ml of lysis buffer (20 mM Tris–HCl, pH 7.5; 150 mM sodium chloride; 1 mM ethylenediaminetetraacetic acid; 1 mM ethyleneglycoltetraacetic acid; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM β-glycerolphosphate; 1 mM sodium orthovanadate; 0.5 mM phenylmethylsulfonyl fluoride; 1 μg/ml leupeptin) on ice. Lysates were clarified by centrifugation at 14,000 rpm at 4°C for 15 minutes. Samples were run in 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Amer sham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked with 5% nonfat dried milk in TBS-T (20 mM Tris–HCl, pH 7.5; 8 g/l sodium chloride; 0.1% Tween 20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies. Horseradish peroxidase–conjugated antirabbit IgG (Cell Signaling Technology, Inc.) was used as secondary antibody. Protein was visualized using the ECL system (Amer sham Pharmacia Biotech).

Immunohistochemistry

Frozen tumor sections (5 μm) obtained from mice with or without CCI-779 treatment (30 days) were stained with hematoxylin and eosin (H&E), Hif-1α, and VEGF antibodies, and were developed using the ABC staining system (Santa Cruz Biotechnology), according to the manufacturer’s instructions. Normal rabbit IgG was used as a negative control for immunohistochemistry.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling (TUNEL) Assay

Apoptosis was measured using an ApopTag Plus In Situ Apoptosis Detection Kit (Chemicon International, Temecula,
CA). Briefly, frozen tumor sections (5 μm) were incubated for 5 minutes in 3% hydrogen peroxide diluted in phosphate-buffered saline. Terminal deoxynucleotidyl transferase was added to the sections for 5 minutes in 3% hydrogen peroxide diluted in phosphate-buffered saline (PBS). Sections were counterstained in 0.5% methyl green and viewed under a microscope. The total number of TUNEL-positive cells was quantified in six randomly selected microscopic fields at ×200 magnification within cell nuclei.

**Angiogenesis Assessment**

Analysis of microvessel density in sections was performed using a commercially available blood vessel staining kit (Chemicon International), according to the manufacturer’s instructions. Frozen tumor sections (5 μm) were processed using CD31 antibody with resultant staining of microvessels and single endothelial cells. For the quantification of mean microvessel density, the total number of CD31-positive microvessels in each section was counted in six random fields at ×400 magnification.

**Statistics**

The results were evaluated using Prism 3.0 statistical software package and were calculated as mean ± SE (n = 8 animals/group). Because data comparing differences were assessed between two groups (control and CCI-779-treated groups), tumor volumes were compared using nonparametric Mann-Whitney t-test and TUNEL-positive cells, and microvessel densities were compared using unpaired Student’s t-test. The level of statistical significance was P < .05.

**Results**

CCI-779 Inhibits the Phosphorylation of S6 and 4E-BP1 in Tumors

To examine the effects of CCI-779 on mTOR signaling in vivo, Rh30 and RD tumor-bearing mice were treated with vehicle or CCI-779 (20 mg/kg), as described in Materials and Methods section. Mice were sacrificed at indicated times, and protein extracts were prepared from tumor samples. On Western blot analysis, treatment with CCI-779 dramatically reduced the phosphorylation of both S6 at Ser235/236 (a physiological downstream target of S6K1) and 4E-BP1 at Ser70, following 24 hours and 30 days of treatment in Rh30 and RD tumor samples, respectively (Figures 1A and 2B). In contrast, CCI-779 had a slight inhibitory effect on S6 expression and had little or no effect on 4E-BP1 expression in both tumor samples (Figure 1A). Furthermore, S6 phosphorylation in Rh30 tumors was inhibited by a single dose of CCI-779 and returned to normal levels 96 hours after treatment, whereas CCI-779-mediated inhibition of 4E-BP1 phosphorylation lasted for more than 96 hours (Figure 1B). These data indicate that CCI-779 had a significant effect on the inhibition of the mTOR pathway in vivo.

Effects of CCI-779 on Caspase-3 Activation In Vivo

Caspase proteases are known to be critical mediators of chemotherapy-induced apoptosis. Caspase-3 is thought to be the major effector. We therefore examined caspase-3 activation in Rh30 and RD tumors following CCI-779 treatment for evidence of apoptosis using Western blot analysis with an antibody for cleaved caspase-3. We observed that caspase-3 was activated in Rh30 and RD tumors after 24 hours of CCI-779 treatment and became obvious after 96 hours of treatment for Rh30 tumor and after 192 hours of treatment for RD tumor (Figure 1C).

**Effects of CCI-779 on the Growth of Xenografts**

To investigate the effects of CCI-779 on tumor growth in RMS in vivo, we injected Rh30 and RD cells orthotopically into the gastrocnemius muscle in the left hind leg of mice. Based on our initial data (Figure 1B) showing that a single dose of CCI-779 (20 mg/kg) mediated the inhibition of S6 phosphorylation for 72 hours, CCI-779 was administered intraperitoneally at a dose of 20 mg/kg every 3 days.
beginning on day 8 after tumor cell injection. All control mice received intraperitoneally an equal volume of carrier solution. Figure 2A demonstrates a significant antitumor effect of 20 mg/kg CCI-779 on both Rh30 and RD tumors compared with controls during 30 days of treatment. The average tumor size of Rh30 xenografts on day 28 was 2228 mm$^3$ in the control group and 475 mm$^3$ in the CCI-779–treated group ($P = .0003$). The average tumor size of RD xenografts on day 28 was 2156 mm$^3$ in the control group and 547 mm$^3$ in the CCI-779–treated group ($P = .0002$).

CCI-779 Induced Tumor Cell Death in RMS Xenografts

To determine tumor response to CCI-779, we examined histology and cell apoptosis in excised tumors. As shown in Figure 3A, histologic examination of tumor xenografts with H&E staining showed that less dense tumor infiltration was observed in CCI-779–treated tumors compared with control tumors, and that a significant nonviable tumor was present. Both Rh30 and RD xenografts from control mice were composed of densely packed cells with hyperchromatic round-to-oval nuclei, whereas the nuclei of cells in tumors treated with CCI-779 became significantly smaller and more condensed ($<200$). To assess the induction of apoptosis, we used in situ TUNEL assay. TUNEL staining confirmed a significant number of apoptotic nuclei in the Rh30 and RD xenograft tumors treated with 20 mg/kg CCI-779 for 30 days, compared with control tumors (Figure 3B). Treatment with CCI-779 significantly increased the number of TUNEL-positive tumor cells from 14 ± 4 in the Rh30 control group to 91 ± 13 in the CCI-779–treated group ($P < .0001$) and from 21 ± 7 in the RD control group to 107 ± 15 in the CCI-779–treated group ($P < .0001$) (Figure 3C).
Effects of CCI-779 on the Dysregulation of Hif-1α and VEGF In Vivo

Hif-1α is a transcription factor that was originally found to be activated in cells in response to hypoxia and is considered an attractive target for cancer therapy because of its pivotal role in cancer development by activating the transcription of genes involved in angiogenesis, tumor metabolism, migration, and invasion [30,31]. To determine whether CCI-779 regulates Hif-1α expression in vivo, we examined the level of Hif-1α protein in vehicle-treated and CCI-779–treated tumors. Hif-1α expression in both Rh30 and RD tumors was inhibited by CCI-779 in a time-dependent manner (Figure 4A). Because Hif-1α is an important upstream mediator of VEGF expression in cancer cells [30,32,33], we next determined the effects of CCI-779 on the level of VEGF protein in Rh30 and RD tumors. CCI-779 inhibited the expression of VEGF protein in a time-dependent manner in both Rh30 and RD tumors (Figure 4A). These findings were further confirmed in both xenograft lines after prolonged treatment with CCI-779 by Western blot analysis and immunohistochemical staining. As shown in Figure 4, A and B, Hif-1α and VEGF were highly expressed in Rh30 and RD tumors, and treatment with CCI-779 every 3 days for 30 days resulted in dramatic reduction in the levels of Hif-1α and VEGF proteins. In Figure 4B, actin was used for loading control. Although some lanes (lanes 1, 2, 7, and 8) have somewhat unequal loading, this does not account for the large differences seen in Hif-1α and VEGF expression. We also used immunohistochemical staining of tumor sections to validate Hif-1α and VEGF protein expression. As expected, treatment with CCI-779 displayed a significant suppression of Hif-1α and VEGF expression in both xenograft tumors (data not shown).

Effects of CCI-779 on Tumor Angiogenesis in RMS Xenografts In Vivo

Because our data demonstrated that CCI-779 inhibited VEGF expression in both RMS xenografts (Figure 4, A

![Figure 3. Effects of CCI-779 on histology and cell death in Rh30 and RD xenografts. (A) H&E–stained tumor sections were evaluated for the growth of Rh30 and RD xenografts after 30 days of vehicle (left panel) or CCI-779 treatment (right panel). Original magnification, ×40 or ×200, as indicated in the upper right corner of each figure. (B) Representative slides of TUNEL-stained tumor sections for Rh30 and RD xenografts treated with vehicle (left panel) or CCI-779 (right panel). Original magnification, ×200. (C) The average number of TUNEL-positive cells was scored in six randomly selected microscopic fields (original magnification, ×200). *P < .0001; **P < .0001.](image-url)
and B), we next sought to determine the effect of CCI-779 on tumor angiogenesis. Tumor sections were stained with CD31 antibody. The degree of angiogenesis in each section was determined by counting the number of CD31-positive microvessels. As shown in Figure 5, in CCI-779–treated mice, intratumoral microvessel density was significantly reduced compared with that of the control group. The average number of CD31-positive microvessels was reduced from 24 ± 5 in the Rh30 control group to 5 ± 3 in the CCI-779–treated group (P < .001) and from 21 ± 4 in the RD control group to 7 ± 2 in the CCI-779–treated group (P < .005) (Figure 5).

**Discussion**

mTOR is a key regulator of cell growth and cell size in mammals and is, therefore, a potential therapeutic target for cancer. An ester analogue of rapamycin (CCI-779) is currently being studied in early clinical trials. Encouraging preliminary results have been reported in abstract form. However, unresolved challenges, such as finding predictive markers for tumor phenotypes that are likely to respond to mTOR inhibitors, defining the optimal dose and schedule, and identifying new targets of mTOR downstream, remain to be determined.

In this study, we demonstrated that S6 (the physiological downstream target of S6K1) and 4E-BP1 are good biomarkers that can be used to evaluate the activities of CCI-779 on the inhibition of mTOR signaling and tumor growth in vivo, as indicated in Figures 1 and 2. CCI-779 rapidly inhibited the phosphorylation of both S6 and 4E-BP1 within 24 hours of treatment in Rh30 and RD tumors. These results suggest that specific effects of CCI-779 on the inhibition of S6 and 4E-BP1 activation can be reproducibly observed in tumors. It should be noted that CCI-779 induces tumor cellular stress response characterized by a rapid activation of caspase-3 in Rh30 and RD tumors, suggesting that caspase-3 activation may also be a potential molecular surrogate marker that can be used to monitor the biologic effects of CCI-779 on tumor cell death in clinical trials.
Tumor extracts derived from mice treated with a single dose of 20 mg/kg CCI-779 demonstrated prolonged inactivation of S6 (=72 hours) and 4E-BP1 (>96 hours) (Figure 1B). The data suggest that specific effects of CCI-779 on S6 and 4E-BP1 occur in tumors and that CCI-779 possesses good bioavailability and efficient tumor penetration. Moreover, the effects of CCI-779 on mTOR signaling given every 3 days for 30 days are sustained and elicit significant tumor suppression and tumor cell apoptosis. These data indicate that frequent CCI-779 administration may be unnecessary in maintaining an antitumor response, although a daily (five times a week) dosing schedule has been used previously [23–25]. The data from preliminary clinical studies have suggested that intermittent dosing allows for differentiation between immunosuppressive and antitumor effects [34,35]. The advantage of administering CCI-779 intermittently may be the avoidance of prolonged CCI-779–induced immunosuppression, which is worthy of further investigation.

The overexpression of HIF-1α has been demonstrated in many human cancers as a result of the adaptation of tumor cells to hypoxia and the correlation between its activity levels in tumor cells and its tumorigenicity/angiogenesis [36,37].

Angiogenesis is essential for tumor growth and metastasis [38,39]. Various angiogenesis inhibitors, including direct and indirect inhibitors, have been developed for clinical application [40,41]. VEGF is one of the most potent stimulators of angiogenesis, and VEGF overexpression has been associated with tumor progression and poor clinical outcome [42–44]. HIF-1α is a major upstream regulator of VEGF. Thus, inhibition of angiogenesis by targeting HIF-1α/VEGF is becoming an important approach for cancer therapy. In this study, we have demonstrated that CCI-779 inhibited both HIF-1α and VEGF expression and tumor angiogenesis in both RMS xenografts. Hyperphosphorylated S6 and 4E-BP1 and overexpressed HIF-1α and VEGF were detected in vehicle-treated tumors, and blockade of mTOR signaling by its specific inhibitor CCI-779 significantly inhibited the levels of HIF-1α and VEGF proteins in Rh30 and RD xenograft tumors. These data suggest that HIF-1α and VEGF are downstream targets of mTOR signaling in Rh30 and RD xenograft tumors.

Consistent with our study, rapamycin has been shown to inhibit the expression of HIF-1α and VEGF in vitro [45,46] and to inhibit primary and metastatic tumor growth by blocking VEGF signaling in vivo [47]. We have recently shown that rapamycin and CCI-779 inhibit ezrin-mediated metastatic behavior in a murine model of osteosarcoma [28]. Stephan et al. [48] demonstrated that rapamycin, alone and in combination with the anti-VEGF antibody 2C3, strongly inhibits primary and metastatic tumor growths in pancreatic cancer in vivo. Taken together, these results suggest that the mTOR inhibitor rapamycin or its analogue CCI-779 can inhibit both primary and metastatic tumor growth by an antiangiogenic mechanism linked to a decrease in the levels of HIF-1α and VEGF proteins, in addition to direct effects on mTOR signaling.

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References


