**CCI-779 inhibits rhabdomyosarcoma xenograft growth by an antiangiogenic mechanism linked to targeting mTOR/Hif-1α/VEGF signaling**

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**INTRODUCTION.** Angiogenesis is one of the critical steps in tumor growth and metastasis. Much attention over the years has been devoted to the notion that tumor blood supply can be targeted with antiangiogenic agents. Our recent study has demonstrated that a mechanism of ezrin-related metastatic behavior is linked to an mTOR pathway, and blockade of this pathway by rapamycin and its analogue CCI-779 led to inhibition of ezrin-mediated metastatic behavior in a murine model of osteosarcoma (1,2). 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 xenografts.

**METHOD.** Female 4-6 week old female beige-SCID mice were used in this study. In initial in vivo experiment, two million cells (rhabdomyosarcoma cells Rh30, RD) were injected orthotopically into the gastronemius muscle in the left hind leg, and after 3 weeks, mice were randomized to control or CCI-779 treatment groups. CCI-779 was prepared in 100% EtOH at 50-mg/ml. On the day of injection, the drug was diluted in 5% Tween-80, 5% polyethylene glycol-400 to final concentration (20 mg/kg). The CCI-779 or vehicle solution was administered intraperitoneally (IP) daily x 5/week for 8 days, or a total of 6 injections. 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. Based on these results, mice were treated with CCI-779 starting on day 8 after injection of cells at 20 mg/kg/IP 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) = a x b², 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.
RESULTS. We demonstrate that CCI-779 rapidly inhibits mTOR activity as indicated by reduction of S6 and 4E-BP1 phosphorylation in 2 xenograft models of rhabdomyosarcoma within 24 h of treatment. Treatment with a single 20 mg/kg dose of CCI-779 suppressed S6 phosphorylation for greater than 72 h and 4E-BP1 phosphorylation for more than 96 h. 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 and immunohistochemical studies demonstrated that the antitumor activity of CCI-779 was associated with decreased angiogenesis as indicated by impaired levels of Hif-1α and VEGF protein expression in Rh30 and RD xenografts.

DISCUSSION. mTOR is a key regulator of cell growth and size in mammals, and therefore is a potential therapeutic target for cancer. An ester analog of rapamycin, CCI-779, is currently in clinical development. Our study demonstrated that S6, the physiological downstream target of S6K1, and 4E-BP1 are good biomarkers to evaluate the activities of CCI-779 on the inhibition of mTOR signaling as well as tumor growth in vivo. Angiogenesis is essential for tumor growth and metastasis (3). VEGF is one of the most potent stimulators of angiogenesis, and VEGF overexpression has been associated with tumor progression and poor clinical outcome. 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 impaired both Hif-1α and VEGF expression in both RMS xenografts. These data suggest that CCI-779 inhibits human rhabdomyosarcoma xenograft growth by an antiangiogenic mechanism associated with targeting mTOR/Hif-1α/VEGF signaling.

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