

# Antitumor Activity of the Rapamycin Analog CCI-779 in Human Primitive Neuroectodermal Tumor/Medulloblastoma Models as Single Agent and in Combination Chemotherapy<sup>1</sup>

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## ABSTRACT

We examined the cytotoxicity of the immunosuppressant agent rapamycin and its analogue CCI-779 in human brain tumor cell lines *in vitro* and *in vivo* as single agents and in combination with standard chemotherapeutic drugs. In the rapamycin-sensitive PNET/MB cell line DAOY, rapamycin exhibited additive cytotoxicity with cisplatin and with camptothecin. *In vivo*, CCI-779 delayed DAOY xenograft growth by 160% after 1 week and 240% after 2 weeks of systemic treatment, compared with controls. Single high-dose treatment induced 37% regression of tumor volume. Growth inhibition of DAOY xenografts was 1.3 times greater after simultaneous treatment with CCI-779 and cisplatin than after cisplatin alone. Interestingly, CCI-779 also produced growth inhibition of xenografts derived from U251 malignant glioma cells, a human cell line resistant to rapamycin *in vitro*. These studies suggest that the rapamycin analogue CCI-779 is an important new agent to investigate in the treatment of human brain tumors, particularly PNET/MB.

## INTRODUCTION

Rapamycin is an immunosuppressant agent that arrests cells in the G<sub>1</sub> phase of the cell cycle and induces apoptosis. This carbocyclic, lactone-lactam macrolide antibiotic is pharmacologically active through binding to ubiquitous, predominantly cytosolic immunophilin receptors (e.g., FK506-binding protein-12). Binding of rapamycin to the mammalian target of rapamycin, also known as RAFT 1, RAPT 1, and FRAP (1–3), inhibits its kinase activity and subsequently decreases phosphorylation and activation of p70 S6 kinase, translation of mRNA-encoding ribosomal proteins and elongation factors [eukaryotic initiation factor 4E binding protein (pH acid stable protein I) and eEF-2], and enzymatic activity of the cyclin-dependent kinase cdk2-cyclin E complex, resulting in a mid-to-late G<sub>1</sub> cell cycle arrest (4–8).

Antitumor activity of rapamycin as a single agent has been described *in vitro* and *in vivo* (9–13). Coadministration enhanced cisplatin-induced apoptosis in human cell lines (14) and produced additive cytotoxicity with 5-fluorouracil and cyclophosphamide in a Colon 38 tumor model (13).

PNET/MB<sup>3</sup> are the most common malignant brain tumors in childhood. With current treatment, including radio- and chemotherapy subsequent to surgical resection, the 5-year survival rate for PNET/MB exceeds 80% (15, 16). However, 30–50% of all patients experience tumor progression or late relapse with very low salvage rates (17). Therefore, improving the long-term survival of patients

with high risk PNET/MB will require therapeutic strategies that augment the effects of current treatment.

Rapamycin and its analogue, CCI-779, are attractive candidates for brain tumor therapy. Their unusual mechanism of action make it unlikely that these agents will interfere with the cytotoxicity of standard chemotherapeutic agents or exhibit cross-resistance. They are highly lipophilic (18) and thus able to penetrate the blood-brain barrier. Finally, rapamycin has minimal systemic toxicity in animals or humans (19–22). *In vitro* studies in our laboratory find that brain tumor cell lines can be exquisitely sensitive to rapamycin. For example, the PNET/MB cell line DAOY has an ID<sub>90</sub> = 102 ng/ml. In contrast, the PNET/MB D283 and malignant glioma U251 cell lines are highly resistant, with ID<sub>90</sub> values of 1.8 × 10<sup>6</sup> and 3.6 × 10<sup>19</sup> ng/ml, respectively.<sup>4</sup>

Here, we describe preclinical investigations of rapamycin and CCI-779 in treatment of human brain tumors. The cytotoxicity of rapamycin was measured using brain tumor cell lines in culture; the cytotoxicity of CCI-779 was measured using s.c. brain tumor xenografts and in combination with cisplatin and CPT. Our results indicated that rapamycin *in vitro* and CCI-779 *in vivo* exhibited additive cytotoxicity in PNET/MB cells when combined with cisplatin or CPT. *In vivo* studies showed that the same dose of CCI-779 given in daily injections over 2 weeks produced greater tumor suppression than when given as a single injection. Four weeks of daily CCI-779 injections were not superior to 2 weeks in the induction of tumor regression and growth retardation of human PNET/MB xenografts. Finally, CCI-779 suppressed the growth of human U251 malignant glioma cells *in vivo* despite the resistance of the cell line to rapamycin cytotoxicity *in vitro*.

## MATERIALS AND METHODS

**Cell Cultures.** DAOY and D283 human medulloblastoma cells were purchased from the American Tissue Culture Collection (Rockville, MD), and the glioma cell line U251 was kindly supplied by Dr. Henry Friedman (Duke University, Durham, NC). The cell lines were grown in Richter's Zinc Option Media (Life Technologies, Inc.) supplemented with 10% FCS (Sigma, St. Louis, MO) and incubated at 37°C in humidified 5% CO<sub>2</sub> (23).

**Drugs.** Rapamycin and CCI-779 were obtained from Wyeth-Ayerst Research Laboratories (Princeton, NJ), CPT from Smith-Kline Beecham Pharmaceuticals, and cisplatin from Sigma Pharmaceuticals. Rapamycin and CCI-779 were stored as dry powder at –20°C and suspended in 100% ethanol the day of use. CPT was stored as a 5-mM solution in DMSO and cisplatin as a 5-mM solution in sterile water at –20°C.

***In Vitro* Assay for Cytotoxicity.** Cytotoxicity of rapamycin, CPT, and cisplatin were assessed for unsynchronized DAOY and D283 cells by MTS tetrazolium Cell Titer 96 AQ Non-Radioactive Cell Proliferation assay (Promega, Madison, WI). DAOY and D283 cells were plated in 96-well plates, 500 cells/well. After 24 h, rapamycin, CPT, and cisplatin, diluted in serum-free Zinc Option Medium immediately before use, were added to the media as single agents or in combination (rapamycin plus CPT or rapamycin plus

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<sup>3</sup> The abbreviations used are: PNET/MB, primitive neuroectodermal tumor/medulloblastoma; CPT, camptothecin; TUNEL, terminal deoxynucleotidyl transferase-mediated dioxynigenin-11-dUTP nick end labeling.

<sup>4</sup> Unpublished data.

cisplatin). Each drug dose or combined drug dose was repeated five times. After 5 days of drug exposure, MTS solution was added to each well, and absorbance was measured at 490 nm. Experimental absorbance was computed by subtracting the absorbance of blank wells (media only). Fractional survival was computed as: experimental absorbance (mean of 5 trials/dose or dose combination)/absorbance for cells treated with drug free media (mean of 15 trials).

**Analysis of *In Vitro* Drug Interaction.** An additivity model was used as described previously (24). The additive cytotoxicity of a drug/dose combination is defined as the sum of cell kill for each agent tested as a single drug, with a 90% confidence interval for this sum. Antagonistic cytotoxicity is defined as a drug interaction that caused less cytotoxicity than the sum of each agent used alone (*i.e.*, less than additivity), and synergy is defined as the combined effect of drugs that is greater than the sum of the cytotoxicity for each agent used alone (*i.e.*, greater than additivity). The treatment effect for each agent at a given dose was identified by means of single-agent dose-effect curves (25, 26). Cytotoxicity of single agents was characterized further by fitting dose-response curves to a linear model by means of regression analysis with transformations of the response (*i.e.*, fractional survival) and the log of the dose. These transformations permit the calculation of 95% confidence intervals for single agent dose-response curves and the subsequent calculation of a 90% confidence interval for the expected additive cytotoxicity. Drug interaction was characterized by comparing the observed cytotoxicity of a drug combination with the expected additive cytotoxicity.

***In Vivo* Human DAOY-MB and U251 Malignant Glioma Xenograft Models.** Female athymic nude mice 4–6 weeks of age were purchased from the National Cancer Institute (Frederick, MD) and maintained in filter-top cages in an aseptic environment with laminar flow filtered ventilation at the Laboratory Animal Facility of the Joseph Stokes, Jr., Research Institute of the Children's Hospital of Philadelphia. DAOY-MB xenograft experiments were performed with DAOY tumors that had undergone at least eight serial passages as xenograft tumors in the flanks of nude mice. The flank xenograft tumors of stock animals were dissected free of connective tissue and external vasculature in a sterile laminar flow hood. The tumors were coarsely minced and then passed through a 20-mesh screen in a tissue press. The xenograft homogenate was passed sequentially through a 20-, 21-, 22-, and 23-gauge needle. The tumor homogenate (0.15 ml) was injected *s.c.* through a 23-gauge needle into the right flank of each nude mouse. For U251 xenografts, U251 cells grown in *in vitro* cell cultures were harvested in log-phase and resuspended in Matrigel (Life Technologies, Inc.). Cells ( $1 \times 10^7$ ) in 0.15 ml per animal were injected into the right flank of athymic nude mice. The length and width (mm) of *s.c.* tumors were measured every 2–3 days with Vernier calipers (Fischer Scientifics). Tumor volume was calculated by the following formula: width (2)  $\times$  length/2 (27).

**Treatment with CCI-779 and/or Cisplatin.** Nude mice bearing *s.c.* DAOY flank xenograft tumors were treated starting on day 10; animals bearing U251 xenografts were treated starting 3 weeks after tumor implantation. Stock dilution of CCI-779 50 mg/kg was diluted to a final concentration of 2 mg/ml using a diluent of 5% Tween 80 and 5% polyethylene glycol 400. The efficacy of different treatment schedules was tested in DAOY/MB xenografts. Each treatment group consisted of five to six animals. CCI-779 (20 mg/kg) was administered daily  $\times$  5 for 1, 2 or 4 weeks; or 100 mg/kg of CCI-779 was administered on days 1 and 12. Drug interaction of CCI-779 *in vivo* was studied by injection of 5 mg/kg cisplatin as a single dose, with or without daily treatment of 20 mg/kg CCI-779  $\times$  5 for 2 weeks. U251 xenograft tumors were treated with daily with 20 mg/kg CCI-779  $\times$  5 for 2 weeks. Control animals bearing *s.c.* flank tumors were treated with diluent at equivalent doses and schedule.

Tumor volumes were measured serially and normalized by the index of observed tumor volume in comparison to initial, pretreatment tumor volume ( $V_0/V_i$ ). Growth delay end points were defined as the posttreatment interval at which tumor volume increased by 5-fold (27). The Wilcoxon-Gehan Test was used to evaluate differences in time to reach 5-fold initial tumor volume (28).

**Histology.** Nude mice with DAOY-MB xenografts were treated on day 10 after tumor inoculation with either 100 mg/kg CCI-779, 5 mg/kg cisplatin, or both, or diluent, as described above in treatment studies. Animals were killed electively 4 days after this treatment. Flank tumors were harvested, fixed in 10% formalin, paraffin-embedded and cut into microsections. Sections were stained with H&E for morphology.

**Proliferation.** Immunohistochemistry for Ki-67 antigen was performed to determine the proliferation indices of the xenograft tumors. A monoclonal MIB-1 antibody (1:50; Immunotech, Marseilles, France) was used, detected by the Animal Research Kit (ARK; DAKO Corporation, Carpinteria, CA) according to the manufacturer's instructions, with biotinylated Fab antimouse antibody, Streptavidin-horseradish peroxidase, and diaminobenzidine tetrahydrochloride (DAKO Corporation). For quantification, tumor cells were counted at  $\times 1000$ . The proliferation index was expressed as the number of MIB-1 positive cells/100 neoplastic cells.

**Apoptosis.** TUNEL was performed to quantify apoptotic cell death. A modified protocol according to Gavrieli *et al.* (29) was used. In brief, tissue sections were deparaffinized, rehydrated, and treated with 10  $\mu$ g/ml Proteinase K (Boehringer Mannheim, Indianapolis, IN) for 15 min at room temperature. DNA strand breaks were labeled by polymerization of 30  $\mu$ M digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) to the 3'-OH sites, catalyzed by 0.3 units/ $\mu$ l terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) for 45 min at 37°C. Labeled DNA breaks were revealed by anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim) at a dilution of 1:200. The labeled DNA strands were visualized using Fast Red (Sigma, St. Louis, MO). Terminal deoxynucleotidyl transferase or the biotinylated dUTP was omitted for negative controls; sections of a postnatal day-8 rat brain were used for positive controls. To quantify TUNEL-positive cells, at least 2000 tumor cells were counted at 400X magnification. Labeled cells displaying compaction or segregation of the nuclear chromatin, or breaking up of the nucleus into discrete fragments, were counted as apoptotic cells. Labeled cells adjacent to or within necrotic areas were not counted. The apoptotic index (AI) was expressed as the number of TUNEL-positive cells/100 tumor cells.

## RESULTS

**Rapamycin Had Additive Effects with Cisplatin or CPT in Human PNET/MB Cell Lines *In Vitro*.** DAOY cells incubated with rapamycin for 96 h demonstrated a dose-dependent reduction of cell viability that reached maximal effects at 1 ng/ml (Fig. 1A). We selected concentrations of rapamycin from 0.001–1 ng/ml to evaluate drug interaction with cisplatin and CPT. Rapamycin augmented cisplatin- and CPT-induced cytotoxicity in DAOY/MB cells in a dose-dependent fashion. To evaluate drug interaction objectively, the additivity model described in "Materials and Methods" was used;  $r^2$  values for single-agent regression curves were 0.867, 0.941, and 0.888 for survival curves of rapamycin, cisplatin, and CPT, respectively. Using this model, simultaneous treatment of DAOY/MB cells with rapamycin and cisplatin resulted in additive cytotoxicity; the efficacy of cisplatin and 1 ng/ml rapamycin was increased 100-fold relative to cisplatin alone (Fig. 1B). Similar results were observed when rapamycin was combined with CPT (Fig. 1C).

Rapamycin did not augment the cytotoxicity of cisplatin or CPT in *in vitro* studies using D283 PNET/MB cells, a cell line resistant to rapamycin (Fig. 2 A and B).

**CCI-779 Induced Growth Delay in DAOY Xenografts.** To evaluate the antitumor activity of CCI-779 in DAOY flank xenografts, athymic nude mice were treated with four different treatment schedules. Results of these studies are summarized in Table 1. CCI-779 administered at 20 mg/kg 5 days/week for 1 and 2 weeks yielded 1.6- and 2.4-fold delayed tumor growth (Fig. 3). Time to reach 5-fold tumor volume was significantly greater in animals treated for 1 week or 2 weeks compared with control animals (see Table 1). Retreatment of large tumors with CCI-779 for 2 weeks (20 mg/kg *i.p.* 5 days/week on days 29 to 42) restored growth inhibition but did not yield tumor regression (Fig. 3). Treatment with CCI-779 (20 mg/kg *i.p.*) 5 days/week for 4 weeks delayed time to reach 5-fold pretreatment volume by 174% compared with controls (see Table 1). Thus, prolonged treatment is not more efficacious than 2-week treatment. A growth delay  $>50$  days was observed in 20% of animals treated 2 or 4 weeks but not in those treated 1 week.

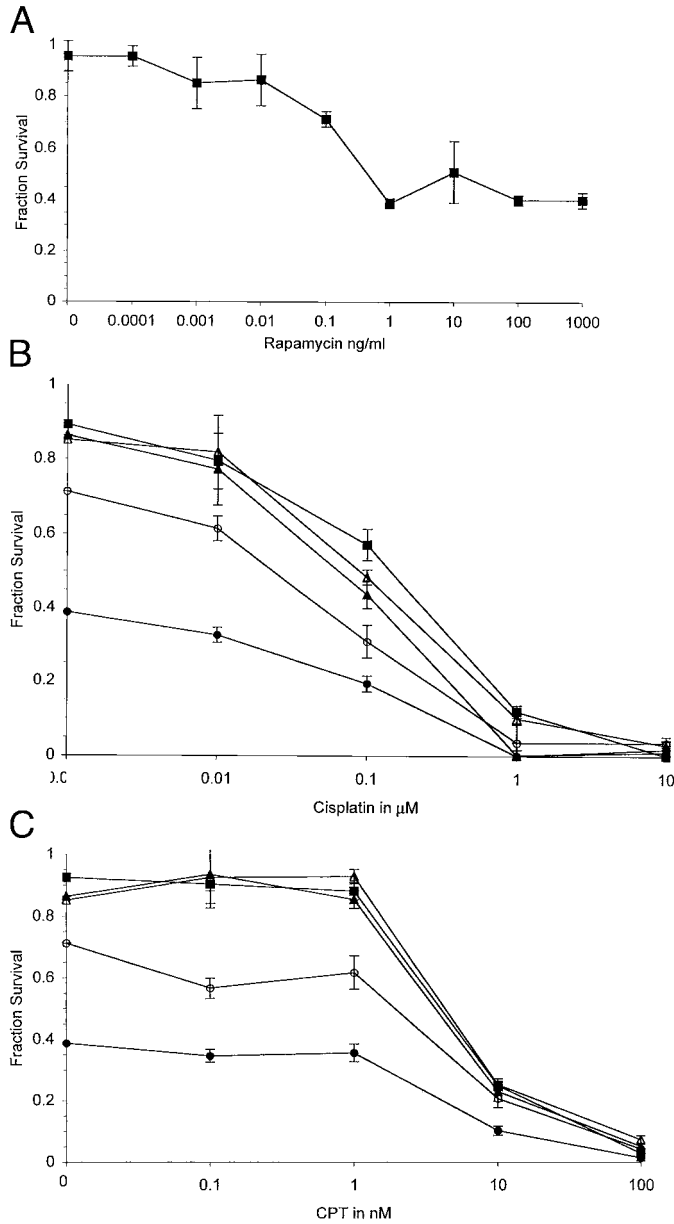


Fig. 1. DA0Y sensitivity to cytotoxic effects of rapamycin *in vitro*. A, dose-response curve of rapamycin as a single agent. Fractional survival of DA0Y cells determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is plotted against the dose of rapamycin (log scale). Data represent the mean of five values. Bars, SE. B, dose-response curves of rapamycin and cisplatin. Fractional survival of DA0Y cells incubated with cisplatin alone (■), with cisplatin and rapamycin 0.001 ng/ml (△), rapamycin 0.01 ng/ml (▲), rapamycin 0.1 ng/ml (○), or rapamycin 1 ng/ml (●). Simultaneous treatment of DA0Y cells with cisplatin and rapamycin increases cytotoxic activity in a dose-dependent and additive fashion. Data are presented as the mean of five values. Bars, SE. C, dose-response curves of rapamycin and camptothecin. Fractional survival of DA0Y cells incubated with CPT alone (■), with CPT and rapamycin 0.001 ng/ml (△), rapamycin 0.01 ng/ml (▲), rapamycin 0.1 ng/ml (○), or rapamycin 1 ng/ml (●). Simultaneous treatment of DA0Y cells with camptothecin and rapamycin increases cytotoxic activity in a dose-dependent and additive fashion. Data are presented as the mean of five values. Bars, SE.

Treatment with a single high dose CCI-779 (100 mg/kg i.p.) induced DA0Y xenograft regression of 37% compared with initial tumor volume within 1 week (Fig. 4). However, unlike tumors treated with daily CCI-779, tumors treated with the single high dose resumed growth after 1 week. Animals bearing these tumors were given a second treatment with 100 mg/kg i.p. on day 12, but did not achieve a second growth regression. Time to reach 5 × pretreatment volume was 14 days in the treatment group compared with 10.5 days in controls (see Table 1).

No significant toxicities were seen in this higher-dosed treatment group, however, weight loss >10% initial body weight and dermatitis were observed in mice treated daily; the percentage of animals exhibiting toxicities increased with the duration of treatment. None of the animals died of chemotherapeutic-induced toxicity.

**CCI-779 Induced Growth Delay in U251 Malignant Glioma Xenografts.** To determine whether the resistance to rapamycin observed *in vitro* occurs *in vivo*, we used xenografts derived from U251 malignant glioma cells, a cell line resistant to rapamycin *in vitro* but reportedly sensitive *in vivo* (12). Animals bearing U251 tumor xenografts were treated with CCI-779 (20 mg/kg i.p. 5 days/week) for 2 weeks. As shown in Table 1 and Fig. 5, time to grow to 5-fold pretreatment tumor volume was 40 days with treatment compared with 27 days in control animals, a delay of 148%.

**CCI-779 and Cisplatin Induced Antitumor Activity in DAOY Xenografts.** To evaluate the interaction of CCI-779 with cisplatin *in vivo*, we treated mice bearing DAOY xenografts with cisplatin (5 mg/kg i.p. once) alone or in combination with CCI-779 (20 mg/kg i.p. 5 days/week) for 2 weeks. Time to reach 5-fold pretreatment volume was 10, 21, and 27 days in control mice, mice treated with cisplatin alone, and mice treated with cisplatin plus CCI-779, respectively (see Table 1 and Fig. 6).

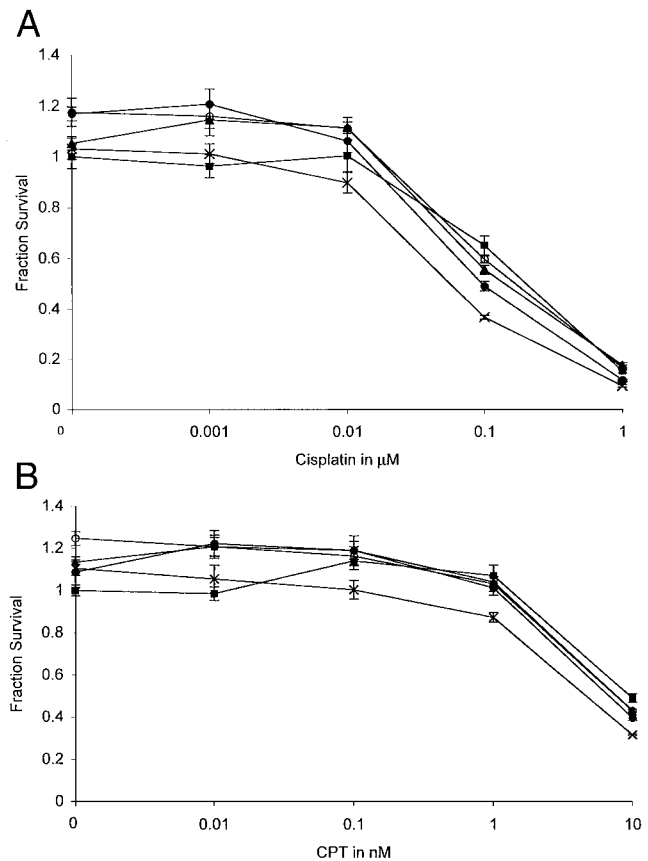


Fig. 2. D283 nonsensitivity to cytotoxic effects of rapamycin *in vitro*. A, dose-response curve of rapamycin and cisplatin. Fractional survival of D283 cells (derived from human PNET/MB) incubated with cisplatin alone (■), with cisplatin and rapamycin 0.01 ng/ml (▲), rapamycin 0.1 ng/ml (○), or rapamycin 1 ng/ml (●) or rapamycin 10 ng/ml (×). Simultaneous treatment of D283 cells with cisplatin and rapamycin did not increase the cytotoxic activity of cisplatin alone. Data represent the mean of five values. Bars, SE. B, dose-response curves of rapamycin and camptothecin. Fractional survival of D283 cells incubated with cisplatin alone (■) with cisplatin and rapamycin 0.01 ng/ml (▲), rapamycin 0.1 ng/ml (○), or rapamycin 1 ng/ml (●) or rapamycin 10 ng/ml (×). Simultaneous treatment of D283-MB cells with camptothecin and rapamycin did not increase the toxic activity of camptothecin alone. Data are presented as the mean of five values. Bars, SE.



Table 1 Summary of human brain tumor xenograft studies

Xenograft cell line	Treatment agent <sup>a</sup>	Dose	Schedule	n	Days to 5-fold pretreatment tumor volume	Range (days)	P <sup>b</sup>
DAOY	Control	0.25 cc i.p.	5 days/wk-2 wk	5	15	16-24	
	CCI-779	20 mg/kg i.p.	5 days/wk-1 wk	6	24	20-30	<0.05
	CCI-779	20 mg/kg i.p.	5 days/wk-2 wk	6	36	25->50	<0.0005
DAOY	Control	0.25 cc i.p.	5 days/wk-4 wk	4	11.5	10.5-16	
	CCI-779	20 mg/kg i.p.	5 days/wk-4 wk	4	20	12->50	<0.05
DAOY	Control	1.5 cc i.p.	Day 1	6	10.5	9-13	
	CCI-779	100 mg/kg i.p.	Day 1	6	14	10-21	<0.05
DAOY	Control	0.25 cc i.p.	5 days/wk-2 wk	5	10	9-12	
	Cisplatin	5 mg/kg i.p.	Day 1	5	21	14-30	<0.05
	Cisplatin + CCI-779	5 mg/kg i.p. + 20 mg/kg i.p.	Day 1 + 5 days/wk-2 wk	5	27	19-32	<0.005
U251	Control	0.25 cc i.p.	5 days/wk-2 wk	4	27	17.5-40	
	CCI-779	5 mg/kg i.p.	5 days/wk-2 wk	6	40	32->50	0.07

<sup>a</sup> Refer to "Materials and Methods" for details of treatment.

<sup>b</sup> Experimental group compared with relevant control.

**CCI-779 Induced Tumor Cell Death in Human PNET/MB Xenografts.** Histological inspection showed that DAOY xenografts from control mice were composed of densely packed cells with hyperchromatic round-to-oval nuclei, nuclei surrounded by scant cytoplasm, and frequent mitotic figures, as many as 20 mitotic figures/field using a 40 × objective. Tumor cells were arranged in solid sheets separated by delicate fibrovascular septae. Small regions of necrosis were often seen at the tumor center (Fig. 7A). The MIB-1 index was 44-48/100 tumor cells, and TUNEL-positive cells occurred in 3.75/100 tumor cells.

Tumors in mice treated with cisplatin alone were smaller, more necrotic (Fig. 7B), and exhibited more proliferation and apoptosis (20-41% of cells stained form MIB-1 and 5% were TUNEL-positive) when compared with tumors from control mice harvested the same day after implantation. DAOY xenografts in mice treated with CCI-779 alone (Fig. 7C) showed lower proliferation activity than those in controls (18 and 17% MIB-1-positive cells), but not increased apoptosis (2.5% TUNEL-positive cells). DAOY tumors from mice treated with cisplatin and CCI-779 were smaller than controls and exhibited large areas of necrosis, hyalinization, and hemosiderin deposition

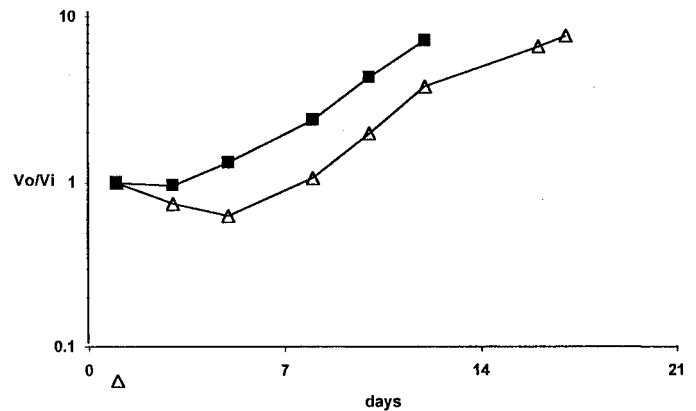


Fig. 4. Suppression of DAOY xenograft growth by a single dose of CCI-779. Athymic mice bearing human DAOY flank xenografts were separated into two treatment groups: control (■) or CCI-779 100 mg/kg i.p. on day 1 (△). Time of treatment is indicated by a symbol beneath the abscissa. Tumor size was normalized by dividing the observed tumor volume by the initial pretreatment tumor volume (Vo/Vi), and this was plotted on a log scale. CCI-779 induced 37% tumor regression within 1 week, but only increased the time to grow 5-fold the initial tumor volume by 131%, as compared with controls.

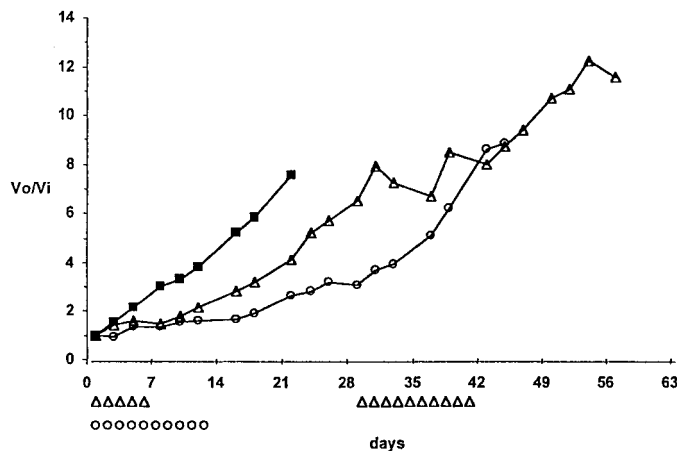


Fig. 3. Suppression of DAOY xenograft growth by CCI-779 used as a single agent. Athymic mice bearing human DAOY flank xenografts were separated into three treatment groups: control (■) and CCI-779 20 mg/kg 5 days/week i.p. for 1 week (△) or for 2 weeks (○). Symbols along the abscissa indicate the time of CCI-779 injection for the latter treatment groups. Tumor size was normalized by dividing the observed tumor volume by the initial pretreatment tumor volume (Vo/Vi), and this was plotted over time with day 1 representing the first day of treatment. CCI-779 treatment delayed growth to 5-fold initial tumor volume by 160% (1 week) and by 240% (2 weeks), compared with controls. Tumors of animals treated with CCI-779 for 1 week received a second course of CCI-779 (20 mg/kg, 5 days/week) beginning day 29 as indicated by the △ along the abscissa. Retreatment resulted in a flattening of the growth curve.

(Fig. 7D). Tumor cells surrounding the central necrosis exhibited 18-38% MIB-1-labeling and 5.5% TUNEL-labeling.

**DISCUSSION**

Rapamycin and its analogue CCI-779 are new cytotoxic agents with the potential to improve the current treatment of pediatric brain tumors. The antitumor activity of rapamycin has been demonstrated in human rhabdomyosarcoma and neuroblastoma tumor cell lines *in vitro* (9, 11) and in B16 melanocarcinoma, Colon 38 tumors, CD8F1 mammary tumors, EM ependymoblastoma, and U251 glioblastoma brain tumors *in vivo* (10, 12, 13).

This report documents the additive cytotoxicity of rapamycin and CCI-779 when combined with a platinating agent or a topoisomerase I inhibitor, classes of drugs currently used clinically for PNET/MB (15, 30). We found that cytotoxic efficacy *in vitro* and tumor regression *in vivo* were modulated by dosing schedule. Furthermore, this report is the first to document the cytotoxicity of rapamycin analogue CCI-779 in human PNET/MB and malignant glioma xenograft tumors both as a single agent and in combination with cisplatin. This supports previous reports that *in vitro* resistance does not accurately predict the efficacy of this agent *in vivo* and demonstrates that tumor toxicity can be increased by using combination chemotherapy without the risk of increased systemic cytotoxicity.

The antitumor activity of CCI-779 *in vivo* was tested using four

distinct treatment schedules. Nude mice engrafted with human DAOY flank xenografts treated systemically with CCI-779 20 mg/kg daily 5 times per week exhibited significant delay of tumor growth. Treatment for 2 weeks was superior to shorter and longer treatment intervals. CCI-779 retreatment during xenograft expansion after treatment-induced growth delay restored growth inhibition, suggesting efficacy even for bulky tumors. Single high-dose treatment with CCI-779 achieved transient tumor regression, however, overall tumor growth delay was inferior to the equivalent dose given daily over a week. Previous studies examining antitumor activity of rapamycin *in vivo* used higher doses and different treatment schedules. For example, Houchens *et al.* (12) used 200, 400, and 800 mg/kg rapamycin i.p. on days 2, 6, and 10 to treat U251 malignant glioma xenografts, whereas Eng *et al.* (13) reported on the antitumor activity of 100 mg/kg i.p. for 9 days or 400 mg/kg i.p. on days 2 and 9 in melanoma, ependyoblastoma, and Colon 38 tumor models. *In vitro* results demonstrated a dose threshold of 1 ng/ml in DAOY, above which additional cytotoxicity is not achieved. *In vivo* results support the absence of a linear dose-response relationship in these tumors. Accordingly, our results indicate that high doses are not necessary for antitumor activity.

*In vitro* studies of rapamycin identify an interesting pattern of tumor cell response: either exquisite sensitivity or profound resistance (9, 11). Evaluation of brain tumor cell lines in our laboratory show a similar pattern. Four PNET/MB cell lines were highly sensitive to growth inhibition by rapamycin *in vitro* ( $IC_{50} \leq 10$  ng/ml), whereas three PNET/MB cell lines and the U251 malignant glioma cell line

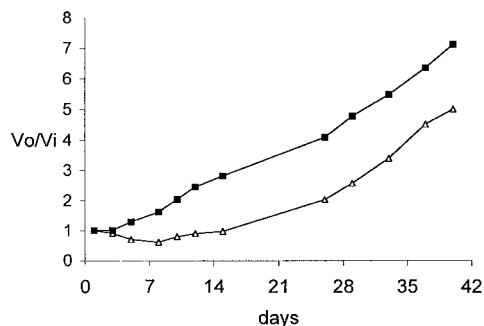


Fig. 5. Suppression of U251 malignant glioma xenograft growth by CCI-779. Athymic mice bearing flank xenografts derived from the *in vitro* nonsensitive human U251 glioma cell line were separated into two groups: control (■) and CCI-779 20 mg/kg 5 days/week i.p. for 2 weeks (△). Symbols along the abscissa indicate time of CCI-779 injection in the treatment group. CCI-779 induced a tumor growth delay of 131% compared with controls.

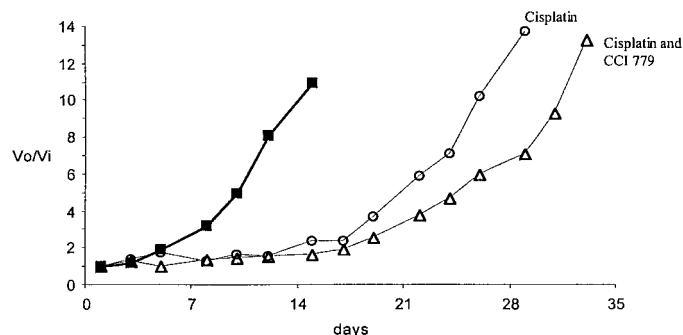


Fig. 6. Suppression of DAOY xenograft growth by cisplatin with or without CCI-779. Athymic mice bearing human DAOY flank xenografts were separated into three treatment groups: control (■), cisplatin (○), and cisplatin plus CCI-779 (△). Cisplatin alone (5 mg/kg i.p., day 1) induced a tumor growth delay of 210%, compared with controls. Simultaneous treatment with cisplatin (5 mg/kg i.p., day 1) and CCI-779 (20 mg/kg i.p., 5 days/week for 2 weeks) enhanced this delay by another 19%.

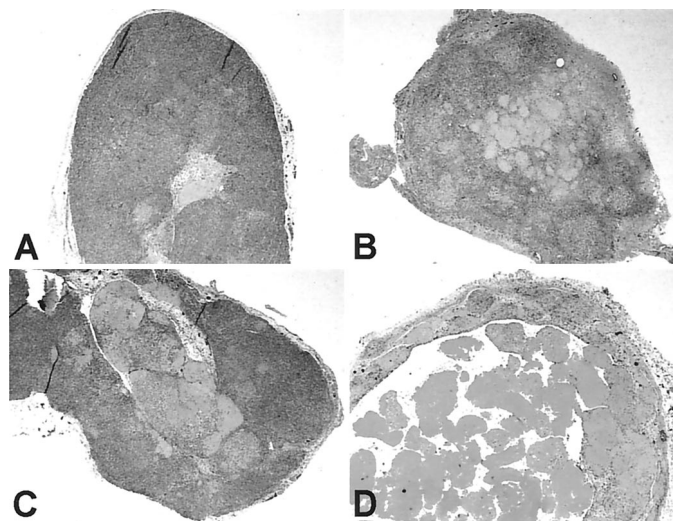


Fig. 7. Impact of systemic cisplatin and/or CCI-779 on DAOY tumor histology. A, control. Micrograph of DAOY flank tumor excised from athymic mouse that had not received antitumor therapy. Tumor volume on day of excision (day 4 after treatment) was 131% larger than the initial tumor volume. The section illustrates homogeneous, cellular tumor cells with large nuclei and little cytoplasm. H&E stain,  $\times 25$ . B, cisplatin. Micrograph of DAOY flank tumor excised from athymic mouse treated with single-dose cisplatin (5 mg/kg i.p.). Tumor volume on the day of excision (day 4 after treatment) was 50% of the initial tumor volume. The tumor exhibits small areas of necrosis. H&E stain,  $\times 25$ . C, CCI-779. Micrograph of DAOY flank tumor excised from athymic mouse treated with a single dose CCI-779 (100 mg/kg i.p.). Tumor volume on the day of excision (day 4 after treatment) was 89% of the initial tumor volume. The tumor exhibits small areas of necrosis comparable with those seen with cisplatin treatment. H&E stain,  $\times 25$ . D, cisplatin plus CCI-779. Micrograph of DAOY flank tumor excised from athymic mouse treated with single-dose cisplatin (5 mg/kg, i.p., day 1) and single-dose of CCI-779 (100 mg/kg, i.p., day 1). Tumor volume on the day of excision (day 4 after treatment) was 32% of the initial tumor volume. The tumor exhibits a large area of central necrosis. H&E stain,  $\times 25$ .

were nearly resistant ( $IC_{50} > 1000$  ng/ml).<sup>5</sup> No cell lines tested exhibited intermediate sensitivity. The mechanism(s) determining resistance or sensitivity to rapamycin is still under investigation. Sensitivity of cancer cell lines may be attributable to the inhibition of insulin-like growth factor-I receptor-mediated signaling by rapamycin, which is essential for proliferation in some tumor cells (*i.e.*, alveolar RMS cell lines; Ref. 11). Cellular resistance to rapamycin has been correlated to paracrine growth factor signaling pathways (9), the failure to inhibit c-Myc induction, mammalian target of rapamycin mutants (11), and GLI zinc finger protein (31). Nevertheless, CCI-779 treatment induced tumor growth delay in nude mice bearing U251 xenografts, a cell line highly resistant to rapamycin *in vitro*. Houchens *et al.* (12) report similar results using rapamycin to treat U251 xenografts, suggesting that resistance to rapamycin and its analogues documented *in vitro* does not accurately predict resistance *in vivo*.

Rapamycin exhibits the same activity in the Colon 38 tumor model when administered i.p., i.v., i.m., and s.c.; upon p.o. administration, its activity was reduced but not abolished (13).

Because of its high lipophilic index (18), rapamycin easily crosses the blood-brain-barrier, which is important for systemic administration in the treatment of brain tumors. Pharmacological studies of rapamycin show that rapamycin is cleared rapidly from the blood after s.c. or p.o. administration and sequestered intracellularly, particularly in erythrocytes. It dissociates slowly from intracellular FK506-binding protein-12 and is metabolized by cytochrome p450 3A enzyme (8). This may explain its long half-life *in vivo* (57–62 h in renal transplant patients; Ref. 32).

Rapamycin induces reduction of tumor growth with little systemic toxicity as compared with other antitumor drugs. In our hands, anti-

<sup>5</sup> Manuscript in preparation.

mals treated with a single dose of 100 mg/kg did not experience any obvious drug toxicity. With daily treatment for >2 weeks (20 mg/kg 5 days/week) animals experienced weight loss and dermatitis. Weight loss, increase in blood glucose concentrations, gastric ulceration, and thrombocytopenia have been described by others (19, 21, 33). Eng *et al.* (13) reported the LD<sub>50</sub> of rapamycin in mice as 587 mg/kg, yet in an earlier study no significant side effects were mentioned when doses up to 800 mg/kg were used (12). Clinical Phase III trials designed to evaluate rapamycin as an immunosuppressant have demonstrated minimal systemic toxicity (20, 22). Two clinical Phase I trials of CCI-779 in patients with advanced tumors are currently ongoing. CCI-779 dose escalation from 7.5–60 mg/kg i.v. in a weekly dose did not induce grade III-IV nor dose-limiting toxicity. Neither significant immunosuppression nor opportunistic infections were observed (34).

Cisplatin- and topoisomerase I-inhibitors such as camptothecin, CPT 11, and topotecan are effective agents in the chemotherapeutic treatment of brain tumors (15, 30). However, high doses lead to severe multiorgan toxicities (35) that limit the ability of dose intensification to increase treatment efficacy and patient survival. CCI-779 is a compelling candidate for novel adjuvant anticancer treatment because of limited toxicity and preclinical studies indicating additive cytotoxicity with several chemotherapeutic agents. Eng *et al.* found that rapamycin enhanced the cytotoxicity of 5-fluorouracil and cyclophosphamide when the drugs were administered sequentially in Colon 38 tumor-bearing mice, and that this combination was more effective than 5-fluorouracil-adriamycin-cyclophosphamide (13). Using DAOY cells, we demonstrated the additive cytotoxicity of rapamycin with cisplatin and camptothecin *in vitro* and the enhanced antitumor effect of CCI-779 with cisplatin *in vivo*. Not only did simultaneous administration of CCI-779 and cisplatin delay xenograft growth, but pathological inspection revealed large areas of central necrosis in the tumors of mice receiving combination chemotherapy far less evident than in the tumors of mice treated with single agent. These data support a role for the use of the rapamycin analogue CCI-779 in combination chemotherapy in the treatment of PNET/MB.

In conclusion, this study documents the cytotoxicity of rapamycin and its analogue, CCI-779, in brain tumor models alone and in combination with other antitumor chemotherapy. This class of agents with a novel mechanism of action, low toxicity, and additive cytotoxicity in combination therapy has great potential in the treatment of human brain tumors including PNET/MB and malignant glioma.

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