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Local delivery of rapamycin: a toxicity and efficacy study in an experimental malignant glioma model in rats

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Rapamycin, an anti-proliferative agent, is effective in the treatment of renal cell carcinoma and recurrent breast cancers. We proposed that this potent mammalian target of rapamycin inhibitor may be useful for the treatment of gliomas as well. We examined the cytotoxicity of rapamycin against a rodent glioma cell line, determined the toxicity of rapamycin when delivered intracranially, and investigated the efficacy of local delivery of rapamycin for the treatment of experimental malignant glioma in vivo. We also examined the dose-dependent efficacy of rapamycin and the effect when locally delivered rapamycin was combined with radiation therapy. Rapamycin was cytotoxic to 9L cells, causing 34% growth inhibition at a concentration of 0.01 μg/mL. No in vivo toxicity was observed when rapamycin was incorporated into biodegradable caprolactone-glycolide (35:65) polymer beads at 0.3%, 3%, and 30% loading doses and implanted intracranially. Three separate efficacy studies were performed to test the reproducibility of the effect of the rapamycin beads as well as the validity of this treatment approach. Animals treated with the highest dose of rapamycin beads tested (30%) consistently demonstrated significantly longer survival durations than the control and placebo groups. All dose-escalating rapamycin bead treatment groups (0.3%, 3% and 30%), treated both concurrently with tumor and in a delayed manner after tumor placement, experienced a significant increase in survival, compared with controls. Radiation therapy in addition to the simultaneous treatment with 30% rapamycin beads led to significantly longer survival duration than either therapy alone. These results suggest that the local delivery of rapamycin for the treatment of gliomas should be further investigated.

Keywords: Glioma, local delivery, rapamycin, sirolimus.

Because of the implementation of combination therapies, the prognosis for patients diagnosed with glioblastoma multiforme (GBM), has increased from 9 months after their initial diagnosis to 20 months during the past decade at the Johns Hopkins Hospital Brain Tumor Center (Baltimore, MD).[1] Despite this success, the response rate of novel targeted therapies has been very low (0%–20%)[2–6] and there is inconclusive evidence regarding their efficacy with respect to survival. Currently, the standard of care for patients with gliomas is surgical debulking when possible, followed by radiation therapy and oral, intravenous, or locally delivered chemotherapeutic agents.[7] As the understanding of receptor-mediated signaling in gliomagenesis grows, there is an increasing number of specific intracellular molecules that are attractive treatment targets for patients with brain tumors.[8] Of the many that have been identified, specific inhibitors of serine/threonine kinases and their associated signaling components have shown great promise.

Rapamycin, also known as FK506 or sirolimus, is a natural product from the soil bacterium *Streptomyces hygrosopius* and is a potent and specific inhibitor of the mammalian target of rapamycin (mTOR).[9] The mTOR serine/threonine kinase lies downstream of PI3K in the PI3K/Akt signaling pathway, which plays a central role in cell cycle progression and protein translation.[10,11] Intracellular rapamycin (FK506) binds to

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cytoplasmic immunophilin FK506–binding protein (FKBP).[12–14] The rapamycin-FK506BP complex then acts to inhibit mTOR. When activated by cytokine-mediated signaling, mTOR phosphorylates 2 major proteins that regulate cell cycle progression and translation.[15] Inhibition of mTOR prevents phosphorylation of the 40S ribosomal protein p70S6k and the eukaryotic translation initiation factor 4E-BP1, resulting in the arrest of the cell cycle at the G1 phase and a halt in protein synthesis.[12,14] Under the trade names of Prograf, Advagraf, and Protopic (Astellas Pharma UA), rapamycin is clinically used as a potent immunosuppressive agent after solid-organ transplantation and also as a possible antitumor agent in renal cell carcinoma and recurrent breast cancer.[16,17]

New evidence suggests that components of the mTOR pathway are elevated in gliomas and contribute to the uncontrolled proliferative properties of these cells, making make them attractive targets of new therapies. Jiang et al.[18] reported that the intracellular binder of rapamycin, FKBP, is over-expressed in glioma and mediates uncontrolled glioma cell growth via the NF-kB pathways. In addition, Masri et al.[19] reported that mTOR complex 2, which is composed of mTOR, rapamycin-insensitive companion of mTOR, GβL, and mammalian stress-activated protein kinase interacting protein, is elevated in gliomas and helps promote tumor cell proliferation and increase invasive potential.

Preclinical studies of systemically delivered rapamycin have shown great promise but this has not been observed clinically for the treatment of malignant gliomas.[20] Analogues of rapamycin have been used as an intravenous infusion in phase I and II clinical trials with only limited, modest, or inconclusive results.[21–25] In a phase I study of CCI-779, an ester of rapamycin, Chang et al.[26] demonstrated that stomatitis, hypercholesterolemia, and hyperglyceridemia toxicities limited dosing to 250 mg intravenously per week in patients with malignant gliomas. A phase II trial by the same group demonstrated that dosing had to be reduced in patients who were receiving enzyme-inducing antiepileptic drugs due to intolerable side effects caused by drug-drug interactions. At this lower dose, the drug was well tolerated, but there was no evidence of efficacy against recurrent GBM.[21] Galanis et al.[22] reported in a phase II trial of CCI-779 in patients with recurrent GBM that 36% of patients demonstrated radiographic improvement without improvement in progression-free survival. Similar to the findings of Chang and colleagues, Galanis and colleagues reported hypercholesterolemia, hypertriglyceridemia, and hyperglycemia as dose-limiting toxicities. Doherty et al.[27] reported a 19% response rate in a pilot study of the combination of epidermal growth factor receptor (EGFR) and mTOR inhibitors in recurrent malignant gliomas that was well tolerated, but long-term progression-free survival was not measured. In a study of the safety and pharmacokinetics of escalating doses of CCI-779, Raymond et al.[28] reported that, in patients with solid-organ cancers (renal, colorectal, and soft-tissue sarcoma), the dose-limiting toxicities were more severe and included manic-depressive syndrome, stomatits, and skin toxicity. Cloughesy et al.[29] conducted a phase I trial of oral neoadjuvant rapamycin in patients with recurrent PTEN-deficient glioblastoma and found evidence of antitumor activity using short-term end points. It was also noted that, despite achieving a sufficient concentration of rapamycin in the tumor, the magnitude of mTOR suppression varied widely. This resistance could not be attributed to cell-intrinsic mechanisms, because it was not found during subsequent in vitro testing. It is possible that the delivery of rapamycin to the tumor cells was actually inadequate and that the concentration measured in the tumor samples was the rapamycin sequestered in the red blood cells of gliomas with high neovascularization.

Local delivery of chemotherapeutic agents has been shown to reduce systemic toxicity and extend survival in patients. Gliadel wafers (Eisai, Inc)—3.8% carmustine in biodegradable polymer wafers—used for the treatment of GBM can prolong survival up to 12 months, compared with standard therapies.[1] We previously demonstrated that local intracranial delivery of temozolomide was superior to conventional oral delivery using a rat glioma model.[30] Many researchers have reported that the development of effective systemic chemotherapy has been difficult, primarily because of the presence of the blood-brain barrier.[31] In addition, it is known that malignant gliomas are most likely to progress or recur, most often within 2 cm of the original site of resection.[32] Therefore, the local delivery of chemotherapeutic agents may be most effective in combating local tumor recurrence by maximizing the concentration of drug at the tumor site and minimizing systemic toxicity.

On the basis of the promising in vitro antitumor activity of rapamycin, the selective upregulation of mTOR components in gliomas, and the disappointing results of phase I and II clinical trials involving systemic delivery of rapamycin and its analogues, we studied the safety and efficacy of locally delivered rapamycin via biodegradable beads in an experimental rat glioma model. In vitro studies demonstrated the cytotoxicity of rapamycin, and in vivo studies examined the safety of locally delivered rapamycin, the efficacy of this agent, and the dose-dependent efficacy on survival. To more closely mimic the clinical situation, we also combined rapamycin with radiation to see whether the combination of these 2 therapies was favorable.

Materials and Methods

In vitro Cytotoxicity Analysis

Rapamycin was investigated for its cytotoxic properties against the 9L rodent gliosarcoma cell line. Rapamycin was obtained from Advanced Technologies & Regenerative Medicine. 9L cells were obtained from the UCSF Brain Tumor Repository. Cells were maintained in DMEM with 10% fetal bovine serum and kept humidified at 37°C with 5% CO_2. Cells were plated in 96-well plates at 2500 cells/well and were allowed to adhere

for 24 h. Cells were then treated with rapamycin at varying concentrations (range, 0.001–10 μg/mL) for 72 h. Ethanol was used as the vehicle solvent in control wells. Cell viability was then assessed by a yellow tetrazolium salt (MTT) assay (Aldrich Chemicals), and optical density was measured at 570 nm.

Incorporation of Rapamycin into Biodegradable Beads

Rapamycin was incorporated into a biodegradable polymer by Advanced Technologies & Regenerative Medicine. The polymer used for the beads was a 35:65 ratio of caprolactone and glycolide (PCL/PGA), and the rapamycin beads were prepared as follows. In brief, a 5% 35/65-PCL/PGA polymer solution in 1,4 dioxane solvent was prepared. Polymer solution was heated to 60°C for 4 h with continuous stirring to ensure complete dissolution of the polymer. The solution was then filtered through an extra coarse Pyrex thimble prior to use. Rapamycin (w/w 0.3%, 3%, or 30%) was then incorporated into the polymer solution at room temperature using continuous stirring. The drug was dissolved in the polymer solution instantaneously. The drug-loaded solution was then added drop-wise through a disposable pipette into liquid nitrogen to form frozen beads. These frozen beads were placed in an aluminum tray and lyophilized to remove the solvent. Residual dioxane was <1 ppm for the 0.3% and 3% beads and was 90 ppm for the 30% rapamycin beads, as determined by high-performance liquid chromatography (HPLC). To evaluate the actual drug content of the beads, three 5-mg beads were cut into 6 to 8 pieces using a scalpel, drug was extracted with 3-mL methanol, and drug content measured by HPLC. Actual drug content of the beads as prepared was 0.23%, 2.27%, and 27.78%. Beads were sterilized using 15-kGy γ-irradiation on dry ice.

In vitro Measurement of Rapamycin Release from Beads

Rapamycin beads (10 mg each) were placed in 1 mL of phosphate-buffered saline (PBS) in triplicate at 37°C. Periodically, PBS was removed and replaced and rapamycin concentrations were determined using HPLC with mass spectrometric detection (LC/MS/MS) over the concentration range of 0.5 to 200 ng/mL. In brief, sample preparation involved a single-step liquid-liquid extraction by the addition of 0.5 mL PBS with 5 mL of acetonitrile-n-butyl chloride (1:4, v/v). Separation of rapamycin and the internal standard was achieved on a Waters X-Terra™ (Waters Corp) C_{18} (50 × 2.1 mm; 3.5 μm inner diameter) analytical column using a mobile phase consisting of acetonitrile-ammonium acetate (pH 2.9; 2 mM) (7:3, v/v) containing formic acid (0.1%, v/v) using isocratic flow at 0.15 mL/min for 7 min. Detection was performed using electrospray ionization MS/MS operating in the positive mode by monitoring the ion transitions from m/z 931.8 to >864.6 (rapamycin) and m/z 470.1 to >148.0 (internal standard). The linear calibration curves were generated over the range of 0.5 to 200 ng/mL. Measurements were completed at 21 days and again at 10 weeks.

In vivo Safety of Intracranially Implanted Rapamycin Beads

Rapamycin was implanted intracranially in 10 mg doses ($n = 5$ per group) into 20 F344 rats, as detailed elsewhere.[30,33] Animals were observed daily for any signs of deterioration, neurotoxicity, and movement disorders and were weighed weekly. On Day 75, rats were euthanized, and their brains were placed in formalin for histological analysis. During the histological analyses, brains were examined for residual beads, intracranial artifact, and any deleterious effects the beads and/or drug may have had on the brain parenchyma.

In vivo Efficacy of Intracranially Implanted Rapamycin

To determine the efficacy of locally implanted rapamycin, rats were randomized into 4 groups and implanted with 9L gliosarcoma, in accordance with previously published methods.[30,33] The 9L gliosarcoma was maintained in the flanks of female F344 rats (Harlan Sprague Dawley). For intracranial implantation, the tumor was surgically excised from the carrier animal, cut into 1-mm^3 pieces, and placed in sterile 0.9% NaCl on ice. Thirty female Fisher 344 rats (150–200 g) were anesthetized with an intraperitoneal injection of 3 to 5 mL/kg of a stock solution containing ketamine hydrochloride, 75 mg/mL (Ketlar; Parke-Davis); xylazine, 7.5 mg/mL (Rompun; Mobay); and 14.25% ethyl alcohol in 0.9% NaCl. All surgical procedures were performed using standard sterile surgical technique. The head was prepped and a midline scalp incision was made, exposing the sagittal and coronal sutures. A small burr hole was drilled, centered 3 mm lateral to the sagittal suture, avoiding the sagittal sinus, and 5 mm posterior to the coronal suture. The dura was incised, and forceps were used to lift off remaining bone and dura. With use of gentle suction, a small area of cortex was resected. Once hemostasis was achieved, a single tumor piece (1 mm^3) was placed in the depths of the cortical resection. Rats received the treatment of rapamycin beads either simultaneously at the time of tumor implantation or 5 days after tumor implant (to more closely mimic the clinical situation where residual tumor remaining after resection must be treated). All groups receiving beads received an implantation weighing 10 mg. The groups were as follows: group 1 ($n = 8$) received no treatment, group 2 ($n = 7$) received blank polymer beads on day 0, group 3 ($n = 7$) received 30% rapamycin beads on day 0, and group 4 ($n = 7$) received 30% rapamycin beads on day 5.

For groups receiving simultaneous implantation, 10 mg of the appropriate concentration of rapamycin beads were implanted into the existing space with the tumor implantation. The skin was then closed with surgical staples. For rats receiving treatment 5 days after tumor implantation, rats were re-anesthetized, and the incision

was re-opened, and 10 mg of beads were placed into the original burr hole. Hemostasis was achieved and the incision was closed with surgical wound clips. Animals were observed daily and were euthanized when moribund. Brains were removed to formalin for analysis.

Dose-Dependent Efficacy of Intracranially Implanted Rapamycin

Eighty F344 rats received an intracranial implant of 9L gliosarcoma as described above. Animals were then divided into 2 groups: those whose treatment was given simultaneously with tumor and those who received treatment 5 days after tumor implantation. Animals in each group were then divided into 1 of 5 treatment groups: (1) placebo beads, (2) 30% rapamycin beads (total dose of rapamycin, 3 mg), (3) 3% rapamycin beads (total dose of rapamycin, 0.3 mg), (4) 0.3% rapamycin beads (total dose of rapamycin, 0.03 mg), or (5) systemically delivered rapamycin (L.C. Laboratories; 2 mg/kg i.p. once per day in dimethyl sulfoxide (DMSO) for 30 days for a total dose of 10.5 mg). Animals were observed daily, and survival was recorded.

Efficacy of Locally Delivered Rapamycin with Radiation Therapy

To mimic clinical therapeutic regimens more closely, we combined our local delivery of rapamycin with radiation therapy (XRT) to treat the rodent tumor model. Fifty-six rats were implanted with the 9L tumor and then were divided into the following groups: (1) control animals that received no treatment ($n = 8$); (2) a placebo group that received placebo beads at the time of tumor implantation ($n = 8$); (3) animals that received placebo beads at the time of tumor implantation, followed 5 days later with XRT ($n = 8$); (4) animals that received 30% rapamycin beads simultaneous with the tumor implantation ($n = 8$); (5) animals that received 30% rapamycin beads 5 days after tumor implantation ($n = 8$); (6) animals that received 30% rapamycin beads simultaneous with the tumor implantation, followed by XRT 5 days after implantation ($n = 8$); and (7) animals that received 30% rapamycin beads and XRT 5 days after tumor implantation ($n = 8$).

Radiation Therapy

For XRT, animals were anesthetized, placed at a fixed distance from the radiation source, and shielded with a square primary collimator (1 cm in diameter) centered over the tumor implantation site. The irradiated animals received external beam single-dose radiation treatment using a ^{138}Cs laboratory irradiator (Mark 1 Irradiator, Model 68) at a dose of 20 Gy.[30]

Animal Care

All animals were housed in standard facilities and given free access to food and water. They were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. On day 120, efficacy studies were terminated with rats deemed as long-term survivors. They were then euthanized and specimens collected to formalin after perfusion.

Statistical Analysis

In vitro results are reported as mean ± SD. For the efficacy experiments, survival was the primary end point. Kaplan-Meier analysis was used to compare survival using GraphPad Prism software, version 5.1. The log-rank (Mantel-Cox) test was used to compare groups, and groups were considered statistically different at $P < .05$. P value analyses were 2-sided.

Results

In vitro Cellular Proliferation Analysis

Rapamycin inhibited the growth and proliferation of 9L cells, causing 34% growth inhibition at 0.01 μg/mL, which increased to 62% inhibition at 10 μg/mL, as compared to vehicle-treated cells. All groups were statistically significantly different from the control ($P < .0001$) using a 1-way analysis of variance, as well as Bartlett's test for equal variances ($P < .0001$).

In vitro Release of Rapamycin from the Biodegradable Beads

In vitro release kinetics demonstrated a burst in drug release during the first 4 days with a steady increase in release for 3 weeks (Fig. 1). Release kinetics were continued for a total of 10 weeks, but by the end

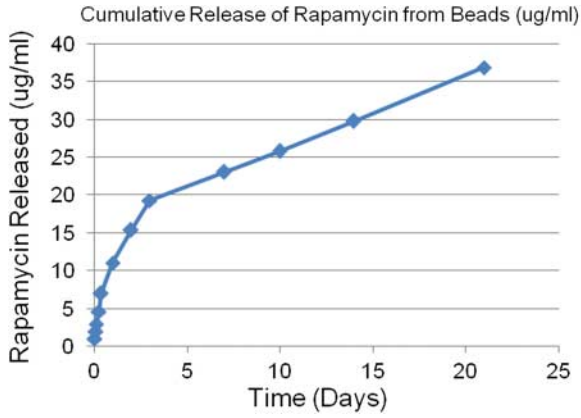

Fig. 1. Cumulative release of rapamycin from beads. Beads (10 mg each) were placed in 1 mL of phosphate-buffered saline (PBS) in triplicate at 37°C. Periodically, PBS was removed and replaced, and rapamycin concentrations were determined using high-performance liquid chromatography with mass spectrometric detection (LC/MS/MS).

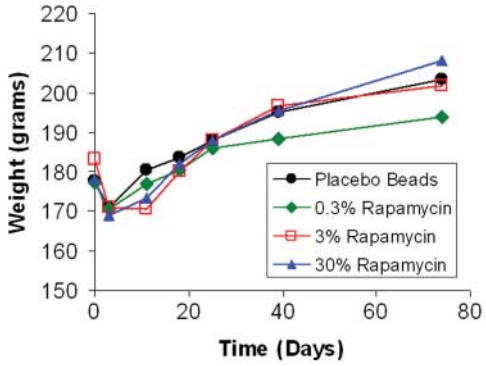

Fig. 2. Weights of rodents implanted intracranially with rapamycin beads. F344 rats ($n = 5$ per group) were intracranially implanted with 10 mg each of 0.3% (closed diamonds), 3% (open squares), or 30% (closed triangles) rapamycin beads or placebo beads (closed circles). There were no deaths due to the implantation or the resulting release of rapamycin. The study was ended 75 days after implantation.

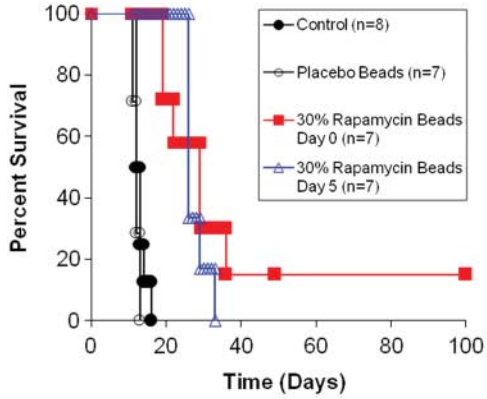

Fig. 3. Efficacy of locally delivered rapamycin against the 9L experimental malignant glioma model. Shown is a Kaplan-Meier plot of F344 rats that were implanted with 9L and either left untreated (closed circles; $n = 8$), treated simultaneously with placebo beads (open circles; $n = 7$) or 30% rapamycin beads (closed squares; $n = 7$; $P < .0001$, compared with the control group), or treated 5 days later with 30% rapamycin beads (open triangles; $n = 7$; $P < .0001$, compared with the control group).

of a period of 2 months, polymer degradation affected the measurement of rapamycin. It has been shown previously that the caprolactone/glycolide polymer is re-absorbed in vivo (at sites other than the brain) by 90 to 120 days (internal unpublished data). γ Irradiation of beads for the purpose of sterilization did not affect the drug content.

Toxicity of Intracranially Implanted Rapamycin

Animals intracranially implanted with increasing doses of rapamycin beads displayed no signs of systemic or intracranial toxicity and displayed normal weight gain

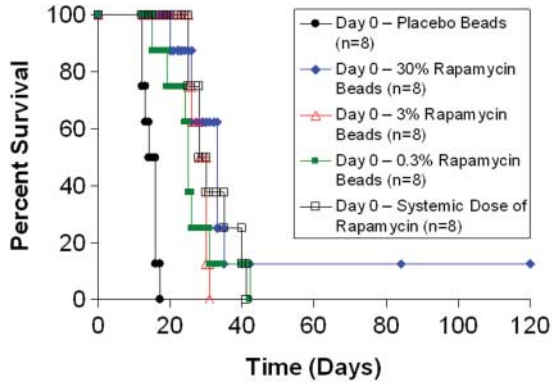

Fig. 4. Rapamycin beads: concurrent treatment and 9L tumor. Kaplan-Meier plot demonstrating survival in animals that received placebo beads, increasing concentrations of rapamycin beads (30%, $P < .0001$ versus the control group; 3%, $P < .0001$ versus the control group; 0.3%, $P < .0006$ versus the control group), or systemic rapamycin (2 mg/kg intraperitoneally per day for 30 days; $P < .0001$, compared with the control group) simultaneously with tumor implantation.

(Fig. 2), with 100% survival. These animals were euthanized 75 days after implantation. Histological analysis of the fixed brains showed no deleterious effects from the implantation.

Efficacy of Intracranially Implanted Rapamycin

Treatment with locally delivered rapamycin at the 30% loading concentration significantly prolonged survival of animals that were intracranially challenged with 9L gliosarcoma (Fig. 3). Animals implanted simultaneously with both tumor and rapamycin beads had a median survival duration of 29 days, compared with 12.5 days for the control group ($P < .0001$) and 12 days for the group that received placebo beads ($P = .0002$). Animals receiving intracranial tumor that were treated 5 days later with locally implanted rapamycin beads had a median survival duration of 26 days; this was considered prolonged compared with controls ($P < .0001$) and placebo recipients ($P = .0002$). There was no statistically significant difference in survival duration between the 2 high-dose rapamycin treatment groups that received rapamycin at different time points.

Dose-Dependent Efficacy of Intracranially Implanted Rapamycin

Animals that received concurrent rapamycin bead therapy at any concentration experienced an increase in survival, compared with controls ($P = .0006$ for 0.3%, $P < .0001$ for 3%, and $P < .0001$ for 30% rapamycin). Animals receiving tumor and concurrent treatment had a median survival duration of 25 days with 0.3% rapamycin, 28 days with 3% rapamycin, 33 days with 30% rapamycin, and 28 days with systemic rapamycin (Fig. 4); the group that received placebo beads had a median survival duration of 15 days. There was

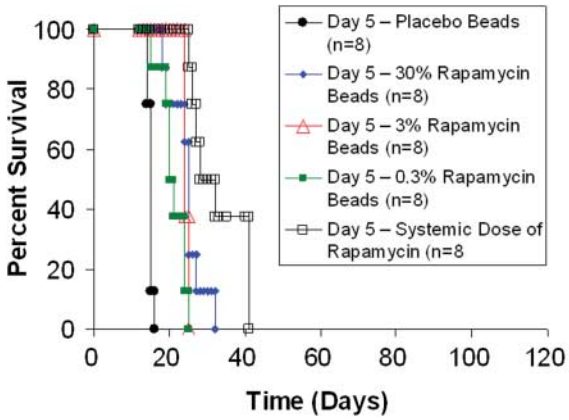

Fig. 5. Rapamycin beads: treatment 5 days after 9L tumor. The figure shows a Kaplan-Meier plot demonstrating survival in animals that received placebo beads, increasing concentrations of rapamycin beads (30%, $P < .0001$ versus the control group; 3%, $P < .0001$ versus the control group; 0.3%, $P < .0001$ versus the control group), or systemic rapamycin (2 mg/kg intraperitoneally per day for 30 days; $P < .0001$, compared with the control group) 5 days after tumor implantation.

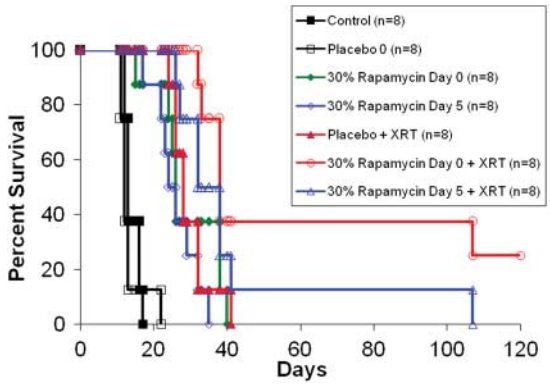

Fig. 6. Efficacy of rapamycin delivered intractranially on day 0 or day 5, with and without radiation therapy (XRT) against 9L tumor. The Kaplan-Meier plot curve shows animals implanted with 9L tumor and receiving either simultaneous placebo beads or 30% rapamycin beads ($P = .0004$, compared with the control group) or delayed treatment with placebo beads, 30% rapamycin beads ($P = .0001$, compared with the control group), XRT (20 Gy; $P = .0001$, compared with the control group), or a combination of the 2 treatment groups: simultaneous 30% rapamycin beads plus XRT ($P = .0045$, compared with XRT; $P = .0283$, compared with simultaneous rapamycin alone) or delayed 30% rapamycin beads plus XRT ($P = .14$, compared with XRT; $P = .01$, compared with delayed rapamycin alone).

a significant increase in survival among animals receiving 30% rapamycin as compared to those receiving 3% rapamycin ($P = .0434$), but no significant difference between the 30% rapamycin and 0.3% rapamycin groups or the group that received 30% rapamycin and the group that received systemically delivered rapamycin ($P = .7869$).

Animals that received delayed treatment with rapamycin beads at any concentration experienced an increase in survival, compared with controls ($P < .0001$ for 0.3%, $P = .0006$ for 0.3%, and $P < .0001$ for 30%). Animals that received treatment 5 days after tumor implantation had a median survival duration of 20 days with 0.3% rapamycin, 24 days with 3% rapamycin, 25 days with 30% rapamycin, and 28 days with systemic rapamycin (Fig. 5); animals that received placebo beads had a median survival duration of 14 days. There was no statistical difference in survival among groups that received rapamycin beads, except for those receiving 3% versus 0.3% rapamycin ($P = .0257$). Systemically delivered rapamycin was associated with a significantly prolonged survival, compared with the 30% ($P = .0133$), 3% ($P = .0003$), and 0.3% ($P = .0001$) groups.

Efficacy of Locally Delivered Rapamycin with Radiation Therapy

All groups that received rapamycin bead implants either simultaneously with tumor or 5 days after tumor implantation or that received XRT, with or without rapamycin, survived significantly longer than the untreated control group and the placebo-treated group (Fig. 6). The control group had a median survival duration of 13 days, and the placebo group had a median survival duration of 12 days. In comparison, the placebo bead group and the XRT group had median survival durations of 28 days ($P < .0001$, compared with controls), the simultaneously treated rapamycin bead group had a median survival duration of 26 days ($P = .0004$, compared with controls), and the group that received the delayed treatment of rapamycin beads had a median survival duration of 25 days ($P = .0001$, compared with controls). The group treated with delayed rapamycin beads and XRT had a mean survival duration of 35 days, which was not significantly different from durations for the placebo bead and XRT group ($P = .1407$) but was significantly better than the group that received delayed rapamycin alone ($P = .01$). However, the group treated with simultaneous rapamycin beads and XRT had a median survival duration of 38 days, which was significantly prolonged when compared with both the placebo and XRT groups ($P = .0045$) and the simultaneous rapamycin alone group ($P = .0283$).

A summary graph has been included to present all of the data from all of the experimental groups in a clear and concise manner (Fig. 7).

Discussion

The recent understanding of the interaction of the metabolism of rapamycin, when used in combination with other drugs frequently given to patients with brain tumors, makes a strong argument for local delivery of this drug. Phase I and II clinical trials have shown that systemically delivered rapamycin metabolized by the liver can alter the metabolic actions of enzyme-inducing antiepileptic drugs, potentially presenting significant

clinical challenges for systemic delivery.[21,26] It has been reported that up to 40% of patients with brain tumors will present with seizures, and as many as 62% will have seizures during the course of their disease.[34] These patients are often treated with common antiepileptic drugs, such as phenytoin, carbamazepine, phenobartibal, oxcarbazapine, and primidone, all of which induce the hepatic cytochrome P-450 enzymes, thereby increasing the metabolism of rapamycin and many other chemotherapeutic agents.[35]

Local delivery allows for the use of a lower dose of chemotherapy to be effective (Table 1). Boni et al.[36] reported that tissue drug levels of temsirolimus were reduced by as much at 67% in patients who were concurrently taking anti-seizure drugs or other agents that induced P450 enzymes. They advised that the dose of rapamycin-analogue drugs should be increased in patients who are exposed to P450 inducers to minimize the risk of subtherapeutic levels.

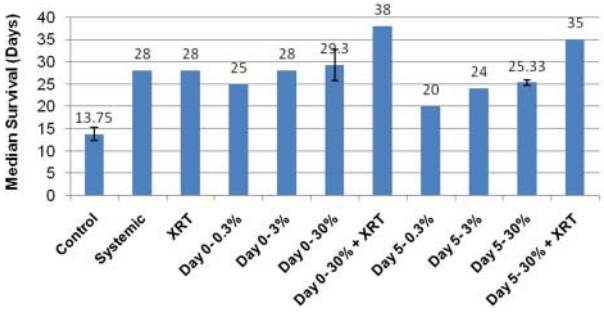

Fig. 7. Summary of median survival durations among all experimental groups. The figure shows the median survival duration (in days) displayed on the Y-axis, with the X-axis showing all groups by dose and treatment modality (concurrent/delayed, with and without radiation therapy, plus controls). Standard error bars are included where applicable. The 4 control groups had a median survival duration of 13.75 ± 1.5 days; systemic delivery of rapamycin resulted in a median survival of 28 days. Radiation therapy (20 Gy) on day 5 was associated with a median survival duration of 28 days; concurrent delivery of 0.3%, 3%, and 30% rapamycin beads was associated with median survival durations of 25, 28, and 29.3 ± 3.5 days, respectively. Delayed treatment (day 5) of 0.3%, 3%, and 30% rapamycin beads was associated with median survival durations of 20, 24, and 25.33 ± 0.57 days, respectively. Radiation therapy plus concurrent 30% rapamycin resulted in a median survival of 38 days, and radiation therapy plus delayed 30% rapamycin resulted in a 35-day median survival duration.

In these experiments, we demonstrated that local intracranial delivery used 71% less drug than systemic administration while resulting in beneficial efficacy as determined by survival. This lower dosage might avoid systemic toxicity and pharmacokinetic interactions with anti-seizure medication when used in patients. Local delivery of rapamycin is an attractive option to avoid the systemic toxicity observed and minimize interference with anti-epilepetic medications and other drugs that are metabolized via hepatic cytochrome p450–induced metabolism.

A second important implication of this work is the potential for a synergistic effect of rapamycin with traditional chemotherapeutic agents for brain tumors and XRT. It has been widely reported that rapamycin enhances the cytotoxicity of DNA-damaging agents in vitro. Recently, Tanaka et al.[37] reported that rapamycin enhances the cytotoxicity induction by ACNU, an alkylating agent, in the U251 human malignant glioma cell line. They reported that rapamycin not only enhanced apoptosis induced by ACNU by inhibition of p21, a cyclin-dependent kinase inhibitor of apoptosis, but that combination therapy with rapamycin and ACNU prolonged survival in a malignant glioma model. A similar effect has been seen with XRT: Eshleman et al.[38] examined the effects of mTOR inhibition on sensitivity to fractionated XRT in the U87 xenograft both in vitro and in vivo. They observed that the combination of rapamycin with XRT was significantly more effective than rapamycin therapy alone, suggesting that the mechanism of rapamycin-mediated radiation sensitization partly depends inhibition of rapamycin-signaling targets in tumor cells.[38] A similar outcome was observed in the efficacy portion of our study, with groups that received either rapamycin alone, XRT alone, or a combination of both. Animals that received rapamycin alone had a median survival duration of 25 days, whereas animals that XRT radiation alone had a median survival duration of 28 days. However, when the 2 therapies were combined, the median survival duration increased to 38 days—a duration significantly greater than that of either therapy alone ($P = .0023$ and .0037, respectively) (Fig. 6). These data suggest that rapamycin will further sensitize cancer cells to traditional therapies (ie, radiation and chemotherapy).

Table 1. Rapamycin dose comparison

Rapamycin dose	Dosage and schedule	Total amount of rapamycin delivered, mg	Comparison with systemic dosing
Systemic delivery (2 mg/kg intraperitonially per day for 30 days)	0.35 mg/day × 30 days	10.5	—
Local delivery (beads)			
0.3%	0.3% × 10 mg	0.03	0.29%
3%	3% × 10 mg	0.3	2.90%
30%	30% × 10 mg	3.0	28.6%

The third important implication is the potential efficacy in patients given the understanding of the behavior of rapamycin targets in glioblastoma. PTEN is a tumor-suppressor gene that negatively regulates the PI3K signaling pathway by inactivating phosphorylation. Mutations in PTEN have been observed in GBM, making inhibitors of its downstream modulators particularly interesting targets. It was first shown that the levels of the p70S6k and phosporylated AKT are elevated in transformed cells of $PTEN^{+/-}$ mice and that these cell-cycle modulators might be playing a key role in the increased proliferative capacity in cells with PTEN mutations.[39] On the basis of this, the rapamycin analogue CCI-779 exhibited preferential mTOR inhibition in PTEN-deficient cancer cells in humans and mice in vitro.[13] As previously shown, the loss of PTEN in these cells leads to increased P70S6K activity, demonstrating that mTOR is overactivated in these cells, and provides the rationale for mTOR inhibition.[13] Finally, an examination of the genetic alteration patterns of 34 patients with GBM showed that the PTEN tumor-suppression gene was mutated in 44% patients studied and in 60% of patients who had a loss of heterozygosity on chromosome 10q; thus, PTEN appears to be the major target of inactivation in GBM.[40,41] A phase I clinical trial with neoadjuvant rapamycin by Cloughesy et al.[29] found a significant reduction in tumor cell proliferation in 7 of 14 patients with recurrent PTEN-deficient GBM; this also correlated with the magnitude of mTOR inhibition. Another finding of this phase I clinical trial was the lack of mTOR inhibition in some patients, despite an adequate intratumoral concentration of rapamycin. The authors hypothesized that rapamycin may be sequestered in red blood cells; thus, the measured intratumoral concentration in some patients may not represent the actual amount of drug that reaches the tumor cells. Variability in blood-brain barrier penetration could be another reason for this differential response. Therefore, local delivery of rapamycin may enhance the anti-tumor effect of rapamycin in such patients.

A recent study by Fan et al.[42] showed that EGFR also signals to mTOR, through a previously under recognized pathway involving PKC, independent of the established Akt pathway in gliomas. This further highlights the importance of inhibiting mTOR in the treatment of gliomas. The results of these studies suggest that a potent inhibitor of a highly mutated and strongly proliferative target in GBM may be of benefit.

Inhibition of mTOR removes the negative feedback control imposed by mTOR via the S6 kinase 1-mediated phosphorylation of insulin receptor substrate 1 and, thus, results in Akt activation in tumor cells.[27,43,44] In a phase I trial, Cloughesy et al.[36] noted that the loss of this feedback control, as identified by induction of PRAS-40 (a downstream marker of Akt activity), was associated with a shorter time-to-progression in 7 of 14 patients with recurrent PTEN-deficient GBM treated with neoadjuvant rapamycin. A combination of mTOR inhibitors with inhibitors of other molecular targets is therefore a promising option.

Doherty et al[27] conducted a pilot study of the combination of EGFR inhibitors (gefitinib or erlotinib) and sirolimus in patients with recurrent malignant gliomas and found encouraging results, with a 19% overall response rate and 6-month progression-free survival rate of 25%. However, they mention that the adverse effects of the combination regimen appeared to be more severe than those seen with monotherapy. Several ongoing trials involving mTOR inhibitors and EGFR have also found toxicity to be a problem. Therefore, local delivery of rapamycin may be a good option in these patients.

In our studies, we investigated the safety and efficacy of locally delivered rapamycin via biodegradable beads in a malignant rat glioma model. We demonstrated that local intracranial delivery of rapamycin is safe and requires a lower dose of rapamycin while prolonging survival in experimental brain tumors. Rapamycin was cytotoxic to glioma cells, with 62% inhibition of growth at a concentration of 0.01 mg/mL. Locally delivered rapamycin was found to be free from any associated systemic or intracranial toxicity at 0.3%, 3%, and 30% bead concentrations of rapamycin. Weight gain and survival in these animals was similar to those of controls. Animals that received concurrent or delayed therapy of rapamycin beads at all concentrations tested experienced an increase in survival, compared with controls ($P = .0006$ for 0.3%, $P = .0001$ for 3%, and $P = .0001$ for 30% rapamycin). In addition, the group treated with simultaneous rapamycin beads and XRT had significantly increased survival over those that received XRT alone ($P = .0045$) and rapamycin alone ($P = .0283$).

We demonstrated that an mTOR inhbitor, rapamycin, can be used effectively to treat gliomas in a rodent model when administered using a local delivery strategy based on a novel biodegradable vehicle. Rapamycin exhibits potent cytotoxic activity against 9L both in vitro and in vivo. Furthermore, rapamycin can be efficiently incorporated and delivered using biodegradable, controlled-release polymeric beads. Local delivery of rapamycin appears to be safe for intracranial delivery, and locally delivered rapamycin prolongs survival in the 9L gliosarcoma intracranial rodent model in a dose-dependent manner. When combined with XRT, simultaneously delivered local rapamycin significantly increases survival, compared with systemic delivery or XRT alone. Additional studies should be performed to test the biodistribution of locally delivered rapamycin through the brain parenchyma and to assess the efficacy of combining locally delivered rapamycin with other potent chemotherapeutic agents to determine its role as an adjuvant therapy.

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