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ORIGINAL ARTICLE

Ridaforolimus for patients with progressive or recurrent malignant glioma: a perisurgical, sequential, ascending-dose trial

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Abstract
Purpose This perisurgical phase 1 study evaluated the pharmacokinetics, pharmacodynamics, and safety of the mammalian target of rapamycin (mTOR) inhibitor ridaforolimus in patients ($N = 10$) with progressive or recurrent primary grade IV malignant glioma, who failed standard therapy. The primary objective of the study was to determine the maximum tolerated dose (MTD) of ridaforolimus.

Methods Treatment was administered intravenously at doses of 12.5 mg ($N = 7$) or 15 mg ($N = 3$) once daily for 4 days prior to surgical resection, then resumed for 5 consecutive days every 2 weeks until disease progression or unacceptable toxicity, following a postsurgical recovery period.

Results The MTD was not determined because the trial was suspended early due to slower than expected patient accrual and postsurgical drug administration challenges. Pharmacokinetic and pharmacodynamic analyses showed that ridaforolimus concentrations declined slowly during the 24-h dosing interval and remained detectable for 10 days after the last infusion in whole blood samples. In peripheral blood mononuclear cells, median levels of the mTOR downstream effector p4E-BP1 were reduced by >80% compared with baseline by 4 h after dosing. Resected brain specimens showed reduced levels of pS6, another mTOR downstream effector, while nuclear staining for $p27^{kip1}$, a protein that functions as a cell cycle inhibitor, increased after treatment. No dose-limiting toxicities were observed, and the reported adverse events were consistent with the previously established safety profile for ridaforolimus. One of 3 patients evaluable for efficacy had stable disease as best response.

Conclusion Results suggest that ridaforolimus can cross the blood–brain barrier in areas of tumor involvement, and may inhibit mTOR activity in advanced gliomas based on decreased pS6 levels. This perisurgical trial design should serve as a template for evaluating intratumoral pharmacokinetics and pharmacodynamics of other targeted agents in this patient population.

Keywords Malignant glioma · Mammalian target of rapamycin · Ridaforolimus · Pharmacodynamics · Immunohistochemistry

Preliminary results from this trial were presented as an abstract at the 2005 AACR-NCI-EORTC international conference.

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Introduction

An estimated 22,020 new cases of primary brain cancer and other nervous system malignancies were diagnosed in the United States in 2010, representing 1.4% of all new cancer diagnoses [1]. Gliomas are the most common primary brain tumors in adults, and are classified by the World Health Organization (WHO) as astrocytic, oligodendroglial, or mixed tumors, based on morphological characteristics [2, 3]. For patients with high-grade invasive astrocytomas, including anaplastic astrocytomas (grade III) and glioblastoma multiforme (GBM, grade IV), surgical resection is the primary treatment approach and is typically followed by fractionated external-beam radiation therapy, with or without chemotherapy [3]. However, these tumors tend to recur frequently—even with a multimodal approach, the 5-year survival rate for patients with primary brain malignancies is only 35% [4], and is particularly poor for those with anaplastic astrocytomas and GBM [2]. This underscores the need for new agents that can effectively cross the blood–brain barrier (BBB) and selectively target pathways involved in glioma pathogenesis, progression, and recurrence.

Activation of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway promotes growth and survival of several types of tumors, including gliomas [5]. Dysregulation of PI3K/Akt/mTOR signaling, either by loss or mutation of the tumor suppressor phosphatase and tensin homolog (PTEN), or by overexpression of receptor tyrosine kinases (such as the epidermal growth factor receptor [EGFR]), is a common finding in gliomas [6–8]. Higher expression of mTOR and its upstream and downstream signaling proteins has been correlated with increasing glioma grade across multiple studies [9–11]. Moreover, mTOR pathway activation has been associated with poorer survival, even among those with low-grade gliomas [11, 12]. These observations suggest that mTOR inhibition may represent an attractive targeted therapy for patients with malignant gliomas.

Ridaforolimus is an investigational mTOR inhibitor that reduces proliferation of cancer cells in vitro and growth of tumor xenografts in vivo, including models derived from GBM cell lines [13–15]. In phase 1 trials, ridaforolimus produced clinical benefit in patients with advanced malignancies, particularly those with soft tissue and bone sarcomas, and was generally well tolerated following intravenous or oral administration [16–18]. On the basis of these studies and a subsequent phase 2 trial [19, 20], ridaforolimus has been explored in a phase 3 trial for use as maintenance therapy in patients with advanced soft tissue and bone sarcomas who did not progress after first-line chemotherapy (Sarcoma Multi-Center Clinical Evaluation of the Efficacy of Ridaforolimus [SUCCEED] trial) [21]. Because the central nervous system (CNS) is functionally separated from the systemic circulation by the BBB, exposure of gliomas to ridaforolimus may differ from the exposure achieved in other solid tumors. Therefore, the present study explored the pharmacokinetics and pharmacodynamics of ridaforolimus in patients with progressive or recurrent gliomas who had failed standard therapy. While the primary objective of the study was to determine the maximum tolerated dose (MTD) of ridaforolimus, immunohistochemical analyses of tumor specimens for proteins involved in the mTOR signaling pathway were also performed to ascertain if pharmacologically effective drug concentrations were achieved at the disease site.

Methods

Patients

Patients aged 18 years or older with radiographically suspected progressive or recurrent glioblastoma or gliosarcoma (WHO grade IV) were eligible if they had failed standard therapy, had not received systemic therapy for the current episode of disease recurrence or relapse, and were candidates for surgical resection or open biopsy of the tumor. Stereotactic biopsy was not permitted. Eligibility also required evidence of neurological stability for at least 1 week prior to the first dose of ridaforolimus, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and adequate hematologic, renal, and liver function. Eligible patients had a tissue-proven diagnosis of malignant glioma, but a previous histologic diagnosis of a lower grade glioma was allowed if there was histologic evidence of progression to a glioblastoma. Enrollment was permitted if at least 4 weeks had elapsed after prior surgical resection, completion of external-beam radiotherapy for the initial primary diagnosis, or receipt of any prior chemotherapy or investigational agents; additionally, patients had to have recovered from any toxicity related to such therapy. Concomitant use of enzyme-inducing anticonvulsants (e.g., phenytoin and carbamazepine) was not allowed in order to exclude potential drug–drug interactions. All patients provided written informed consent before any procedures specifically related to this trial were performed.

Pregnant or lactating women were excluded, and patients of childbearing potential agreed to use a medically effective method of contraception. Other exclusion criteria included serum albumin less than 2.5 g/dL; total cholesterol greater than 350 mg/dL; triglycerides greater than 400 mg/dL; creatinine clearance less than 50 mL/min/1.73 m^2; known hypersensitivity to macrolide antibiotics; significant cardiovascular disease; any active infection requiring intervention; HIV infection; prior treatment with rapamycin, a rapamycin analog, or tacrolimus; or current treatment with

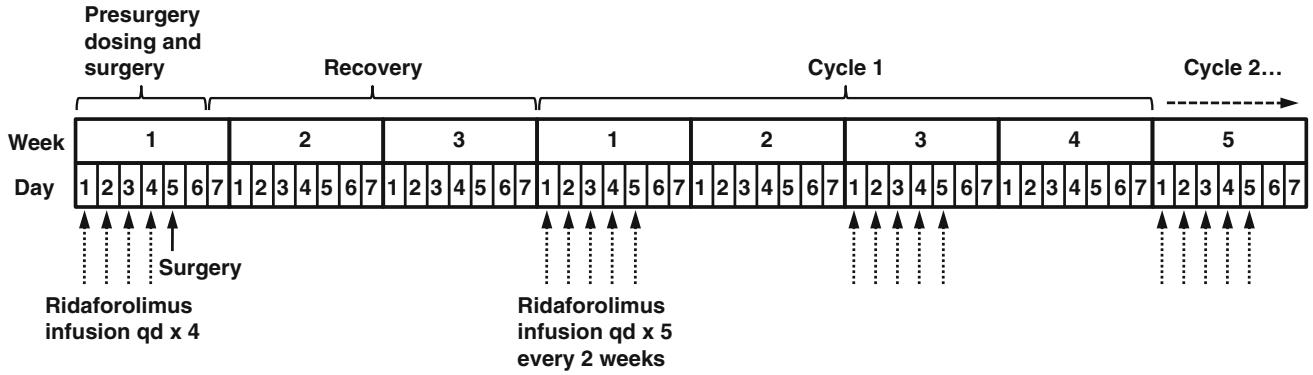

Fig. 1 Dosing schedule for ridaforolimus before and after surgery; qd, once daily

an immunosuppressive agent other than a stable dose of a corticosteroid.

Study design

This open-label, nonrandomized, sequential, dose-escalation cohort trial utilized a classical 3 + 3 dose-escalation design with 3–6 patients planned per dose level. Ridaforolimus was administered once daily as a 30-min intravenous infusion for 4 days; then within 24–48 h of the last dose, patients underwent surgical resection or open biopsy (Fig. 1). Gadolinium (Gd)-enhanced magnetic resonance imaging (MRI) was performed within the 48 h following surgical resection. Patients were to recover from surgery for at least 2 weeks, with complete resolution of any toxicity that may have occurred during resection or open biopsy, and then started postsurgical treatment with ridaforolimus once daily for 5 consecutive days every 2 weeks. Each treatment cycle consisted of two 2-week courses. Tumor evaluations were performed by Gd-MRI after every 2 treatment cycles, and ridaforolimus treatment was continued unless there was evidence of disease progression or unacceptable toxicity.

The starting dose of ridaforolimus was 12.5 mg, which was selected based on its safety and tolerability in phase 1 studies conducted in patients with advanced malignancies [22, 23]. Dose escalation was to be performed in 20% increments (i.e., 15, 18, 20, 22, and 26 mg) until the MTD was identified. The MTD was defined as the highest dose level identified on which fewer than 2 patients in a cohort of at least 6 patients experienced dose-limiting toxicities (DLTs) during cycle 1 of treatment. Three patients were to be enrolled at each dose level, and the cohort expanded to 6 patients in the event of a DLT. Dose escalation to the next level was planned if none of the first 3 patients (or no more than one of 6 patients) at the preceding level had a DLT.

This study was conducted in accordance with the principles originating in the Declaration of Helsinki, and in compliance with the guidelines of the International Conference on Harmonization, Good Clinical Practices, and all applicable regulatory requirements. The protocol, its amendments, and the informed consent form were approved by the institutional review boards at each study site before patients were enrolled at that site.

Pharmacokinetics

Blood samples (~3.0 mL) for pharmacokinetic analysis were collected during the presurgical treatment period and during postsurgical cycle 1. During the presurgical period, samples were collected before and 4 and 24 h after the ridaforolimus infusion on day 1, before and 4 h after the infusion on day 4, and at the time of surgery. On days 1 and 5 of cycle 1 after surgery, samples were collected prior to and at the end of the infusion, and then 5, 30, 60, 120, and 240 min after the infusion (also at 360 min post-infusion on day 5). Additional samples were collected during cycle 1 after surgery before the infusions on days 2, 3, 4, 6, 8, 11, and 15 (and 4 h post-infusion on day 15), as well as prior to the infusion starting cycle 2.

Blood concentrations of ridaforolimus were analyzed using a high performance liquid chromatography/mass spectrometry/mass spectrometry (HPLC/MS/MS) method with a lower limit of detection of 0.5 ng/mL. The measured whole-blood ridaforolimus concentrations were used to estimate pharmacokinetic parameters including time of maximum concentration (T_{max}), maximum concentration (C_{max}), area under the concentration–time curve (AUC), clearance (CL), volume of distribution at steady state (V_{ss}), and terminal half-life ($t_{1/2}$). Pharmacokinetic parameters (population means and 95% confidence intervals) were estimated using nonlinear modeling software.

Pharmacodynamics

Levels of phosphorylated 4E-BP1 (p4E-BP1) in peripheral blood mononuclear cells (PBMCs), relative to levels of total 4E-BP1, were evaluated as a surrogate of mTOR

activity. Blood samples were collected prior to and 4 h after the ridaforolimus infusion on days 1 and 4 of the presurgical treatment period, and on days 1, 5, and 15 of postsurgical cycle 1. Additional samples were collected at screening; prior to the infusion on presurgical day 2; on days 2, 8, and 11 of cycle 1 post-surgery; and before the first dose of cycle 2. Protein extracts were prepared from the PBMCs and analyzed by Western blot using antibodies specific for total 4E-BP1 or for 4E-BP1 phosphorylated at Ser65/Thr70.

An archival tumor specimen from a previous biopsy or resection and a tumor specimen collected at the surgery or open biopsy following treatment with ridaforolimus for 4 days were sent to Ventana Medical Systems Inc (Tucson, AZ) for immunohistochemical analysis of proteins involved in the mTOR signaling pathway. The following antibodies were used: murine monoclonal anti-vascular endothelial growth factor (VEGF; Santa Cruz clone C-1), rabbit polyclonal anti-4E-BP1 (Novus), rabbit polyclonal anti-p4EBP1 (pThr 70) and anti-pS6 (pSer235/pSer236, Cell Signaling Technology), murine monoclonal anti-cyclin D1 (Cell Marque clone DCS-6), and anti-$p27^{kip}$ (Lab Vision/Neomarkers). An antibody to vimentin was used as a positive control and to assess specimen quality, and antibodies to rabbit and murine immunoglobulins and the antibody diluent served as negative controls. Enzymatic detection and localization of each antibody was accomplished with a streptavidin-horseradish peroxidase conjugate followed by reaction with hydrogen peroxide in the presence of diaminobenzidine and copper sulfate. Staining intensity was scored on a semiquantitative scale ranging from 0 (negative) to 3 (strong) by a pathologist who was blinded to the identity of the treatment conditions. The percentage of cells with positive staining, as well as the subcellular localization of the stain, was also evaluated. A combined immunohistochemistry score was obtained by multiplying the staining intensity by the percentage of cells with positive staining. If staining was not consistent for the entire specimen, then the score for the region with the best staining was reported. The optical density of staining was also evaluated using the ARIOL Imaging System (Applied Imaging, San Jose, CA).

Efficacy and safety assessments

Tumor status was assessed by Gd-enhanced MRI at screening, within 48 h after surgery, and after every 2 postsurgical cycles of ridaforolimus using Macdonald Response Criteria [24]. Patients were monitored throughout the study for the occurrence of adverse events and disease progression. Safety assessments included physical examination, vital signs, ECOG performance status, 12-lead electrocardiogram (ECG), slit-lamp ophthalmologic examination, and clinical laboratory testing.

Adverse events were graded using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events, Version 3.0 [25]. For the purpose of this trial, DLTs were defined as any grade 4 non-hematologic toxicity; grade 4 neutropenia that was sustained; grade 3 or 4 neutropenia associated with fever; thrombocytopenia; and dosing delays due to any toxicity thought to be associated with study drug taking place greater than 2 weeks from the scheduled "next dose" (excluding post-surgery recovery period). Clinical judgment was utilized when determining whether it was advisable to continue a patient onto the next cycle of dosing.

Statistics

Pharmacokinetic parameters and changes in pharmacodynamic parameters were analyzed descriptively by dosage cohort. All patients receiving at least one dose of ridaforolimus were considered evaluable for safety. Adverse events were coded by body system and preferred term using the Medical Dictionary for Regulatory Activities (MedDRA, version 8.1) and characterized by severity and relationship to study treatment by the investigator. The incidence of adverse events and other safety parameters were evaluated using descriptive statistics. Patients completing 2 cycles of ridaforolimus or discontinuing within the first 2 cycles for progressive disease were evaluable for antitumor activity using descriptive statistics.

Results

Patient characteristics and study progression

The study was terminated early by the sponsor due to a slower than expected rate of patient enrollment and challenges of postoperative drug administration. As a result, not all primary and secondary objectives of the trial were met, including determining the MTD of ridaforolimus administered intravenously to patients with advanced gliomas. Eleven patients were enrolled: 7 patients received ridaforolimus at a dose of 12.5 mg, 3 patients received a dose of 15 mg, and one patient withdrew consent before administration of study drug. The demographic and baseline clinical characteristics of the study cohort are shown in Table 1. The median age was 50.5 years (range 29–70 years), all patients were white, and the majority were male (70%) and had good PS (90% ECOG PS 0 or 1). All 10 patients had grade IV disease, including 9 patients with a histological diagnosis of GBM and one patient with a glioneuronal neoplasm. None of the patients were receiving enzyme-inducing anticonvulsants (EIACs) at the time of enrollment. All patients had undergone prior surgery (including 4 patients

Table 1 Patient demographics and baseline characteristics

Characteristic	Ridaforolimus 12.5 mg ($N = 7$)	Ridaforolimus 15 mg ($N = 3$)	Total ($N = 10$)
Age, years			
Mean (SD)	49.1 (6.26)	52.7 (21.22)	50.2 (11.36)
Range	37–57	29–70	29–70
Gender, *n* (%)			
Male	6 (85.7)	1 (33.3)	7 (70.0)
Female	1 (14.3)	2 (66.7)	3 (30.0)
Race, *n* (%)			
White	7 (100)	3 (100)	10 (100)
ECOG PS, *n* (%)			
0	1 (14.3)	0 (0.0)	1 (10.0)
1	5 (71.4)	3 (100.0)	8 (80.0)
2	1 (14.3)[a]	0 (0.0)	1 (10.0)
Diagnosis at study entry, *n* (%)			
Glioblastoma multiforme	6 (85.7)	3 (100.0)	9 (90.0)
Malignant glioneuronal neoplasm	1 (14.3)	0 (0.0)	1 (10.0)
Tumor grade, *n* (%)			
Grade IV	7 (100)	3 (100)	10 (100)

SD Standard deviation, *ECOG* eastern cooperative oncology group, *PS* performance status

[a] Patient had a PS of 1 at screening but a PS of 2 prior to the first dose of study treatment

who had 2 or more previous procedures), and all had received prior radiotherapy and chemotherapy (including 6 patients who had received 2 or more drug regimens). Eight patients were treated previously with temozolomide, whereas CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea], Gliadel wafers, 06-benzylguanine, cloretazine, etoposide, and irinotecan were also given to patients. None of the patients had received prior bevacizumab.

Of the 10 patients who received at least one dose of study drug after enrolling in the trial, 3 patients received ridaforolimus only in the presurgical setting before withdrawal based on an investigator's decision—one patient to prevent exacerbation of preexisting medical conditions, one patient due to prolonged postoperative course complicated by multiple factors that prevented restarting ridaforolimus treatment, and one patient due to clinical decline. Ridaforolimus was discontinued post-surgery due to progressive disease in 6 patients, including 2 patients in the 15-mg cohort. One patient discontinued ridaforolimus treatment from the 15-mg cohort due to a grade 4 CNS hemorrhage that was considered by the investigator to be unrelated to study drug. During post-study follow-up, 9 patients died of progressive disease, including 2 who died within 30 days of the last dose of study drug. The remaining patient from the 12.5-mg cohort was monitored through the 6-month follow-up visit, but was not monitored for survival thereafter. Nine patients underwent surgery following ridaforolimus therapy, including 4 patients with gross total resection of visible disease, one with a near-total resection, and 4 with partial resections. The one patient who discontinued the trial to prevent exacerbation of a preexisting condition did not undergo surgery.

Pharmacokinetics

During the presurgical period, blood concentrations of ridaforolimus were collected in 10 patients, but pharmacokinetic parameters could not be calculated due to the limited sampling times. In the 12.5-mg cohort, the mean ± SD blood concentrations were 155.5 ± 29.0 ng/mL ($N = 6$) and 141.8 ± 30.8 ng/mL ($N = 5$) at 4 h after the ridaforolimus infusion on day 1 and 4, respectively. Mean trough levels of ridaforolimus measured prior to the infusions on day 2, day 4, and at the time of surgery were 44.4 ± 12.3 ng/mL ($N = 5$), 68.6 ± 23.0 ng/mL ($N = 7$), and 76.9 ± 38.8 ng/mL ($N = 4$), respectively. Presurgical blood concentration data were available for only one patient in the 15-mg cohort; mean trough levels at 4 h after the infusions on day 1 and 4 were 142.0 and 211.0 ng/mL, respectively. Prior to the infusions on day 2, day 4, and at the time of surgery, trough levels were 76.5, 175.0 and 74.7 ng/mL, respectively.

Blood samples were collected during cycle 1 following surgery in 5 patients in the 12.5-mg cohort and one patient in the 15-mg cohort. Ridaforolimus concentrations at the end of the infusion on day 1 of cycle 1 ranged from 333 to 1,140 ng/mL in the 12.5-mg cohort (mean ± SD: 534.4 ± 341.0 ng/mL); in the patient who received the 15-mg dose, the concentration of ridaforolimus was 737 ng/mL. Blood concentrations declined gradually following the infusion, reaching a mean trough level of 49.4 ± 29.8 ng/mL ($N = 3$) at 24 h post-infusion (Fig. 2). Ridaforolimus remained detectable in blood for a long period after the last infusion on day 5: mean concentrations on days 8, 11, and 15 in the 12.5-mg cohort were 24.1 ± 8.0, 8.2 ± 2.2 and 3.6 ± 0.8 ng/mL (all $N = 3$), respectively. The mean elimination half-life for the slow phase was calculated to be 52 h, suggesting that ridaforolimus was being slowly released from a deep compartment.

A noncompartmental pharmacokinetic analysis was carried out for 4 patients (3 from the 12.5-mg cohort and one from the 15-mg cohort) with sufficient blood concentration data over the 0 to 24-h time period following the first infusion in cycle 1 (Table 2). The $AUC_{0-24\,h}$ and $AUC_{0-\infty}$ were 2.92 ± 0.86 and 3.92 ± 1.71 h μg/mL, respectively, and

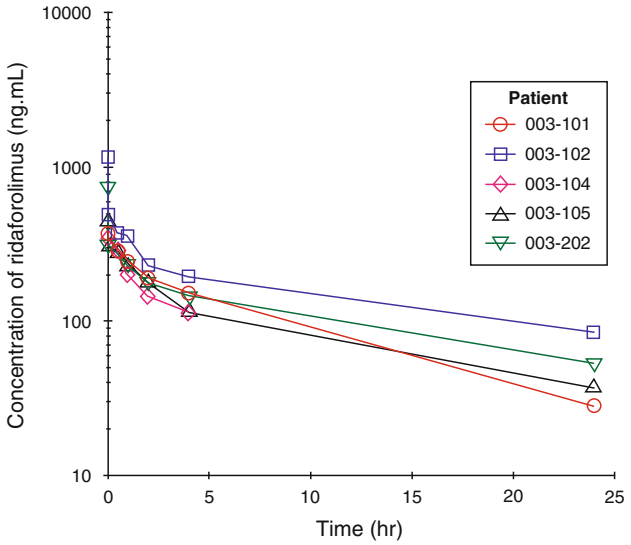

Fig. 2 Whole-blood concentration–time profile for ridaforolimus following intravenous infusion on day 1 of cycle 1. All patients received a 12.5-mg dose, except for patient 003–202, who received a 15-mg dose

the CL and V_{ss} were 3.6 ± 1.2 L/hr and 48.1 ± 12.4 L, respectively. An elimination half-life was not determined for this time interval because it did not include a slow elimination phase (Fig. 2).

Pharmacodynamics

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells isolated from blood samples collected prior to and at a maximum of 15 time points after the initiation of ridaforolimus dosing, which spanned the presurgical dosing cycle through the beginning of postsurgical cycle 2, were analyzed from all 10 patients. Potent inhibition of mTOR activity was observed in 9 of 10 patients. Median levels of p4E-BP1 were reduced by greater than 80% relative to baseline by 4 h after dosing, and this degree of inhibition was maintained at all time points within 24 h of an infusion. Similar levels of p4E-BP1 inhibition were observed in patients who received the 12.5 and 15-mg doses.

Tumor specimens

Both archival and surgical tumor specimens were received from all 6 patients in the 12.5-mg cohort and all 3 patients in the 15-mg cohort who underwent resection. Staining with hematoxylin and eosin (H&E) showed that cell viability was greater than or equal to 70% in all archival specimens (except for one specimen with 5% viability) and 90–100% in all resection samples obtained after 4 days of ridaforolimus dosing (except for one specimen with 10% viability). As a positive control, strong cytoplasmic vimentin staining (score 3) was evident in all specimens, except for one surgical specimen with moderate staining (score 2); in all cases, staining for vimentin was seen in 100% of the cells. As negative controls, no staining with a control antibody to murine immunoglobulin was evident in any tumor specimens; however, 2 surgical specimens showed staining to a control antibody to rabbit immunoglobulin, which was characterized as strong staining in 70% of cells (patient 203:15-mg cohort) and moderate staining in 5% of cells (patient 106:12.5-mg cohort), respectively. Therefore, the measurement of pharmacodynamic markers detected with rabbit polyclonal antibodies (i.e., pS6, 4E-BP1, and p4E-BP1) may be confounded in these 2 cases.

Moderate to strong staining of pS6, a downstream effector of mTOR, was identified in most archival and surgical tumor specimens, and in the 6 patients from the 12.5-mg cohort, the percentage of cells with pS6 staining declined from a mean of 85% (range 30–100%) in archival samples to 18% (range 0–40%) following presurgical ridaforolimus dosing. If patient 106 is excluded from the analysis due to negative control antibody staining, then the percentage of cells with pS6 staining declined from 86% (range 30–100%) to 13% (range 0–30%). Levels of pS6 based on the combined immunohistochemistry score declined by twofold or more in each patient in the 12.5-mg cohort (Fig. 3a). Photomicrographs of pS6 staining for

Table 2 Pharmacokinetic parameters following infusion of ridaforolimus on day 1 of cycle 1

Cohort/patient	Concentration at end of infusion (ng/mL)	$AUC_{0-24\ h}$ (μg hr/mL)	$AUC_{0-\infty}$ (μg hr/mL)	CL (L/h)	V_{ss} (L)
12.5-mg cohort					
101	380.00	2.62	2.95	4.23	37.43
102	1,140.00	3.89	5.89	2.12	45.19
105	436.00	2.26	2.92	4.28	61.67
Mean (SD)	652.00 (244.50)	2.92 (0.86)	3.92 (1.71)	3.55 (1.23)	48.10 (12.37)
15-mg cohort					
202	737.00	2.81	3.86	3.89	66.51

AUC Area under the curve, *CL* clearance, V_{ss} volume of distribution, *SD* standard deviation

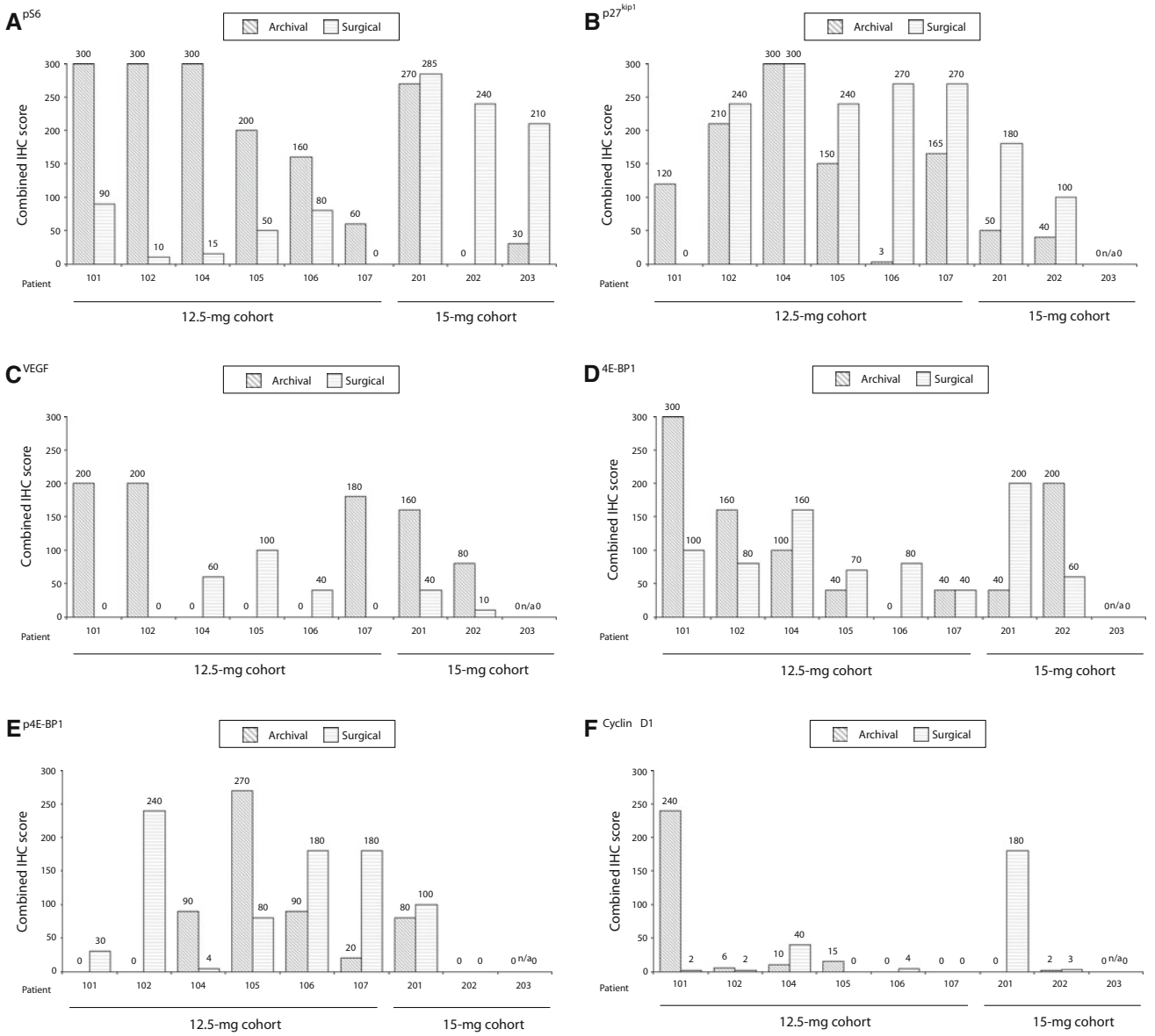

Fig. 3 Combined immunohistochemistry score for **a** pS6, **b** $p27^{kip1}$, **c** VEGF, **d** 4E-BP1, **e** p4E-BP1, and **f** cyclin D1 in archival tumor specimens and surgical tumor specimens following presurgical dosing with ridaforolimus. The combined score represents the product of immunohistochemistry score multiplied by the percentage of cells with positive staining. For patient 107, the archival score represents the mean of 2 specimens analyzed. The specimens from patient 203 were only analyzed for pS6 expression; *IHC* immunohistochemistry, *VEGF* vascular endothelial growth factor

patient 105, whose combined immunohistochemistry score declined from 200 in the archival specimen to 50 in the tumor sample, were obtained at resection following ridaforolimus and are shown in Fig. 4a, b. The reductions in pS6 levels were confirmed for 5 pairs of tumor specimens, in which the optical density of staining was measured using the ARIOL Imaging System. Reductions in pS6 levels were not evident in tumor specimens from 2 patients in the 15-mg cohort, nor in patient 203, whose postsurgical specimen showed strong negative control antibody staining.

Moderate to strong nuclear staining of $p27^{kip1}$ was seen in most archival samples, with a mean of 46% (range 1–100%) of cells having positive staining. Following presurgical dosing with ridaforolimus, $p27^{kip1}$ expression increased to some extent in 6 of the 8 patients (Fig. 4c, d), including both patients from the 15-mg cohort who were analyzed (Fig. 3b). In contrast, one postsurgical specimen from the 12.5-mg cohort showed loss of $p27^{kip1}$ staining after ridaforolimus. Expression of the other 4 proteins examined (VEGF, cyclin D1, 4E-BP1, and p4E-BP1) did not vary in any consistent manner between archival and surgical samples (Fig. 3c–f).

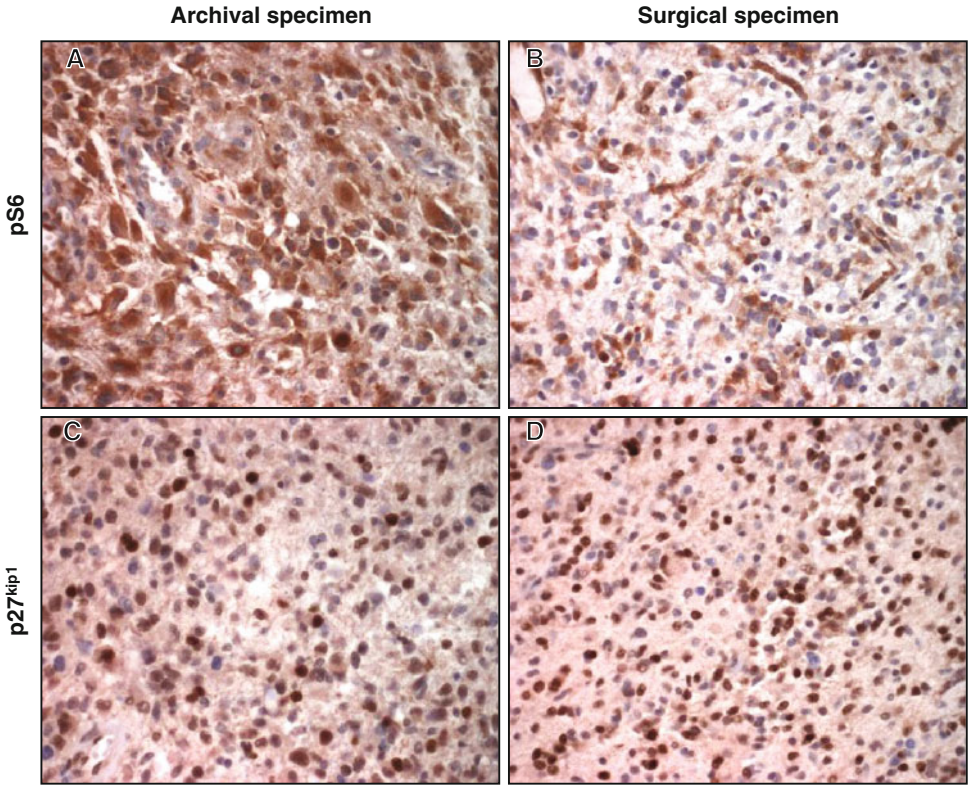

Fig. 4 Photomicrograph from patient 105 of pS6 and $p27^{kip1}$ staining in archival tumor specimens (**a**, **c**) and in surgically resected tumor samples following presurgical treatment with ridaforolimus 12.5 mg (**b**, **d**). Moderate pS6 staining (score 2) was seen in 100% of cells in the archival sample and in 25% of cells in the resected specimen, while strong $p27^{kip1}$ staining (score 3) was seen in 50% of cells in the archival sample and in 80% of cells in the resected specimen

Antitumor activity

Two of 7 patients in the 12.5-mg cohort and one of 3 patients in the 15-mg cohort received at least 2 cycles of ridaforolimus after surgery. One of the patients who received the 12.5-mg dose achieved stable disease for a duration of 2 months as the best response, whereas the other 2 had progressive disease at the first postsurgical response assessment.

Safety

All 10 patients who received ridaforolimus experienced at least one treatment-emergent adverse event, but no DLTs (as defined for this study) were observed. Most adverse events were mild or moderate in severity. Hypercholesterolemia, hyperglycemia, hypoalbuminemia, hypertriglyceridemia, hypocalcemia, and thrombocytopenia were the most common adverse events overall, and in most cases were considered by the investigator to be related to study treatment (Table 3). Four patients experienced grade 4 adverse events: one patient in the 15-mg cohort had cerebral hemorrhage and hemiplegia on the day of surgery and developed aspiration pneumonia 13 days later; one patient each had hyperglycemia and disease progression after receiving 2 cycles of ridaforolimus at a dose of 12.5 and 15 mg, respectively; and one patient assigned to the 12.5-mg cohort had vena cava thrombosis 5 days after surgery. None of the

Table 3 Treatment-emergent adverse events, considered to be related to treatment or not, occurring in 3 or more patients

Adverse event	All casuality[a] *n* (%)	Treatment-related, *n* (%)
Hypercholesterolemia	7 (70)	6 (60)
Hyperglycemia	6 (60)	4 (40)
Hypoalbuminemia	6 (60)	3 (30)
Hypertriglyceridemia	5 (50)	5 (50)
Hypocalcemia	5 (50)	4 (40)
Thrombocytopenia	5 (50)	5 (50)
Hemoglobin decreased	4 (40)	4 (40)
Hyponatremia	4 (40)	2 (20)
Diarrhea	4 (40)	3 (30)
ALT increased	3 (30)	2 (20)
AST increased	3 (30)	3 (30)
Fatigue	3 (30)	0 (0)
Nausea	3 (30)	2 (20)
Procedural pain	3 (30)	0 (0)
Rash	3 (30)	1 (10)
Vomiting	3 (30)	1 (10)

[a] The following events were reported in 2 patients each: anemia, asthenia, atelectasis, brain edema, confusional state, convulsion, cough, dehydration, disease progression, dysphagia, hypernatremia, oral candidiasis, peripheral edema, pyrexia, and stomatitis, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase

grade 4 events were considered by the investigator to be related to treatment. Four patients had treatment-related grade 3 events (decreased hemoglobin, diarrhea, increased alanine aminotransferase [ALT], and brain edema). No safety issues were identified in the pre-infusion versus post-infusion analyses of vital signs, physical examinations, slit-lamp ophthalmological examinations, or 12-lead ECG recordings.

Four patients experienced adverse events that led to treatment interruption or discontinuation; these adverse events were all considered by the investigator not to be related to treatment. These included the aforementioned patient with grade 4 cerebral hemorrhage and aspiration pneumonia and 2 patients with progressive disease listed as an adverse event, all of whom discontinued study treatment. The fourth patient experienced grade 2 dehydration, diarrhea, nausea, abdominal pain, and vomiting, which were considered possibly related to study treatment and resolved without sequelae after interruption of treatment.

Discussion

This study evaluated perisurgical treatment with ridaforolimus in patients with progressive or recurrent gliomas who had failed standard therapy. Starting ridaforolimus before surgery and then collecting tumor specimens at resection allowed a pharmacodynamic assessment of whether ridaforolimus crosses the BBB in areas with tumor-related enhancement and then achieves concentrations in tumor cells sufficient to impact downstream effectors of mTOR. Another mTOR inhibitor and rapamycin analog, temsirolimus, was previously shown to be present in glioma tissue obtained at the time of surgery but pharmacodynamic analyses were not performed in the study [26]. In the present study, attention was focused on analysis of pS6, as this is the downstream readout of mTOR activity that has thus far been observed to best correlate with mTOR activity in tissue specimens [27, 28]. When compared with archival tumor specimens, presurgical treatment with ridaforolimus for 4 days reduced pS6 levels measured in the freshly resected tumor specimens in all 6 patients analyzed in the 12.5-mg cohort. This finding suggests that ridaforolimus crosses the BBB in areas with tumor-related enhancement and achieves pharmacologically effective concentrations.

The reduction in pS6 levels seen in the 12.5-mg cohort was not evident among the 3 patients in the 15-mg cohort. At this higher dose, pS6 levels were higher in the surgical resection samples than in the archival specimen in 2 cases and unchanged in the third case. The apparent difference in effect on pS6 between the 2 dose levels may reflect the small number of patients who received the 15-mg dose, which included patient 203, whose surgical specimen exhibited strong staining to the negative control antibody to rabbit immunoglobulins. Moreover, the archival sample from patient 202 exhibited no staining for pS6, unlike the archival specimens from the other patients. The quality of the archival tumor samples obtained from the patients may have affected the overall tissue staining procedure, although strong vimentin and H&E staining suggested good tissue and cell viability. Alternatively, the higher ridaforolimus dose may have produced greater mTOR inhibition than the 12.5-mg dose, leading to activation of a feedback regulatory loop that enhanced signaling in the PI3K/Akt/mTOR pathway [29]. Unfortunately, this hypothesis cannot be confirmed; in the current study, Akt expression and activity were not measured in the tumor specimens.

Five other proteins besides pS6 were assessed by immunohistochemistry in the archival and surgical tumor specimens. Among them, $p27^{kip1}$ expression was higher in the surgical specimens obtained following treatment with ridaforolimus for 4 days compared with the archival specimens in 6 of 8 patients, including both patients from the 15-mg cohort who were evaluated. The expression of $p27^{kip1}$, a protein that functions as a cell cycle inhibitor, has been observed to increase following inhibition of mTOR in some preclinical studies [30]. However, control of $p27^{kip1}$ expression is multiple steps downstream from mTOR, and therefore it is difficult to conclude that higher levels of this protein reflect a direct pharmacodynamic effect of ridaforolimus on mTOR activity. Notably, variable effects were seen on the expression of 4E-BP1 and p4E-BP1 in the tumor specimens, even though ridaforolimus was shown to markedly reduce p4E-BP1 levels in PBMCs. The activation of mTOR leads to hyperphosphorylation of 4E-BP1, thereby promoting its dissociation from eukaryotic initiation factor 4E (eIF-4E) to drive expression of several key proteins involved in tumor growth, including VEGF and cyclin D1 [29, 31]. These latter proteins were also variably affected by ridaforolimus therapy in the tumor specimens. The differing effects of ridaforolimus on p4E-BP1 in PBMCs versus tumor specimens suggests that higher ridaforolimus concentrations may need to be achieved within glioma cells in order to see meaningful reductions in this mTOR downstream effector.

The effect of another mTOR inhibitor, rapamycin, has been assessed in patients with recurrent PTEN-deficient GBM using a similar trial design [32]. Treatment with rapamycin at daily doses of 2–10 mg resulted in measurable intratumoral drug concentrations in the resected samples, which in most cases were above those associated with antiproliferative activity. Consistent with the present findings, rapamycin reduced pS6 levels compared with levels in archival samples when measured by immunohistochemistry or immunoblot techniques. In contrast, pS6 levels were not modified in a cohort of GBM patients who did not receive

the mTOR inhibitor. Notably, the reduction in pS6 with rapamycin was associated with a decrease in tumor cell proliferation measured by Ki-67 staining, and it helped distinguish tumors as either rapamycin-sensitive or rapamycin-resistant. Even though both rapamycin-sensitive and rapamycin-resistant tumors achieved detectable intratumoral drug concentrations, the authors suggested that rapamycin may have been sequestered in red blood cells in highly vascular tumors and not delivered in sufficient concentrations to the tumor cells in the resistant subset. This observation may also help to explain the lack of pS6 inhibition observed in the present study in the 15-mg cohort. Alternatively, differences in signaling pathways may account for the apparent lack of response to mTOR inhibitors.

Intratumoral concentrations of ridaforolimus were beyond the scope of this study and were not determined. However, intratumoral pharmacokinetic assessments should be included as an important component of future studies of targeted agents that use the current perisurgical study design. While there is great interest in pursuing intratumoral pharmacokinetic assessments in patients treated with an experimental drug, these measurements most often are contaminated by the contribution of drug concentrations present in the blood contained within the tumor specimen. Gliomas are microscopically heterogeneous; therefore, attempts to mathematically correct for the intravascular drug component, which require assumptions regarding the microvascular density, are imprecise at best. For example, in experimental orthotopic human glioma xenografts, clinically significant changes in intratumoral concentrations of erlotinib depended on the removal of blood by saline perfusion before tumor harvesting [33].

The pharmacokinetic data obtained in the present study were limited, but differed to some extent with findings from a previous phase 1 study in which ridaforolimus was also administered intravenously for 5 days every 2 weeks to patients with non-CNS advanced malignancies [16]. Although the CL rates for the 12.5-mg dose were comparable, the $AUC_{0-24\,h}$ and V_{ss} were lower in the present study compared with the earlier report. It remains to be determined whether exposure to ridaforolimus by patients with recurrent gliomas differs from those with other advanced malignancies, or more importantly, whether effective drug concentrations achieved in respective tumors differ. In the study by Mita et al., the MTD for ridaforolimus was identified as 18.75 mg, a dose level that was not reached in the present investigation. Dose-limiting toxicities were not seen in the present study, and since only a limited number of dose levels were evaluated (i.e., 12.5 and 15 mg), an MTD was not identified.

The safety profile observed in patients with recurrent glioma treated with ridaforolimus was consistent with previous reports in patients with sarcomas and other solid tumors [16, 19]. Treatment was generally well tolerated, with metabolic abnormalities (including hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and hypocalcemia) as the most common adverse events. Notably, mucositis (mouth sores), which was commonly reported in previous trials of ridaforolimus, was identified in this study as the preferred term of stomatitis in only 2 patients in the present trial (mild in one case and moderate in the other).

The antitumor activity of ridaforolimus monotherapy in patients with recurrent gliomas appears to be limited at the doses tested. One patient achieved stable disease as a best response. Because mTOR inhibitors are cytostatic rather than cytotoxic agents, ridaforolimus might be expected to stabilize disease rather than producing tumor shrinkage. In a phase 2 trial conducted in 212 patients with advanced soft tissue or bone sarcomas, ridaforolimus at the 12.5-mg dose schedule produced a clinical benefit rate of 29% consisting mostly of stable disease [20]. Notably, patients with such responses to ridaforolimus achieved improved overall survival compared with the entire study cohort. On the basis of these findings, ridaforolimus was evaluated as maintenance therapy in patients with metastatic sarcomas who have an objective response or stable disease after a minimum of 4 cycles chemotherapy in the phase 3 SUCCEED trial [21]. This trial, which enrolled 711 patients, met the primary endpoint of improved progression-free survival (PFS) in comparison with placebo (hazard ratio = 0.72, $P = 0.0001$), demonstrating that treatment with ridaforolimus may provide a benefit by prolonging stable disease in cancer patients. Although this phase 3 trial utilized an oral formulation of ridaforolimus, it has been shown that the 40-mg oral formulation of ridaforolimus has a similar pharmacokinetic profile to intravenous ridaforolimus [34]. Other mTOR inhibitors have also been evaluated in patients with recurrent malignant gliomas, with clinical benefit consisting mostly of stable disease, and in rare cases, partial responses [32, 35, 36]. Recent efforts to improve the clinical benefit of mTOR inhibitors have focused on combination therapy with other targeted agents, such as the EGFR tyrosine kinase inhibitors erlotinib and gefitinib, but early results appear comparable to those with mTOR inhibitors alone [37] Interestingly, a recent phase 3 trial of the combination of everolimus and exemestane in locally advanced or metastatic breast cancer was stopped because interim analysis results demonstrated a significant difference in PFS after 6 weeks [38], supporting the notion that mTOR inhibitors may be used in combination with other agents to improve outcomes in cancer patients. Future prospects for ridaforolimus and other mTOR inhibitors in recurrent glioma may rely on the ability to select patients most likely to benefit—either those with high tumoral levels of pS6, or using a maintenance paradigm for those whose disease is stabilized by another intervention.

Conclusions

Our study provides pharmacodynamic evidence consistent with an intratumoral effect of the mTOR inhibitor ridaforolimus among recurrent grade 4 malignant glioma patients. The BBB is a distinguishing challenge for patients with tumors within the CNS since therapeutic agents must successfully penetrate the BBB at concentrations that are sufficient to inhibit target activity. The current study provides a clinical trial paradigm that can be utilized in order to assess delivery across the BBB and actual intratumoral pharmacodynamics and pharmacokinetics. Future applications of such study designs are critical to the development of potential therapeutics for CNS tumor patients.

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