Cancer Chemother Pharmacol (2012) 69:1369–1377
DOI 10.1007/s00280-011-1813-7

CLINICAL TRIAL REPORT

Analysis of the pharmacodynamic activity of the mTOR inhibitor ridaforolimus (AP23573, MK-8669) in a phase 1 clinical trial

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Received: 14 September 2011 / Accepted: 22 December 2011 / Published online: 10 January 2012

Abstract
Purpose As part of a phase 1 dose-escalation trial, the pharmacodynamic activity of the mammalian target of rapamycin (mTOR) inhibitor ridaforolimus was assessed in multiple tissues by measuring levels of phosphorylated 4E binding protein-1 (p-4E-BP1) or S6, two downstream markers of mTOR activity.
Methods 32 patients (pts) were dosed intravenously with ridaforolimus once daily for 5 consecutive days (QD × 5) every 2 weeks. The pharmacodynamic activity of ridaforolimus was assessed in peripheral blood mononuclear cells (PBMCs; 32 pts), skin (28 pts), and tumor specimens (3 pts) collected before and after dosing by measuring levels of p-4E-BP1 by immunoblot analysis or pS6 by immunohistochemistry. Levels of these markers were assessed in up to 19, 5, and 2 pre- and post-dose time points in PBMC, skin, and tumor specimens, respectively.
Results In preclinical models, ridaforolimus induced a dose-dependent inhibition of p-4E-BP1 in PBMCs that was associated with antitumor activity. Rapid and potent inhibition of mTOR was observed in PBMCs from all 32 pts dosed, with a median level of inhibition of 96% observed within 1 h after the first dose. Inhibition of mTOR (>90%) was sustained during the entire QD × 5 dosing period, and substantial inhibition was still observed after the 9-day holiday between dosing courses. Evidence of mTOR inhibition was also obtained in skin in pts from all dose cohorts, although it did not persist through the break between courses. After two to three doses of ridaforolimus, inhibition of mTOR was detected in the tumor from one of three pts analyzed.
Conclusions Ridaforolimus was shown to inhibit its intended target, mTOR, in PBMCs, skin, and tumors. In PBMCs and skin, inhibition was observed at all dose levels tested, thus supporting but not driving the selection of a recommended phase 2 dose.

Keywords Ridaforolimus · mTOR · Pharmacodynamics · Peripheral blood mononuclear cells (PBMCs)

Introduction

Ridaforolimus (AP23573, MK-8669, formerly deforolimus) is a novel nonprodrug analog of rapamycin currently under clinical investigation as an anticancer agent. Ridaforolimus is a potent and selective inhibitor of mammalian target of rapamycin (mTOR), a serine/threonine kinase member of the PI3K/AKT pathway known to play a central role in controlling cell growth and proliferation, protein translation, angiogenesis, and metabolism [1, 2]. Accordingly, inhibition of mTOR by ridaforolimus has been shown to inhibit the proliferation of a wide variety of cancer cell lines as well as result in cell cycle arrest, tumor cell shrinkage, and antiangiogenic effects [3]. The PI3K/

Electronic supplementary material The online version of this article (doi:10.1007/s00280-011-1813-7) contains supplementary material, which is available to authorized users.

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AKT pathway is frequently altered in human cancers, leading to upregulation of mTOR activity and making mTOR an attractive therapeutic target.

The effects of mTOR on protein translation are mediated via the phosphorylation of multiple downstream effectors, including eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and ribosomal protein S6 kinase-1 ($p70^{S6K}$). Mammalian target of rapamycin-mediated phosphorylation of 4E-BP1 promotes the selective translation of certain mRNAs involved in cell proliferation, angiogenesis, and nutrient metabolism. 4E binding protein-1 is phosphorylated on multiple residues, including serine 65 and threonine 70, which have been shown to be sensitive to mTOR inhibition [4, 5]. Similarly, activation of $p70^{S6K}$ and subsequent phosphorylation of its substrate ribosomal protein S6, which together control cell growth and size, have been shown to be mTOR-dependent. Exposure to an mTOR inhibitor has been shown to inhibit phosphorylation of 4E-BP1, $p70^{S6K}$, and S6 in multiple preclinical models, confirming their utility as readouts of mTOR activity [5–8].

An important component of the clinical evaluation of a targeted agent, such as ridaforolimus, is the analysis of the effect of the agent on its intended target in patients. This allows one to determine whether the levels of exposure of the agent that are sufficient to inhibit its intended target can be achieved and, if done with sufficient sampling, what the kinetics and duration of inhibition are. This type of analysis can potentially play an important role in the selection of an optimal dosing regimen. While such an analysis ideally involves assessment of pharmacodynamic activity in the tumor being treated, the requirement for serial analysis (i.e., before and multiple time points after treatment) and the inaccessibility of many tumors necessitate the assessment of pharmacodynamic activity in surrogate tissues as well. Two such useful surrogates are peripheral blood mononuclear cells (PBMCs) and skin, both of which have previously been used to assess the pharmacodynamic activity of other mTOR inhibitors [7, 9–11].

Ridaforolimus was evaluated in a phase 1, dose-escalation trial for safety, tolerability, and identification of a maximum tolerated dose (MTD) [12]. Patients with advanced or metastatic cancers that were recurrent or refractory to standard therapy were administered ridaforolimus once daily, by intravenous injection, for five consecutive days (QD × 5) every 2 weeks on a 4-week cycle. As reported previously, the MTD was determined to be 18.75 mg/day and evidence of antitumor activity was observed [12].

A secondary objective of the trial was to evaluate markers of pharmacodynamic activity of ridaforolimus. Here, we describe the complete results of these analyses. The pharmacodynamic activity of ridaforolimus was examined via the assessment of levels of phosphorylation of S6 in tumors collected from a small subset of patients and in skin from a majority of patients, before and after dosing. The most comprehensive analysis, in all patients, was performed in PBMCs via the measurement of levels of phosphorylation of 4E-BP1 in samples collected at up to 19 time points before and after dosing. Preclinical development of this assay is also described.

Methods

In vitro studies

The human leiomyosarcoma cell line SK-LMS-1 was obtained from the American Type Culture Collection and maintained in modified Eagle's medium with 10% FBS at 37°C in 5% (v/v) CO_2. Ridaforolimus (AP23573; MK-8669) was synthesized at ARIAD Pharmaceuticals and prepared in ethanol to a 1 mM stock concentration. SK-LMS-1 cells were plated in 100-mm dishes at a density of 7×10^4/mL and treated for 2 h at 37°C in 5% (v/v) CO_2 with ridaforolimus at concentrations ranging from 0.3 to 1,000 nM. Cells were harvested by rinsing in ice-cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche, Sigma).

Xenograft tumor preparation

Female SCID mice were purchased from The Jackson Laboratory and treated under a protocol approved by the Institutional Animal Care Committee. Mice were implanted subcutaneously with 2×10^6 SK-LMS-1 cells. Following tumor establishment, mice were treated with a single intraperitoneal dose of ridaforolimus at 0.1 or 10 mg/kg and tumors harvested 18 h later. Individual tumors were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche, Sigma) and clarified by centrifugation. Whole blood from 5 mice per dose group was pooled to allow isolation of sufficient numbers of PBMCs for analysis. PBMCs were extracted as described for human PBMCs below.

Human volunteer peripheral blood mononuclear cell collection

Blood samples from healthy volunteers were collected into 10-mL Vacutainer tubes containing EDTA as an anticoagulant using standard venipuncture techniques. Ridaforolimus was added to the blood ex vivo by preparing an appropriate concentrated stock in 100% ethanol and then diluting the stock 1,000-fold directly into the blood in the Vacutainer tube. After the addition of drug or vehicle alone, the blood was mixed by inverting and incubated at 37°C for 2 h. Briefly, PBMCs were isolated by diluting the

blood in PBS and centrifugation over a Ficoll gradient. The isolated mononuclear cells were washed in PBS, and contaminating erythrocytes were lysed in a hypotonic buffer. The PBMCs were then washed, counted, and lysed in MPer lysis buffer (Roche) containing protease and phosphatase inhibitors.

Clinical trial design

As described previously [12], this was an open-label, single-center (Cancer Therapy and Research Center, San Antonio, Texas) phase 1, dose-escalation trial with flat-fixed dosing of ridaforolimus. Patients with documented recurrent or refractory, advanced or metastatic malignancies (including myeloma and lymphoma) that were measurable or evaluable and not amenable to standard therapy or surgical intervention, were eligible. Ridaforolimus was administered as a 30-min intravenous infusion QD × 5 every 2 weeks on a 4-week cycle with six dose levels tested (3-, 6.25-, 12.5-, 15-, 18.75-, and 28-mg dose cohorts). Patient response was evaluated every 8 weeks according to the Response Evaluation Criteria In Solid Tumors (RECIST). The primary objectives of the trial were to determine the safety, tolerability, and MTD of ridaforolimus, with one of the secondary objectives being to evaluate pharmacodynamic markers of ridaforolimus activity. Written informed consent for pharmacodynamic sampling was obtained from all patients at the time of patient screening. All human studies were approved by an institutional review board and performed in accordance with the Declaration of Helsinki. Pharmacokinetic analysis was also performed as described previously [12].

Peripheral blood mononuclear cell pharmacodynamic analysis in patients

Twenty milliliters of blood was collected into EDTA-containing Vacutainer tubes from patients at the following time points: at the time of screening; during cycle 1 on day 1 immediately before and 1, 4, and 8 h after the initial infusion; on days 2–4 immediately prior to infusion (i.e., 24 h after the previous infusion); on day 5 prior to and 1, 4, 8, 24, 48, and 72 h following the fifth infusion; and prior to and at 1 and 4 h after the sixth dose on day 15 of cycle 1. A single blood sample was also drawn immediately prior to the start of cycle 2. Blood specimens were collected into EDTA-containing Vacutainer tubes and shipped on ice packs overnight from the clinical site to ARIAD Pharmaceuticals for processing and analysis. Peripheral blood mononuclear cell protein lysates were generated using the Ficoll gradient method described above, and the lysates were stored at −80°C until analysis.

After all samples were collected for a given patient, 15 μg of each protein lysate was loaded onto four SDS-PAGE gels under denaturing conditions and transferred to PVDF membranes. Membranes were blocked in 1:1 NapBlock (GenoTechnology)/TBS-Tween overnight at 4°C. Membranes were incubated in 1:3 NapBlock/TBS-Tween in duplicate with either phosphorylated (p)-4E-BP1 (Ser65/Thr70; Santa Cruz Biotechnology) or total 4E-BP1 (Cell Signaling Technology) for 1 h at room temperature, followed by incubation with a goat antirabbit horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected using the enhanced chemiluminescence method, and autoradiography was performed by exposure to X-ray film. Band intensities were quantified using Kodak 1D software. For each sample, levels of p-4E-BP1 were normalized to total 4E-BP1 and expressed as a normalized percentage relative to either vehicle or the sample collected at baseline, as appropriate.

Skin and tumor pharmacodynamic analysis in patients

Skin specimens (3-mm punch biopsy) for pharmacodynamic analysis were collected during cycle 1 on day 1 immediately prior to and 4 h after the initial infusion; on day 3 prior to infusion; and on day 15, prior to and 4 h after infusion. Tumor specimens (3-mm punch biopsy) were collected prior to infusion on day 1 of cycle 1 and on either day 3 or 4 of cycle 1 from patients with accessible tumors. Immunohistochemical analysis of p-S6 and total S6 was performed on 4-μm formalin-fixed paraffin-embedded sections using rabbit polyclonal antibodies (Ser235/236; Cell Signaling Technology). The negative controls were incubated with immunoglobulin fraction (normal rabbit for polyclonal antibodies) in place of polyclonal primary antibody. The degree of staining was evaluated by a pathologist, blinded, and scored by determining the intensity of staining (0–3 scale; 1 being weak staining intensity; 2, moderate staining; and 3, strong staining) and the percentage of positive staining cells; the staining index was determined as the product of these values (0–300 scale). Statistical analysis between time points was done using an ANOVA one-way test.

Results

Identification of a robust marker of mammalian target of rapamycin activity in human peripheral blood mononuclear cells

We first evaluated several downstream targets of mTOR in PBMCs for their potential to be used as pharmacodynamic

markers of ridaforolimus activity. The ideal marker needed to satisfy several requirements. The activated, phosphorylated, protein should be readily detected in PBMCs, with a strong signal in the baseline state to allow a decrease in signal over a substantial dynamic range to be detected. In addition, treatment with ridaforolimus should result in a reduction in the level of phosphorylated protein with a dose dependence that recapitulates its antitumor activity. Analysis using an immunoblotting-based assay was preferred since it would allow all samples collected from a patient to be analyzed side-by-side, with appropriate controls and because its sensitivity would allow relatively small amounts of sample to still be analyzed in duplicate to increase precision of the assay. To begin to assess candidate markers for these properties, PBMCs were obtained from healthy donors and treated with either vehicle or with a concentration of ridaforolimus previously shown to give maximal mTOR inhibition [3]. Protein extracts were prepared and analyzed using antibodies specific for phosphorylated forms of each protein, with an antibody that recognizes the total protein used as a control. The proteins examined included $p70^{S6K}$, ribosomal protein S6, and 4E-BP1, all of which contain multiple phosphorylation sites previously shown to be sensitive to inhibition of mTOR (e.g., by rapamycin; data not shown).

Of these proteins, only 4E-BP1 phosphorylated at amino acids Ser65 and Thr70 (p-4E-BP1) was found to meet our criteria. As shown in Fig. 1, we were able to detect a basal level of p-4E-BP1 in human PBMCs and a concentration-dependent inhibition of p-4E-BP1 following 2 h of ridaforolimus exposure ex vivo. Exposure to 3.3 nM ridaforolimus led to an approximately 50% reduction in p-4E-BP1 levels (IC_{50}); nearly complete inhibition was seen at concentrations above 10 nM. Based on these results, we next used preclinical models to assess the suitability of measuring p-4E-BP1 in PBMCs as a surrogate marker for antitumor activity.

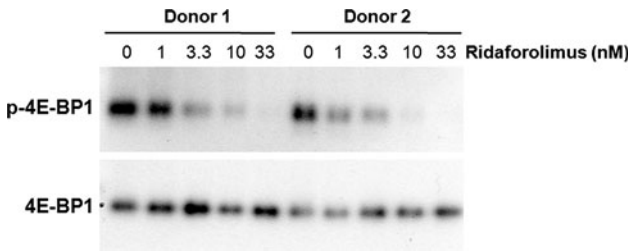

Fig. 1 Sensitivity of p-4E-BP1 to ridaforolimus in human PBMCs. Whole blood from two healthy donors was treated ex vivo with the indicated concentration of ridaforolimus, or vehicle, for 2 h. Isolated PBMCs were subjected to immunoblotting to assess the levels of p-4E-BP1 (Ser65/Thr70) (*upper panel*) and total 4E-BP1 (*lower panel*). *PBMC* peripheral blood mononuclear cell; *p-4E-BP1* phosphorylated 4E binding protein-1

Ridaforolimus-mediated inhibition of 4E-BP1 phosphorylation in PBMCs is associated with antitumor effects in preclinical models

To evaluate the modulation of 4E-BP1 phosphorylation as a marker of mTOR inhibition by ridaforolimus, we used the sarcoma cell line SK-LMS-1, which we have previously shown to be sensitive to ridaforolimus in in vitro proliferation assays [13]. Exposure of SK-LMS-1 cells to increasing concentrations of ridaforolimus for 2 h resulted in a dose-dependent inhibition of phosphorylation of 4E-BP1 (Fig. 2a), with an IC_{50} of 5.3 nM, and no change in total 4E-BP1 levels. In contrast to human PBMCs, complete loss of p-4E-BP1 was not observed, possibly reflecting the fact that 4E-BP1 can also be phosphorylated by an mTOR-independent mechanism in some cell lines [14].

We next investigated whether p-4E-BP1 could be measured in PBMCs in vivo and whether inhibition in this surrogate was associated with a similar pharmacodynamic effect in the tumor. Mice bearing SK-LMS-1-derived xenografts were administered a single dose of either 0.1 or 10 mg/kg ridaforolimus; tumors and PBMCs were collected

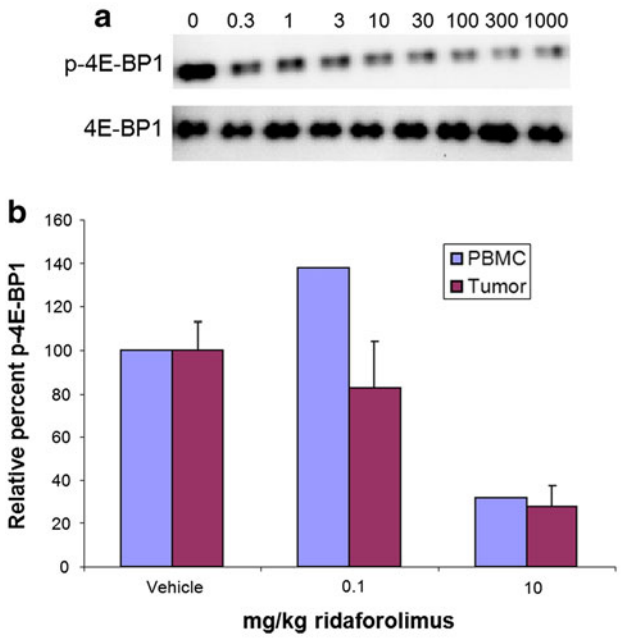

Fig. 2 Validation of p-4E-BP1 in PBMCs as a pharmacodynamic marker in a mouse model. **a** Levels of phosphorylated and total 4E-BP1 in SK-LMS-1 cells determined by immunoblotting following 2-h treatment with the indicated concentrations of ridaforolimus or vehicle. **b** Mice bearing SK-LMS-1 xenografts were administered a single 0.1- or 10-mg/kg dose of ridaforolimus or vehicle. After 18 h, tumors (5 mice per dose group analyzed separately) and PBMCs (pooled from 5 mice per dose group) were harvested and subjected to immunoblotting. Levels of p-4E-BP1 were normalized to levels of total 4E-BP1 and plotted relative to levels measured in vehicle-treated mice. *PBMC* peripheral blood mononuclear cells; *p-4E-BP1* phosphorylated 4E binding protein-1

18 h later. We have previously shown that 10 mg/kg, but not 0.1 mg/kg, ridaforolimus leads to significant inhibition of tumor growth when administered on an intermittent schedule (QD × 5 every 2 weeks) [3]. As shown in Fig. 2b, substantial inhibition of p-4E-BP1 (by ~70%) was only seen in tumors from mice dosed with 10 mg/kg ridaforolimus. Importantly, similar results were observed in PBMCs collected from the same mice. These data suggest that ridaforolimus-induced inhibition of p-4E-BP1 can be measured at efficacious dose levels in PBMCs and support the use of PBMCs as surrogate for measuring the effects of ridaforolimus on mTOR signaling in the tumor.

Pharmacodynamic activity of ridaforolimus in patient peripheral blood mononuclear cells

Having established the feasibility of using p-4E-BP1 in PBMCs as a pharmacodynamic marker of ridaforolimus activity, this assay was used to measure target inhibition in the clinical setting. The ease of PBMC collection from patients allowed an assessment of the pharmacodynamic activity of ridaforolimus at many time points, and the sensitivity of the immunoblotting-based assay allowed each blood sample to be assayed in duplicate. In this phase 1, dose-escalation trial, patients received intravenous doses of ridaforolimus (3–28 mg) QD × 5 every 2 weeks (i.e., on days 1 through 5 and 15 through 19 of each 4-week cycle). PBMCs were scheduled to be collected at 2 time points prior to the administration of ridaforolimus (i.e., at the time of screening and prior to the first dose on day 1), and at multiple time points during and after the first 5 days of dosing (i.e., 1, 4, and 8 h post-dose on days 1 and 5, as well as pre-dose on days 2, 3, 6, 7, and 8). PBMCs were also collected at "trough" time points that were 10 days removed from the prior dose (i.e., pre-dose on day 15 and day 1 of cycle 2), as well as at several post-dose time points on day 15 (i.e., 1 and 4 h). The dosing and pharmacodynamic sampling scheme is depicted in Fig. 3a.

All 32 patients in the trial who were dosed with ridaforolimus had a pre-dose and multiple post-dose PBMC samples collected. For each sample, levels of p-4E-BP1 were normalized to the levels of total 4E-BP1 and expressed as a percentage of the cycle 1 day 1 pre-dose sample. The results for all patients are shown in Supplementary Table 1, and representative data from a single patient are shown in Fig. 3b. As seen by examination of the immunoblots and the normalized results plotted in the bar graphs, similar, high levels of p-4E-BP1 were detected in the two pre-dose samples. Levels of p-4E-BP1 were strongly reduced within 1 h after the administration of the first dose of ridaforolimus. This strong degree of mTOR inhibition was maintained throughout the first 5 days of dosing and persisted for at least 72 h following the fifth infusion (day 8). Moreover, evidence

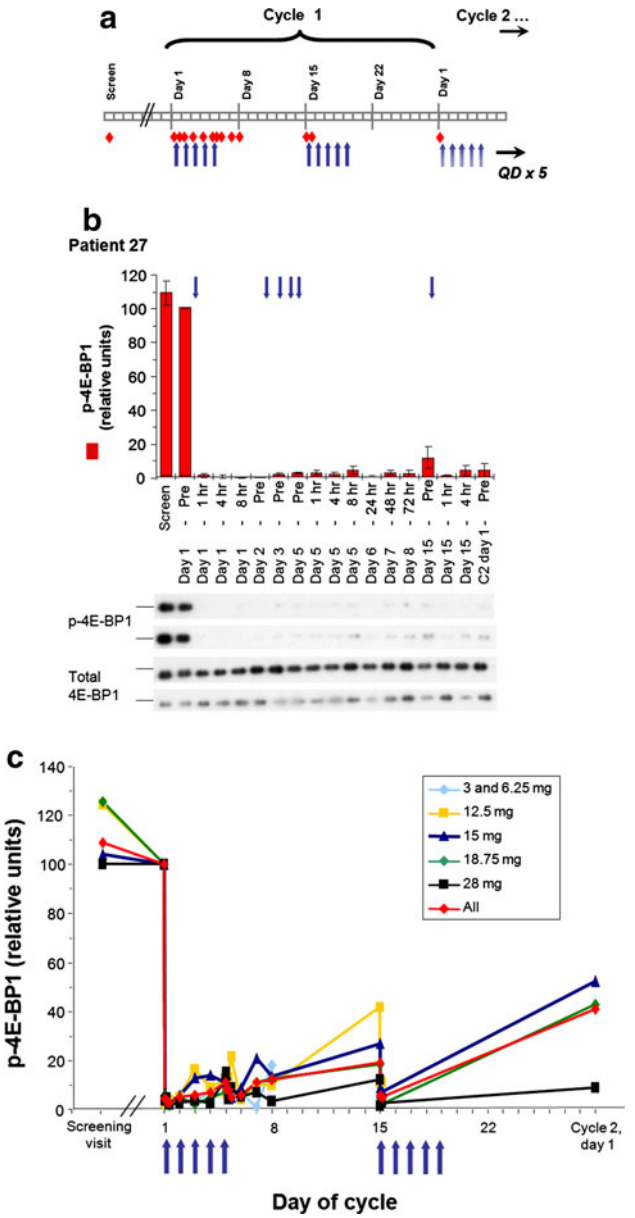

Fig. 3 Evaluation of p-4E-BP1 in PBMCs from patients dosed with ridaforolimus. **a** Dosing and pharmacodynamic sampling scheme for the phase 1 trial exploring a once daily for 5 consecutive days (QD × 5) every 2 weeks administration of ridaforolimus. *Arrows* indicate the timing of drug administration. *Diamonds* indicate time points at which a pharmacodynamic sample was collected. A *single diamond* is shown for 1, 4, and 8-h post-dose collections for days 1, 5, and 15. **b** Representative pharmacodynamic data for patient 27. Immunoblot analysis of phosphorylated and total 4E-BP1 levels, measured in duplicate, is shown. Normalized results are plotted in the bar graph. *Arrows* indicate the timing of ridaforolimus administration. **c** Plot of median relative p-4E-BP1 levels at each dose level (3- and 6.25-mg cohorts combined) and for all 32 patients combined. *Arrows* indicate timing of drug administration. Data from time points for which there were evaluable samples from fewer than two patients (e.g., cycle 2 day 1 for patients at the 3-, 6.25-, and 12.5-mg dose levels) were not plotted. *p-4E-BP1* phosphorylated 4E binding protein-1; *PBMC* peripheral blood mononuclear cell; *C2* cycle 2

of prolonged mTOR inhibition was observed at time points that followed the 9-day holiday between QD × 5 dosing courses (i.e., at day 15 and at day 1 of cycle 2).

A comprehensive assessment of the pharmacodynamic activity of ridaforolimus was made by determining the median relative level of p-4E-BP1 at each time point and for each dose level (Supplementary Table 1). For this analysis, samples collected following a dosing interruption or reduction were considered nonevaluable and were excluded. Since the 3-mg dose cohort contained only one patient, the results were combined with the three patients from the 6.25-mg cohort. The 12.5-mg cohort also contained three patients, and the remaining cohorts of 15, 18.75, and 28 mg contained 6, 13, and 6 patients, respectively.

The summary of these data (Fig. 3c) revealed the following: first, relative p-4E-BP1 levels were generally very similar (within 20%) in PBMCs collected at the two different pre-dose time points (i.e., at the time of screening and pre-dose on day 1). This close similarity indicated that the substantial reductions in phosphorylation observed in post-dose samples reflected drug effects and not variability due to sample handling or processing. Second, in all 32 patients, there was potent, rapid inhibition of mTOR activity by 1–4 h of ridaforolimus treatment irrespective of dose (Supplementary Table 1), with a median level of ~96% inhibition of p-4E-BP1 levels observed across all patients and dose levels both 1 and 4 h after ridaforolimus administration. Greater than 90% inhibition was observed 24 h after dosing (i.e., at days 2, 3, 4, and 5 pre-dose time points), and at least 85% inhibition was maintained up to 72 h after dosing (i.e., at day 8, which is 3 days removed from the day 5 dose).

Importantly, there was evidence that inhibition of mTOR activity was also maintained during the 9-day holiday between dosing courses in this QD × 5 schedule. At the day 15 and cycle 2 day 1 time points, median levels of inhibition of 81 and 58% were observed, indicating a strong pharmacodynamic effect even 10 days removed from the previous dose. Overall, with high levels of p-4E-BP1 inhibition observed at all dose levels, no clear association between ridaforolimus dose and degree of mTOR inhibition was observed. While the degree of inhibition at different dose levels observed 10 days post-dosing suggested some dose dependency (Fig. 3c; day 15 and day 1 of cycle 2), the small number of patients in the lower dose cohorts who had evaluable samples at these time points (Supplementary Table 1) precluded meaningful statistical analysis of this observation.

Pharmacodynamic activity of ridaforolimus in patient skin and tumor

In order to assess the pharmacodynamic activity of ridaforolimus in a specimen that required tissue penetration, but which could be obtained from a majority of patients, skin biopsies were collected from patients pre- and post-dose. We chose p-S6 as the readout of mTOR activity based on an earlier report that successfully measured the pharmacodynamic activity of another mTOR inhibitor, everolimus, using this marker in skin and tumor samples [11]. The product of the p-S6 staining intensity and the percentage of positive cells were used to derive a staining index for each sample (0–300 scale).

Skin biopsies were scheduled to be collected at 5 time points: prior to dosing on day 1, 4 h after dosing on day 1, 24 h after the second dose (day 3 pre-dose), 10 days after the fifth dose (day 15 pre-dose), and 4 h after the day 15 dose. A pre-dose and at least one post-dose sample were available from 28 of the 32 dosed patients. Evidence of mTOR inhibition in the skin was not observed in every patient at all post-dose time points; however, the majority of patients (24/28) showed a reduction in p-S6 levels at one or more post-dose time points (Supplementary Table 2). Representative immunohistochemical images for two patients are shown in Fig. 4a. A high level of p-S6 staining was observed in the pre-dose samples with staining indices of 200 and 300 in patients 32 and 31, respectively. In the skin sample collected on day 3 from patient 32, levels of p-S6 were reduced by 85% relative to the pre-dose sample. In contrast, no reduction in p-S6 was observed in the post-treatment specimen collected from patient 31. A comprehensive analysis of the data from all patients and dose levels combined is shown in Fig. 4b. Mean p-S6 levels were significantly reduced, relative to baseline, 4 h after the initial ridaforolimus administration ($P = 0.007$), and further reduction was observed at day 3, 24 h after the second dose. At the trough time point 10 days removed from the prior does (i.e., pre-dose on day 15), p-S6 levels were somewhat reduced relative to baseline, but the difference was not statistically significant ($P = 0.06$). However, significant levels of inhibition were again observed 4 h following the day 15 infusion ($P = 0.00014$).

There was no apparent relationship between the level of p-S6 inhibition and the dose of ridaforolimus, with substantial inhibition seen in patients in low- and high-dose cohorts (Supplementary Table 2). Likewise, no association was detected between mTOR inhibition and ridaforolimus pharmacokinetic parameters (i.e., maximum concentration [C_{max}] and area under the concentration-time curve [AUC]) measured in each patient [12] (Supplementary Table 2). Finally, there was also no detectable association between mTOR inhibition in skin and the antitumor activity of ridaforolimus in each patient [12] (Supplementary Table 2).

Lastly, a limited analysis of the pharmacodynamic activity of ridaforolimus in the target tumor tissue was also performed via the measurement of p-S6 levels in tumor biopsies. Tumor specimens collected prior to infusion on

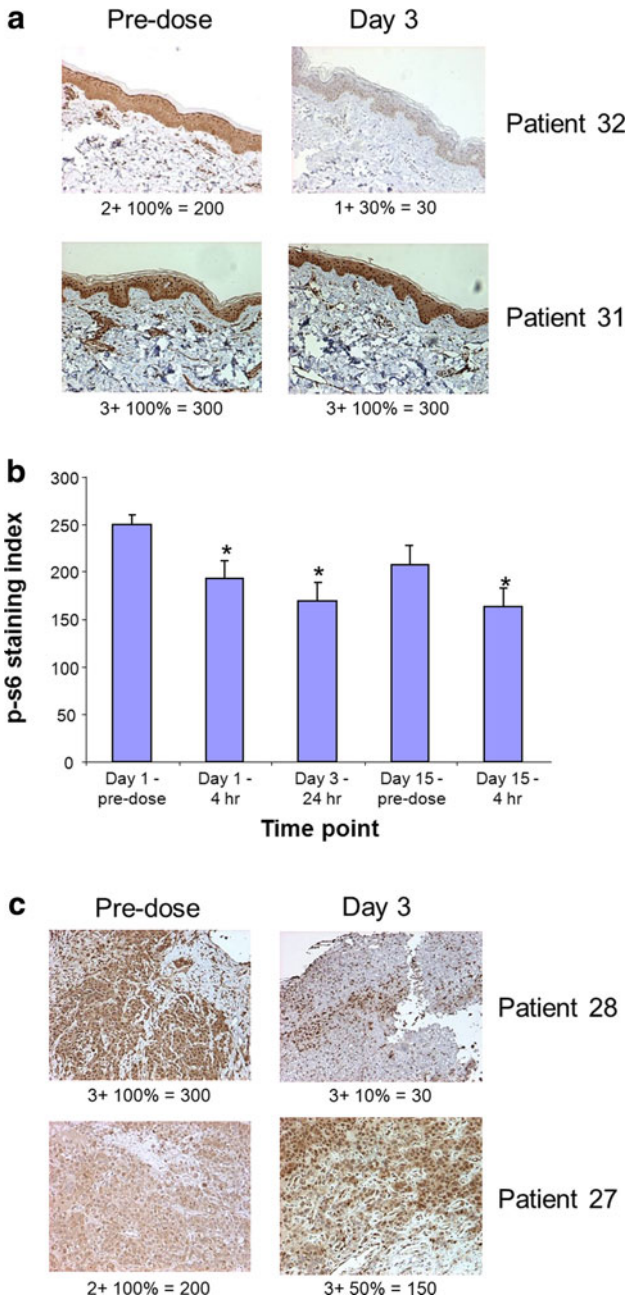

Fig. 4 Evaluation of p-S6 in skin and tumor from patients dosed with ridaforolimus. **a** Immunohistochemical staining of p-S6 in skin samples from two patients. Biopsies were taken immediately prior to the initial infusion of ridaforolimus on day 1 (pre-dose) and before infusion on day 3 (i.e., 24 h following the day 2 dose). The staining index value shown below each image was determined as the product of the staining intensity (1+ to 3+) and the percentage of cells staining positive (0–100%). (**b**) Mean p-S6 staining index for all patients at each time point. Time points shown are pre- and 4-h post-dose on day 1, pre-infusion on day 3, and pre- and 4-h post-dose on day 15. *$P < 0.05$. **c** Immunohistochemical staining of p-S6 in tumor biopsies from two patients with accessible tumors. Staining index values determined as in (**a**)

day 1 and post-infusion on day 3 or 4 were available from three patients who had received the maximal dose of ridaforolimus (Supplementary Table 2). Representative staining images for two of these patients are shown in Fig. 4c. In one of the three patients, a major reduction in the p-S6 staining index of 90% was observed following ridaforolimus administration (Fig. 4c, patient 28). In the other two patients, little or no reduction in the overall staining index was seen. In one of these patients, a decrease in the percentage of cells staining positive was detected, although an increase in the staining intensity resulted in little change to the overall staining index (Fig. 4c, patient 27).

Discussion

Alterations in the mTOR signaling pathway are common in cancer and have triggered the development of multiple mTOR inhibitors, with analogs of rapamycin having advanced the furthest. Clinical validation of mTOR as a cancer target and rapamycin analogs as therapeutics has come from the approval of temsirolimus and everolimus for the treatment of renal cell carcinoma [15], with promising activity also observed in other indications [16]. Given that these agents are also approved as immunosuppressants when administered daily [17], their clinical development has included the exploration of a broad variety of doses and dosing regimens aimed at maximizing their effects on tumors. In the case of temsirolimus, which is administered by intravenous injection, two intermittent dosing regimens, QD × 5 every 2 weeks and once weekly (QW), have been explored. In the case of everolimus, which is administered orally, both intermittent (i.e., QW) and continuous (i.e., daily) dosing regimens have been explored. A key aspect of these studies has been analysis of the pharmacodynamic activity of the agents in the target tumor, as well as in surrogate tissues [7, 9, 11]. In the case of everolimus in particular, the results of these studies have been used to help guide selection of the dose and regimen used for further evaluation [18, 19].

Ridaforolimus is a novel nonprodrug analog of rapamycin that has been shown to have promising anticancer activity in a number of indications [20]. During its initial clinical development, a phase 1 trial was conducted in which ridaforolimus was administered intravenously, at doses between 3 and 28 mg, on a QD × 5 every 2 weeks dosing regimen. Important questions that were addressed in the correlative studies described here are whether dose levels could be achieved that led to the inhibition of mTOR and, if so, what was the duration of inhibition, with the

outcome of these studies having the potential to inform, or at least support, the selection of a recommended phase 2 dose. To address these questions, the trial was designed to allow the analysis of the pharmacodynamic activity of ridaforolimus in the target tumor as well as in two surrogate tissues, skin, and PBMCs. Our experience and the results we obtained illustrate some of the advantages and limitations of these varied approaches.

Analysis of the pharmacodynamic activity of ridaforolimus in PBMCs yielded by far the most extensive dataset. Pre- and multiple post-treatment specimens (from up to 19 total time points) were obtained from all patients in the trial, allowing us to examine the degree and kinetics of target inhibition in great detail. This analysis was conducted by measuring levels of 4E-BP1 phosphorylated at Ser65/Thr70, a marker that we selected based on its ease of detection in human PBMCs and the association between ridaforolimus-induced inhibition of phosphorylation in PBMCs and anti-tumor activity in preclinical models. The results provided evidence of rapid (within 4 h) and strong (generally $> 90\%$) target inhibition in all 32 patients dosed with ridaforolimus. We also found that substantial target inhibition (>85%) was still observed through 72 h after dosing. Furthermore, substantial target inhibition (58–81%) was still observed even 10 days after dosing (i.e., between QD $\times$ 5 regimens). Although the degree of inhibition observed after 10 days of dosing was not as substantial as that observed at time points closer to dosing, overall, the results obtained in PBMCs indicate that a QD $\times$ 5 every 2 weeks dosing regimen can lead to substantial, continuous inhibition of mTOR activity, at least in this surrogate tissue. However, since strong inhibition was observed in all patients, including the four patients administered the lowest doses studied, no association between the dose of ridaforolimus and the pharmacodynamic effect in PBMCs could be discerned. It is important to note that this robust assay, the development of which we describe here, was subsequently used to examine the pharmacodynamic activity of ridaforolimus in PBMCs in other phase 1 trials with results obtained that were generally similar to those reported here [21–23].

Examination of pre- and post-treatment skin biopsies also provided evidence that ridaforolimus inhibits mTOR in patients. In this case, p-S6 levels were measured by immunohistochemistry. Evidence of a decrease in pS6 levels was observed in 24 out of 28 patients and, overall, significant decreases in pS6 levels were detected at all three time points examined that were 4 or 24 h removed from dosing. However, in contrast to the results observed in PBMCs, mTOR inhibition was not observed in all patients, the magnitude of inhibition was smaller, and inhibition of mTOR signaling did not appear to be sustained to a significant degree over the course of the 9 days between QD $\times$ 5 dosing. Although differences in the methodologies used and targets being assessed in PBMCs and skin make a direct comparison of the results difficult, these results suggest that examination of pharmacodynamic effects in a surrogate that does not require tissue penetration (i.e., PBMCs) may be less likely to recapitulate the pharmacodynamic activity observed in a solid tumor. As was the case in PBMCs, there was no clear association between the dose administered, or the ridaforolimus pharmacokinetic parameters measured in individual patients, and the degree of target inhibition in the skin.

Finally, evidence was also obtained that ridaforolimus can inhibit its target in tumor tissue. Not unexpectedly, tumor specimens were able to be obtained from far fewer patients than skin and PBMC specimens (3, 28, and 32 patients, respectively). These limitations also allowed the analysis of tumor tissue collected from only one post-treatment time point (day 3 or 4). A strong reduction in levels of pS6, relative to those in a tumor sample collected prior to treatment, was observed in one of the three patients analyzed. The lack of inhibition seen in tumors from two patients may reflect either the inability of ridaforolimus to inhibit mTOR signaling in those tumors or a reduced sensitivity that could be overcome with additional dosing with ridaforolimus (i.e., beyond the 2–3 doses delivered here).

Overall, these studies demonstrate that ridaforolimus can be dosed to levels in patients that lead to inhibition of mTOR activity, as measured by a reduction in levels of p-4E-BP1 or pS6 in tumor and surrogate tissues. Since pharmacodynamic activity was observed in surrogate tissues at all dose levels, these results could not be used to guide the selection of a recommended phase 2 dose, other than to indicate that any of the doses tested here are sufficient to induce mTOR inhibition in PBMCs. If skin is taken to be a better surrogate for the tumor than PBMCs, then these results suggest that the 9-day holiday between QD $\times$ 5 doses may be too long to allow continuous inhibition of the target. If that goal is to be achieved, for example, by shortening the gap between QD $\times$ 5 doses, for reasons of convenience an oral dosing regimen may be preferred over the intravenous regimen used here.

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