Preclinical Development

Antitumor Activity of Ridaforolimus and Potential Cell-Cycle Determinants of Sensitivity in Sarcoma and Endometrial Cancer Models

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Abstract

Ridaforolimus is a nonprodrug rapamycin analogue that potently inhibits mTOR and has shown significant activity in patients with metastatic sarcoma and endometrial cancer, two diseases where high unmet need remains. Here, we evaluated the activity of ridaforolimus in preclinical models of these tumor types and used these models to explore molecular correlates of sensitivity. The in vitro sensitivity of a panel of sarcoma and endometrial cancer cell lines was established by measuring the effect of ridaforolimus on cell proliferation rate, revealing broad inhibition at low nanomolar concentrations. Additional benefit was found when ridaforolimus was combined with agents used to treat sarcoma and endometrial cancer patients. In vivo, potent antitumor activity of ridaforolimus associated with inhibition of mTOR signaling was observed in sarcoma and endometrial xenograft models. Immunoblot analysis was conducted to assess the expression and activation state of multiple signaling proteins in the phosphoinositide-3-kinase/AKT/mTOR and cell-cycle pathways. In endometrial but not sarcoma cell lines, the absence of PTEN or elevated levels of phosphorylated or total AKT was associated with greater sensitivity. However, in both tumor types, the proportion of cells in the G0–G1 phase before treatment correlated significantly with ridaforolimus sensitivity. Consistent with this, expression of several G1 phase cell-cycle proteins, notably p21 and p27, was higher in more sensitive lines. These results underscore the promise of ridaforolimus as a single agent or combination treatment of these tumor types and suggest novel potential predictive biomarkers of sensitivity to an mTOR inhibitor based on cell-cycle status. Mol Cancer Ther; 10(10): 1959–68. ©2011 AACR.

Introduction

Ridaforolimus (AP23573, MK-8669, formerly known as deforolimus) is a nonprodrug analogue of rapamycin currently in clinical development as a novel therapeutic for the treatment of cancer (1). Ridaforolimus potently inhibits mTOR (2), a serine/threonine kinase that acts as a checkpoint for both cellular nutritional status and cell-cycle control (3–5). mTOR is a downstream effector of phosphoinositide-3-kinase/AKT/mTOR and cell-cycle pathways. In endometrial but not sarcoma cell lines, the absence of PTEN or elevated levels of phosphorylated or total AKT was associated with greater sensitivity. However, in both tumor types, the proportion of cells in the G0–G1 phase before treatment correlated significantly with ridaforolimus sensitivity. Consistent with this, expression of several G1 phase cell-cycle proteins, notably p21 and p27, was higher in more sensitive lines. These results underscore the promise of ridaforolimus as a single agent or combination treatment of these tumor types and suggest novel potential predictive biomarkers of sensitivity to an mTOR inhibitor based on cell-cycle status. Mol Cancer Ther; 10(10); 1959–68. ©2011 AACR.

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deletions or defects in PTEN can be hypersensitive to inhibition of mTOR (14, 15). Consistent with this, ridaforolimus has shown promising single agent activity in a phase 2 clinical trial in patients with advanced, progressive endometrial cancer (16). Bone and soft tissue sarcomas are rare tumors and represent a heterogeneous collection of malignancies with diverse characteristics in terms of pathology, molecular and genetic composition, and clinical presentation (17, 18). Ridaforolimus treatment resulted in significant therapeutic benefit in a phase 2 study of patients with advanced bone or soft tissue sarcoma (19). A phase 3 pivotal trial of single agent ridaforolimus in this indication is currently underway.

There remains a clear unmet medical need for superior treatment options for both of these cancer types. Chemotherapy and hormonal therapy play important roles in first-line management of endometrial tumors; however, the majority of patients with advanced endometrial cancer relapse and face a dismal prognosis (20). Similarly, advanced and metastatic sarcoma is rarely curable, as standard chemotherapy has a low response rate, and consequently patient prognosis remains poor (21). Because of its clinical activity and favorable safety profile, the combination of ridaforolimus with standard-of-care agents for these indications might be expected to improve patient outcome. In this regard, recent preclinical evidence suggests that combinations of mTOR inhibitors with chemotherapeutics, targeted therapies, or hormonal therapies hold substantial promise for improved antitumor efficacy (22). Moreover, this approach may represent a means to overcome drug resistance to conventional cytotoxic agents (23–25).

An ongoing challenge for the optimal application of mTOR inhibitors is the identification of biomarkers that are predictive of favorable outcome (26). Candidate biomarkers have been assessed in a number of clinical trials, typically through retrospective analysis of archival samples (reviewed in ref. 27). Unfortunately, the clinical evaluation of specific mTOR pathway components, such as PTEN, has yielded inconsistent findings. In part, this may be linked to technical challenges. These include the question of whether archival specimens accurately reflect the genetic makeup of the current tumor being treated. In addition, the reproducibility and sensitivity of the methodologies used, such as immunohistochemistry, remain obstacles for predictive marker development. Because of factors such as these, no robust molecular markers predictive of response to an mTOR inhibitor have been clinically validated to date.

In this study, we evaluated the activity of ridaforolimus in preclinical models of sarcoma and endometrial cancer, both as a single agent and in combination with standard-of-care chemotherapeutics, doxorubicin, carboplatin, and paclitaxel. With these studies as a foundation, we then explored potential molecular correlates of sensitivity to ridaforolimus in these tumor types. Specifically, we examined the expression and activation of multiple signaling proteins in the PI3K/AKT/mTOR pathway. Furthermore, given the essential regulatory role of mTOR in cell-cycle progression, we also evaluated potential biomarkers related to this process. Our results support the use of ridaforolimus as a viable therapeutic option for these diseases and also suggest potential determinants of sensitivity across tumor types, linked to cell-cycle status, that warrant further investigation.

Materials and Methods

Cell lines, antibodies, and reagents

All human sarcoma and endometrial tumor lines used in this study were obtained from the American Type Culture Collection (ATCC). All ATCC cell lines were authenticated by the company routine Cell Biology Program and were used within 6 months of receipt for this study. Cells were maintained and cultured according to standard techniques at 37°C in 5% (v/v) CO₂ using culture medium recommended by the supplier. Ridaforolimus (AP23573; MK-8669; Supplementary Fig. SI) was synthesized at ARIAD Pharmaceuticals and prepared in ethanol to a 1 mmol/L stock concentration. For in vitro cellular assays, ridaforolimus was diluted in the optimal medium. For in vivo experiments, ridaforolimus was diluted in a vehicle of 4% ethanol, 5% Tween-80, and 5% propylene glycol. Paclitaxel and carboplatin were purchased from Sigma and doxorubicin obtained from LKT Laboratories. The following antibodies were used: mTOR, phospho-mTOR (Ser2448), 4E-BP1, S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), PTEN, AKT, phospho-AKT (Ser473), p70S6 kinase, phospho-p70S6 kinase (Thr389), TSC2, RICTOR, RAPTOR, RHEB, eIF-4E, CDK2, and CDK4 from Cell Signaling Technology; PTEN, AKT, phospho-AKT (Ser473), p70S6 kinase, phospho-p70S6 kinase (Thr389), TSC2, RICTOR, RAPTOR, RHEB, eIF-4E, pan-actin, p21, cyclin A, cyclin D1, cyclin E, CDK2, and CDK4 from Cell Signaling Technology; and GAPDH from Abcam.

In vitro proliferation assays

Exponentially growing cell lines were plated into 96-well plates and incubated overnight at 37°C. Twenty-four hours after plating, 1 plate (D1) was aspirated and stored at −80°C. The other plate (D4) was treated with 10-fold serial dilutions of ridaforolimus (1,000–0.001 nmol/L) or vehicle (ethanol). Following 72 hours culture at 37°C, the plates were aspirated and stored at −80°C. The D1 and D4 plates were assessed simultaneously for cell growth using the CyQUANT Cell Proliferation Assay Kit (Invitrogen). Doubling time (DT) = \[0.301 \times (72)/\log(\text{day 4}/\text{day 1})\]. Doublings = 72/DT. Cell growth rate (%) = doublings ridaforolimus/doublings vehicle × 100. \[I_{\text{max}} = 100 - \text{cell growth rate (}%\right)\] at the dose whereby maximum inhibition is observed. \[I_{\text{max}}\] was used to determine relative sensitivity of each cell line.

Multiple drug effect analysis

An effective but submaximal dose was determined for each compound tested and defined as 1×. The drug
concentrations used ranged from 0.125 x to 8 x at a fixed ratio. Percentage of cell growth inhibition was calculated as \([100 - (\text{fluorescence treated/fluorescence untreated}) \times 100]\) at each compound concentration. Combinatorial effects on cell growth were then analyzed using the Chou and Talalay Median-Effects method (28) to determine the combination index (CI) values (CalcuSyn software, Biosoft).

**Flow cytometric analysis**

Cell lines were cultured for 48 hours to achieve exponential growth phase before harvest for cell-cycle analysis. Ridaforolimus-treated cells were incubated with 100 nmol/L compound for 24 hours before harvest. Cells were fixed with ice cold 70% ethanol/30% PBS overnight at 4°C. Following fixation, cells were washed, resuspended in PBS/2% FBS, and then treated with 50 μg/mL RNase A for 30 minutes at 37°C. Cells were then stained with 20 μg/mL propidium iodide for 30 minutes at room temperature, protected from light. After staining, cells were passed through a 30-μm mesh filter and then maintained in the dark on ice until analyzed by flow cytometry within 1 to 2 hours of processing. Ten thousand cells per sample were analyzed and the percentage of cells in each phase of the cell cycle (sub-G₀-G₁, G₀-G₁, S, and G₂-M) was determined from the FL2-A histogram using ModFit LT software. The relationship between ridaforolimus sensitivity, as determined by \(I_{\text{macu}}\) and the percentage of the cell population present in the G₀-G₁ phase of the cell cycle during exponential growth phase was determined by linear regression analysis conducted using Excel Fit software.

**In vivo growth inhibition studies**

All animal research protocols were conducted in accordance with the guidelines of the American Association of Laboratory Animal Care. Endometrial xenografts were established by the subcutaneous implantation of AN3-CA cells (2 x 10⁶ cells) at the right flank area of 6- to 8-week-old female nude mice (nu/nu strain; Charles River Laboratories). Animals were randomly assigned to individual treatment groups (10 animals per cohort). Ridaforolimus at dosages of 0.1, 0.3, 1, 3, and 10 mg/kg/dose was administered intraperitoneally using the same 5 continuous days every week schedule. The control group was treated with vehicle alone.

Treatments were initiated in both the endometrial and sarcoma xenograft studies when the average tumor volume reached approximately 200 mm³. All mice were monitored daily and weighed twice per week. Tumor size was evaluated twice per week by caliper measurements using the following formula: tumor volume = \((\text{length} \times \text{width}^2)/2\). Tumor growth inhibition (TGI) was calculated when the treatment period was finished, \(\text{TGI} = (1 - \Delta\text{T}/\Delta\text{C}) \times 100\), where \(\Delta\text{T}\) stands for mean tumor volume change of each treatment group and \(\Delta\text{C}\) for mean tumor volume change of control group. The tumor volume data were collected and overall differences between the multiple study groups analyzed by 1-way ANOVA test. Each ridaforolimus treatment group was further compared with the vehicle control group for statistical significance using Dunnett’s test. A value of \(P < 0.05\) was considered to be statistically significant.

**Results**

**Antiproliferative activity of ridaforolimus in sarcoma and endometrial cancer lines**

To assess the sensitivity of cells to ridaforolimus \(\text{in vitro}\), a panel of 11 sarcoma and 6 endometrial cancer cell lines was used to evaluate the effects of treatment on the rate of cellular proliferation. Changes in proliferation rate, rather than absolute cell number, were examined because the relative effect a cytostatic drug, such as ridaforolimus, has on cell number is influenced by the doubling time of the cells being studied (2). In addition, 2 separate parameters were measured to assess the sensitivity of cell lines: the maximal inhibitory effect on rate of proliferation (i.e., the \(I_{\text{macu}}\)) and the concentration of ridaforolimus that induced half the maximal effect (i.e., the \(EC_{50}\)). We have previously shown that this assay methodology allows a more informative evaluation of cytostatic agents (2). As shown in Fig. 1, low nanomolar concentrations of ridaforolimus were found to suppress cellular proliferation of all 17 cell lines examined. Maximal levels of inhibition ranged from approximately 20% to 60%, with \(EC_{50}\) values between 0.1 and 1 nmol/L for nearly all cell lines (Table 1). These results show that ridaforolimus has broad inhibitory activity against sarcoma and endometrial cancer cell lines \(\text{in vitro}\) but that the level of sensitivity of cell lines varies.

**TGI of endometrial and sarcoma xenografts by ridaforolimus**

To examine the antitumor effects of ridaforolimus treatment \(\text{in vivo}\), mice bearing sarcoma- and endometrium-derived xenografts were treated with the recommended phase 2 oral dosing regimen of 5 continuous days each week (29). We have previously shown the \(\text{in vivo}\) efficacy and pharmacodynamic activity of ridaforolimus in a sarcoma xenograft model, using an alternate dosing regimen (2). To extend those observations and to
confirm the efficacy of the 5 continuous days dosing schedule, mice bearing tumors grown from an SK-LMS-1–derived sarcoma line were treated with doses ranging from 0.1 to 3 mg/kg (Fig. 2A). Ridaforolimus exposure resulted in a dose-dependent inhibition of tumor growth, with significant inhibition observed at the 1 and 3 mg/kg levels, a result consistent with our previous observations. Similar effects were seen in an AN3-CA endometrial xenograft model in which mice were treated with drug doses ranging from 0.1 to 10 mg/kg (Fig. 2B). A dose-dependent inhibition of tumor growth was observed, with the minimal and maximal efficacious doses being 0.3 and 3 mg/kg, respectively. In both models, TGI was observed as opposed to tumor regressions, consistent with a predominantly cytostatic mechanism of action.

To determine whether the antitumor activity of ridaforolimus was associated with inhibition of mTOR signaling in the target tumor tissue, the phosphorylation status of the critical downstream effectors 4E-BP1 and ribosomal protein S6 was examined in AN3-CA xenografts. A strong reduction was seen in levels of both phospho-4E-BP1 and phospho-S6 18 hours after administration of either 1 or 10 mg/kg ridaforolimus (Fig. 2C).

![Graphs showing cell proliferation rate vs. ridaforolimus concentration for sarcoma and endometrial cancer cell lines.]

**Figure 1.** Ridaforolimus reduces the rate of proliferation of sarcoma and endometrial cancer cell lines. Determination of ridaforolimus sensitivity of a panel of sarcoma (A) and endometrial (B) cancer cell lines over a 0.0001 to 1,000 nmol/L concentration range, analyzed 72 hours after drug exposure.

| Table 1. EC$_{50}$ and I$_{max}$ values of sarcoma and endometrial lines to ridaforolimus |
|-----------------|-----------------|-----------------|
| Line            | Cancer type     | EC$_{50}$, nmol/L | I$_{max}$ |
| SK-ES-1         | Ewing’s sarcoma | 1.0             | 19          |
| U-2OS           | Osteosarcoma    | 4.7             | 20          |
| HT-1080         | Fibrosarcoma    | 0.5             | 22          |
| SK-UT-1         | Leiomyosarcoma  | 1.0             | 22          |
| SW872           | Liposarcoma     | 0.2             | 28          |
| Saos-2          | Osteosarcoma    | 3.6             | 31          |
| SW982           | Synovial sarcoma| 0.5             | 35          |
| SJSA-1          | Osteosarcoma    | 0.8             | 39          |
| SK-LMS-1        | Leiomyosarcoma  | 0.2             | 40          |
| MG-63           | Osteosarcoma    | 0.7             | 42          |
| VA-ES-BJ        | Epithelioid sarcoma | 0.4       | 52          |
| HEC-1-A         | Endometrial adenocarcinoma | 2.5 | 18          |
| HEC-1-B         | Endometrial adenocarcinoma | 1.0 | 31          |
| KLE             | Endometrial adenocarcinoma | 6.0 | 34          |
| AN3-CA          | Endometrial adenocarcinoma | 0.5 | 40          |
| RL95-2          | Endometrial adenocarcinoma | 0.1 | 52          |
| SK-UT-1B        | Endometrial/leiomyosarcoma | 0.4 | 61          |
doses that led to significant inhibition of tumor growth (Fig. 2B). In contrast, a dose of 0.1 mg/kg, which did not lead to significant inhibition of tumor growth, did not lead to substantial inhibition of either target. Therefore, the potent antitumor activity of ridaforolimus in the endometrial xenograft system correlated with the degree of mTOR inhibition within the tumor, substantiating the proposed mode of action of the agent. Taken together, these findings show that ridaforolimus exhibits broad antiproliferative activity across both sarcoma and endometrial cancer cell lines in vitro and in vivo.

Ridaforolimus activity in combination with standard-of-care agents in sarcoma and endometrial cancer cells

Next, we examined the potential for achieving enhanced therapeutic benefit by combining ridaforolimus with standard-of-care agents (30, 31). To begin to evaluate this, 6 sarcoma cell lines were treated in vitro with a combination of ridaforolimus and doxorubicin, and 3 endometrial cancer lines were treated with the triple combination of ridaforolimus, carboplatin, and paclitaxel (Carbo-Tax). Combinatorial activity was assessed using the median-effects method (28) to establish whether the combinations exhibited antagonistic, additive, or synergistic activity. Table 2 summarizes the sensitivity of the cell lines to the chemotherapeutic agents and ridaforolimus. In sarcoma, the combination of ridaforolimus and doxorubicin was found to be either additive or moderately synergistic in all 6 cell lines evaluated. Similarly, the effects of the ridaforolimus and Carbo-Tax combination in endometrial lines were generally additive, although RL95-2 cells displayed a modest synergistic interaction. Overall, these results show at least additive benefits of combining standard-of-care treatments with ridaforolimus in both sarcoma and endometrial cancer models.

Relationship between the mTOR signaling pathway and ridaforolimus sensitivity

The signaling pathways that regulate mTOR activity are frequently activated in human tumors, raising the possibility that alterations in these signaling molecules might predict sensitivity to ridaforolimus. To examine this, the expression level and/or activation state of multiple signaling proteins in the PI3K/AKT/mTOR pathway were measured, by immunoblot analysis, in the same panel of sarcoma and endometrial cell lines whose sensitivity to ridaforolimus was described above (Fig. 1 and Table 1). Signaling proteins examined included upstream regulators of mTOR (PTEN, AKT, TSC2, and RHEB), components of the mTOR signaling complex (RICTOR, RAPTOR, and mTOR), and downstream effectors of mTOR activity (ribosomal protein S6 kinase, S6, 4E-BP-1, and elf4E; ref. 3). Levels of each protein were then compared with the level of ridaforolimus sensitivity of each cell line (Fig. 3). In the sarcoma cell line panel, no clear associations were observed between the expression or activation of PI3K/AKT/mTOR pathway components and sensitivity to ridaforolimus. However, in the endometrial model
in the G0–G1 phase of the cycle following treatment. The initial analysis compared the percentage of cells that entered mitosis, before and after ridaforolimus treatment (Fig. 4).

Endometrial cancer lines was assessed by flow cytometry, to determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle progression (2).

Table 2. Ridaforolimus activity in combination with standard-of-care agents in sarcoma and endometrial cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>Ridaforolimus EC_{50} nmol/L</th>
<th>Doxorubicin EC_{50} ng/mL</th>
<th>Carboplatin EC_{50} μmol/L</th>
<th>Paclitaxel EC_{50} nmol/L</th>
<th>Mean CI (±SD)</th>
<th>FA, 0.2–0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-LMS-1</td>
<td>Sarcoma</td>
<td>0.2</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
<td>0.97 (±0.17)</td>
<td>Additivity</td>
</tr>
<tr>
<td>SK-UT-1</td>
<td>Sarcoma</td>
<td>0.5</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
<td>0.92 (±0.01)</td>
<td>Additivity</td>
</tr>
<tr>
<td>SW982</td>
<td>Sarcoma</td>
<td>0.5</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
<td>0.81 (±0.07)</td>
<td>Additivity</td>
</tr>
<tr>
<td>VA-ES-BJ</td>
<td>Sarcoma</td>
<td>0.2</td>
<td>0.05</td>
<td>–</td>
<td>–</td>
<td>0.65 (±0.01)</td>
<td>Moderate synergy</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Sarcoma</td>
<td>5</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
<td>0.66 (±0.10)</td>
<td>Moderate synergy</td>
</tr>
<tr>
<td>U-2OS</td>
<td>Sarcoma</td>
<td>5</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
<td>0.63 (±0.09)</td>
<td>Moderate synergy</td>
</tr>
<tr>
<td>AN3-CA</td>
<td>Endometrial</td>
<td>0.5</td>
<td>–</td>
<td>75</td>
<td>3</td>
<td>1.07 (±0.01)</td>
<td>Additivity</td>
</tr>
<tr>
<td>HEC-1-B</td>
<td>Endometrial</td>
<td>1</td>
<td>–</td>
<td>100</td>
<td>16</td>
<td>0.86 (±0.04)</td>
<td>Additivity</td>
</tr>
<tr>
<td>RL95-2</td>
<td>Endometrial</td>
<td>1</td>
<td>–</td>
<td>37.5</td>
<td>9</td>
<td>0.72 (±0.04)</td>
<td>Moderate synergy</td>
</tr>
</tbody>
</table>

NOTE: An effective but submaximal dose was determined for each compound tested and defined as 1 ×. The drug concentrations used ranged from 0.125/C_{2} to 8/C_{6} at a fixed ratio. Strict criteria were applied to drug interaction analysis, where synergism was defined as CI < 0.75, additivity as 0.75 < CI < 1.25, and antagonism as CI > 1.25. Data expressed as mean CI (±SD), determined for a range of drug concentrations and a fractional effect (FA) of 0.2 to 0.8. Abbreviations: CI, combination index; FA, fractional effect.

panel, the absence of PTEN, or elevated levels of either phospho-AKT or total AKT, appeared to associate with sensitivity to ridaforolimus. Of the 6 lines examined, the 3 PTEN-null lines (SK-UT-1B, RL95-2, and AN3-CA). Catalog of Somatic Mutations in Cancer database; ref. 32) exhibited the greatest degree of drug sensitivity. This finding suggests that PTEN, or AKT, status is a potential molecular marker of ridaforolimus sensitivity in endometrial cancer that warrants further investigation.

Inherent G0–G1 cell phase proportion correlates with tumor cell sensitivity

We have shown previously that ridaforolimus treatment inhibits progression through the G1 phase of the cell cycle (2). To determine whether a relationship exists between ridaforolimus sensitivity and cell-cycle status, the cell phase distribution of 8 sarcoma and 4 endometrial cancer lines was assessed by flow cytometry, before and after ridaforolimus treatment (Fig. 4). The initial analysis compared the percentage of cells in the G0–G1 phase of the cycle following treatment with ridaforolimus for 24 hours. A highly significant correlation was found between the percentage of cells present in the G0–G1 phase of the cell cycle following treatment and the sensitivity of cells to ridaforolimus as calculated on the basis of the effect on cell proliferation (I_{max}). This is shown by the linear relationship between those parameters by linear regression analysis (Fig. 4A; R^{2} = 0.821, P = 0.000048). Given that ridaforolimus arrests cells in the G1 phase of the cell cycle, it is not surprising that cell lines whose proliferation is most affected by ridaforolimus have a higher percentage of cells in the G0–G1 phase at the end of treatment. But, interestingly, no correlation was found between the magnitude of change in cell phase distribution and I_{max} (Fig. 4B; R^{2} = 0.002, P = 0.8832). That is, the percentage increase in cells in the G0–G1 phase, relative to the percent in G0–G1 before treatment, was no greater in cell lines that were highly sensitive to ridaforolimus than in those that were less sensitive. However, when the basal cell phase distribution of the lines was examined, a clear linear relationship between G0–G1 distribution and sensitivity was observed (Fig. 4C; R^{2} = 0.733, P = 0.00037). Consistent with this observation, enrichment of S-phase at baseline was also significantly associated with ridaforolimus insensitivity (Fig. 4D; R^{2} = 0.4978, P = 0.01). These data strongly suggest that it is the inherent cell-cycle distribution of each line that serves as a primary determinant of sensitivity to ridaforolimus. In contrast to the results for PTEN and AKT expression described above, this marker of responsiveness was observed across both tumor types.

G1 phase cell-cycle markers correlate with ridaforolimus sensitivity in untreated sarcoma and endometrial lines

The association between ridaforolimus sensitivity and the proportion of cells in the G0–G1 phase of the cell cycle suggested that expression of cell-cycle–related proteins might serve as an indicator of drug responsiveness. To test this, the basal status of various cell-cycle markers was examined by immunoblot analysis (Fig. 5). The markers examined included 4 proteins expressed predominantly in the G1 phase of the cell cycle (p21, p27, cyclin D1, and CDK4), and 3 proteins expressed during either the G1–S or S–G2 phases of the cell cycle (cyclin E, CDK2, and cyclin A; ref. 33). Across both indications, increased sensitivity was
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linked to elevated levels of the G1 markers, p21 and p27. This association between G1 protein expression and ridaforolimus sensitivity was more evident in the endometrial panel, where the 2 most sensitive cell lines (SK-UT-1B and RL95-2) also showed elevated levels of CDK4 and cyclin D1. In contrast, there was no apparent relationship between sensitivity and expression of any non-G1 marker examined. These data are in concordance with the cell phase distribution findings obtained by flow cytometry (Fig. 4). The observation that levels of G1 cell-cycle regulatory proteins are associated with sensitivity to ridaforolimus in 2 different tumor types provides further evidence of a common cell-cycle–based mechanism that determines responsiveness.

Discussion

Ridaforolimus has shown encouraging clinical activity in sarcoma and endometrial cancer patients in early trials (10, 16, 19). Despite this, the activity of ridaforolimus has not been broadly examined in preclinical models of these diseases, and predictive biomarkers of sensitivity remain to be defined. Here, we show that single agent ridaforolimus inhibited the in vitro proliferation of all 11 sarcoma and all 6 endometrial cancer cell lines examined, though to varying degrees. Furthermore, ridaforolimus was shown to exhibit potent antitumor activity in vivo in representative tumor xenograft models when dosed on a 5 continuous days weekly schedule, the recommended clinical phase 2 dosing regimen (29). Importantly, pharmacodynamic analysis confirmed that ridaforolimus inhibited mTOR signaling in tumors as evidenced by a reduction in phosphorylation of the downstream effectors 4E-BP1 and ribosomal protein S6. Significantly, blockade of mTOR function was only observed at dose levels that inhibited tumor growth. Taken together, these findings are consistent with the observed antitumor activity of ridaforolimus in sarcoma and endometrial cancer patients.

One strategy to improve objective response rates in patients is through the use of combination regimens. The mTOR inhibitor rapamycin has been shown to potentiate the cytotoxic activity of multiple chemotherapeutic agents in a number of cancer cell types in vitro (25, 34, 35). Therefore, an important objective of this study was to determine whether ridaforolimus enhanced the efficacy of chemotherapeutics commonly used in sarcoma and endometrial cancer treatment. We evaluated the combinatorial antiproliferative activities of ridaforolimus with doxorubicin in sarcoma cell lines and with carboplatin/paclitaxel (Carbo-Tax) in endometrial cancer cell lines. As assessed by median-effects analysis, the antiproliferative activity of ridaforolimus against sarcoma and endometrial cancer cell lines was found to be generally additive or moderately synergistic with the respective chemotherapeutic agents. Depending on the cellular context, doxorubicin and Carbo-Tax both inhibit outside of the G1 phase. In light of the cell-cycle relationship we uncovered, this may account for why more potent synergistic effects were not observed. Similar combinatorial benefit has been reported for rapamycin with these same cytotoxic agents in breast, hepatocellular carcinoma, and head and neck cancer lines (34–36).

Despite the broad use and promise of mTOR inhibitors as anticancer agents and the extensive study of upstream regulators and downstream effectors of mTOR signaling, no markers have been identified that reliably predict sensitivity of cancer cells to mTOR inhibitors. This consideration prompted a comprehensive investigation of potential molecular correlates of ridaforolimus sensitivity using a panel of 11 sarcoma and 6 endometrial cancer cell lines. aberrant hyperactivation of the PTEN/AKT/mTOR pathway has been suggested to be predictive of sensitivity to mTOR inhibition in vitro. Of the 11 components of the PTEN/AKT/mTOR pathway analyzed, ridaforolimus activity was only associated with PTEN loss and high basal p-AKT levels in the endometrial cell lines examined but not in the sarcoma panel. Genotyping studies have identified PTEN as the most frequently mutated gene in endometrial carcinomas, with approximately...
50% of tumors harboring a double allelic mutated PTEN gene (37). Moreover, the combination of PTEN/p-AKT expression has been reported to be a prognostic indicator for patients with advanced endometrial cancer, as patients with PTEN-positive and p-AKT-negative expressing tumors exhibited a higher overall survival rate (38). It has been reported that PTEN loss may predict clinical benefit response in advanced endometrial cancer patients in a trial using everolimus (RAD001), another rapamycin analogue (39). Conversely, however, PTEN status appeared not to be predictive of response in a separate trial of this disease with the mTOR inhibitor temsirolimus (CCI-779; ref. 40). These conflicting observations suggest that further assessment of PTEN functional status (such as a clinically relevant marker for ridaforolimus sensitivity across both the endometrial and sarcoma cell line panels. It is known that the regulation of p27 levels is associated with the antiproliferative activity of rapamycin (41, 42): Murine myogenic cells selected for resistance to rapamycin exhibit an intact mTOR signaling pathway but greatly reduced p27 expression (41). Furthermore, it has been suggested that basal levels of p27 are a limiting factor in determining rapamycin sensitivity in T cells (42). In the case of p21, Huang and colleagues (43) have shown that loss of this protein decreased the ability of human rhabdomyosarcoma and mouse embryonic fibroblast cells to arrest in G1 following treatment. D, association between sensitivity and magnitude of G₀–G₁ enrichment following ridaforolimus treatment. C, association between sensitivity and percentage of basal G₀–G₁ population without ridaforolimus treatment. Figure 4. Relationship between ridaforolimus sensitivity and the G₀–G₁ cell population. The relationship between ridaforolimus sensitivity, as determined by Imax, and the percentage of the cell population present in the G₀–G₁ or S-phase of the cell cycle was assessed by linear regression analysis. Eight sarcoma (VA-ES-BJ, MG-63, SK-LMS-1, SJSA-1, Saos-2, HT-1080, SK-UT-1, and U-2OS) and 4 endometrial cancer lines (SK-UT-1B, AN3-CA, HEC-1-B, and HEC-1-A) were examined. R² and P values are shown. A, association between sensitivity and percentage of G₀–G₁ population posttreatment. B, association between sensitivity and magnitude of G₀–G₁ enrichment following ridaforolimus treatment.
rapamycin treatment. Overall, the relationship between G1 gene expression and mTOR inhibitor activity may reflect a general mechanism of sensitivity to mTOR inhibition and suggests that markers of G1 status may be valuable predictors of drug responsiveness across tumor types.

There are several potential clinical applications of these findings that are worthy of exploration. For example, expression of G1 markers in tumor biopsy material may serve as positive predictive markers of sensitivity to an mTOR inhibitor. These results also suggest the potential for a noninvasive approach to predict sensitivity via the use of positron emission tomography (PET) imaging. Specifically, uptake of the thymidine analogue [18F]fluorothymidine ([18F]FLT), a substrate of thymidine kinase-1 (TK1), can be used to evaluate the cell phase distribution of tumor cells in situ. TK1 expression is tightly regulated throughout the cell cycle, peaking during S-phase and becoming greatly reduced or nearly undetectable in G1 and nondividing cells (44, 45). Wei and colleagues (46) have recently shown that [18F]FLT uptake in xenografts is decreased by rapamycin, which is expected as rapamycin treatment leads to an increase in cells in the G1 phase. By extension, baseline levels of [18F]FLT in a tumor should provide a measure of the relative percentage of cells that are not in the G1 phase and may therefore serve as a marker of resistance to an mTOR inhibitor. Though not addressed by Wei and colleagues, the rapamycin-sensitive xenograft displayed an approximately 3-fold lower baseline level of [18F]FLT uptake in comparison to a resistant xenograft. Although only evaluated in 2 models, this result is consistent with the hypothesis that resistant tumors comprise a relatively higher proportion of cells in S-phase than sensitive tumors.

In summary, preclinical studies using sarcoma and endometrial cancer cell lines established the broad antiproliferative activity of rapamycin as a single agent in these tumor types in vitro and in vivo. In addition, the capacity for rapamycin to potentiate the cytotoxic effects of standard-of-care chemotherapeutics provides a molecular rationale for combining this targeted agent with established drug protocols. The identification of robust molecular markers of clinical responsiveness to mTOR inhibitors has proven to be a major challenge. Our findings support the utility of PTEN/p-AKT as a predictive marker of rapamycin sensitivity in endometrial cancer but not sarcoma. Most importantly, our results show that the relative percentage of cells in the G1 phase of the cycle, or the expression of G1 cell-cycle proteins, may be a more general predictor of sensitivity to rapamycin. Overall, these findings support the encouraging clinical results for rapamycin seen in sarcoma and endometrial cancer and suggest new molecular markers that may be predictive of response to mTOR inhibition, which warrant further investigation and validation in additional studies.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees of ARIAD Pharmaceuticals, Inc.

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References


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