Preclinical Characterization of OSI-027, a Potent and Selective Inhibitor of mTORC1 and mTORC2: Distinct from Rapamycin

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Abstract

The phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway is frequently activated in human cancers, and mTOR is a clinically validated target. mTOR forms two distinct multiprotein complexes, mTORC1 and mTORC2, which regulate cell growth, metabolism, proliferation, and survival. Rapamycin and its analogues partially inhibit mTOR through allosteric binding to mTORC1, but not mTORC2, and have shown clinical utility in certain cancers. Here, we report the preclinical characterization of OSI-027, a selective and potent dual inhibitor of mTORC1 and mTORC2 with biochemical IC50 values of 22 nmol/L and 65 nmol/L, respectively. OSI-027 shows more than 100-fold selectivity for mTOR relative to PI3Kα, PI3Kβ, PI3Kγ, and DNA-PK. OSI-027 inhibits phosphorylation of the mTORC1 substrates 4E-BP1 and S6K1 as well as the mTORC2 substrate AKT in diverse cancer models in vitro and in vivo. OSI-027 and OXA-01 (close analogue of OSI-027) potently inhibit proliferation of several rapamycin-sensitive and -insensitive nonengineered and engineered cancer cell lines and also, induce cell death in tumor cell lines with activated PI3K–AKT signaling. OSI-027 shows concentration-dependent pharmacodynamic effects on phosphorylation of 4E-BP1 and AKT in tumor tissue with resulting tumor growth inhibition. OSI-027 shows robust antitumor activity in several different human xenograft models representing various histologies. Furthermore, in COLO 205 and GEO colon cancer xenograft models, OSI-027 shows superior efficacy compared with rapamycin. Our results further support the important role of mTOR as a driver of tumor growth and establish OSI-027 as a potent anticancer agent. OSI-027 is currently in phase I clinical trials in cancer patients. Mol Cancer Ther; 10(8); 1394–406. ©2011 AACR.

Introduction

mTOR is a serine/threonine protein kinase related to the phosphoinositide 3-kinase (PI3K) family (1). The mTOR signaling pathway integrates both extracellular and intracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, and survival (2–6). Many cancer-driving mutations in genes encoding receptor tyrosine kinases, Ras, PI3K, and PTEN stimulate cell proliferation, growth, and survival through activation of mTOR kinase signaling. mTOR resides in 2 distinct multiprotein complexes called mTORC1 and mTORC2 (3). mTORC1 directly phosphorylates ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor eIF4E-binding protein 1 (4E-BP1) and AKT in tumor tissue with resulting tumor growth inhibition. OSI-027 shows robust antitumor activity in several different human xenograft models representing various histologies. Furthermore, in COLO 205 and GEO colon cancer xenograft models, OSI-027 shows superior efficacy compared with rapamycin. Our results further support the important role of mTOR as a driver of tumor growth and establish OSI-027 as a potent anticancer agent. OSI-027 is currently in phase I clinical trials in cancer patients. Mol Cancer Ther; 10(8); 1394–406. ©2011 AACR.
agents in a limited number of tumor types (e.g., renal cell carcinoma and mantle cell lymphoma), the mode of action of these drugs does not completely exploit the antitumor potential of fully inhibiting mTOR signaling (17). The emerging role of mTORC2 in the activation of AKT in cancer is now considered important to tumor maintenance and progression, but this complex is generally resistant to rapalogs. In addition, inhibition of mTORC1 by rapamycin and its analogues has been shown to result in hyperactivation of AKT through the release of negative feedback loop between S6K1 and IRS-1 (18). Indeed, Cloughesy and colleagues have recently shown that hyperactivation of AKT following rapamycin treatment was associated with shorter time to progression in PTEN-deficient glioblastoma patients, suggesting that TORC1-specific inhibition and associated AKT activation limits anticancer activity (19). Consequently, intense efforts are now underway to develop inhibitors of the PI3K/AKT/mTOR pathway, including ATP-competitive small molecule mTOR kinase inhibitors targeting both mTORC1 and mTORC2 (20).

Several recent reports have described biochemical and cellular properties of selective ATP-competitive inhibitors of mTORC1 and mTORC2 (21–26). These chemically distinct compounds all show suppression of both mTORC1- and mTORC2-mediated downstream signaling in various tumor cell lines. These inhibitors have been shown to induce a stronger suppression of protein synthesis and cell growth than rapamycin. More recently, antitumor activity and tumor pharmacodynamic effects were reported with the selective mTORC1 and mTORC2 inhibitors, AZD8055 and WYE-132. In this article, we describe the preclinical characterization of OSI-027, an orally bioavailable, potent, and specific dual inhibitor of mTORC1 and mTORC2. We show broad-spectrum antitumor activity in several xenograft models, in vivo differentiation from rapamycin, PK/PD/efficacy correlations in P13K-wt versus mutant models and tumor growth inhibition (TGI) by intermittent and once weekly dosing schedules. OSI-027 is currently in phase I clinical development in cancer patients.

Materials and Methods

Synthesis of OSI-027 and OXA-01

OSI-027 is a 4,5,7-trisubstituted imidazo[5,1-f]triazine and OXA-01 is a 1,3,8-trisubstituted imidazo[1,5-alpyrazine. These were synthesized in patent application US-20070112005 (27). Compound identity and purity (>99%) were verified by ‘H and 13C nuclear magnetic resonance, mass spectrometry, and high-performance liquid chromatography by using Bruker Avance 400, Waters Micromass ZQ, and Waters LC Module I Plus instruments, respectively, as well as by elemental analysis. OSI-027 was dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L for use in biochemical or cellular in vitro assays. For in vitro studies, OSI-027 was dissolved in 20% Trappsol at an appropriate concentration to deliver the described dose in 10 mL/kg by oral gavage.

Cell lines

Cancer cell lines were obtained from the American Type Culture Collection or other sources, as indicated in Supplementary Materials and Methods, banked after receipt, and passaged for less than 6 months before use in experiments. All cell lines were cultured as directed. Rh1, Rh1mTORrr, Rh30, Rh30/mTORrr, and Rh30/Rapa10K cells were grown in culture medium previously described (28).

Western blot analysis

Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail, 1 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride (all reagents purchased from Sigma). Lysates were cleared by centrifugation and 20 μg protein was loaded per well. Lysates were fractionated on 4% to 12% tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes by using a semidry apparatus. Membranes were blocked with 5% nonfat dry milk in TBS and incubated overnight with primary antibody in 3% bovine serum albumin. The following primary antibodies from Cell Signaling Technology were used at 1:1,000 dilution: phospho-4E-BP1[T37/46], phospho-4E-BP1[S65], 4E-BP1, phospho- AKT[S473], phospho-AKT[T308], AKT, phospho-PRAS40 (T246), PRAS40, phospho-S6K1[T389], S6K1, phospho-S6 [S235/236], S6, cyclin D1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). β-Actin antibody was purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Labs or GE Healthcare. Horseradish peroxidase-conjugated secondary antibodies were incubated in nonfat dry milk for 1 hour. SuperSignal chemiluminescent reagent (Pierce Biotechnology) was used according to the directions and blots were imaged by using the Alpha Innotech image analyzer.

Animals

Female nu/nu CD-1 mice (6–8 weeks, 22–25 g) were purchased from Charles River Laboratories and allowed to acclimate for a minimum of 1 week before initiation of a study. Throughout the studies, animals were allowed sterile rodent chow and water ad libitum. All in vivo studies were conducted at OSI facilities with the approval of the Institutional Animal Care and Use Committee in an American Association for Accreditation of Laboratory Animal Care accredited vivarium and in accordance with guidelines from the Institute of Laboratory Animal Research.

Cell proliferation assays, apoptosis assays, and cell-cycle analysis

For assays of cell proliferation, cells were seeded into 96-well plates and incubated for 3 days in the presence of OSI-027 or OXA-01 at various concentrations. Inhibition
of cell growth was determined by luminescent quantification of intracellular ATP content by using CellTiterGlo (Promega). Proliferation on day 0 versus 72 hours was used to plot dose–response curves for IC50 calculations and to determine cell death. For apoptosis induction (caspase-3/7) assay, cells were seeded into 96-well plates and incubated for 48 hours in the presence of OSI-027 at various concentrations. Induction of caspase-3/7 activity was determined by luminescent quantification by using CaspaseGlo reagent (Promega). Fold induction of apoptosis was calculated as compared with DMSO-treated controls. For cell-cycle analysis, cells were treated with DMSO, rapamycin, or OSI-027 for 48 hours and stained with propidium iodide for analysis by flow cytometry (BD FACS Caliber). Percent of total cells in subG1, G0–G1, S, and G2–M were calculated by using fluorescence-activated cell sorting (FACS) analysis software.

**Pharmacokinetic analysis**

Blood from mice dosed with OSI-027 was collected in EDTA tubes and separated plasma was extracted by protein precipitation with methanol followed by centrifugation (10,000 × g for 10 minutes at 4°C). Extracted plasma samples were analyzed by high-performance liquid chromatography–tandem mass spectrometry. All pharmacokinetic parameters were obtained by noncompartmental analysis.

**In vivo pharmacodynamic analysis**

To assess the ability of OSI-027 to inhibit phosphorylation of 4E-BP1 (T37/T46) or AKT (S473) in tumor tissue, female nu/nu CD-1 mice were implanted in the mammary fat pad with MDA-MB-231 tumor cells harvested from cell culture flasks. Animals with established tumors of 300 ± 50 mm3 size were dosed orally with OSI-027 dissolved in 20% Trappsol (CTD Inc.). Tumor samples were collected at specified time points and snap frozen in liquid nitrogen. Tumor lysates were prepared by homogenizing samples in a Precellys instrument (BD FACS Caliber). Percent of total cells in subG1, G0–G1, S, and G2–M were calculated by using fluorescence-activated cell sorting (FACS) analysis software.

**In vivo antitumor efficacy studies**

Cells were harvested and implanted s.c. in the right flank of nu/nu CD-1 mice as described previously (29). Tumors were allowed to establish to 200 ± 50 mm3 in size before randomization into various treatment groups with 8 mice per group. OSI-027 was administered orally dissolved in 20% Trappol or rapamycin administered intraperitoneally (i.p.) in an aqueous solution of 4% ethanol, 5% Tween 80, and 5% PEG400 at indicated doses. Rapamycin was purchased from LC Laboratories. Tumor volumes were determined from caliper measurements by using the following formula: %TGI = [1 – (Vt/Ct0)/(Ct0/Ct)]/1 – [Ct0/Ct] × 100, in which Vt = tumor volume of treated animal × at time t, T0 = tumor volume of treated animal × at time 0, Ct = median tumor volume of control group at time t, and C0 = median tumor volume of control group at time 0. Median% TGI was calculated and reported for the entire dosing period for each group. Significant antitumor activity is defined as achievement of a median% TGI of at least 50%. Regressions in tumor volume were calculated as: % regression = 100 × (V0 – Vt)/Vt0, in which V0 = mean tumor volume of treated group at time 0, and Vt = mean tumor volume of that group at time t. Rank ANOVA with Dunnett’s comparison was used to compare treatment groups with the control group. All comparisons were deemed statistically significant if P ≤ 0.05.

**Results**

**OSI-027 is a selective inhibitor of mTORC1 and mTORC2**

To establish the potency and selectivity of OSI-027 against mTOR and related kinases, native protein complexes were immunoprecipitated from HeLa cell lysates and assayed for inhibition of mTOR by OSI-027. The IC50 value for OSI-027 was 4 nmol/L (Supplementary Table S1). None of those kinases were inhibited by OSI-027. OSI-027 is a selective inhibitor of mTORC1 and mTORC2.

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OSI-027 potently inhibits mTORC1 and mTORC2 activity in cells

Structure of OSI-027, OXA-01, and rapamycin is shown in Fig. 1A. Inhibition of mTORC1 and mTORC2 substrate phosphorylation and downstream signaling by OSI-027 in BT-474 (PIK3CA-mut), IGR-OV1 (PTEN-null), and MDA-MB-231 (PIK3CA-wt) cell lines were determined by immunoblot analysis. OSI-027 inhibited phosphorylation of AKT at S473 and T308 in these 3 cell lines in a concentration-dependent manner in BT-474 and IGR-OV1 cells under normal culture conditions, but it was detected after IGF-1 stimulation (see Supplementary Fig. S2). The IC_{50} of OSI-027 in BT-474 cells was determined with a pAKT S473 sandwich ELISA and was found to be 0.3 \mu mol/L (Supplementary Fig. S3). In addition, OSI-027 inhibited phosphorylation of mTORC2-mediated PRAS40 in these 3 cell lines in a dose-dependent manner. In BT-474, IGR-OV1, and MDA-MB-231 cells, OSI-027 inhibited phosphorylation of the mTORC1 substrate S6K1 at T389 in a concentration-dependent manner with potency similar to pAKT [S473] inhibition, suggesting equipotent cellular inhibition of both mTORC1 and mTORC2 by OSI-027 (Fig. 1B). Similarly, phosphorylation of S6 at S235/236 downstream of S6K1 was inhibited by OSI-027 in these 3 cell lines. Moreover, OSI-027 inhibited phosphorylation of the mTORC1 and mTORC2 substrate 4E-BP1 at T37/T46 and S65 in a concentration-dependent manner in these 3 cell lines (Fig. 1B) with IC_{50} of 0.4 \mu mol/L in BT-474 cells (Supplementary Fig. S3). In addition, treatment with OSI-027 inhibited cyclin D1 expression (translation known to be regulated in a cap-dependent manner) in a concentration-dependent manner in IGR-OV1, BT-474, and MDA-MB-231 cells (Fig. 1B) and shows reasonable correlation with...
p4E-BP1 (both T37/36 and S65) inhibition. These data suggest that OSI-027 inhibits both mTORC1 and mTORC2 potently in cancer cell lines with diverse genetic backgrounds.

**OSI-027 inhibits 4E-BP1 and AKT phosphorylation and prevents feedback to AKT in majority of cancer cell lines**

To survey the ability of OSI-027 to inhibit mTORC1- and mTORC2-mediated downstream signaling, we used Western blot analysis to assess the inhibition of p4E-BP1 (T37/46) and AKT (S473) phosphorylation by OSI-027 (20 μmol/L) and rapamycin (20 μmol/L) after 24 hours of treatment in 12 or 24 cancer cell lines representing various tumor types, respectively. These concentrations were chosen on the basis of the plasma exposure of OSI-027 at efficacious doses in preclinical models. OSI-027 significantly inhibited p4E-BP1 (T37/46) phosphorylation in the majority of cell lines tested and representative data are shown in Fig. 1C. In contrast, rapamycin failed to significantly inhibit p4E-BP1 in the majority of cell lines even at 20 μmol/L, which is more than 200-fold higher than clinically relevant plasma concentrations. Moreover, OSI-027 strongly inhibited pAKT (S473) in 83% of the 12 cell lines tested, whereas rapamycin inhibited pAKT (S473) in only 13% of the 24 cell lines tested (Fig. 1C and Table 1). More importantly, rapamycin stimulated pAKT (S473) in 62% of total 24 cell lines (Fig. 1C and Table 1) or in 58% of same 12 cancer cell lines tested for OSI-027. These data suggest that OSI-027 can effectively inhibit pAKT and prevent feedback activation of AKT in the majority of tumor cell lines tested, which stands in clear contrast to the observed effects of rapamycin. Furthermore, OSI-027 inhibited IGF-1–induced pAKT (S473), whereas rapamycin was ineffective (Supplementary Fig. S4). We also determined whether OSI-027 can inhibit pAKT (S473) induced by rapamycin treatment in the NCI-H23 NSCLC cell line and, indeed, OSI-027 fully inhibited pAKT (S473) induced by rapamycin treatment (Fig. 1D), suggesting the likely role of mTORC2 activation in the stimulation of pAKT by rapamycin. Overall, these data suggest that OSI-027 can inhibit both mTORC1 and

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**Figure 1.** (Continued) C, U-87 MG, RL95-2, NCI-H2122, C-33A, and IGR-OV1 cells were treated with OSI-027 (20 μmol/L) or rapamycin (20 μmol/L) for 24 hours and total lysates were analyzed by Western blotting for p4E-BP1 (T37/46), 4E-BP1, pAKT (S473), AKT, and GAPDH. D, NCI-H23 NSCLC cells treated with DMSO, rapamycin (20 μmol/L), OSI-027 (20 μmol/L), and rapamycin + OSI-027 (20 μmol/L each) for 24 hours and total lysates were analyzed by Western blotting for pAKT (S473).
mTORC2 activity in the majority of tumor cell lines, whereas rapamycin only inhibits mTORC1 and generally stimulates or has no effect on mTORC2-mediated signaling.

**OSI-027 and OXA-01 inhibits cell proliferation and induces cell death: superiority versus rapamycin**

mTOR is important in the control of cell proliferation. On the basis of the potency of rapamycin in the inhibition of proliferation, we defined cell lines as either rapamycin sensitive (IC_{50} < 0.075 μmol/L) or rapamycin insensitive (<30% inhibition at 20 μmol/L). We then examined the antiproliferative potency of OSI-027 among these rapamycin-sensitive and -insensitive subgroups. Inhibition of cell proliferation was determined by quantifying cellular ATP levels after 72 hours of exposure to increasing concentrations of OSI-027. Cell proliferation on day 0 was used as a background to calculate IC_{50} for inhibition of cell proliferation and to determine whether additional cell death occurred. OSI-027 potently inhibited cell proliferation in rapamycin-insensitive cell lines, with IC_{50} values ranging from 0.4 to 4.5 μmol/L (Fig. 2A, Supplementary Table S2). Moreover, OSI-027 induced 10% to 50% cell death in majority of rapamycin-sensitive cell lines (Fig. 2B) but not rapamycin (Supplementary Fig. S5). Taken together, these data suggest that OSI-027 is superior to rapamycin in inhibiting cell proliferation and inducing cell death in a large panel of tumor cell lines. We further tested sensitivity to rapamycin and the mTORC1/ mTORC2 inhibitor OXA-01 (a close analogue of OSI-027) in parental Rh1 and an Rh1/mTORrr cell line in which S2035I mutation was introduced in FRB domain of mTOR to prevent rapamycin-FKBP12 binding. Rh1 cells are highly sensitive to rapamycin, whereas Rh1/mTORrr cells show a decrease in rapamycin potency of at least 3 orders of magnitude (28). In contrast to rapamycin, both Rh1 and Rh1/mTORrr cells show similar sensitivity to the mTOR kinase inhibitor OXA-01, consistent with the direct action of this inhibitor on the mTOR catalytic protein rather than the FRB domain (Fig. 2C). Similarly, OXA-01 inhibited proliferation of Rh30 (rapamycin-sensitive parental), Rh30/mTORrr (FRB domain mutant), and Rh30/Rapa10K (selected *in vitro* for resistance to 10 μmol/L rapamycin) cells with similar IC_{50} values, whereas rapamycin showed a significant potency shift of more than 10,000-fold (Fig. 2C and Table 2). These data suggest that ATP-competitive mTORC1/2 inhibitors, such as OXA-01 maintain potency in rapamycin-insensitive cell lines and inhibit mTOR by a different mechanism as compared with rapamycin. Furthermore, these data predict that some patients with rapamycin-refractory cancers might still respond to mTOR kinase inhibitors such as OXA-01 or OSI-027.

**OSI-027, but not rapamycin, strongly induces apoptosis in a PTEN-null cell line**

To study proapoptotic effects more directly, we next investigated whether OSI-027 and rapamycin can induce caspase-3/7 activation in multiple cancer cell lines with or without PIK3CA or PTEN mutation. OSI-027 induced caspase-3/7 activity by more than 2-fold in 3 of 22 cell lines, whereas rapamycin failed to induce caspase-3/7 activity in all cell lines tested (Supplementary Table S3). Caspase-3/7 induction was concentration dependent in IGR-OV1 and BT-474 cells (Fig. 3A). Furthermore, we quantified sub-G1 cells following treatment with OSI-027 (20 μmol/L), rapamycin (20 μmol/L), or DMSO (control; Fig. 3B). OSI-027, but not rapamycin, induced a significant sub-G1 fraction (40%) indicating apoptotic cell death (Fig. 3B).

**Pharmacodynamic effects of OSI-027 on p4E-BP1 and pAKT in MDA-MB-231 xenografts**

Pharmacokinetic analysis following oral administration of OSI-027 to mice revealed that plasma levels of OSI-027 (C_{max} and AUC) increased approximately linearly between 20 and 200 mg/kg doses with oral bioavailability of 65% to 95% (Supplementary Fig. S6).

Pharmacodynamic studies were carried out in the MDA-MB-231 breast cancer xenograft model to evaluate effects on phosphorylation of the mTORC1/mTORC2 substrate 4E-BP1 and the mTORC2 substrate AKT in relation to plasma drug concentrations after a single oral dose of 25 or 65 mg/kg of OSI-027. A single oral dose of 65 mg/kg resulted in considerable inhibition (>75%) of 4E-BP1 phosphorylation that was evident for at least 16 hours (Fig. 4A, Supplementary Fig. S7). At 24 hours postdose in the 65 mg/kg dose group, 47% inhibition of p4E-BP1 was still maintained with a corresponding plasma OSI-027 concentration of 2.2 μmol/L. Such extended target suppression was associated with significant efficacy corresponding to 100% median TGI in the MDA-MB-231 xenograft model (Fig. 4C). In comparison, once daily administration of OSI-027 at 25 mg/kg for 14 days resulted in only moderate antitumor activity corresponding to 64% median inhibition of MDA-MB-231 tumor growth (TGI) compared with control vehicle.

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**Table 1. Summary table showing effect of OSI-027 (20 μmol/L) and rapamycin (20 μmol/L) on pAKT (S473) in 12 and 24 cancer cell lines, respectively**

| Percentage of cell lines showing pAKT (S473) |
|-----------------|-----------------|-----------------|-----------------|
|                  | Stimulation     | No change       | Inhibition      |
| Rapamycin       | 15/24 (62)      | 6/24 (25)       | 3/24 (13)       |
| (20 μmol/L, 24 h) |                 |                 |                 |
| OSI-027         | 0/12 (0)        | 2/12 (17)       | 10/12 (83)      |
| (20 μmol/L, 24 h) |                 |                 |                 |
treated animals for 14 days (Fig. 4C), whereas administration of OSI-027 on a twice daily schedule of 25 mg/kg for 14 days resulted in robust 90% median TGI (data not shown). Pharmacodynamic analysis showed that although the lower dose of 25 mg/kg showed significant inhibition (>79%) of 4E-BP1 phosphorylation, it only lasted for about 8 hours with inhibition of p4E-BP1 completely reversed by 16 hours postsingle dose of OSI-027 at 25 mg/kg, corresponding to plasma concentrations of less than 0.13 μmol/L (Supplementary Fig. S8). These data suggest that sustained inhibition of tumor p4E-BP1 by more than 50% seems to be necessary for substantial TGI in this model.

Tumor samples from the same animals were also used to quantify the in vivo effects of OSI-027 on phosphorylation of the mTORC2 substrate AKT (S473). After a single 25 mg/kg dose, greater than 60% inhibition of tumor pAKT was observed for up to 4 hours with a rebound of pAKT content as drug plasma levels decreased (Supplementary Fig. S8). A single dose of 65 mg/kg resulted in marked inhibition (>50%) of pAKT for up to 16 hours with a rebound of pAKT content at 24 hours (Fig. 4B).
In vivo antitumor activity of OSI-027 and rapamycin in colorectal tumors

The effects of OSI-027 were initially evaluated in the human colorectal xenograft models GEO and COLO 205, selected in part due to insensitivity to rapamycin in vitro (data not shown). In the COLO 205 tumor model, oral OSI-027 treatment at 65 mg/kg once daily for 12 days resulted in 100% median TGI with 37% regression, whereas rapamycin treatment (20 mg/kg i.p., d1-5, d8-12) resulted in 79% median TGI (Fig. 4D). Statistical analysis showed significant difference between the efficacy of rapamycin and OSI-027 treatments ($P < 0.001$). At the end of dosing, tumor samples were removed at 8 and 24 hours and analyzed for phospho-S6, phospho-4E-BP1, and phospho-AKT. OSI-027 treatment resulted in significant inhibition of both mTORC1 and mTORC2 effectors at 8 hours with recovery by 24 hours (Fig. 4E and Supplementary Fig. S9). The corresponding median plasma concentrations at 8 and 24 hours were 22 and 0.77 μmol/L, respectively.

On the other hand, rapamycin treatment showed sustained inhibition of only the mTORC1 effector pS6, but had little to no effect on mTORC2 signaling, as assessed by tumor p4E-BP1 (T37/46) and pAKT (Ser473; Fig. 4E and Supplementary Fig. S9). These data show that an mTORC1/mTORC2-specific inhibitor such as OSI-027 can achieve superior inhibitory effects on pAKT and p4E-BP1 in vivo compared with an mTORC1-selective inhibitor such as rapamycin, and this inhibition of tumor pAKT and p4E-BP1 correlated with improved antitumor activity.

Similarly, improved tumor growth inhibitory activity of OSI-027 was observed in the GEO xenograft model as

Table 2. Summary table showing differentiation of OXA-01 from rapamycin in Rh1, Rh1/mTORrr, Rh30, Rh30/mTORrr, and Rh30/Rapa10K cell lines

<table>
<thead>
<tr>
<th>Proliferation IC50, μmol/L</th>
<th>Rapamycin</th>
<th>OXA-01</th>
</tr>
</thead>
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<tr>
<td>Rh1</td>
<td>0.0005</td>
<td>0.5</td>
</tr>
<tr>
<td>Rh1/mTORrr</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Rh30</td>
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<td>0.4</td>
</tr>
<tr>
<td>Rh30/mTORrr</td>
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<td>0.4</td>
</tr>
<tr>
<td>Rh30/Rapa10K</td>
<td>&gt;10</td>
<td>1.0</td>
</tr>
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</table>

Figure 3. Proapoptotic effect of OSI-027, OXA-01, and rapamycin. A, induction of caspase-3/7 was measured in BT-474 and IGR-OV1 cancer cell lines by treatment with drugs for 48 hours by using CaspaseGlo reagent. Data are from 2 separate experiments and presented as mean ± SD. B, IGR-OV1 cells treated with OSI-027 (20 μmol/L), rapamycin (20 μmol/L), and DMSO for 48 hours and stained with propidium iodide to determine cell death (sub-G$_1$ fraction) by FACS analysis.
compared with the efficacy of rapamycin (Fig. 4F). Oral OSI-027 administration at 65 mg/kg once daily for 14 days resulted in 95% median TGI, whereas rapamycin treatment (20 mg/kg i.p., d1-5, d8-12) resulted in 75% median TGI (Fig. 4F). Statistical analysis showed significant difference between the rapamycin and OSI-027 treatment groups ($P < 0.05$).

**Evaluation of in vivo efficacy of OSI-027 in mTOR pathway–dependent xenograft models**

The antitumor efficacy of OSI-027 was evaluated in PIK3CA mutant SKOV-3 and KRAS mutant OVCA-5 human ovarian cancer xenograft models. In the SKOV-3 model, OSI-027 at 50 mg/kg once daily for 14 days resulted in significant TGI (100% median TGI) with 15% regression.
Figure 5. OSI-027 inhibits tumor growth in mTOR-activated tumor models. A, efficacy of OSI-027 in PIK3CA mutant SKOV-3 ovarian xenograft model. SKOV-3 tumors were treated with OSI-027 at 50 mg/kg once daily and 25 mg/kg twice daily for 14 days by oral gavage. Alternatively, steady-state concentrations of OSI-027 were delivered by using 14-day mini pumps implanted s.c. in tumor-bearing animals. Steady-state concentrations of 0.5 and 2 μmol/L were achieved by using a dose of 0.12 mg/kg/h and 0.49 mg/kg/h, respectively. B, actual plasma concentrations achieved by using mini-pumps on days 5 and 13 in tumor-bearing animals (n = 4). C, efficacy of OSI-027 in KRAS mutant OVCAR-5 ovarian xenograft model. OVCAR-5 tumors were treated with OSI-027 at 50 mg/kg once daily and 25 mg/kg twice daily for 14 days by oral gavage and at 6 μmol/L steady-state concentrations by using mini-pumps. D, pharmacodynamic effects of OSI-027 delivered by using mini-pumps. Phosphorylation of 4E-BP1 (T37/46) was evaluated in satellite OVCAR-5 tumor-bearing animals at various steady-state concentrations on day 11. E, efficacy of intermittent dosing of OSI-027 in PTEN-null IGR-OV1 ovarian xenograft model. IGR-OV1 tumors were treated with OSI-027 at 50 mg/kg once daily for 14 days, 150 mg/kg once every 3 days (Q3D x 5) doses, or 300 mg/kg once every 7 days (Q7D x 2). F, Ki67 nuclear staining determined by immunohistochemistry in SKOV-3 xenografts collected 8 hours after 3 daily doses of OSI-027 at 65 mg/kg. BID, twice daily; QD, once daily; Q3D, once every 3 days; Q7D, once every 7 days.
In addition, twice daily dosing at 25 mg/kg twice daily corresponding to a total daily dose of 50 mg/kg resulted in comparable TGI (100% median TGI with 12% regression; Fig. 5A). In contrast, treatment of OVCAR-5 (KRAS mutant) tumors with 50 mg/kg once daily or 25 mg/kg twice daily was only marginally efficacious showing 47% and 51% median TGI, respectively (Fig. 5C). Furthermore, to better understand the relationship between TGI and drug pharmacokinetics, continuous dosing of OSI-027 delivered via 14-day osmotic pumps was evaluated. In the OSI-027-sensitive tumor model, SKOV-3 maintenance of a steady-state concentration of approximately 0.5 μmol/L for 14 days was sufficient to achieve maximal efficacy equivalent to 50 mg/kg once daily dosing (100% median TGI with 20% regression; Fig. 5A). Figure 5B shows the actual plasma drug concentration on days 5 and 13 for estimated 0.5 and 2 μmol/L levels delivered via osmotic pump. Interestingly, in the OVCAR-5 model, which is less sensitive to OSI-027, a higher steady-state plasma concentration of 6 μmol/L was necessary to achieve efficacy equivalent to 50 mg/kg once daily dosing (Fig. 5C). Steady-state concentrations of 0.5 and 2 μmol/L were inactive in this model (data not shown). Pharmacodynamic evaluation in additional OVCAR-5 tumor-bearing mice on day 11 of treatment showed that tumor phospho-4E-BP1 inhibition in tumor samples was achieved only at 6 μmol/L steady state (Fig. 5D), and this pharmacodynamic inhibition correlated with efficacy (Fig. 5C).

We further evaluated the effects of intermittent dosing of OSI-027 in the PTEN null IGR-OV1 ovarian carcinoma xenograft model. OSI-027 administration at 50 mg/kg once daily for 14 days resulted in 100% TGI (Fig. 5E). OSI-027 administration at 150 mg/kg once every 3 days × 5 doses, corresponding to the same cumulative dose of 50 mg/kg per day, resulted in equivalent TGI (100% median TGI with 17% regression). Similarly, a high intermittent dose of 300 mg/kg administered once weekly resulted in comparable efficacy (100% TGI with 45% regression). Similar results were observed with the SKOV-3 model (data not shown). Pharmacokinetic analysis at the 300 mg/kg dose showed that more than 20 μmol/L OSI-027 plasma concentrations were maintained for at least 96 hours, with a decline to 0.58 μmol/L by 120 hours.

Finally, we determined the level of proliferation in SKOV-3 tumors by staining tumor samples for Ki67. OSI-027 was administered at 65 mg/kg once daily for 3 days and tumor samples were collected 8 hours post last dose. OSI-027 treatment resulted in 51.8% ± 4.2% inhibition of Ki67 staining compared with vehicle-treated control tumors (Fig. 5F), confirming the antiproliferative effects of OSI-027.

**Table 3. Broad-spectrum antitumor activity of OSI-027**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>PTEN/PIK3CA/KRAS/BRAF mutation status</th>
<th>OSI-027 dose, daily</th>
<th>Tumor growth inhibition (%)</th>
<th>Maximum regression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>PIK3CA mt</td>
<td>65 mg/kg</td>
<td>87</td>
<td>9</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Breast</td>
<td>PTEN null</td>
<td>50 mg/kg</td>
<td>99</td>
<td>26</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colon</td>
<td>PIK3CA mt, KRAS mt</td>
<td>65 mg/kg</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>NCI-H292</td>
<td>Lung</td>
<td>wt</td>
<td>50 mg/kg</td>
<td>61</td>
<td>8</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Lung</td>
<td>PIK3CA mt, KRAS mt</td>
<td>50 mg/kg</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>DU145</td>
<td>Prostate</td>
<td>wt</td>
<td>65 mg/kg</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>RL</td>
<td>Lymphoma</td>
<td>wt</td>
<td>65 mg/kg</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>FADU</td>
<td>HNSCC</td>
<td>wt</td>
<td>50 mg/kg</td>
<td>86</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: OSI-027 was administered orally once daily at indicated dose for 14 days. The percentages of TGI and regression were calculated as described in Materials and Methods. n = 8 animals per group in all studies.

Abbreviation: HNSCC, head and neck squamous cell carcinomas.

Discussion

Clinical studies with rapamycin and rapalogs have validated mTOR as an anticancer target. However, the effectiveness of rapalogs may be limited by lack of inhibition of mTORC2 signaling functions and activation of AKT via release of the S6K–IRS-1 negative feedback loop. These considerations led to intensive efforts to develop mTOR-selective kinase inhibitors that completely suppress both mTORC1 and mTORC2 activities in cancer cells. In this article, we provide biochemical, cellular, and in vivo evidence that OSI-027 is a potent and selective dual inhibitor of mTORC1 and mTORC2 and is mechanistically and functionally distinct from rapamycin.
As predicted, OSI-027 potently inhibited phosphorylation of AKT on Ser 473 in the majority of cell lines tested. Surprisingly, phosphorylation of AKT on Thr 308, a PDK1 phosphorylation site was also inhibited by OSI-027 in several tumor cell lines that we have investigated and similar inhibition of PAKT (T308) has been reported with mTORC1/mTORC2-selective inhibitors such as AZD-8055, Ku-0063794, PP242, and INK128 (15, 21–22, 31). But our observation differs from results with the selective mTORC1/mTORC2 inhibitor WYE-132 or genetic ablation of mTORC2, which did not inhibit AKT phosphorylation on T308. It is unclear whether this distinction is associated with differences in selectivity profile or differences in the cell lines used. More detailed comparisons of these mTORC1/mTORC2-selective inhibitors are clearly needed to understand underlying difference in their mechanisms of action.

OSI-027 profoundly inhibits proliferation of both rapamycin-sensitive and -insensitive cancer cell lines with IC50 values in low micromolar range. Inhibition of 4E-BP1 (T37/46) phosphorylation by OSI-027 correlates with inhibition of cell proliferation in rapamycin-insensitive MDA-MB-231 and rapamycin-sensitive BT-474 breast cancer cell lines (data not shown). The molecular mechanism for rapamycin sensitivity and insensitivity in these cell lines is not yet well understood and needs further investigation. Treatment with OSI-027, but not rapamycin, results in profound inhibition of 4E-BP1 (T37/46) phosphorylation, and it seems to translate into robust antiproliferative effects in the majority of cell lines tested. OSI-027 and other selective mTORC1/2 inhibitors have been shown to inhibit rapamycin-insensitive functions of mTORC1 (15, 21–26). Surprisingly, OSI-027 induced substantial cell death in several cell lines with PI3K/AKT pathway activation. We hypothesized that this cell death is likely due to induction of autophagy and/or apoptosis. The inhibition of phosphorylation of 4E-BP1 at T37/46 has been associated with a greater inhibition of cap-dependent translation and cellular proliferation with concomitant induction of autophagy (7). In most of the cell lines studied in vitro, rapamycin and rapalogs induce a partial growth inhibition and limited autophagy (32). mTORC1 has been shown to control autophagy by direct and indirect mechanisms (32, 33). Recently, OSI-027, but not rapamycin, has been shown to profoundly stimulate autophagy in K562 leukemic cells and RCC cell lines (34, 35). Alternatively, we have measured caspase-3/7 activation upon OSI-027 or rapamycin treatment to determine whether cell death is mediated by apoptosis. Rapamycin failed to induce caspase-3/7 in the majority of cell lines, whereas OSI-027 induced apoptosis in some cell lines with PI3K/AKT pathway activation. Indeed, quantitation of the sub-G1 fraction by flow cytometry indicates that OSI-027 induced apoptotic cell death in IGR-OV1 cells. However, the observation of caspase-3/7 induction in PTEN-deficient IGR-OV1 and U-87 MG, but not PTEN-deficient MDA-MB-468, indicates that there is not a simple correlation between PTEN status and apoptosis induction. These collective results suggest that OSI-027 can cause cell death by inducing both autophagy and apoptosis in a cell type-specific manner.

OSI-027 was well tolerated in vivo and induced dose-dependent growth inhibition and/or regression in multiple tumor xenografts models. Efficacy was associated with a dose-dependent pharmacodynamic inhibition of both p4E-BP1 and pAKT in tumors. These studies highlight the critical importance of mTOR as a driver of tumor proliferation in vivo. OSI-027 was effective against tumors from diverse genetic backgrounds showing significant TGI and regression in tumors harboring PTEN deficiency (IGR-OV1, MDA-MB-468), mutant PIK3CA (MCF-7, SKOV-3), and mutant KRAS (MDA-MB-231, H460). Steady-state modeling of OSI-027 drug concentration by using osmotic pumps in PIK3CA mutant (SKOV-3) and PIK3CA wild-type (OVCAR-5) xenograft models indicates that PIK3CA mutant models are more sensitive to OSI-027 treatment. Interestingly, in a PTEN-null IGR-OV1 model, a dose of 300 mg/kg once weekly was well tolerated and as efficacious as once daily dosing at 50 mg/kg. This efficacy was attributed to sustained plasma drug concentrations greater than 20 µmol/L for at least 4 days after dosing. Taken together, these data suggest that in sensitive tumors, maintenance of trough drug concentrations of 0.5 µmol/L achieved through daily or intermittent oral dosing are needed for therapeutic efficacy. The efficacy of OSI-027 and rapamycin was compared in GEO and COLO 205 colorectal cancer xenografts dosing both agents at MTD with rapamycin dosed i.p to compensate for its poor oral bioavailability. In these studies, OSI-027 induced significantly greater TGI than rapamycin. Interestingly, both of these cell lines have an active IGF-II/IGF-1R autocrine loop, thereby activating the PI3K/AKT pathway. The limited efficacy of rapamycin compared with OSI-027 in these models is likely to be due to the fact that OSI-027 inhibits both mTORC1 and mTORC2, whereas rapamycin only inhibits mTORC1. In all (>10) in vivo xenografts tested, the efficacy of OSI-027 was not restricted to PTEN deficiency, PIK3CA mutation, or PTEN mutation status.

In conclusion, specific and global targeting of mTOR in cancer cells by dual mTORC1 and mTORC2 inhibitors has further validated an essential role of mTOR in cell proliferation, survival, and tumor growth. OSI-027 is mechanistically and functionally distinct from rapalogs and inhibits growth of numerous models of breast, colon, ovary, lung, and prostate cancer. The preclinical pharmacology, broad-spectrum efficacy, and feasibility of multiple dosing schedules have supported the use of OSI-027 as an anticancer agent and justified its entry into phase I clinical development as a promising new anticancer therapy for potential treatment of solid tumors and hematologic malignancies.

Disclosure of Potential Conflicts of Interest

Employment by OSI Pharmaceuticals for: S.V. Bhagwat, P.C. Gokhale, A.P. Crew, A. Cooke, Y. Yao, C. Mantis, J. Kahler, J. Workman, M. Bittner,
N.W. Gibson, L.D. Arnold, R. Wild, D.M. Epstein, and J.A. Pachter at the time this work was conducted. Inventorship on patents for A.P. Crew and L.D. Arnold.

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References

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