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

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mTOR, rapamycin, mTORC1, mTORC2, OSI-027, OXA-01
Abbreviations List

mTOR (mammalian target of rapamycin), mTORC1 (mTOR complex 1), mTORC2 (mTOR complex 2), PI3K (phosphoinositide 3-kinase), TGI (tumor growth inhibition)

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Abstract

The PI3K/AKT/mTOR pathway is frequently activated in human cancers, and mTOR (mammalian target of rapamycin) 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 analogs 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 >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 analog of OSI-027) potently inhibit proliferation of several rapamycin-sensitive and –insensitive non-engineered 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 demonstrates robust anti-tumor 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 anti-cancer agent. OSI-027 is currently in Phase I clinical trials in cancer patients.

Introduction

Mammalian target of rapamycin (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 two 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), both involved in protein translation (7). The more recently discovered mTORC2 phosphorylates the survival kinase AKT on Ser 473, thus leading to activation of the AKT pathway, which drives several cancer-related cellular responses, including increased cell growth, proliferation and survival, increased cell migration and a shift to glycolytic metabolism (8-12). Evidence also suggests that mTORC2 phosphorylates PKCα and regulates the organization of the actin cytoskeleton (9-10).

Rapamycin and rapamycin-related analogs (rapalogs) are allosteric inhibitors of mTORC1 (13, 14). In addition to lacking a direct mTORC2 inhibitory effect, rapalogs do not inhibit the function of mTORC1 completely (15, 16). Although rapalogs (e.g. temsirolimus and everolimus) have demonstrated clinical benefits as single 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 analogs has been shown to result in hyperactivation of AKT through the release of negative feedback loop between S6K1 and IRS-1 (18). Indeed, Cloughesy
et al 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 anti-cancer 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, anti-tumor activity and tumor pharmacodynamic effects were reported with the selective mTORC1 and mTORC2 inhibitors, AZD8055 and WYE-132. In this report, we describe the preclinical characterization of OSI-027, an orally bioavailable, potent and specific dual inhibitor of mTORC1 and mTORC2. We demonstrate broad-spectrum anti-tumor activity in several xenograft models, in vivo differentiation from rapamycin, PK/PD/efficacy correlations in PI3K-wt vs. mutant models and tumor growth inhibition 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-a]pyrazine. These were synthesized by the methods described in patent application US-2007112005 (27). Compound identity and purity (>99%) were verified by $^1$H and $^{13}$C nuclear magnetic resonance, mass spectrometry, and high performance liquid chromatography 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 DMSO at 10 mmol/L for use in biochemical or cellular in vitro assays. For in vivo studies, OSI-027 was dissolved in 20% Trapsol 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 < 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 RIPA buffer supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail, 1 mM sodium orthovanadate and 10 mM sodium fluoride (all reagents purchased from Sigma, St Louis, MO). Lysates were cleared by
centrifugation and 20 μg protein was loaded per well. Lysates were fractionated on 4-12% tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes using a semidry apparatus. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, and incubated overnight with primary antibody in 3% bovine serum albumin. The following primary antibodies from Cell Signaling Technology (Danvers, MA) were used at 1:1000 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 GAPDH. β-Actin antibody is purchased from Sigma (St Louis, MO). HRP-conjugated secondary antibodies were obtained from Jackson Labs or GE Healthcare. HRP-conjugated secondary antibodies were incubated in nonfat dry milk for one hour. SuperSignal chemiluminescent reagent (Pierce Biotechnology, Rockford, IL) was used according to the directions and blots were imaged 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 (Wilmington, MA) and allowed to acclimate for a minimum of one week prior to 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 (AAALAC) accredited vivarium and in accordance with guidelines from the Institute of Laboratory Animal Research (Bethesda, MD).
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 using CellTiterGlo® (Promega). Proliferation on Day 0 vs. 72h was used to plot dose-response curves for IC\textsubscript{50} calculations and to determine cell death. For apoptosis induction (Caspase3/7) assay, cells were seeded into 96-well plates and incubated for 48h in the presence of OSI-027 at various concentrations. Induction of caspase3/7 activity was determined by luminescent quantification using CaspaseGlo® reagent (Promega). Fold induction of apoptosis was calculated as compared to DMSO-treated controls. For cell cycle analysis, cells were treated with DMSO, rapamycin or OSI-027 for 48h 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 using 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 x 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 non-compartmental analysis.

\textit{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 $300 \pm 50 \text{ mm}^3$ size were dosed orally with OSI-027 dissolved in 20% Trappsol (CTD Inc., High Springs, FL). Tumor samples were collected at specified time points and snap frozen in liquid nitrogen. Tumor lysates were prepared by homogenizing samples in a Precellys 24 homogenizer (MO Bio Laboratories, Inc., CA) with tumor lysis buffer [50 mM HEPES (pH 7.4) containing 1% Triton X-100, 10% glycerol, 50 mM, 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EDTA supplemented with fresh protease inhibitor cocktail (Sigma, MO), phosphatase inhibitor cocktail (Sigma, MO), 10 mM NaF and 1 mM sodium orthovanadate]. Tissue homogenates were clarified by centrifugation at 14,000g for 5 min at 4°C and supernatants were then analyzed by western blot as indicated. Plasma samples were also collected at specified time points for OSI-027 concentration analysis. Phospho-4E-BP1 (T37/46), total 4E-BP1, Phospho-AKT (S473), total AKT or β-actin were quantified from western blots using Multi Gauge software program (FujiFilm). Ki67 nuclear staining was carried out using formalin-fixed, paraffin-embedded SKOV-3 xenografts. Ki67 immunohistochemical nuclear staining was quantified using ACIS II imager scoring ten fields.

**In vivo Anti-Tumor 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 $200 \pm 50 \text{ mm}^3$ in size before randomization into various treatment groups with 8 mice/group. OSI-027 was
administered orally dissolved in 20% Trappsol or rapamycin administered i.p. in an aqueous solution of 4% ethanol, 5% Tween 80 and 5% PEG400 at indicated doses. Rapamycin was purchased from LC Laboratories (Woburn, MA). Tumor volumes were determined from caliper measurements using the formula $V = (\text{length} \times \text{width}^2)/2$. Tumor sizes and body weights were measured twice weekly. Tumor growth inhibition (TGI) was determined at different time points for each animal by the following formula: 

$$\%\text{TGI} = \left\{1 - \left[\frac{T_t}{T_0} / \frac{C_t}{C_0}\right] / \left[1 - \frac{C_0}{C_t}\right]\right\} \times 100,$$

where $T_t =$ tumor volume of treated animal x at time t, $T_0 =$ tumor volume of treated animal x at time 0, $C_t =$ median tumor volume of control group at time t, and $C_0 =$ median tumor volume of control group at time 0. Median % TGI was calculated and reported for the entire dosing period for each group. Significant anti-tumor activity is defined as achievement of a median % TGI of at least 50%. Regressions in tumor volume were calculated as: 

$$\%\text{regression} = 100\left(\frac{V_0 - V_t}{V_0}\right),$$

where $V_0 =$ mean tumor volume of treated group at time 0, and $V_t =$ mean tumor volume of that group at time t. Rank ANOVA with Dunnett’s comparison was used to compare treatment groups to the control group. All comparisons were deemed statistically significant if $P \leq 0.05$.

## 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 IC$_{50}$ value for OSI-027 was 4 nM.
Immune precipitation with anti-Raptor antibodies to evaluate mTORC1 activity in the presence of drug, or anti-Rictor antibodies to assess mTORC2 activity indicated that OSI-027 is equipotent in the inhibition of both functional complexes. Greater than 100-fold selectivity was observed for mTOR relative to other PI3K-related kinases in biochemical assays. To determine the selectivity profile, OSI-027 (1 μM) was tested against 101 kinases using Caliper kinase profiling assays and the data are shown in Table S1. None of those kinases were inhibited by >50%. In addition, OSI-027 activity was tested against 37 kinases at Invitrogen at 100 μM ATP and none of them were significantly inhibited (Supplementary Table S1). Additional selectivity data for OSI-027 in cell assays and an Ambit KinomeScan has been published recently by our colleagues (30). These data indicate that OSI-027 is a potent and selective inhibitor of mTORC1/mTORC2.

**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 a concentration-dependent manner in BT-474 and IGR-OV1 cells under normal culture conditions (Fig 1B), and in MDA-MB-231 cells upon insulin stimulation (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 μM (Supplementary Fig. S3). In addition, OSI-027 inhibited phosphorylation of mTORC2
mediated PRAS40 in these three 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 three 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 three cell lines (Fig. 1B) with IC$_{50}$ of 0.4 μM 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 fashion in IGR-OV1, BT-474 and MDA-MB-231 cells (Fig. 1B) and shows reasonable correlation with p-4E-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 4E-BP1 (T37/46) and AKT (S473) phosphorylation by OSI-027 (20 μM) and rapamycin (20 μM) after 24h treatment in 12 or 24 cancer cell lines representing various tumor types respectively. These concentrations were chosen based on the plasma exposure of OSI-027 at efficacious doses in preclinical models. OSI-027 significantly inhibited 4E-BP1
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 μM, which is >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 1D). More importantly, rapamycin stimulated pAKT (S473) in 62% of total 24 cell lines (Fig. 1C and 1D) 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. Further, OSI-027 inhibited IGF-1 induced pAKT (S473), while 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. 1E) 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 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 vs. Rapamycin**

mTOR is important in the control of cell proliferation. Based on the potency of rapamycin in the inhibition of proliferation, we defined cell lines as either rapamycin-
sensitive (IC$_{50}$ <0.07 μM) or rapamycin-insensitive (<30% inhibition at 20 μM). 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 72h 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 μM (Fig. 2A, Supplementary Table S2). Moreover, OSI-027 induced 10-50% 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 analog 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-FKB12 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 μM rapamycin) cells with similar IC$_{50}$ values while rapamycin demonstrated a significant potency shift of >10000 fold (Fig. 2C and 2D). 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 to 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 pro-apoptotic 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 out 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 μM), rapamycin (20 μM) 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 65-95% (Supplementary Fig. S6).
Pharmacodynamic studies were performed 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 post-dose 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 μM. Such extended target suppression was associated with significant efficacy corresponding to 100% median tumor growth inhibition (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 to control vehicle treated animals for 14 days (Fig. 4C) while administration of OSI-027 on a twice daily schedule of 25 mg/kg for 14 days resulted in robust 90% median TGI (data not sown). Pharmacodynamic analysis showed that while the lower dose of 25 mg/kg demonstrated significant inhibition (> 79%) of 4E-BP1 phosphorylation, it only lasted for about 8 hours with inhibition of p4E-BP1 completely reversed by 16 hours post single dose of OSI-027 at 25 mg/kg corresponding to plasma concentrations of less than 0.13 μM (Supplementary Fig S8). These data suggest that sustained inhibition of tumor p4E-BP1 by more than 50% appears to be necessary for substantial tumor growth inhibition 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 h (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 *qd* for 12 days resulted in 100% median TGI with 37% regression, whilst rapamycin treatment (20 mg/kg ip, 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 h 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 h with recovery by 24 h (Fig. 4E and Suppl Fig S9). The corresponding median plasma concentrations at 8 and 24 h were 22 and 0.77 μM, respectively. On the other hand, rapamycin treatment demonstrated 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 demonstrate that an mTORC1/mTORC2 specific inhibitor such as OSI-027 can achieve superior inhibitory effects on pAKT and p4E-BP1 *in vivo* as compared to 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 compared to the efficacy of rapamycin (Fig. 4F). Oral OSI-027 administration at 65 mg/kg \textit{qd} for 14 days resulted in 95% median TGI, whilst rapamycin treatment (20 mg/kg \textit{ip}, 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 \textit{PIK3CA} mutant SKOV-3 and \textit{KRAS} mutant OVCAR-5 human ovarian cancer xenograft models. In the SKOV-3 model, OSI-027 at 50 mg/kg \textit{qd} for 14 days resulted in significant tumor growth inhibition (100% median TGI) with 15% regression. Additionally, twice daily dosing at 25 mg/kg \textit{bid} 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 (\textit{KRAS} mutant) tumors with 50 mg/kg \textit{qd} or 25 mg/kg \textit{bid} was only marginally efficacious showing 47% and 51% median TGI, respectively (Figure 5C). Further, to better understand the relationship between tumor growth inhibition 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 $\mu$M for 14 days was sufficient to achieve maximal efficacy equivalent to 50 mg/kg \textit{qd} 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 $\mu$M 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 μM was necessary to achieve efficacy equivalent to 50 mg/kg qd dosing (Fig. 5C). Steady-state concentrations of 0.5 and 2 μM were inactive in this model (data not shown). Pharmacodynamic evaluation in additional OVCAR-5 tumor-bearing mice on day 11 of treatment demonstrated that tumor phospho-4E-BP1 inhibition in tumor samples was achieved only at 6 μM steady state (Figure 5D) and this pharmacodynamic inhibition correlated with efficacy (Figure 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 qd for 14 days resulted in 100% TGI (Figure 5E). OSI-027 administration at 150 mg/kg q3d x 5 doses, corresponding to the same cumulative dose of 50 mg/kg per day, resulted in equivalent tumor growth inhibition (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 >20 μM OSI-027 plasma concentrations were maintained for at least 96 h, with a decline to 0.58 μM by 120 h.

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 qd for 3 days and tumor samples were collected 8 h post last dose. OSI-027 treatment resulted in 51.8 ± 4.2% inhibition of Ki67 staining compared to vehicle-treated control tumors (Fig 5F) confirming the anti-proliferative effects of OSI-027.

**Broad spectrum antitumor activity of OSI-027**
The anti-tumor efficacy of OSI-027 was further evaluated in a wide variety of human cancer xenograft models. OSI-027 administered orally at a 50 or 65 mg/kg daily dose demonstrated significant inhibition of tumor growth and induced tumor regression in several models of breast, colon, lung, prostate, lymphoma and head & neck cancer (Table 1). In all studies, OSI-027 was well tolerated with <10% body weight loss observed during the treatment period.

Discussion

Clinical studies with rapamycin and rapalogs have validated mTOR as an anti-cancer 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 report, 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 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 IC$_{50}$ 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 appears to translate into robust anti-proliferative 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 Caspase3/7 activation upon OSI-027 or rapamycin treatment to determine whether cell death is mediated by apoptosis. Rapamycin failed to induce Caspase3/7 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 (PD) 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 tumor growth inhibition 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 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 μM for at least 4 days after dosing. Taken together, these data suggest that in sensitive tumors, maintenance of trough drug concentrations of 0.5 μM 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 tumor growth inhibition 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 to OSI-027 in these models is likely to be due to the fact that OSI-027 inhibits both mTORC1 and mTORC2 while 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 hematological malignancies.
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Disclosure of Potential Conflicts of Interest

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<table>
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<th>Cell line</th>
<th>Tumor type</th>
<th>PTEN/PIK3CA/KRAS/BRAF mutation status</th>
<th>OSI-027 Dose, QD</th>
<th>Tumor growth inhibition (%)</th>
<th>Maximum regression (%)</th>
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<td>HCT-116</td>
<td>Colon</td>
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OSI-027 was administered orally once daily at indicated dose for 14 days. % tumor growth inhibition and % regression were calculated as described in ‘materials and methods’ section. N=8 animals/group in all studies.
Legends to Figures

**Figure 1.** OSI-027 is a potent inhibitor of mTORC1 and mTORC2 in cells. *A,* Structures of Rapamycin, OXA-01 and OSI-027. *B,* BT-474, IGR-OV1 and MDA-MB-231 cells were treated with OSI-027 in a dose-dependent manner for 2h and total cell lysates were analyzed by western blotting. In MDA-MB-231 cells, pAKT (T308) was not detected in normal culture conditions but it was detected after IGF-1 stimulation (see Supplementary Fig. S2). *C,* U-87 MG, RL95-2, NCI-H2122, C-33A and IGR-OV1 cells were treated with OSI-027 (20 μM) or rapamycin (20 μM) for 24h and total lysates were analyzed by western blotting for p4E-BP1 (T37/46), 4E-BP1, pAKT (S473), AKT and GAPDH. *D,* Summary table showing effect of OSI-027 (20 μM) and rapamycin (20 μM) on pAKT (S473) in 12 and 24 cancer cell lines respectively. *E,* NCI-H23 NSCLC cells treated with DMSO, rapamycin (20 μM), OSI-027 (20 μM) and rapamycin + OSI-027 (20 μM each) for 24h and total lysates were analyzed by western blotting for pAKT (S473).

**Figure 2.** Anti-proliferative effects of OSI-027 and rapamycin. *A,* Rapamycin-insensitive cell lines (IC$_{50} > 20$ μM) were treated with OSI-027 for 72h in a dose-dependent manner. Relative cell viabilities are measured using Cell TiterGlo and plotted as % DMSO control using Prism. *B,* Rapamycin-sensitive (IC$_{50} < 0.075$ μM) cell lines were treated with OSI-027 for 72h and dose response curve is plotted as % DMSO control using Prism. *C,* Rh1, Rh1/mTORrr, Rh30, Rh30/mTORrr and Rh30/Rapa10K cells were treated with OXA-01
and rapamycin for 72h and cell viability is measured using Cell TiterGlo reagent. D,
Summary table showing differentiation of OXA-01 from rapamycin in above cell lines.

**Figure 3.** Pro-apoptotic effect of OSI-027, OXA-01 and rapamycin. A, Induction of Caspase3/7 was measured in BT-474 and IGR-OV1 cancer cell lines by treatment with drugs for 48h using CaspaseGlo reagent. Data is from 2 separate experiments and presented as Mean ± SD. B, IGR-OV1 cells treated with OSI-027 (20 μM), Rapamycin (20 μM) and DMSO for 48h and stained with propidium iodide to determine cell death (sub-G1 fraction) by FACS analysis.

**Figure 4.** Pharmacodynamic effects and efficacy of OSI-027 in tumor xenograft models. Correlation of mean plasma drug concentrations (red line) and percent p4E-BP1 (T37/46) (blue line) (A) and pAKT (Ser473) (green line) (B) content over time (n=4/time point) following 65 mg/kg oral single dose of OSI-027 in MDA-MB-231 xenograft model. C, dose-dependent efficacy of OSI-027 in MDA-MB 231 model. Efficacy of OSI-027 (oral dose) and rapamycin (intraperitoneal dose) in COLO 205 (D) and GEO (F) models. OSI-027 treatment was significantly more effective than rapamycin treatment (P < 0.001 in COLO 205 and P < 0.05 in GEO). E, Pharmacodynamic effects of OSI-027 and rapamycin in COLO 205 tumors. COLO 205 tumors were collected at 8 and 24 h after 12 days of dosing (n=2) and immunoblotted for p4E-BP1 (T37/46), pS6 (S235/236) and pAKT (S473). The bands were quantified and plotted as a percentage of vehicle-treated controls.
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 qd and 25 mg/kg bid for 14 days by oral gavage. Alternatively, steady state concentrations of OSI-027 were delivered using 14-day mini pumps implanted subcutaneously in tumor bearing animals. Steady state concentrations of 0.5 and 2 μM were achieved using a dose of 0.12 mg/kg/h and 0.49 mg/kg/h, respectively. B, Actual plasma concentrations achieved 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 qd and 25 mg/kg bid for 14 days by oral gavage and at 6 μM steady-state concentrations using mini-pumps. D, Pharmacodynamic effects of OSI-027 delivered 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 qd for 14 days, 150 mg/kg q3d x 5 doses or 300 mg/kg q7d x 2 doses. F, Ki67 nuclear staining determined by immunohistochemistry in SKOV-3 xenografts collected 8 h after 3 daily doses of OSI-027 at 65 mg/kg.
Fig 1

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Fig 1

C

20 μM, 24h

DMSO  OSI-027  Rapa

U-87 MG

p4E-BP1 (T37/46)

4E-BP1

pAKT (S473)

AKT

GAPDH

RL95-2

p4E-BP1 (T37/46)

4E-BP1

pAKT (S473)

AKT

GAPDH

NCI-H2122

p4E-BP1 (T37/46)

4E-BP1

pAKT (S473)

AKT

GAPDH

C-33A

p4E-BP1 (T37/46)

4E-BP1

pAKT (S473)

AKT

GAPDH

IGR-OV1
### Fig. 1

#### D

<table>
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<th>Percentage of Cell Lines Showing</th>
<th>pAKT (S473) Stimulation</th>
<th>pAKT (S473) No Change</th>
<th>pAKT (S473) Inhibition</th>
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<tr>
<td>Rapamycin (20 μM, 24h)</td>
<td>15/24 (62%)</td>
<td>6/24 (25%)</td>
<td>3/24 (13%)</td>
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<tr>
<td>OSI-027 (20 μM, 24h)</td>
<td>0/12 (0%)</td>
<td>2/12 (17%)</td>
<td>10/12 (83%)</td>
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</table>

#### E

- Rapamycin, 20 μM
- OSI-027, 20 μM

<table>
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<th>24h Treatment</th>
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<tbody>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
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<td>+</td>
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</tbody>
</table>

- pAKT (S473)
- AKT
- GAPDH
Fig 2

A

![Graph A](image)

B

![Graph B](image)

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
**Figure 2**

**C**

- Graph shows the fraction of maximal proliferation as a function of log [Rapamycin] for Rh1 and Rh1/mTORrr.
- Graph shows the fraction of maximal proliferation as a function of log [OXA-01] for Rh1 and Rh1/mTORrr.

**D**

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<td></td>
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<tr>
<td>Rh1</td>
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<tr>
<td>Rh1/mTORrr</td>
<td>1.5</td>
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<tr>
<td>Rh30</td>
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<tr>
<td>Rh30/mTORrr</td>
<td>4.4</td>
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<tr>
<td>Rh30/Rapa10K</td>
<td>&gt;10</td>
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</table>

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**Fig 3**

**A**

IGR-OV1

- OSI-027
- OXA-01
- Rapamycin

BT-474

- OSI-027
- OXA-01
- Rapamycin

Log [Compd], μM

Induction (fold)

**B**

Percent of Total Cells

- sub G1
- G0/G1
- S/G2/M

DMSO

20 μM Rapamycin

20 μM OSI-027

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**Fig 4**

(A) Plasma [OSI-027] (µM) and p4E-BP1 (%) over time for OSI-027 and p4E-BP1.

(B) Plasma [OSI-027] (µM) and pAKT (%) over time for OSI-027 and pAKT.

(C) % Tumor Volume over time for MDA-MB-231: Control vehicle, OSI-027 25 mg/kg QD (1-14), OSI-027 65 mg/kg QD (1-14).

(D) % Tumor Volume over time for COLO 205: Control, OSI-027 65 mg/kg QD (1-14), Rapamycin 20 mg/kg QD (1-15, 8-12).

(E) Phosphorylation (% control) over time for p4E-BP1, pAKT, and pS6 for OSI-027 and Rapamycin at 6 h and 24 h.

(F) % Tumor Volume over time for GEO: Control, Rapamycin 20 mg/kg (QD 1-8-12), OSI-027 65 mg/kg (QD 1-14).

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**Fig 5**

A. SKOV-3

- Control
- OSI-027, 50 mg/kg QD (1-14)
- OSI-027, 25 mg/kg BID (1-14)
- OSI-027, 0.5µM (1-14)
- OSI-027, 2µM (1-14)

B. Actual OSI-027 plasma conc. (µM)

- Day 5
- Day 13

C. OVCAR-5

- Control
- OSI-027, 50 mg/kg QD (1-14)
- OSI-027, 25 mg/kg BID (1-14)
- OSI-027, 6µM (1-14)

D. % p4E-BP1 content

- Control
- 0.5 µM
- 2.0 µM
- 6.0 µM

E. IGR-OV1

- Control
- OSI-027, 50 mg/kg (QD 1-14)
- OSI-027, 150 mg/kg (Q3D x 5)
- OSI-027, 300 mg/kg (Q7D x 2)

F. Bhagwat SV et al
Preclinical Characterization of OSI-027, a Potent and Selective Inhibitor of mTORC1 and mTORC2: Distinct from Rapamycin

Shripad V Bhagwat, Prafulla C Gokhale, Andrew P Crew, et al.

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