P7170: A Novel Molecule with Unique Profile of mTORC1/C2 and Activin Receptor-like Kinase 1 Inhibition Leading to Antitumor and Antiangiogenic Activity


Abstract

The mTOR pathway is often upregulated in cancer and thus intensively pursued as a target to design novel anticancer therapies. Approved and emerging drugs targeting the mTOR pathway have positively affected the clinical landscape. Recently, activin receptor-like kinase 1 (ALK1), belonging to the TGFβ receptor family, has been reported as an emerging target for antiangiogenic cancer therapy. Here, we describe a novel orally efficacious compound, P7170, that inhibits mTORC1/mTORC2/ALK1 activity with a potent cell growth inhibition. In cell-based assays, P7170 strongly inhibited (IC50 < 10 nmol/L) the phosphorylation of p70S6K (T389) and pAKT (S473). In many cancer cell lines, such as prostate, ovarian, colon, and renal, P7170 treatment resulted in marked cell growth inhibition. Furthermore, it induced G1-S cell-cycle arrest and autophagy. In vitro HUVEC tube formation, in vivo Matrigel plug, and rat aorta ring assays demonstrated that P7170 exhibited significant antiangiogenic activity. In addition, ALK1 knockdown studies in HUVEC confirmed that the antiangiogenic activity of P7170 was primarily due to ALK1 inhibition. Strong inhibition of ALK1 in addition to mTORC1/mTORC2 differentiated P7170 in its mechanism of action in comparison with existing inhibitors. In vivo mouse xenograft studies revealed P7170 to exhibit a significant dose-dependent tumor growth inhibition in a broad range of human tumor types when administered orally at 10 to 20 mg/kg doses. The distinctive pharmacological profile with favorable pharmacokinetic parameters and in vivo efficacy makes P7170 an attractive candidate for clinical development. It is currently being tested in phase I clinical studies. Mol Cancer Ther; 14(5): 1095–106. ©2015 AACR.

Introduction

The PI3K/AKT/mTOR pathway is a foremost signaling pathway regulating various hallmarks of cancer that include sustained proliferative signaling, evading growth suppressors, resistance to programmed cell death, consequently enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (1). The significance of this pathway stems from the abundant evidence that it is frequently deregulated by various genetic and epigenetic mechanisms in a wide range of tumor types. Aberrant PI3K activation due to activating point mutations or amplification of the PIK3CA gene, genetic loss or loss of function mutations within tumor suppressor PTEN (2) has been associated with increased activity of downstream kinases AKT and mTOR that regulate a myriad of cellular processes (3). Extensive evidence is now available validating diverse components of this pathway as molecular targets in cancer (4).

A highly conserved and ubiquitously expressed serine/threonine (Ser/Thr) kinase that is a member of the PI3K-related super kinase (PIKK) family, the mTOR, is a fundamental regulator of cell proliferation, differentiation, growth, and survival. mTOR is a central node that functions as a sensor of the nutrients, energy, insulin, growth factors, and environmental cues and also acts as a negative regulator of autophagy (5). These mitogenic signals are transmitted to mTOR via PI3K and AKT, which in turn relays it to downstream targets to effectuate the cellular and metabolic responses (6, 7). Two physically and functionally distinct signaling multiprotein complexes are formed by mTOR, mTORC1 (containing Raptor and PRAS40) and mTORC2 (containing Rictor and Protor; ref. 8). mTORC1, which is the molecular target of rapamycin, regulates protein translation by phosphorylating downstream proteins p70S6K (56k) and 4E-BP1 (9), whereas mTORC2 increases the enzymatic activity of AKT by 10-fold by phosphorylating it on Ser473 (10). As the mTOR pathway has
emerged as an essential hub in regulating various cellular processes, its activity is tightly regulated in normal physiology. Phosphorylation of S6K by mTOR induces the degradation of IRS1, which in turn decreases insulin-driven AKT activity and, consequently, mTOR activity (11, 12). Akt destabilizes the TSC1/TSC2 complex and increases mTOR activity. Inhibition of mTORC1 results in the release of the negative feedback loop between S6K and IRS1, leading to hyperactivation of Akt (13, 14). The absence of AKT inhibition due to lack of mTORC2 activity is reported to limit the use of rapamycin and rapa-analouges in several cancers (15). Moreover, inhibition of both TORC1 and TORC2 negates a recognized feedback mechanism of resistance to rapalogues (16).

Recent reports have emphasized the role of Activin receptor like kinase-1 (ALK1) in regulating cell type–specific transcriptional modulators which collaborate to activate or repress transcription of specific target genes in the angiogenic response (18). ALK1 is an endothelial cell-restricted receptor of the large TGF-β superfamily receptor family and is found to be widely present on tumor blood vessels, most notably in lymphomas and numerous solid cancers (19, 20).

Here, we describe P7170, a small-molecule inhibitor of mTORC1/mTORC2/ALK1. Preclinical pharmacology illustrates P7170, an orally bioavailable, potent inhibitor of mTORC1 and mTORC2 with its distinctive activity against TGFβ superfamily kinase ALK1. P7170 demonstrates profound growth inhibitory activity in vitro and significant antitumor activity in multiple human xenograft models. Its compelling antiangiogenic activity, both in vitro and in vivo, offers a unique opportunity to deliver a best-in-class therapeutic for cancers that have high unmet medical need. P7170 is currently in phase 1 clinical development.

Materials and Methods

Cell lines, antibodies, and compounds

All the cell lines (Huh7, 786-O, A549, A2780, PC3, H460, HCT15, A431, SW480, HCT116, MDA MB 231, Panc1, HepG2, MCF7, MDA MB 468, AsPc-1), until and unless stated, were obtained from the ATCC and cultured in ATCC recommended media at 37°C. Patient-derived cell lines were procured from academia and industry and cultured in cell-specific media. In the present study, to keep experimental consistency, we used MCF7, MDA MB 468, AsPc-1, until and unless stated, were obtained from the ATCC and cultured in ATCC recommended media at 37°C.

Cell growth inhibition assay

Various cancer cell lines were seeded at a density of 3,000 cells/well in a 96-well plate and treated with test compound or vehicle (DMSO) for 48 hours. Following incubation, cell viability was assayed using propidium iodide (PI) and percent inhibition was calculated using GraphPad Prism software 6.0.

3D clonogenic assay

The test tumor panel comprised 13 different human tumor histology types, namely bladder cancer, colon, gastric, head and neck, liver, non–small cell lung adenocarcinoma, squamous cell and large cell, mammary, ovary, pancreatic, prostate, renal cancer, malignant melanoma, pleuromesothelioma, and sarcoma. In addition, the compounds were tested against three preparations of hematopoietic stem cells as representatives of nonmalignant tissue. The clonogenic assay was performed in a 24-well format according to a modified two-layer soft agar assay. The bottom layer consisted of 0.2 mL/well IMDM supplemented with 20% (v/v) FCS, 0.01% (v/v) gentamicin and 0.75% (w/v) agar. Of note, 0.8×10^4 to 4×10^4 cells were added to 0.2 mL of the same culture medium supplemented with 0.4% (v/v) agar and plated in 24-well plates onto the bottom layer. The test compounds were applied by continuous exposure (drug overlay) in 0.2 mL culture medium. The drug overlay was added 24 hours after seeding the cells as 3-fold concentrated solution. Every plate included six untreated control wells and drug-treated groups in triplicate at six concentrations. Cultures were incubated at 37°C and 7.5% CO2 in a humidified atmosphere for up to 20 days and monitored closely for colony growth using an inverted microscope. Within this period, in vitro tumor growth led to the formation of colonies with a diameter of >50 μm. At the time of maximum colony formation (based on internal historical data, range 4–21 days), counts were performed with an automatic image analysis system (BIOREADER 5000-W, Biosys GmbH). Twenty-four hours before evaluation, vital colonies were stained with a sterile aqueous solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/mL, 100 μL/well).

KINOMEscan kinase assays

For most assays, kinase-tagged T7 phage strains were grown in parallel in 24-well blocks in an Escherichia coli host derived from the BL21 strain. E. coli were grown to log phase and infected with T7 phage from a frozen stock (multiplicity of infection = 0.4) and incubated with shaking at 32°C until lysis (90–150 minutes). The lysates were centrifuged (6,000 × g) and filtered (0.2 μm) to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small-molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer [1% BSA, 0.05% Tween 20, 1 mM DTT] to remove unbound ligand and to reduce nonspecific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1× binding buffer (20% SeaBlock, 0.17× PBS, 0.05% Tween 20, 6 mM DTT). Test compounds were prepared as 40× stocks in 100% DMSO and directly diluted into the assay. All reactions were performed in polypropylene 384-well plates in a final volume of 0.04 mL. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer...
(1 × PBS, 0.05% Tween 20). The beads were then resuspended in elution buffer (1 × PBS, 0.05% Tween 20, 0.5 μmol/L non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR (21).

Cell-cycle analysis

Cells were seeded in 6-well plates at a density of 0.5 to 1 × 10^5 cells and treated with varying concentrations of P7170 (30–1,000 nmo/L) or vehicle for 48 hours. After incubation, cells were trypsinized, washed twice with ice-cold PBS, and fixed overnight in 70% ethanol at 4°C. Cells were subsequently suspended in PBS and treated with 1 μg/μL of RNase A and 1 μg/μL of PI in PBS for 45 minutes. Cell-cycle distribution was evaluated using flow cytometer (BD FACS Calibur).

Western blot analysis

Cells were seeded at a density of 5 × 10^3 in 35 mm dishes. After 24 hours of incubation, cells were serum starved for 16 hours and then treated with the corresponding compounds or vehicle (DMSO) for 2 hours followed by serum stimulation for 30 minutes. At the end of stimulation, cells were lysed in lysis buffer containing protease and phosphatase inhibitors. Equal amount of protein was resolved on SDS-PAGE and membranes were probed for various proteins. Densitometric analysis of immunoblot analyses was performed by ImageJ analysis software (NIH).

Autophagy analysis

Fifty thousand cells/well were seeded in a 6-well plate and treated with various concentrations of P7170, BEZ235, and rapamycin for 24, 48, 72 hours. Media were replaced with PBS containing 1 μg/mL Acridine Orange (AO) solution and incubated at 37°C for an additional 15 minutes. Cells were harvested, resuspended in PBS, and analyzed for autophagy using a flow cytometer (BD FACS Calibur). For fluorescence microscopy, PC3 cells were treated with test compounds for 72 hours and observed under fluorescence microscope (Zeiss LSM) after staining acidic vesicles with acridine orange.

In-cell Western assay

A 96-well cell-based assay was developed to evaluate mTORC1 and mTORC2 activity in PC3 cells. Cells were seeded at a density of 5,000 cells per well and treated with test compounds as described in Western blot analysis. At the end of the incubation period, cells were fixed, washed, and probed with antibodies for either protein or phospho-protein of AKT (S473) and S6 (S235/236). Percent inhibition was calculated, and the IC50 values were drawn using GraphPad Prism software 6.0.

Animal xenograft studies

The use of athymic nude (nu/nu) mice and their treatment was approved by the Institutional Animal Care and Use Committee (IACUC) and all the experiments were carried out in strict compliance with their regulations. Exponentially growing PC3 cells (5 × 10^6) were injected into the flanks of the nude mice and once they reached 100 mm^3, animals were randomized into test and control groups (n = 10). Test groups were treated with various doses of P7170 (3, 10, 12.5, 15 mg/kg) and 25 mg/kg of BEZ235 dosed orally, once daily whereas control group received vehicle alone. Tumor volumes were measured thrice a week and animal body weight was recorded on alternate days.

Pharmacokinetic-pharmacodynamic (PK-PD) studies

Xenograft-bearing mice were randomized into test and control groups and treated with P7170 for 3 days (oral, once daily). Tumor and blood samples were collected at different time intervals after the last dose administration of test compound. Tumors were lysed and analyzed by immunoblotting, whereas blood samples were analyzed for drug content by high-performance liquid chromatography/mass spectrometry (UFLC Shimadzu-Sciex API 4000).

Purified ALK1 enzyme kinase assay

Kinase reactions were carried out in a 384-well plate format in a final volume of 20 μL. The standard enzyme reaction buffer consisted of 50 mmol/L Tris HCl (pH: 7.4), 1 mmol/L EGTA, 10 mmol/L/MgCl2, 2 mmol/L/DTT, 0.01% tween-20, 20 nmol/L of ALK1 kinase enzyme, 50 nmol/L of DNA topoisomerase 2 alpha (Thr 1342) peptide substrate (PerkinElmer), and 20 μmol/L of ATP. Final concentrations of P7170 and BZ 235 in DMSO were 20 nmol/L to 20 μmol/L. ALK1 enzyme at a concentration of 20 nmol/L and various concentrations of P7170/BEZ235 and vehicle were preincubated for 10 minutes at 23°C followed by the addition of 50 nmol/L (final concentration) of the peptide. Reaction was initiated with the addition of 20 μmol/L of ATP. After 1-hour incubation at 23°C, kinase reaction was stopped with the addition of 5 μL EDTA (final concentration of 10 mmol/L in 20 μL). Eu cryptate–anti–phospho-Topoisomerase 2-alpha (Thr 1342) at a final concentration of 2 nmol/L was added, and the mixture was allowed to equilibrate for 1 hour at 23°C. After irradiation of the kinase reaction at 320 or 340 nm, the energy from the Europium donor gets transferred to its acceptor (APC), which, in turn, generates light at 665 nm. The intensity of the light emission is proportional to the level of the peptide phosphorylation. The IC50 values were determined by a four-parameter sigmoidal curve fit (GraphPad Prism).

Tube formation assay

Human umbilical vein endothelial cells (HUVEC) were grown in endothelial medium (Promocell) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL basic fibroblast growth factor, and 5 U/mL heparin at 37°C under a humidified 5% (v/v) CO2. For the assay, 250 μL of growth factor-reduced Matrigel (BD Biosciences) was pipetted into a 24-well tissue culture plate and polymerized for 30 minutes at 37°C. ALK-1 siRNA or scrambled siRNA transfected or nontransfected HUVECs incubated in endothelial media containing 1% FBS for 6 hours were harvested after trypsin treatment and suspended in endothelial medium containing 1% FBS. Cells were plated onto a Matrigel layer at a density of 2 × 10^4 cells/well and treated with P7170, BEZ235, and sorafenib each at 100 nmol/L concentration for 30 minutes. The culture media were supplemented with 40 ng/mL VEGF and incubated for additional 18 hours. Tube formation was photographed under a microscope (Carl Zeiss).

In vivo Matrigel plug assay

Nude (nu/nu) mice were injected subcutaneously with 0.5 mL of Matrigel containing 200 ng VEGF and 10 U heparin. After 5 days of implantation, mice were treated with test compounds sorafenib (20 mg/kg), BEZ235 (25 mg/kg), and P7170 (10 mg/kg) for 3 days. At the end of the experiment, plugs were excised and photographed. Hemoglobin content was used as a marker for angiogenesis.
**Rat aorta ring assay**

The rat aortic ring assay was performed as described by Nicosia and colleagues (22) with minor changes. Healthy rats were humanely euthanized by overdose of anesthesia followed by cervical dislocation. Animal’s thoracic cavity was opened by scalpel blade, and these rings were washed in ice cold PBS. In a 24-well plate, 200 μL of Matrigel (BD Sciences) premixed with 1:1 RPMI was coated and incubated at 37°C for 1 hour. Rings were carefully arranged in every well and preplated Matrigel (50 μL) supplemented with VEGF (40 ng/mL) was added to the wells. After 20 minutes of incubation, RPMI media (500 μL) supplemented with 4% FBS, 40 ng/mL VEGF, and antibiotics (penicillin-streptomycin–neomycin) were added and incubated for 4 hours to initiate the sprouting. Once initiation of sprouting (day 2) was observed, test compound and positive controls at various concentrations (30, 100, and 300 nmol/L) were added in triplicate after replenishing the RPMI media with all the supplements mentioned above and incubated for 4 days. Microvasculature on aortic rings was photographed under microscope (Carl Zeiss) on the day of compound treatment and 4 days later. Photographed vasculature was quantified using AxioVision software (Carl Zeiss).

**Statistical analysis**

Results were expressed as mean ± SD for at least triplicates. Data were compared by the Student t test and/or one-way ANOVA followed by Bonferroni post hoc analysis for multiple comparisons. Differences were considered statistically significant at P < 0.05 (n = 3).

**Results**

**P7170 is a potent inhibitor of mTORC1, mTORC2, and ALK1 enzymes**

P7170, a compound belonging to imidazoquinoline series of pharmacophore (Fig. 1A), is a potent small-molecule inhibitor of class I PI3K isoforms and mTOR kinase and displays excellent selectivity against a large panel of other kinases in biochemical assays (PCT patent application no. WO 2012/007926 A1). Besides the PI3K–mTOR pathway, it also inhibits ALK1, an important enzyme involved in angiogenesis. Using radiometric protein kinase assay (3PanQinase activity assay), P7170 was shown to potently inhibit mTOR and ALK1 activity with an IC50 value of 4.4 and 47 nmol/L (Fig. 1B), respectively. In addition, the inhibitory activity of P7170 against PI3K was identified when screened against a commercially available panel of 320 kinases (Ambit Biosciences and ProQinase). P7170 displayed a pan-PI3K inhibitory activity with IC50 values ranging from 2.2 to 203 nmol/L (Supplementary Table S1A). P7170 also inhibited DNA-PK, MAP4K2, JAK2, ALK2, and all PIK3CA-mutant kinases (C420R, E542K, E545A, E545K, H1047L, H1047Y, I800L, M1043I, and Q546K) as tabulated in Supplementary Table S1B and 1C. Few kinases, such as ABL1 (H396P), CK1, HIPK2, LKB1, MEK2, and PIK3GC, were also inhibited on P7170 treatment at 250 nmol/L (Supplementary Table S1C). However, upon further evaluation of their IC50 values, P7170 did not inhibit these set of kinases as efficiently as mTORC1/C2 or ALK-1 (Supplementary Table S1D).

**P7170 downregulates the mTOR pathway**

To further evaluate the cellular PI3K/mTOR inhibitory activity of P7170, Western blot analysis was carried out in the A2780 cell line (Fig. 1C). A substantial proportion of ovarian tumors has been associated with deregulation of mTOR (23). Furthermore, A2780, an ovarian cancer cell line, is reported to have amplified PIK3CA resulting in an activated PI3K/mTOR pathway (24). As shown in Fig. 1C, P7170 strongly inhibited phosphorylation of Ser240/244 and Ser473, which are substrates of mTORC1 (9), on ribosomal S6 protein. Complete inhibition of a direct target of mTORC1, phosphorylated Thr389 on p70S6K, was observed with treatment as low as 10 nmol/L of P7170. Furthermore, a decrease in phosphorylation of 4EBP1 protein at Ser65 and Thr37/46 was observed with P7170 and other mTORC1/mTORC2 inhibitor AZD8055 but not, as expected, with the selective mTORC1 inhibitor everolimus (9). Similarly, phosphorylation of AKT at Ser473, an mTORC2 substrate, was completely abolished on treatment with P7170 and AZD8055, but not with everolimus. Interestingly, P7170 weakly inhibited phosphorylation of AKT at Thr308, a PI3K substrate. The potent biochemical activity of PI3K did not translate in intact cells most likely because of feedback mechanism of mTOR inhibition (Fig. 1C). As demonstrated, IC50 = 2.2 nmol/L P3Kx biochemical activity translated into weak IC50 = >100 nmol/L) inhibitory activity in ovarian cancer (A2780) cells (Fig. 1C). Some of the other kinase biochemical activities seem to be cell specific (unpublished data), which is being explored intensively in our laboratories. P7170 activity was further evaluated in other cancer cell lines associated with the activated PI3K/mTOR pathway (Fig. 1D). Treatment with P7170 resulted in strong inhibition of the mTORC1/C2 pathway in HCT116 (colon), 786-O (renal), and PC3 (prostate) cancer cell lines, as shown in Fig. 1D. Using a high-throughput in-cell Western screening assay in PC3 cells, cellular IC50 values for P7170 were calculated as 2.7 nmol/L against mTORC1 indirect substrate pS6(e235/236) and 12.4 nmol/L against mTORC2 substrate pAKT(S473) (Fig. 1E).

**P7170 exhibits antiproliferative activity toward various cancer cell lines and patient-derived cancer cell lines**

The PI3K/mTOR pathway is known to play a central role in regulating fundamental cellular processes like proliferation, growth, and survival (25). Therefore, the impact of P7170 treatment was evaluated in a panel of cancer cell lines using a cell viability assay (Fig. 2A). P7170 treatment resulted in a dose-dependent reduction in the number of viable cancer cells with IC50 ranging between 2 nmol/L and 22 nmol/L across a wide array of cancer cell types. No toxicity was observed in normal cells, hPBMcs, even on treatment with the highest concentration (10 μmol/L) of P7170, demonstrating a safe therapeutic window between cancer and normal cell types (Fig. 2A). P7170 was then tested for its effect on the clonogenic growth of 39 patient-derived cancer cell lines using a clonogenic assay. P7170 exhibited absolute mean IC50 of 115 nmol/L (Fig. 2B). On the basis of the mean IC50 determined within the different histotypes, the most sensitive tumor types were kidney cancer (RKF), pleuramesothelioma (PXF), liver cancer (LIJF), and breast cancer (MAXF). Growth curves for each tumor type are summarized in Supplementary Fig. S1.
P7170 induces G₁–S cell-cycle arrest and autophagy in vitro

PTEN-null PC3 prostate cancer cells exhibit elevated intrinsic PI3K/mTOR signaling associated with increased AKT kinase activity resulting from hyper phosphorylation of T308 and S473 residues (26). Therefore, PC3 cells were evaluated for cell-cycle content analysis on treatment with increasing concentrations of P7170 (30–1,000 nmol/L; Fig. 3A). P7170 treatment induced G₁–S cell-cycle arrest with an increase in G₁ population from 52.3% (DMSO controls) to 65.9% (30 nmol/L P7170-treated cells) and sub-G₁ population increased from 1.1% to 63.3% (1,000 nmol/L P7170-treated cells), an observation consistent with the growth arrest and cell death expected with PI3K/AKT/mTOR signal inhibition (27). This considerable increase in the sub-G₁ population observed following P7170 treatment was dose- and time dependent (Fig. 3B). A 62% increase (over DMSO control) in sub-G₁ (apoptotic) cell population upon treatment with P7170 led us to investigate potential outcome of this treatment on apoptotic pathways. However, P7170, when treated
at 100 nmol/L, had no significant impact on PARP cleavage, a known apoptotic marker (Fig. 3C). However, a dose-dependent increase in PARP cleavage was observed at increasing concentration of P7170.

mTOR inhibitors, in particular, are known to induce autophagy in various cancer cells (9). Using acridine orange as an indicator of autophagy, the formation of cytoplasmic acidic vesicles was examined to evaluate the effect of P7170 on autophagosome formation in PC3 cells (Fig. 3D). P7170 induced a concentration- and time-dependent increase in acridine orange staining with maximum accumulation observed with 300 nmol/L in P7170-treated cells for 72 hours (Fig. 3E). A similar pattern of acridine staining was seen in A2780 and HCT116 cancer cell lines (Supplementary Fig. S2). Detection of microtubule-associated protein 1A/1B-light chain 3 (LC3) by immunoblotting has been reported as a reliable autophagosome marker for monitoring autophagy and autophagy-induced cell death (28). P7170 demonstrated a dose-dependent increase in the expression of LC3-I and, in particular, LC3-II (Fig. 3E). Tracking the level of conversion of LC3-I to LC3-II is indicative of autophagic activity as the levels of LC3-II correlate with autophagosome formation. Furthermore, induction of autophagy by P7170 was comparable with the activities of known PI3K/mTOR inhibitor BEZ235, or mTOR inhibitor everolimus (Fig. 3E).

P7170 potently inhibits ALK1 activity and angiogenic sprouting in vitro and in vivo

Inhibitory activity of ALK1 by P7170 was noted when the compound was screened against a commercially available panel of 330 kinases by an independent outfit as described in Material and Methods (Supplementary Table S1C). ALK1 has been implicated in vascular development and pathologic angiogenesis by numerous genetic and molecular mechanisms (19, 29). Therefore, our subsequent efforts were focused on understanding the effect of P7170 on ALK1 activity and its implication on...
P7170, a Novel Potent mTORC1/C2 and ALK1 Inhibitor

angiogenic processes. In comparison with BEZ235 and everolimus, P7170 strongly inhibited ALK1 kinase activity with IC\textsubscript{50} of 47 nmol/L (Fig. 1B). Both BEZ235 and everolimus did not demonstrate any inhibition of ALK1 up to 2 \mu mol/L, the highest concentration tested (Fig. 1B). Furthermore, functional analyses in \textit{in vivo} and \textit{ex vivo} angiogenesis models (Fig. 4A and B, respectively) demonstrated P7170 to significantly impair angiogenic response toward VEGF. The antiangiogenic activity of P7170 \textit{in vivo} was assessed in a Matrigel plug assay generated in nude mice and treated with 10 mpk of P7170 for 5 days. P7170 strongly inhibited angiogenesis as seen by diminished formation of blood vessels (Fig. 4A). In addition, VEGF at a concentration of 40 ng/mL significantly (\(P < 0.05\)) induced microvasculature on rat aortic rings starting on day 2 (data not shown) and reached maximum on day 7 (Fig. 4B and C). This VEGF-induced aortic microvasculature was inhibited by P7170 in a dose-dependent manner (Fig. 4B). However, everolimus and BEZ235 had no significant effect on microvasculature formation (Fig. 4B). The average microvasculature on control rings was 1327 \mu m\(^2\)/\mu m and reduced to 42 \mu m\(^2\)/\mu m upon P7170 treatment, indicating 97% inhibition (Fig. 4C). Moreover, it may be noted that the plasma PK resulting from a dose of 10 mg/kg of P7170 is sufficient to inhibit ALK1 (Fig. 5D).

Although PI3K/mTOR activity is reported to be absolutely required for microvessel formation as everolimus (a potent mTOR inhibitor) or BEZ235 (a PI3K/mTOR dual inhibitor) was ineffective in inhibiting microvessel formation in this \textit{ex vivo} model.

In parallel, P7170 treatment resulted in the complete loss of tube formation ability of HUVEC cells upon VEGF induction (Fig. 4D) at concentrations that were not cytotoxic and did not inhibit endothelial cell proliferation (data not shown). Also, in HUVECs, tube formation was compromised upon ALK1 knockdown by shRNA but not with scrambled shRNA (Fig. 4E). ALK1 knockdown was confirmed by immunocytochemistry (Fig. 4F). No significant attenuation in tube formation was observed by P7170 in HUVEC cells that lack ALK1, suggesting that ALK1 was the direct target of P7170.

P7170 induces significant tumor growth inhibition, coupled with a strong pharmacodynamic effect on both pS6 and pAKT, in a broad range of human tumor xenografts

In nude mice bearing PC3 (\textit{PTEN} null) xenografts, oral administration of P7170 once a day resulted in a dose-dependent tumor growth inhibition of 28%, 67% (\(P < 0.002\)), 60% (\(P < 0.001\)), and 76% (\(P < 0.0001\)) with 3, 10, 12.5, and 15 mg/kg,
respectively (Fig. 5A). P7170 also demonstrated substantial inhibition of tumor growth in renal (68%), ovarian (78%), colon (80%), and hepatocellular (53%) xenograft models when administered orally once daily at 5, 10, 15, and 20 mg/kg, respectively (Table 1). P7170 at a dose of 15 mg/kg (76% TGI) was as efficacious as the mice treated with 25 mg/kg of BEZ235.
(71%) in a prostate xenograft model. No significant body-weight loss was observed with increasing doses of P7170 (Fig. 5A, inset). Furthermore, the pharmacodynamic effects of P7170 were evaluated in mice bearing PC3 xenografts after 3 doses of oral administration of P7170 at 10 mg/kg at varying time intervals (Fig. 5B). P7170 markedly inhibited the phosphorylation of pAKT (S473) and pS6 (S235/236) within 1 hour of compound administration. IHC staining of pS6 (S235/236) in these tumor samples also demonstrated complete inhibition of phosphorylation on treatment with P7170 at early time points, which recovers slightly by 16 hours (Fig. 5C). This pharmacodynamic activity correlated well with the concentrations of the compound in plasma (Fig. 5D).

**Discussion**

The PI3K–AKT–mTOR pathway has emerged as a prime strategic target of “drugging the cancer kinome” that has led to the development of several novel targeted anticancer therapeutic agents (16, 17, 30, 31). mTOR acts as a sensor protein that...
assimilates extracellular and intracellular events from many cancer-driving proteins such as PI3K, AKT, EGFR, HER2/neu, and BCR–ABL (32). It thus lies at the nexus of the regulatory network coordinating cellular proliferation, growth, and survival (6). Despite an increasing armament of targeted agents that inhibit key components of the PI3K–AKT–mTOR pathway (5, 6), there is still an unmet medical need to identify effective agents that would minimize toxicities and maximize clinical benefit, to improve and transform their care.

In the present work, we describe P7170, a novel, orally bioavailable, potent, mTORC1/mTORC2 kinase inhibitor with distinctive inhibitory activity against activin-like receptor kinase ALK1. P7170 activity profile has been confirmed in a broad range of human cancer cell models both in vitro and in vivo. P7170 potently inhibited phosphorylation of AKT on the mTORC2 site S473, phosphorylation of S6 on S235/236, and phosphorylation of 4EBP1 on mTORC1 site T37/46 in all cancer cell types studied. The inhibition of phosphorylation on both mTORC1 and mTORC2 sites has been associated with an enhanced effect on cap-dependent translation, growth inhibition, and potentially autophagy. The results of the present study show that P7170 significantly decreases the phosphorylation of 4E-BP1 on the T37/46 sites at low nanomolar concentrations. It is interesting to note that similar concentrations of P7170 also inhibit proliferation. Furthermore, targeting mTOR signaling by P7170 attacks two fundamental mechanisms of treating cancer, that is, induction of cell death (through autophagy) and inhibition of cell-cycle progression. Inhibiting mTOR blocks the phosphorylation of two key downstream effectors, p70S6 kinase and pEBP1. Both proteins have been implicated in translational regulation and inhibition of expression of the G1/S cell-cycle regulator protein. Consistent with the published reports that the PI3K/AKT/mTOR pathway is indeed critical for clonalogenic cell growth (33, 34), it was observed that P7170 inhibits soft-agar colony formation of various patient-derived tumor cell lines. The growth inhibitory effect of P7170 in these cell lines was independent of mutation status or histologic subtypes. Inhibition of cell proliferation and colony formation of various cancer and patient-derived cancer cells by P7170 is not solely through PARP cleavage, suggesting that classical apoptosis is not the only mechanism of cell death. In contrast, P7170-induced cell death was associated with several characteristics of autophagy, including an increase in acidic vesicular organelle content, conversion of cytosolic LC3-I to membrane-bound LC3-II (35). Numerous reports have proved that mTORC1 is a negative regulator of autophagy. Following mTOR inhibition, autophagosome formation is primed directly by phosphorylating ULK1 thus preventing ULK1–Atg13–FIP200 complex formation and indirectly by phosphorylating S6K and 4E-BP1 (36). Reports on the involvement of mTORC2 protein rictor in regulating autophagy through a mechanism independent of mTORC1 have also started to emerge (35), further emphasizing the advantage of having a dual mTORC1 and mTORC2 inhibitor.

Recent literature has emphasized on the importance of angiogenesis in the progression of cancer development and use of antiangiogenic agents as crucial therapeutic agents in cancer therapy (19, 37). Interestingly, P7170, in addition to potently inhibiting the mTORC1/C2 pathway, also effectively inhibits AKL activity (IC50 47 nmol/L). AKL1 is a type I receptor of the TGFβ receptor family, that is found on proliferating endothelial cells (38). Hu-Lowe and colleagues have demonstrated using ALK1−/− mice and zebrafish harboring a loss-of-function mutation that ALK1 plays a fundamental role in vasculogenesis, particularly in vessel maturation and in the organization of neo-angiogenic vessels (19, 38). In addition, overexpression of AKL1 has been observed in a wide variety of tumor blood vessels, including lymphomas, prostate, skin, thyroid, kidney, ovarian, lung, pancreatic, and liver cancers (39, 40). Notably, recent development of two agents targeting AKL1; an antibody developed by Pfizer, PF-03446962 (Clinical Trial Identifier: NCT01911273; ref. 40), and an ALK1 ligand trap, ACE-041, by Acceleron has accentuated the significance of targeting ALK1 in cancer drug discovery (Clinical Trial Indentifier: NCT00996957). It has been reported in animal studies that treatment with ACE-041 inhibits tumor angiogenesis and growth. Intriguingly, ACE-041 treatment was generally well tolerated and antitumor activity was observed, resulting in tumor shrinkage and stabilization of disease in a clinical study of patients with advanced, refractory solid tumors (Clinical Trial Identifier: NCT00996957). Both ACE-041 and PF-03446962 are being studied in phase II clinical trials (41, 42). On the basis of these encouraging clinical activities, inhibition of ALK1 may turn out to be a promising novel antiangiogenic strategy to treat certain cancers. Our data from in vitro and ex vivo studies suggested that ALK1 is the direct target of P7170. Although PI3K/mTOR dual activity is reported to be important for VEGF signaling and angiogenesis, mTOR does not seem to be absolutely required for microvessel formation as everolimus (a potent mTOR inhibitor) or BEZ-235 (a PI3K/mTOR dual inhibitor) was ineffective in inhibiting microvessel formation in the ex vivo model as described in Fig. 4B. This strongly suggests that ALK1 inhibitory activity by P7170 may be responsible for its effect on inhibition of microvessel formation.

Our results also demonstrate that P7170 was well tolerated and induced a dose-dependent growth inhibition in a broad range of tumor xenografts. This antitumor activity was associated with a rapid and dose-dependent pharmacodynamic effect on both pS6 and pAKT providing evidence for target engagement by P7170 in vivo studies. Furthermore, the plasma PK resulting from a dose of 10 mg/kg P7170 is sufficient to achieve ALK1 inhibition.

In summary, having antiangiogenesis (ALK1 Inhibition) activity in addition to potent activity against mTOR enzymes, P7170 may turn out to be of considerable therapeutic advantage in clinical setting. Therefore, P7170 is currently being evaluated in phase I studies in a variety of cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

<table>
<thead>
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<th>Table 1. Antitumor activity of P7170 in various mouse xenograft models</th>
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<td><strong>Cell Line</strong></td>
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<td>PC3</td>
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<td>786-O</td>
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<tr>
<td>A2780</td>
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<td>HCT116</td>
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Abbreviation: QD, once daily.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.R. Bhatia, A. Joshua, N. Vewalkar, A. Damre, V. Agarwal

Study supervision: J. Bose, S. Kumar, A. Damre, V. Agarwal

Other (provided the ALK-1 activity of the molecule): J. Bose

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