P7170: a novel molecule with unique profile of mTORC1/C2 and Activin Receptor-Like Kinase 1 inhibition leading to anti-tumor and anti-angiogenic activity

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Abstract

Mammalian target of Rapamycin kinase (mTOR) pathway is often up regulated in cancer and thus intensively pursued as a target to design novel anti-cancer therapies. Approved and emerging drugs targeting mTOR pathway have positively impacted the clinical landscape. Recently, Activin receptor-like kinase 1 (ALK1), belonging to the TGF-β receptor family, has been reported as an emerging target for anti-angiogenic cancer therapy. Herein, 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 nM) 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 anti-angiogenic activity. Additionally, ALK1 knockdown studies in HUVEC confirmed that the anti-angiogenic activity of P7170 was primarily due to ALK1 inhibition. Strong inhibition of ALK1 in addition to mTORC1/ mTORC2 differentiates P7170 in its mechanism of action in comparison to 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 10-20 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.

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 which is a member of the PI3K related super kinase (PIKK) family, the mammalian target of rapamycin (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 multi protein complexes are formed by mTOR; mTORC1 (containing Raptor and PRAS40) and mTORC2 (containing Rictor and Protor) (8). mTORC1, which is the molecular target of rapamycin, regulates protein translation by phosphorylating downstream proteins p70S6K (S6K) and 4E-BP1 (9), whereas mTORC2 increases the enzymatic activity of AKT by 10-fold by phosphorylating it on Ser473 (10). As 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 hyper activation of AKT (13, 14). Absence of AKT inhibition due to lack of mTORC2 activity is reported to limit the use of rapamycin and rapa-analogues in several cancers (15). Moreover, inhibition of both TORC1 and TORC2 negates a recognized feedback mechanism of resistance to current rapalogs (16). Therefore, a potential for a complete signaling blockade would be achieved by inhibition of both mTORC1 and mTORC2. An increasing panorama of targeted agents inhibiting PI3K/AKT/mTOR pathway are already in clinical trials (17). Only a couple of mTORC1 inhibitors Everolimus and Temsirolimus have been commercialized. Therefore, there is still a need for effective agents with the potential to maximize clinical benefit, minimize toxicities and aimed at targets complementary to the ones currently available. As a result of these issues, it will be beneficial to have a compound with activity against both mTORC1 and mTORC2. Since angiogenesis plays an important role in tumor growth, having anti-angiogenesis activity will be advantageous. 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 receptor family, is found to be widely present on tumor blood vessels, most notably in lymphomas and numerous solid cancers (19, 20).
Herein, 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 TGF-β superfamily kinase ALK1. P7170 demonstrates profound growth inhibitory activity in vitro and significant anti-tumor activity in multiple human xenograft models. Its compelling anti-angiogenic 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 I clinical development.

**Material 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 American Type Culture Collection (ATCC) and cultured in ATCC recommended media at 37°C under 5% CO2. Patient derived cell lines were developed and maintained at Prof. Fiebig’s lab, ONCOTEST, Germany. All cells were used within 20 passages from receipt and less than 6 months from authentication. All the primary antibodies were procured from Cell Signaling Technology (Danvers, MA), whereas secondary antibodies were procured from Santacruz Biotechnology (Dallas, TX). P7170 was synthesized at Piramal Enterprises Ltd., India under patent number WO-2012007926A1. BEZ235, AZD8055 and Everolimus were obtained from AXON Medchem, and LC Laboratories, respectively.
**Cell growth inhibition assay:** Various cancer cell lines were seeded at a density of 3000 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 Graph Pad Prism software 6.0 (Graph Pad, San Diego, CA).

**3D clonogenic assay:** The test tumor panel comprised of 13 different human tumor histology types, namely bladder cancer, colon, gastric, head and neck, liver, non-small cell lung adeno, squamous cell and large cell, mammary, ovary, pancreatic, prostate, renal cancer, malignant melanoma, pleuramesothelioma, and sarcoma. In addition, the compounds were tested against three preparations of hematopoietic stem cells as representatives of non-malignant 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) fetal calf serum, 0.01% (w/v) gentamicin) and 0.75% (w/v) agar. 0.8 x 10^4 to 4 x 10^4 cells were added to 0.2 ml of the same culture medium supplemented with 0.4% (w/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 hrs after seeding the cells as 3-fold concentrated solution. Every plate included six untreated control wells and drug-treated groups in triplicate at 6 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 prior to 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 *E. 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 x g) and filtered (0.2µm) 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 (SeaBlock (Pierce), 1 % BSA, 0.05 % Tween 20, 1 mM DTT) to remove unbound ligand and to reduce non-specific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20 % SeaBlock, 0.17x PBS, 0.05 % Tween 20, 6 mM DTT). Test compounds were prepared as 40x 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 (1x PBS, 0.05 % Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5 µM 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 0.5-1x10^5 cells and treated with varying concentrations of P7170 (30-1000 nM) or vehicle for 48 h. After incubation, cells were trypsinised, washed twice with ice cold PBS and fixed overnight in 70% ethanol at 4°C. Cells were subsequently suspended in PBS and treated with 1µg/µl of RNase A and 1µg/µl of propidium iodide (PI) in PBS for 45 min. Cell cycle distribution was evaluated using flow cytometer (BD FACS Calibur).

Western blot analysis: Cells were seeded at a density of 5x10^5 in 35mm dishes. After 24 h of incubation, cells were serum starved for 16 h and were then treated with the corresponding compounds or vehicle (DMSO) for 2 h followed by serum stimulation for 30 min. 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 Immunoblots was performed by Image J analysis software (NIH).

Autophagy analysis: 50000 cells/well were seeded in a 6-well plate and treated with various concentrations of P7170, BEZ235 and Rapamycin for 24, 48, 72 h. Media was replaced with PBS containing 1µg/ml Acridine Orange (AO) solution and incubated at 37°C for an additional 15 min. Cells were harvested, re-suspended 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 h and observed under fluorescence microscope (Zeiss LSM) after staining acidic vesicles with acridine orange.
**In-cell-western:** A 96-well cell-based assay was developed to evaluate mTORC1 and mTORC2 activity in PC3 cells. Cells were seeded at a density of 5000 cells per well and treated with test compounds as described above under the heading *western blot*. At the end of the incubation period, cells were fixed, washed and probed with antibodies for either protein or phosphoprotein of AKT (S473) and S6 (S235/236). Percent inhibition was calculated and the IC$_{50}$ values were drawn using Graph Pad Prism software 6.0 (Graph Pad, San Diego, CA).

**Animal xenograft studies:** The use of athymic nude 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 ($5 \times 10^6$) were injected into the flanks of the nude mice and once tumor volume 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 three days (oral, QD). Tumor and blood samples were collected at different time intervals after 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 mM Tris HCl (pH: 7.4), 1mM EGTA, 10 mM MgCl₂, 2 mM DTT, 0.01% tween-20, 20 nM of ALK1 kinase enzyme, 50 nM of DNA topoisomerase 2 alpha (Thr 1342) peptide substrate (Perkin Elmer) and 20 µM of ATP. Final concentration of P7170 and BEZ 235 in DMSO were 20 pM to 20 µM. ALK1 enzyme at a concentration of 20 nM and various concentration of P7170 / BEZ235 and vehicle were pre-incubated for 10 mins at 23°C followed by the addition of 50 nM (final concentration) of the peptide. Reaction was initiated with the addition of 20 µM of ATP. After 1 hr incubation at 23°C, kinase reaction was stopped with the addition of 5 µl EDTA (final concentration of 10 mM in 20 µl). Eu cryptate - anti-phospho-Topoisomerase 2-alpha (Thr 1342) at a final concentration of 2 nM was added and the mixture was allowed to equilibrate for 1hr 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 (Graph pad Prism).

**Tube formation assay:** Human umbilical vein endothelial cells (HUVECs) were grown in endothelial medium (Promocell) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 3 ng/ml basic fibroblast growth factor and 5 units/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 min at 37°C. ALK-1 SiRNA or Scrambled siRNA transfected or non-transfected HUVECs incubated in endothelial media containing 1% FBS for 6 h were harvested after trypsin treatment and suspended in endothelial medium.
containing 1% FBS. Cells were plated onto a matrigel layer at a density of 2x10^4 cells/well and treated with P7170, BEZ235 and sorafenib each at 100 nM concentration for 30 min. The cultures media was supplemented with 40 ng/ml VEGF and incubated for additional 18 h. Tube formation was photographed under microscope (Carl Zeiss, Germany).

**In vivo matrigel plug assay.** Nude mice were injected subcutaneously with 0.5 ml of matrigel containing 200 ng VEGF and 10 units 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:** Rat aortic ring assay was performed as described by Nicosia *et al* (22) with minor changes. Healthy rats were humanely euthanized by overdose of anesthesia followed by cervical dislocation. Animal’s thoracic cavity was opened and using sterile fine forceps aorta was separated from vertebral column. A segment of aorta was excised between heart and thoracic aorta and kept in ice cold PBS supplemented with Penicillin-Streptomycin. Fine 1.0 mm aorta rings were prepared from aorta using scalpel blade and these rings were washed in ice cold PBS. In a 24-well plate 200µL of matrigel (BD Sciences, Bedford, MA) pre-mixed with 1:1 RPMI was coated and incubated at 37°C for 1 h. Rings were carefully arranged in every well and pre-diluted matrigel (50µL) supplemented with VEGF (40 ng/mL) was added to immerse rings. After 20 minutes of incubation, RPMI media (500µL) supplemented with 4% FBS, 40 ng/mL VEGF and antibiotics (Penicillin-Streptomycin-Neomycin) were added and incubated for 24 h to initiate the sprouting. Once initiation of sprouting (day 2) was observed, test compound and
positive controls at various concentrations (30, 100 and 300 nM) were added in triplicate after replenishing the RPMI media with all the supplements mentioned above and incubated for four days. Microvasculature on aortic rings was photographed under microscope (Carl Zeiss, Germany) on the day of compound treatment and four days later. Photographed vasculature was quantified using AxioVision software (Carl Zeiss, Germany).

**Statistical analysis:** Results were expressed as mean ± SD for at least triplicates. Data were compared by Student’s t-test and/or one way ANOVA followed by Bonferroni’s 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). Other than PI3K-mTOR pathway it also inhibits ALK1, an important enzyme involved in angiogenesis. Using radiometric protein kinase assay (**33PanQinase activity assay**), P7170 was shown to potently inhibit mTOR and ALK1 activity with an IC\textsubscript{50} value of 4.4 nM and 47 nM (Fig. 1B), respectively. Additionally, the inhibitory activity of P7170 against PI3K was identified when screened against a commercially available panel of 320 kinases (Ambit Biosciences, USA and ProQinase, Germany), P7170 displayed a pan-PI3K inhibitory activity with IC\textsubscript{50} values ranging from 2.2 nM-203 nM (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 Table1 (Supplementary Table S1B, C). Few kinases such as ABL1 (H396P), CLK1, HIPK2, LKB1, MEK2 and PIK3GC were also inhibited on P7170 treatment at 250 nM (Supplementary Table S1C). However, upon further evaluation of their IC\textsubscript{50} values, P7170 did not inhibit these set of kinases as efficiently as mTORC1/C2 or ALK-1 (Supplementary Table S1D).

**P7170 down regulates mTOR pathway.** To further evaluate the cellular PI3K/mTOR inhibitory activity of P7170, western blot analysis was carried out in A2780 cell line (Fig 1C). A substantial proportion of ovarian tumors have been associated with deregulation of mTOR (23). Further,
A2780, ovarian cancer cell line, is reported to have amplified PIK3CA resulting in an activated PI3K/mTOR pathway (24). As shown in Figure 1C, P7170 strongly inhibited phosphorylation of Ser<sup>240/244</sup> and Ser<sup>235/236</sup>, that are substrates of mTORC1 (9), on ribosomal S6 protein. Complete inhibition of a direct target of mTORC1, phosphorylated Thr<sup>389</sup> on p70S6K, was observed with treatment as low as 10 nM of P7170. Furthermore, a decrease in phosphorylation of 4EBP1 protein at Ser<sup>65</sup> and Thr<sup>37/46</sup> was observed with P7170 and other mTORC1/mTORC2 inhibitor AZD8055 but not, as expected, with selective mTORC1 inhibitor Everolimus (9). Similarly, phosphorylation of AKT at Ser<sup>473</sup>, a mTORC2 substrate, was completely abolished on treatment with P7170 and AZD8055 but not with Everolimus. Interestingly, P7170 weakly inhibited phosphorylation of AKT at Thr<sup>308</sup>, a PI3K substrate, an observation that further emphasizes it to be a potent dual mTORC1/mTORC2 inhibitor and a weak PI3K inhibitor. 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, the (IC<sub>50</sub>= 2.2 nM) PI3Kα biochemical activity translated into weak (IC<sub>50</sub> = >100 nM) inhibitory activity in ovarian cancer (A2780) cells (Fig.1C). Some of the other kinase biochemical activities seem to be cell specific (manuscript in preparation) which is being explored intensively in our laboratories. P7170 activity was further evaluated in other cancer cell lines associated with activated PI3K/mTOR pathway (Fig. 1D). Treatment with P7170 resulted in strong inhibition of mTORC1/C2 pathway in HCT116 (colon), 786-O (renal) and PC3 (prostate) cancer cell lines as shown in Figure 1D. Using high-throughput in-cell western screening assay in PC3 cells, cellular IC<sub>50</sub>s for P7170 were calculated as 2.7nM against mTORC1 indirect substrate pS6<sup>Ser235/236</sup> and 12.4 nM against mTORC2 substrate pAKT<sup>Ser473</sup> (Fig. 1E).
**P7170 exhibits anti-proliferative activity towards various cancer cell lines and patient-derived cancer cell lines.** 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 IC₅₀s ranging between 2-22 nM across a wide array of cancer cell types. No toxicity was observed in normal cells, hPBMCs, even on treatment with highest concentration (10 µM) 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 99 patient derived cancer cell lines using a clonogenic assay. P7170 exhibited absolute mean IC₇₀ of 115 nM (Fig. 2B). Based on the mean IC₇₀ determined within the different histotypes, the most sensitive tumor types were kidney cancer (RXF), pleuramesothelioma (PXF), liver cancer (LIXF), 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 (30-1000 nM) (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 nM P7170 treated cells) and sub-G1 population increased from 1.1 % to 63.3% (1000 nM 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 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 nM 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 300nM in P7170 treated cells for 72 h (Fig. 3E). A similar pattern of acridine staining was seen in A2780 and HCT116 cancer cell lines (Suppl. 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. Further, induction of autophagy by P7170 was comparable to 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 activin like kinase 1 (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 & Methods (Suppl. 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 angiogenic processes. In comparison to BEZ 235 and Everolimus, P7170 strongly inhibited ALK1 kinase activity with IC$_{50}$ of 47 nM (Fig.1B). Both BEZ 235 and Everolimus did not demonstrate any inhibition of ALK1 up to 2 µM, the highest concentration tested (Fig.1B). Further, functional analyses in, in vivo and ex-vivo angiogenesis models (Fig.4A&B, respectively) demonstrated P7170 to significantly impair angiogenic response toward vascular endothelial growth factor (VEGF). The anti-angiogenic activity of P7170 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). Also, 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-C). This VEGF induced aortic microvasculature was inhibited by P7170 in a dose dependent manner (Fig. 4B). However, Everolimus and BEZ-235 had no significant effect on microvasculature formation (Fig. 4B). The average microvasculature on control rings was 1327 µm$^2$/µm and reduced to 42 µm$^2$/µm 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 important
for VEGF signaling and angiogenesis, neither mTOR, and/or PI3K seems to be absolutely required for microvessel formation as Everolimus (a potent mTOR inhibitor) or BEZ235 (a PI3K/mTOR dual inhibitor) were ineffective in inhibiting microvessel formation in this *ex vivo* model.

In parallel, P7170 treatment resulted in 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 is observed by P7170 in HUVEC cells that lack ALK1 suggesting ALK1 is 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 (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 dose of 15 mg/kg (76% TGI) was as efficacious as the mice treated with 25 mg/kg of BEZ235 (71%) in prostate xenograft model. No significant body weight loss was observed with increasing doses of P7170 (Fig. 5A, Inset). Furthermore, the
pharmacodynamic effects of P7170 was 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 1h of compound administration. Immunohistochemical 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 h (Fig. 5C). This pharmacodynamic activity correlated well with the concentrations of the compound in plasma (Fig. 5D).

Discussion

Phosphatidylinositol (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway has emerged as a prime strategic target of ‘drugging the cancer kinome’ that has led to the development of several novel targeted anti-cancer 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, epidermal growth factor receptor (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 army of targeted agents that inhibit key components of 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, in order 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 \textit{in vitro} and \textit{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 Everolimus-insensitive T37/46 has been coupled 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 i.e. 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 cell cycle regulatory protein. Consistent with the published reports that PI3K/AKT/mTOR pathway is indeed critical for clonogenic 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. By 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 anti-angiogenic agents as crucial therapeutic agents in cancer therapy (19, 37). Interestingly, P7170, in addition to potently inhibiting mTORC1/C2 pathway, also effectively inhibits ALK1 activity (IC₅₀ 47 nM). Activin receptor-like kinase 1 (ALK1) is a type I receptor of the transforming growth factor beta (TGF-β) receptor family, that is found on proliferating endothelial cells (38). Hu-Lowe et al 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 ALK1 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 ALK1; an antibody developed by Pfizer, PF-03446962 (Clinical Trial Identifier: NCT01911273) (40), and an ALK1 ligand trap, ACE-041, by Acceleron has accentuated the significance of targeting ALK1 in cancer drug discovery (Clinical Trial Identifier: 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 2 clinical trials (41, 42). Based on these encouraging clinical activities, inhibition of ALK1 may turn out to be a promising novel anti-angiogenic 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 doesn’t seem to be absolutely required for microvessel formation as Everolimus (a potent mTOR inhibitor) or BEZ-235 (a PI3K/mTOR dual inhibitor) were ineffective in inhibiting microvessel formation in the ex vivo model as described in Figure 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 anti-tumor 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 anti-angiogenesis (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.

Acknowledgement: Colony forming assays were performed at Oncotest, Germany. This is an extension of the data presented at AACR in 2012 (In: Proceedings of the 103rd Annual Meeting
of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2012;72 (8 Suppl):Abstract nr 3759

References


Figure Legends

**Figure 1. P7170 down regulates PI3K/mTOR signaling proteins.**  
A) Structure of P7170.  B) Kinetic profile of P7170 on mTOR inhibition and IC$_{50}$s of P7170, BEZ235 and Everolimus in ALK1 kinase assay.  C) Serum starved A2780 cells were treated with various concentrations of test compounds, P7170, AZD8055 and Everolimus, for 4 hrs and stimulated with serum for 30 min. Whole cell lysates were prepared and probed for PI3K/mTOR signaling proteins by western blot.  D) Similar experiments were performed in colon (HCT116), renal (786-O) and prostate (PC3) cancer cell lines.  E) Cellular IC$_{50}$s of P7170 against mTORC1 and mTORC2 substrates, pS6$_{\text{Ser235/236}}$ and pAKT$_{\text{Ser473}}$, respectively, determined using high-throughput in-cell western screening assay in PC3 cells.

**Figure 2. Antiproliferative activity of P7170 across various cell lines and patient derived cell lines.**  
A) Cells were seeded in a 96-well plate at a density of 3000 cells/well and treated with various concentration of P7170 for 48 h. Viability of the cells was determined by propidium iodide assay.  B) P7170 was tested for clonogenic growth inhibition of 99 patient derived cancer cell lines using soft-agar colony forming assay.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumor type</th>
<th>Doses (in mg/Kg/ QD)</th>
<th>Tumor growth inhibition (%TGI)</th>
</tr>
</thead>
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<td>PC3</td>
<td>Prostate</td>
<td>3, 10, 12.5, 15</td>
<td>28, 67, 60, 76</td>
</tr>
<tr>
<td>786-O</td>
<td>Renal</td>
<td>5</td>
<td>68</td>
</tr>
<tr>
<td>A2780</td>
<td>Ovarian</td>
<td>10</td>
<td>78</td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon</td>
<td>15</td>
<td>80</td>
</tr>
<tr>
<td>Huh-7</td>
<td>Hepatocellular</td>
<td>20</td>
<td>53</td>
</tr>
</tbody>
</table>

*QD: once daily

**Table 1: Antitumor activity of P7170 in various mouse xenograft models**
**Figure 3. P7170 induces cell cycle arrest, apoptosis and autophagy.** PC3 cells were seeded in 6-well plates at a density of 0.5-1x10^5 cells and treated with various concentrations of P7170 for 48 hr. Cell cycle profile was evaluated by flowcytometry (A). PC3 cells were treated with P7170 and apoptosis was evaluated by flowcytometry (B) and by western blot analysis of apoptosis marker, cleaved PARP (C). PC3 cells were seeded at a density of 2x10^5 Cells in a 6-well plate and treated with P7170, BEZ235 or Everolimus for 24 and 48 hrs. Cells were either stained with acridine orange for autophagy vacuoles (D) or analyzed by flowcytometry for autophagy quantification (E) or subjected to western blot for LCII - define expression (F).

**Figure 4. P7170 inhibits angiogenesis through ALK1 inhibition.** A) Nude mice were injected subcutaneously with 0.5 ml of matrigel containing 200 ng VEGF and 10 units heparin and mice treated with P7170 (10 mg/Kg), BEZ235 (25 mg/Kg) and Sorafenib (20 mg/Kg). Plugs were excised from animals, photographed; a representative photograph is presented. B) Aorta slices were excised from rats and submerged in matrigel containing 40 ng/mL VEGF. Rings were treated with test compounds at various concentrations and photographed under microscope on day 7. Microvessel formations on aortic rings were quantitated and are presented as bar diagram, (C). D) ALK1 ShRNA transfected and non-transfected HUVECs were seeded on a matrigel bed and treated with test compounds. After 24 h, tube formation was imaged under microscope and representative photographs are presented. E) ALK1 knockdown in HUVECs. HUVECs were transfected with 1 µg of ALK1 ShRNA and stained for ALK1 and nucleus. Knockdown levels of ALK1 were imaged under microscope and DAPI staining was used as an internal control.

**Figure 5. In vivo efficacy of P7170.** A) Five million PC3 cells suspended in 1:1 Matrigel and PBS were implanted subcutaneously in to nude mice. Once tumor reached 100 mm^3, mice were randomized and treated with P7170 at doses of 3, 10, 12.5, 15 mg/Kg or BEZ235 at a dose of 25 mg/Kg whereas control mice received vehicle alone. Tumor size was measured twice a week and weight was recorded every day (Inset: mice body weight). B) Pharmadynamic studies were performed after 3 doses of 10 mg/Kg of P7170. Tumors were excised from animals and evaluated for PI3K/mTOR signaling molecules by western blot. C) Immunohistochemistry showing time
kinetics of pS6 (S235/236) expression after 1 hr of P7170 administration. DAPI was used as internal control for viable cells. D) PK-PD analysis of P7170. Tumor bearing mice were treated with P7170 and tumor and blood samples were collected at different time points. Tumor lysates were evaluated for pS6, pAKT and p4EBP1 where drug content was estimated from plasma and plotted against the pS6, pAKT and p4EBP1 expression.
**FIGURE 1**

A

![Chemical structure of P7170](image)

B

**mTOR**

\[
\begin{align*}
[\text{ATP}] &= 1 \mu\text{M} \\
\text{IC}_{50} &= 4.4 \text{ nM}
\end{align*}
\]

**Activity, % of control**

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<th>Concentration (nM)</th>
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<th>300</th>
<th>1000</th>
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<tr>
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C

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ALK-1 Kinase (IC_{50} nM)</th>
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<tr>
<td>P7170</td>
<td>47</td>
</tr>
<tr>
<td>BEZ 235</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Everolimus</td>
<td>&gt;2000</td>
</tr>
</tbody>
</table>

D

![Blot images of protein expression](image)

E

![Graphs of % pS6 inhibition](image)

\[ \text{IC}_{50} : 2.7 \text{nM} \]

![Graphs of % pAKT inhibition](image)

\[ \text{IC}_{50} : 12.4 \text{nM} \]
FIGURE 2

A

![Graph showing IC₅₀ (nM) for various cell lines]

Huh-7, 786-O, A549, A2780, PC3, H460, A431, SW480, HCT116, A431, SW480, HCT15, A549, A2780, PC3, H460, A431, SW480, HCT116, MDA MB 231, HepG2, MCF7, MDA MB 468, AsPc1, hPBMC

B

Kidney [4]
Pleuramesoth. [4]
Liver [1]
Breast [4]
HSC [3]
Melanoma [11]
Lung epid. [6]
Sarcoma [3]
Bladder [5]
Prostate [2]
Head & Neck [7]
Lund adeno [16]
Lung, large [4]
Pancreas [19]
Colon [7]
Ovary [3]
Gastric [3]

Abs. IC70, (Geom. Mean = 0.115 μM)

#Tissue Types: 17   #Tumors: 102   MOA/Targets: PI3K/mTOR inhibitor   Aliases: P7170   OT-Key: 19620

log   log/2   Geom. Mean Over All   Geom. Mean of Tumor   Geom. Mean of Tissue Types
FIGURE 3

A) Cell Cycle Phase Distribution (in %) vs. P7170 Concentration (nM)

B) Apoptosis (% sub G1) vs. P7170 Concentration (nM)

C) Western Blot Images for Cleaved PARP and β-Actin with concentrations:
- DMSO
- 100 nM
- 300 nM
- 1000 nM

D) Images showing cell morphology with treatments:
- Control
- P7170
- Everolimus

E) Graph showing % of Autophagy vs. Concentration (nM) at 24HR, 48HR, and 72HR

F) Western Blot Images for LC3 A/B I and LC3 A/B II with concentrations:
- DMSO
- 30 nM
- 100 nM
- 300 nM

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### FIGURE 4

**A**

<table>
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<tr>
<th>Compounds</th>
<th>ALK-1 Kinase (IC\textsubscript{50} nM)</th>
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<tr>
<td>P7170</td>
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<td>&gt;2000</td>
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<tr>
<td>Everolimus</td>
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**B**

+ VEGF Control  | Sorafenib (30mpk)  
BEZ 235 (25mpk) | P7170 (10mpk)  

**C**

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<tr>
<td>300 nM</td>
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**D**

![Graph of Percent Microvasculature](image5)  

Values are mean ± SEM of triplicates. *p<0.01

**E**

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**F**

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<tr>
<td>ALK-1 shRNA + ALK-1</td>
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FIGURE 5

A

![Graph showing tumor weight and treatment period](image)

B

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<th>Treatment</th>
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<td>β-actin</td>
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C

Timepoint (hr)

D

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<tbody>
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Molecular Cancer Therapeutics

P7170: a novel molecule with unique profile of mTORC1/C2 and Activin Receptor-Like Kinase 1 inhibition leading to anti-tumor and anti-angiogenic activity


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