Inhibition of PI3Kβ signaling with AZD8186 inhibits growth of PTEN deficient breast and prostate tumours alone and in combination with docetaxel

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Running title: AZD8186 inhibits PI3Kβ dependent tumour growth

Key words: AZD8186, PI3Kβ, PTEN, Prostate Cancer, Breast Cancer

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Abbreviations: PTEN - Phosphatase and tensin homolog; AKT - PKB (Protein kinase B); TNBC - Triple Negative Breast Cancer; PI3K - Phosphoinositide 3 Kinase

Conflict of Interest/Financial Statement:
All authors are current or former AstraZeneca employees and shareholders. There are no other conflicts of interest to disclose.
Abstract

Loss of PTEN protein results in upregulation of the PI3K/AKT pathway, which appears dependent on the PI3Kβ isoform. Inhibitors of PI3Kβ have potential to reduce growth of tumours in which loss of PTEN drives tumour progression. We have developed a small molecule inhibitor of PI3Kβ and PI3Kδ (AZD8186) and assessed its anti-tumour activity across a panel of cell lines. We have then explored the anti-tumour effects as single agent and in combination with docetaxel in triple negative breast (TNBC) and prostate cancer models. In vitro AZD8186 inhibited growth of a range of cell lines. Sensitivity was associated with inhibition of the AKT pathway. Cells sensitive to AZD8186 (GI50<1μM) are enriched for, but not exclusively associated with, PTEN deficiency. In vivo AZD8186 inhibits PI3K pathway biomarkers in prostate and TNBC tumours. Scheduling treatment with AZD8186 shows anti-tumour activity required only intermittent exposure, and that increased tumour control is achieved when AZD8186 is used in combination with docetaxel. AZD8186 is a potent inhibitor of PI3Kβ with activity against PI3Kδ signalling, and has potential to reduce growth of tumours dependent on dysregulated PTEN for growth. Moreover AZ8186 can be combined with docetaxel, a chemotherapy commonly used to treat advance TBNC and prostate tumours. The ability to schedule AZD8186 and maintain efficacy offers opportunity to combine AZD8186 more effectively with other drugs.
Introduction

Signalling through the PI3K pathway plays an important role in regulating the growth and survival of many tumour types (1, 2). The PI3K family consists of four isoforms p110α, p110β, p110δ and p110γ. PI3Kα and β are expressed in most normal tissues, while PI3Kδ is thought to be restricted to the immune system. Activation of Class 1a PI3Ks results in the formation of phosphatidyl inositol 3,4,5 phosphate (PIP3) by phosphorylation of phosphatidyl inositol 4,5 phosphate (PIP2) (2). Formation of PIP3 is critical for activation of AKT and other PIP3 dependent effectors, which in turn leads to the activation of a number of various downstream signalling events. PI3K can be activated by extracellular stimuli via Receptor Tyrosine Kinases (RTKs), G-protein coupled receptors (GPCRs) or B-cell receptor (BCR) complexes (3). Activation of the PI3K pathway is negatively regulated by the lipid phosphatase PTEN, a tumour suppressor PTEN that controls the levels of intracellular PIP3 by dephosphorylating PIP3 to regenerate PIP2. Hyper-activation of PI3K signalling is a feature of subsets of many different tumour types. PI3Kα is commonly directly activated by mutations in the catalytic p110α subunit (4). Many tumours in which hyper-activation of RTK has occurred are dependent on PI3Kα (5). In contrast PI3Kβ is rarely mutated, however loss of PTEN protein expression creates a dependency on the PI3Kβ isoform (6,7,8). Although the mechanism that creates this dependency is unclear the switch is thought to be related to modulation of the basal PIP3 levels in the cells.

Loss of PTEN protein can occur in different ways, through deletion of one or both copies of the gene, through mutation resulting in loss of function, epigenetic regulation or by down regulation of protein by micro-RNA (2). Tumours with lower levels of PTEN protein have been observed in a number of tumour types including triple negative breast cancer (9) and prostate cancer (10,11). Tumours with reduced PTEN protein are commonly associated with poor prognosis, suggesting that inhibitors of PI3Kβ may be useful for treating subsets of a range of different cancers (8).

We describe AZD8186 a small molecule inhibitor of PI3Kβ and PI3Kδ. AZD8186 inhibits growth of a range of tumour cell lines, regulating signalling through the AKT signalling pathway. We show that
AZD8186 effectively inhibits growth of prostate and TNBC tumours, both as a single agent and in combination with docetaxel, a chemotherapy commonly used to treat both diseases.
Material and Methods

Cell Line Culture

Cells lines were grown in RPMI 1640 buffer + 10% FCS + 2mM glutamine at 37°C 5% carbon dioxide. Cell lines used for primary in vitro and in vivo experiments are listed in Supp. Table 1. Cell line identification procedures are indicated per cell line in Supp. Table 1. Cell panel screen details and associated cell line identification procedures are summarized in Davies et al (12). All cell lines were authenticated at AstraZeneca cell banking using DNA fingerprinting short-tandem repeat (STR) assays. All revived cells were used within 15 passages, and cultured for less than 6 months.

In vitro Enzyme assays

Inhibition of recombinant PI3Kβ, PI3Kα, PI3Kγ and PI3Kδ was evaluated in a Kinase Glo based enzyme activity assay. Detailed method is shown in Supplementary Methods. To evaluate a broader selectivity profile, AZD8186 was also tested across the Dundee Kinase Panel (MRC Protein Phosphorylation Unit, University of Dundee, UK) and across the panel of enzymes in the KinomeScan (DiscoveRx, Fremont, CA).

Assaying PI3K pathway suppression in cell lines

BT474c cells were plated at 5600/well in 384 well plates in DMEM (Invitrogen 1966-025) supplemented with 10% FCS and 1% glutamine (Sigma, Dorset, UK). Cells were incubated with compound dosed by Acoustic dispensing) for 2 hours. Medium was then aspirated and cell lysis buffer (25mM Tris-HCl, 3mM EDTA, 3mM EGTA, 50mM sodium fluoride, 2mM sodium orthovanadate, 0.27M sucrose, 10mM β-glycerophosphate, 5mM sodium pyrophosphate, 0.5% Triton X-100) added for 20 min incubation on ice. Cell lysates were transferred to ELISA plates pre-coated with an anti-total AKT antibody (part of PathScan pAKT (Thr308) Antibody Pair kit, Cell Signaling Technology (CST, Danvers, MA) (7144) using the Plate Mate Plus (Matrix, ThermoFisher Scientific, Loughborough, UK). The ELISA plates are then incubated overnight at 4°C before being washed & incubated with a phospho-AKT Thr308 specific antibody. Plates were washed again before addition of an anti-mouse-HRP conjugated secondary antibody. Following incubation at room temperature, plates were washed and QuantaBlu substrate working solution (Pierce, Thermo Scientific, Loughborough, UK) was added.
to each well. Development of fluorescent product was stopped by addition of stop solution to the wells & the plates read using a Safire plate reader (Tecan, Mammendorf Switzerland).

MDA-MB-468 cells plated in 384 well plates were incubated with compound for two hours. Cells were then fixed in PBS + 3.7% formaldehyde, blocked and permeabilised in one step with addition of PBS containing 0.5% Tween 20 and 1% Marvel then stained using a phospho specific AKT Ser473 antibody (CST3787) and AlexaFluor 488 conjugated secondary antibody. Inhibition was measured on the Accumen HCS system (TTP Labtech, Cambridge, UK) by a decrease in the number of fluorescently labeled cells containing phosphorylated AKT Ser473 counted per well.

JEKO cells were plated in 96 well plates and pre-incubated with compound for 1 hour at 37°C (RPMI containing 1% glutamine) before stimulating with anti-IgM F(ab’)_2 fragment goat anti-human IgM, (109-006-129, (Stratech, Newmarket, UK) (0.06μg/ml or an equivalent EC90 dose) at 37°C for 10 min. Plates were placed on ice and centrifuged at 12000rpm for 4min. On ice, media were carefully removed and 40μl lysis buffer (25mM Tris/HCL pH6.8, 3mM EDTA, 3mM EGTA, 50mM NaF, 2mM sodium orthovanadate, 270mM sucrose, 10mM beta-glycerophosphate, 5mM sodium pyrophosphate and 0.5% Triton X-100 plus protease and phosphatase inhibitors (Sigma)). Plates were incubated on ice for 5min and stored at -80°C. AKT was assayed using the phospho (Ser473)/Total AKT whole cell lysate kit according to manufacturer’s instructions (Mesoscale Diagnostics (MSD), Gaithersburg, US Kit K11100D-3).

**Western Blot analysis of pathway inhibition**

Cells were exposed to AZD8186 at concentrations ranging from 3 to 0.01μmol/L for 2 hours. Cells were then lysed on ice with a buffer containing 25mM Tris/HCL pH6.8, 3mM EDTA, 3mM EGTA, 50mM NaF, 2mM sodium orthovanadate, 270mM sucrose, 10mM beta-glycerophosphate, 5mM sodium pyrophosphate and 0.5% Triton X-100 and protease and phosphatase inhibitors (Sigma, Dorset, UK). Lysates were diluted with sample loading buffer (Invitrogen, Carlsbad, CA), separated on 4-12% Bis-Tris Novex gels, transferred onto nitrocellulose membranes and probed with primary antibodies overnight (Supp. Table 2). After a washing step, membranes were incubated with horseradish peroxidase-tagged secondary antibodies and visualised on a Syngene ChemiGenius with Super-Signal West Dura Chemiluminescence Substrate.
LPA stimulation of serum starved cells

MDA-MB-468 and PC3 cells were cultured to 70% confluence in RPMI 1640 (Life Technologies, Inc) 10% heat-inactivated FCS (Sigma, Dorset, UK), 1% L-glutamine (Sigma). Cells were serum-starved in RPMI 1640 overnight then pre-incubated with vehicle (0.1% DMSO) or AZD8186 for 1 hour, stimulated for 15 minutes with 10 μM LPA (1-oleoyl lysosphatidic acid Sodium Salt stock solution, Tocris, R&D Systems) diluted in serum-free medium (SFM) containing 1% delipidated BSA (Sigma). Cell lysates were prepared in RIPA cell lysis buffer (89900 Pierce, ThermoFisher Scientific, Loughborough, UK) containing Mini Complete Protease Inhibitor (Roche) and Halt Phosphatase Inhibitor (Pierce) and analysed by Western blotting as described above.

FOXO3a Translocation Assay

Cells were seeded into a clear bottom, black wall 96-well plate and incubated overnight at 37°C, 5% CO₂ before exposure to AZD8186 for 2 hours. Cells were then fixed with 3.7% formaldehyde, permeabilised and blocked in 0.5% Triton-X-100/5% BSA and probed with an antibody against FOXO3a (CST2497) overnight at 4°C. Following washing in PBS-Tween 0.05%, cells were incubated with a secondary antibody conjugated to an Alexa Fluor 488 dye (A11008, Invitrogen) and Hoescht nuclear stain (H3570, Invitrogen) before washing and imaging using a Cellomics Arrayscan. An algorithm measuring the ratio of nuclear to cytoplasmic fluorescence intensity was used to analyse the cells.

Cell Panel Proliferation Assays

Culture conditions, source and identity testing of cell lines included in the large cell panel are provided in (12 (Supp. Table S1)) and experimental procedures are outlined in Supplementary Methods.

Histopathological staining

See Supplementary Methods for detailed methods. Briefly formalin fixed paraffin embedded samples were sectioned, subjected to antigen retrieval and stained for phospho-AKT Thr308 (CST, 2965), phospho-AKT Ser473 (Dako, M3628), phospho-PRAS40 (CST, 299), FOXO3a (CST, 2497) Following peroxidase blocking (H₂O₂) and incubation with serum free block (Dako, X0909) for 20min to block
non-specific binding. Primary antibodies were applied for 1h (RT) and binding detected using Rabbit Envision + System-HRP-labelled Polymer (Dako) (30min). Antibodies were detected by incubation with 3,3’-diaminobenzadine (DAB) chromagen (Dako) for 10min and then counterstained with Carazzi’s haematoxylin.

Anti-tumour experiments

All animal experiments were performed to the according to the local regulations Home Office UK; Direction des Services Vétérinaires, Ministère de l’Agriculture et de la Pêche, France and in USA an AAALAC-accredited facility, by the AstraZeneca R&D Boston Institutional Animal Care and Use committee. PC3 cells (1x10^6 cells in Iscove’s serum free medium mixed 50:50 with matrigel) or HCC70 cells (1x10^6 cells in RPMI serum free medium mixed 50:50 with matrigel) tumours were implanted in the flank of female nude mice (nu/nu:Alpk) (AstraZeneca, Alderley Park, UK) between the ages of 8 and 12 weeks. MDA-MB-468 cells (ATCC) were implanted into #3 mammary fat pad (10^7/mouse) in 0.05 ml of medium without serum and Matrigel (Beckton Dickinson) at a 1:1 ratio. Experiments were performed at AstraZeneca (Gatehouse Park, USA) using female CB17 SCID mice aged 6 to 8 weeks were obtained from Charles River (Wilmington, MA). HID28 in vivo experiments were performed under contract by Xentech, 4 Rue Pierre, Fontaine, 91000 Evry, France) HID28 tumour fragments (approximately 40 mm^3) from donor animals were aseptically implanted subcutaneously in at the level of the interscapular region. Outbred athymic (nu/nu) male mice (HSD: Athymic Nude-Foxn1nu) weighing 18-25 grams (Harlan Laboratories, Gannat, France). For all animals studies groups were powered with a minimum of 8 animals per group.

AZD8186 was generally formulated once weekly as a suspension in HPMC/Tween and dosed once or twice daily (0 and 6-8 hours). For groups where ABT was administered. AZD8186 was formulated once weekly either alone in 10% DMSO / 60% TEG/ 30% WFI or in the presence of ABT at 10 mg/ml. For twice daily dosing (0 and 6-8 hours) AZD8186 was co-dosed with ABT at 0 hour and administered alone as the single formulation at 6-8 hours. Docetaxel (Sanofi-Aventis) was formulated fresh in physiological saline at 1.5mg/ml and dosed as a single i.v. bolus dose at a rate of 0.1ml/10g on Day 0, 24 hours prior to the administration of AZD8186.
Tumour volume was calculated twice weekly from bilateral caliper measurements using the formula (Length x width x width) x \( \pi/6 \). Growth inhibition from the start of treatment was assessed by comparison of the geometric mean change in tumour volume for the control and treated groups. Statistical significance was evaluated using a one-tailed, two-sample t-test.

**Pharmacodynamic studies**

When mean tumour size reached \( \sim 0.4 \text{ cm}^3 \) (PC3, HCC70) and 150mm\(^3\) (HID28, MDA-MB-468). Half the tumour was snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) the other half was fixed in 10% formalin buffer for 24 hours and then embedded in paraffin for immunohistochemical (IHC) staining. Total blood was collected by intracardiac puncture and plasma prepared and immediately frozen at \(-20^\circ\text{C}\) for pharmacokinetic analysis. For each time point a minimum of 4 or 5 tumours were used for the analysis.

Frozen tumors were homogenized using Fastprep methodology lysis matrix A (MP Biomedicals) and lysates generated using adjusted lysis buffer (1% Triton X-100). Equivalent amounts of protein (35-45µg/well) were analysed. The AKT(Ser473)/total AKT whole cell lysate Kit (Mesoscale Discovery K151OOD) was used to detect phosphorylated and total AKT. Two Life Technologies ELISAs (KHO0421 and KHO0411) were used to detect total and phospho-PRAS40. The effect of AZD8186 was calculated by comparing the geometric mean of treated groups to control for phosphorylated and total signal separately. Mouse tissue was analysed using the same AKT plates and separate plates for the PRAS ELISA (KMO0421 and KMO0411). Phosphorylated (Ser235/236) and total S6 were analysed with Mesoscale Discovery kits (K150DFD and K150DHD). All kits were used as per manufacturers’ instructions.
Results

AZD8186 selectively inhibits PI3Kβ and δ mediated signaling through AKT in vitro

AZD8186 (Fig. 1A) is an isoform specific small molecule PI3Kβ inhibitor (Barlaam et al manuscript in preparation). In biochemical assays AZD8186 potently inhibits PI3Kβ (IC50 4nM) and PI3Kδ (12 nM) with selectivity over PI3Kα (IC50 35nM) and PI3Kγ (IC50 675nM) (Table 1). Tight binding kinetics of AZD8186 means biochemical assays under-estimate the absolute selectivity profile for PI3Ks. In a broad panel of protein and lipid kinase assays selectivity for PI3Kβ and δ was > 100 fold versus 74 protein and lipid kinases. At 10μM AZD8186 had no significant binding to 442 other kinases in an Ambit screen. AZD8186 shows selectivity for PI3K family kinases, no other off-target activity was detected. In the PTEN null line MDA-MB-468 AZD8186 inhibited PI3Kβ dependent activation of pAKT (Ser473) with an IC50 of 3nM (Table 1). Potency in the PI3KCA mutant line BT474c was 752nM demonstrating selectivity for PI3Kβ over PI3Kα (Table 1; Supp. Fig.1A). IgM mediated stimulation of B cells results in phosphorylation of AKT through activation of PI3Kδ. (Supp. Fig. 1C). AZD8186 inhibited IgM stimulated phosphorylation of pAKT (Ser473) activation in JEKO cells with an IC50 of 17nM. (Table 1; Supp. Fig.1C). These data show that in cells AZD8186 is a potent inhibitor of PI3Kβ with additional activity against PI3Kδ. In cell proliferation assays AZD8186 inhibited proliferation of MDA-MB-468 cells with a GI50 of 65nM, IgM stimulated JEKO cell growth with an IC50 of 228nM. It only inhibited BT474c cell growth with an IC50 of 1.981μM (Supp. Table 3) consistent with its selectivity for PI3Kβ over PI3Kα.

PI3Kβ mediates GPCR receptor signaling, regulating thrombin and ADP mediated platelet aggregation (13, 14), as well as LPA receptor mediated signals via the small GTPase rac (15). Consistent with this AZD8186 inhibits ADP induced human platelet aggregation with a mean IC50 of 186nM (Fig.1B). In PTEN null MDA-MB-468 and PC3 cells AZD8186 inhibited LPA mediated activation of AKT (Fig.1C; Supp. Fig.2A), while the selective PI3K inhibitor BYL719 did not. In PTEN null MEF cell lines genetic ablation of PI3Kβ function does not inhibit EGF mediated activation of AKT (6). Interestingly AZD8186 inhibited EGF mediated activation of pAKT following serum starvation in MDA-MB-468 cell lines. The PI3Kα selective inhibitor BYL719 had no effect on AKT activation in this
Acute stimulation assay. This suggests that AZD8186 has the potential to inhibit signaling downstream of both GPCR and RTK activation in PTEN null lines (Supp. Fig.2A). PI3Kβ is thought to be involved in signaling downstream of certain GPCRs, and mutant rac. (16). To further assess signaling via the LPA receptor pathway inhibition in the PTEN WT mutant rac lines HT1080 and MDA-MB-157 was assessed. AZD8186 inhibited LPA mediated activation of AKT in these cell lines. Interestingly it also inhibited basal AKT activation in both lines, perhaps related to the rac mutation status. (Supp. Fig.2B). Collectively these data demonstrate that AZD8186 not only inhibits PI3Kβ dependent AKT activation resulting from PTEN deletion, but also ligand-mediated activation of AKT.

**AZD8186 inhibits AKT pathway activation and growth of multiple tumour cell lines in vitro**

To assess pathway modulation in more detail the activity of AZD8186 was tested in a range of breast and prostate cancer cell lines (Fig.2A,B). AZD8186 inhibited pathway activation in the PTEN null prostate lines LNCAP, PC3 (Fig.2A) and breast lines MDA-MB-468, HCC70 (Fig.2B). AZD8186 suppressed phosphorylation of AKT, PRAS40, S6 and FOXO with IC50s in the range of <10nM to 300nM, and full pathway inhibition at 300nM to 3μM. AZD8186 inhibited the growth of the PTEN deficient cells lines with GI50s less than 1μM (Fig.2A,B,D, Supp. Table 3). AZD8186 was less effective at reducing pathway activation in the PTEN WT prostate cell line DU145 and the breast cell line BT474, with activity only seen at higher doses and possibly reflecting PI3Kα activity in the compound. PI3K activation is reported to regulate the transcription factor FOXO, AKT mediated phosphorylation of FOXO excluding it from the nucleus (17). AZD8186 promoted translocation of FOXO to the nucleus in the PTEN deficient HCC70 and LNCAP cells at concentrations related to the potency in each cell line (Fig.2C). In LNCAP cells treatment with 500nM AZD8186 induced association of FOXO3a with the chromatin fraction (Supp. Fig.3). In contrast FOXO translocation in the BT474 cell line was only induced at the highest concentrations of AZD8186 (Fig.2C) and the pan-PI3K inhibitor GDC0941 and PI3Kα selective inhibitor BYL719 induced translocation at lower doses (Supp. Fig.1D).

Understanding mechanisms that potentially determine sensitivity is important to select the patients appropriate for treatment with AZD8186. In a broad cross-disease in vitro cell panel a cut off of GI50 < 1μM highlighted a subset of lines that were sensitive to AZD8186 (Fig.2D; Supp. Fig.4, Supp. Table 3). The AZD8186 sensitive subset was enriched for PTEN null cells. PTEN deficient cells represent
52% (13/25) of the sensitive lines, but only 8% (9/106) of the insensitive lines. Interestingly a number of lines with genetically WT PTEN were sensitive to AZD8186 (48% (12/25)), implying PTEN loss is not the only mechanism through which cells become dependent on PI3Kβ. The activity of AZD8186 in breast and prostate lines was compared with the AKT inhibitor AZD5363 (Fig.2E,F). Across the breast panel while activity was seen in cells where PTEN protein is lost, AZD8186 was also active in some PTEN WT lines. Interestingly two of these lines, MDA-MB-157 and MDA-MB-436 were not responsive to AZD5363. SGK-1 expression activation has recently been shown to be associated with reduction in sensitivity to AKT inhibition in breast cancer cell lines (18). While a relationship is seen as expected between SGK-1 expression and sensitivity to AZD5363, the relationship is not as clear for AZD8186 (Fig. 2E), and in particular MDA-MB-157 and MDA-MB-436 express SGK-1. This suggests that there are situations where there are differences the dependency on PI3Kβ or AKT signaling. In the smaller prostate sub-panel AZD8186 was active in cells lines where PTEN is lost, and had a profile closer to that of AZ5363 (Fig.2F). Full blots are shown in Supp. Fig.5. Collectively these data confirm that AZD8186 is effective in breast and prostate lines, but that in breast lines in particular activity is not restricted to PTEN protein null lines.

**AZD8186 modulates pathway biomarkers and inhibits growth of breast and prostate tumour models**

To assess single agent efficacy of AZD8186 *in vivo*, anti-tumour activity was assessed in the PTEN null TNBC models HCC70, MDA-MB-468 (Fig.3A,E), and the prostate models PC3, HID28 (Fig.3I,J). At 50mg/kg and 25mg/kg BID AZD8186 inhibited the growth of all four models. At 25mg/kg and 50mg/kg HCC70 were inhibited 62% (p<0.001) and 85% (p<0.001) respectively, MDA-MB-468 were inhibited at 47% (p<0.001) and 76% (p<0.001) respectively at end at of study, with regression early in the study (Fig.3A,E). Efficacy in the PTEN null prostate model PC3 was less pronounced with 25mg/kg and 50mg/kg giving maximal growth inhibition of 59% (p<0.001) and 64% (p<0.001) respectively (Fig.3I). In contrast AZD8186 gave 79% (P<0.001) growth inhibition in the PTEN null prostate explant model HID28 (Fig.3J). In mouse AZD8186 has a short half-life delivering a pharmacokinetic profile which results in intermittent cover over a 24 hours dosing interval (Fig.3K). To increase the time of exposure animals bearing PC3 tumours were co-dosed with AZD8186 in the presence of the Cyt P450 inhibitor ABT, which resulted in significantly increased exposure (Fig.3K).
This also increased the efficacy in the PC3 model with 86% (p<0.005) reduction in tumour growth achieved with 30mg/kg AZD8186 + ABT.

Changes in pathway pharmacodynamic biomarkers were investigated. AZD8186 inhibits pAKT (Ser473 and Thr308) and pPRAS40 following acute or chronic dosing in HCC70 (Fig.3B,C,D) and MDA-MD-468 (Fig.3F,G,H) in a dose and time dependent manner. Pathway modulation in HID28 and PC3 was similar (Supp. Fig.6A,B). Co-dosing with ABT increased the time of pathway suppression in PC3 (Supp. Fig.6C). Analysis of multiple biomarkers by ELISA demonstrates that AZD8186 gives dynamic biomarker modulation consistent with the pharmacokinetic profile. In addition to pathway biomarkers the ability of AZD8186 to induce translocation of the transcription factor FOXO to the nucleus was investigated (Fig.4). IHC analysis shows that FOXO translocation correlates with the inhibition of pAKT.

To determine whether chronic or daily pathway inhibition was required to maintain anti-tumour activity HCC70 tumours were treated with AZD8186 for 4, 5 and 7 days out of a 7 day cycle (Fig. 3N, M). Dosing for 4 days at 50mg/kg bid reduced tumour growth by 75% (p<0.05) vs. 88% (p<0.05) with constant dosing. When dosed for 5 days at 25mg/kg bid reduced tumour growth by 87% (p<0.05) compared to 93% (p<0.05) with constant dosing. A 2 days on, 5 days off schedule to AZD8186 was inactive. AZD8186 selectively modulates AKT activation in PTEN null tumour tissues. Both AZD8186 and the pan-PI3K inhibitor GDC-041 reduce growth of HCC70 and PC3 tumours (Supp. Fig.7A,C) and phosphorylation of AKT (Supp. Fig.7B,D), however only GDC-0941 gave significant inhibition of pAKT in lung tissue (Supp. Fig.7B,D). Collectively these data establish that AZD8186 is capable of modulation PI3K pathway activation in PTEN null TNBC and Prostate tumour models, resulting in decreased tumour growth. Monotherapy activity is retained with a minimum of 4-5 days drug exposure.

**AZD8186 combines with docetaxel to give improved tumour control**

TNBC and late stage prostate cancer are commonly treated with docetaxel. HCC70 and PC3 tumour xenografts were treated with a single dose of docetaxel, and with a continuous daily dose of 25 and 50 mg/kg AZD8186 (Fig.5A,B). In HCC70 tumours combining 25 and 50mg/kg AZD8186 BID with 15 mg/kg docetaxel gave greater tumour control compared to each monotherapy (Fig.5A). Likewise in
PC3 tumours 10mg/kg and 30mg/kg AZD8186 with 15 mg/kg docetaxel showed regressions. Both continuous and intermittent dosing (5 days on 2 days off) of AZD8186 combination with docetaxel in HCC70 tumours gave equal benefit in combination with docetaxel (Fig.5C,E). Benefit was achieved with 2 days exposure of AZD8186 in combination with docetaxel, but this was less effective than 5 days or continuous dosing. Collectively these data demonstrate that in PTEN null TNBC and prostate tumours AZD8186 can be combined effectively with docetaxel resulting in tumour regression or stasis.
Discussion

AZD8186 is a potent inhibitor of PI3Kβ with additional activity versus the PI3Kδ isoform. Although biochemical assays suggest a modest selectivity window within the Class I PI3K family, cell based assays show a clear selectivity for PI3Kβ and PI3Kδ. There is a 5 fold difference in relative potency versus PI3Kδ. Despite this PI3Kδ is likely to be relevant both in vitro and in vivo at concentrations that inhibit PI3Kβ. The selectivity for PI3Kβ over PI3Kα is mirrored in vivo with AZD8186 modulating AKT and associated pathway biomarkers in a range of PTEN null tumour models, but not in PTEN WT tumour models where pan PI3K family inhibitors are active. Moreover AZD8186 showed limited effects on normal tissues compared to the pan-PI3K inhibitors.

While AZD8186 showed preferential activity in cells with mutation or deficiency in PTEN protein, some WT PTEN cells were also sensitive. It is possible that other mechanisms regulate PTEN function in these apparently WT cell lines. For example inactivation by oxidative stress and/or loss of TXNIP expression, which restores PTEN function inactivated by oxidative stress, could create a “PTEN-mediated” dependency (19, 20), inducing PI3Kβ sensitivity. PI3Kβ activation by GPCRs for LPA, SIP and thrombin via rac / DOCK180 (15) may also be important in these lines. Interestingly one of the more sensitive lines, MDA-MB-157, expressed mutant rac (16). Loss of PTEN expression is common across many tumour types, TNBC (9), prostate (10,11), head and neck (21), CRC (22,23) and squamous lung (24) suggesting inhibitors of PI3Kβ are relevant in many diseases. Gaining insight into the why PTEN WT cells are sensitive will broaden the application of these differentiated PI3K inhibitors.

A number of PTEN null lines were insensitive to AZD8186, consistent with the hypothesis that multiple factors can influence the dependency on PI3Kβ. Understanding which pathways render the PTEN protein null cells less dependent on PI3Kβ will inform both patient selection, and combination opportunities to maximise the benefit of these agents. Candidate pathways include IGFR, EGFR, alternate PI3K pathway drives and the presence of RAS and RAF mutations (25, 26, 27). It cannot be excluded that the additional PI3Kδ activity also contributes.
In vivo AZD8186 inhibits the growth of multiple PTEN deficient tumour models (unpublished data). Here we show inhibition in models of TNBC and prostate cancer. In mouse AZD8186 has a short exposure profile which we have exploited to determine how target cover influences the efficacy. In most models robust anti-tumour activity is seen with a PK profile that does not achieve 24hr cover. Extending the exposure in PC3 by co-dosing with ABT increased efficacy suggesting that in this model longer pathway suppression is required to drive a single agent anti-tumour effect. While it is possible to drive greater efficacy with increased cover it appears that in the majority of sensitive models intermittent inhibition of the pathway is sufficient. The anti-tumour effects are largely through inhibition of tumour cell signalling as in the tumour models tested AZD8186 did not impact blood vessels, stroma, or inflammatory infiltrate.

Achieving anti-tumour effect with intermittent pathway suppression is advantageous. Given treatment with AD8186 has the potential to induce feedback through FOXO regulated genes activating other pro-proliferative signalling pathways (21, 23), intermittent pathway suppression could reduce the feedback loop induced resistance as a result of up-regulation of IGF-R, IRS-2 or ERB mediated signaling as seen with AKT inhibitors (25, 26, 27). Moreover when combining with other agents, being able to schedule each therapy to maximise therapeutic index is also an advantage (3). AZD8186 can achieve anti-tumour activity when dosed for 4 or 5 days out of a 7 day cycle, reducing the dose intensity of therapy, and establishing drug holidays. This allows effective combination with chemotherapy, or other targeted agents with known side effects. Further work exploring the degree of pathway suppression required achieve optimal therapeutic activity across a range of models will be required to optimise the most effective clinical schedule (3).

In triple negative breast and prostate cancer, docetaxel is a standard of care chemotherapy. AZD8186 together with a single dose of docetaxel gave improved tumour growth inhibition in both PC3 and HCC70 models. It is interesting that growth inhibition achieved following a single dose of docetaxel in combination with AZD8186 is sufficient to give prolonged tumour control when treatment is maintained. A possible explanation is that given AD8186 does not induce a profound cell cycle arrest in these cell lines then additional stress of the chemotherapy and inhibition of the PI3Kβ pathway induces a greater effect on the tumour. Interestingly again the reduced dosing schedule was sufficient.
to maintain this improved tumour control in the HCC70 tumours. It is likely that agents targeting PI3Kβ will give greatest benefit when used in combination, therefore combination with androgen receptor therapy and other molecular targeted therapies will be explored.

AZD8186 also inhibits PI3Kδ, however this study has not explored the potential for AZD8186 in tumour types sensitive to PI3Kδ inhibition. Although PI3Kδ is reported to play a role in some solid tumour settings, in our models inhibitors with preferential activity to PI3Kδ are not active. PI3Kδ signals downstream of the B cell receptors (28) and PI3Kδ inhibitors show more profound activity in some haematological disease (29, 30) including Chronic Lymphocytic Leukaemia (31, 32), and Mantle Cell Lymphoma (33). The PI3Kβ/δ profile of AZD8186 may provide benefit in haematological disease.

In summary AZD8186, a small molecule inhibitor of PI3Kβ/δ, shows promising activity in PTEN protein deficient lines, with activity seen in PTEN WT lines suggesting broad potential for PI3Kβ inhibitors in a number of tumour settings. AZD8186 is currently being tested in a Phase I clinical trial.
ACKNOWLEDGMENTS

We would like to thank Kathryn Cronin and Stefan Symeonides for comments on the manuscript, and acknowledge the support of the staff in Block 41 for in vivo studies.
References


Table 1. Potency of AZD8186 in biochemical and cell based assays.

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<thead>
<tr>
<th>Biochemical assay</th>
<th>IC50 mM +/- SEM (n)</th>
<th>Cell Isoform selectivity: cell line</th>
<th>Modulation of pAKT IC50 mM ± SEM</th>
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<tbody>
<tr>
<td>PI3Kα</td>
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<td>PI3Kα: BT474</td>
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<td>PI3Kγ</td>
<td>0.675±0.114</td>
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<td>NT</td>
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</table>
Figure Legends

Figure 1 Activity of AZD8186. (A) AZD8186. (B) Inhibition of ADP-induced platelet aggregation, representative curve and mean IC50 186 ± 36nM (SEM) (n=3). (C) LPA (10mM) activation of serum starved MDA-MB-468 and PC3 cells in the presence or absence of 250nM AZD8186 as indicated. Western blots for pAKT (Ser473), pERK, total AKT and total ERK, with vinculin as a loading control. Representative of 3 similar experiments.

Figure 2 AZD8186 inhibits phosphorylation of AKT and downstream pathway proteins inducing nuclear translocation of FOXO3a in vitro. (A) Western blot of protein phosphorylation reduction by AZD8186 in three prostate cancer cell lines. Inhibition of proliferation shown as GI50 (μM ±SEM). (B) Western blot analysis of protein phosphorylation reduction by AZD8186 in three breast cancer cell lines. Inhibition of proliferation shown as GI50 (μM±SEM). (C) Nuclear accumulation of FOXO3a in HCC70, LNCAP and BT474 cells. Representative images show Hoescht (blue) and FOXO3a (green) localization at 20X magnification in control cells and cells treated with 0.1μM and 0.5μM AZD8186 for 2 hours. A representative dose response plot (of 3 experiments) of relative nuclear translocation for each line is shown (mean relative Foxo translocation (±SD)). (D) AZD8186 activity in a 72hr MTS cell proliferation assay; relationship of most sensitive lines (classified as <1μM GI50) with PTEN genetic status (green = WT, red = MT, blue = increased copy number, grey = incomplete data) (E,F) Sensitivity of AZD8186 and AZD5363 (AKTi) in MTS cell proliferation assay and relationship to SGK-1 mRNA expression in a panel of (E) breast cancer cell lines and (F) prostate cancer cell lines. SGK expression: blue low, red high expression. GI50 data, red bars resistant, green bars sensitive to compound. Western blot of PTEN protein expression for each cell line is shown. NT not tested.

Figure 3 In vivo activity of AZD8186. (A) HCC70 tumours treated with vehicle (closed circles) 50mg/kg (closed triangles) and 25mg/kg (open circles) bid. Modulation of the biomarkers (B) pAKT Ser473 (C) pAKT Thr308 (D) pPRAS40 in HCC70 tumours by 50mg/kg and 25mg/kg AZD8186 the times indicated. Mean % inhibition (±SD) is shown. (E) MDA-MB-468 tumours treated with vehicle (closed circles) 50mg/kg (closed triangles) and 25mg/kg (open circles) bid. Modulation of the biomarkers (F) pAKT Ser473 (G) pAKT Thr308 (H) pPRAS40 in MDA-MB-468 tumours by 50mg/kg
and 25mg/kg AZD8186 at times indicated. Mean % inhibition (±SD) is shown. (I) PC3 tumours treated with vehicle (closed circles) AZD8186 at 25mg/kg (open triangles) and 50mg/kg (closed triangles) bid. (J) HID28 tumours were treated with vehicle (closed circles) AZD8186 at 50mg/kg (closed triangles) bid. (K) Representative PK profile of AZD8186 dosed bid with and without ABT (once in a 24 hours dosing cycle). (L) PC3 tumours treated with vehicle + ABT (closed circles) and 30mg/kg AZD8186 + ABT (open circles). (M) HCC70 tumours treated with vehicle (closed circles), AZD8186 at 50mg/kg BID (closed triangles), 50mg/kg bid (4days on, 3days off) (inverted closed triangles), or (N) treated with vehicle (closed circles), AZD8186 at 25mg/kg bid (closed triangles), 25mg/kg bid (5days on, 2days off) (inverted open triangles). Geometric mean tumour volume +/- SEM are shown. Statistical significance relative to control * p <0.05, ** p <0.01, *** p < 0.001.

Figure 4. AZD8186 modulates pAKT and nuclear translocation of FOXO3a. Representative images of pAKT Ser473 and FOXO3a staining in HCC70, MDA-MB-468, PC3 and HID-28 tumours. Vehicle treated and AZD8186 treated sample are shown. PC3 samples, vehicle control (8h post-dose) and 30mg/kg AZD8186 (30min post-dose). For HID28, vehicle and 50mg/kg AZD8186 (2h post-dose). For HCC70, vehicle and 50mg/kg AZD8186 (30min post-dose). For MDA-MB-468, vehicle and 50mg/kg AZD8186 (2 hours post-dose). Images were taken at 20x magnification. (A) pAKT Ser473 staining. (B) Foxo3a staining.

Figure 5. AZD8186 combines with docetaxel. (A) HCC70 tumours treated with control (closed circles), single dose 15mg/kg docetaxel (closed star), and single dose (arrow) 15mg/kg docetaxel with 50 mg/kg (closed triangles) or 25mg/kg (inverted open triangles) bid. Right hand graph, single agent AZD8186 50 mg/kg (closed triangles) and 25mg/kg (inverted open triangles). (B) PC3 tumours treated with control (closed circles), single dose (arrow) 15mg/kg docetaxel (closed star) and single dose 15mg/kg docetaxel with 30 mg/kg (closed triangles) and 10 mg/kg (inverted open triangles) bid. The single agent effect for 30 mg/kg (closed triangles) and 10 mg/kg (inverted open triangles) are shown in the right hand graph. (C) HCC70 tumours treated with vehicle (closed circles) and 25mg/kg AZD8186 bid (inverted open triangles); (D) with vehicle (closed circles), single dose (arrow) docetaxel (15mg/kg) (closed stars) alone or in combination with 25mg/kg AZD8186 (bid) (inverted closed triangles), or 25mg/kg 2 days on 5 days off (inverted open triangles); (E) with vehicle (closed circles),
single dose (arrow) docetaxel (15mg/kg) (closed stars) alone or in combination with 25mg/kg AZD8186 (bid) (inverted closed triangles), or 25mg/kg 5 days on 2 days off (inverted open triangles). Geometric mean tumour volume +/- SEM are shown. Statistical significance: on combination studies * indicates comparison with the docetaxel alone control and # indicates comparison with the corresponding AZD8186 monotherapy control. * p <0.05, ** p <0.01, *** p < 0.001.
Figure 1

**A**

Chemical structure of AZD8186.

**B**

Graph showing concentration IC50 of AZD8186: 186 ± 36nM.

**C**

Western blot analysis of MDA-MB-468 and PC3 cells with the following treatments:
- 0.1% DMSO ctrl
- 250nM AZD8186
- 10μM LPA
- pAKT Ser473
- pERK 1/2 Thr202/Tyr204
- Total AKT
- Total ERK 1/2
- Vinculin

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Figure 3

A. Tumour volume (cm$^3$) of HCC70 cells treated with AZD8186 at different doses and treatment schedules.

B. % normalised control of pAKT: S473 for HCC70 cells.

C. % normalised control of pAKT: T308 for HCC70 cells.

D. pRAS40: T246 for HCC70 cells.

E. Tumour volume (cm$^3$) of MDA-MB-468 cells treated with AZD8186 at different doses and treatment schedules.

F. % normalised control of pAKT: S473 for MDA-MB-468 cells.

G. % normalised control of pAKT: T308 for MDA-MB-468 cells.

H. pRAS40: T246 for MDA-MB-468 cells.

I. Tumour volume (cm$^3$) of PC3 cells treated with AZD8186 at different doses and treatment schedules.

J. Tumour volume (cm$^3$) of HD28 cells treated with AZD8186 at different doses and treatment schedules.

K. Free plasma concentration of AZD8186 at different doses and treatment schedules.

L. Tumour volume (cm$^3$) of PC3 cells treated with AZD8186 at different doses and treatment schedules.

M. Tumour volume (cm$^3$) of HCC70 cells treated with AZD8186 at different doses and treatment schedules.

N. Tumour volume (cm$^3$) of HCC70 cells treated with AZD8186 at different doses and treatment schedules.
Figure 4

A  pAKT Ser473
Vehicle control  AZD8186

HCC70

MDA-MB-468

PC3

HID-28

B  FOXO3a
Vehicle control  AZD8186

50µm

50µm
Figure 5

**A** HCC70 Continuous Tumour volume (cm$^3$) Days of treatment

**B** PC3 Continuous Tumour volume (cm$^3$) Days of treatment

**C** HCC70 Intermittent Tumour volume (cm$^3$) Days of treatment

**D**

**E**

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Molecular Cancer Therapeutics

Inhibition of PI3Kβ signaling with AZD8186 inhibits growth of PTEN deficient breast and prostate tumour alone and in combination with docetaxel

Urs Hancox, Sabina Cosulich, Lyndsey Hanson, et al.

Mol Cancer Ther Published OnlineFirst November 14, 2014.

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0406

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