Title: AZD4547: An orally bioavailable, potent and selective inhibitor of the Fibroblast Growth Factor Receptor tyrosine kinase family.

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EDITED PRÉCIS: This study offers preclinical proof that AZD4547, a potent and highly selective small molecule inhibitor of the FGFR tyrosine kinase family, shows significant efficacy in a xenograft tumour model harboring deregulated FGFR pathway signaling, with implications for the treatment of FGFR-dependent tumors.
Abstract

The fibroblast growth factor (FGF) signaling axis is increasingly implicated in tumorigenesis and chemoresistance. Several small molecule FGF-receptor (FGFR) kinase inhibitors are currently in clinical development, however, the predominant activity of the most advanced of these agents is against the kinase insert domain receptor (KDR), which compromises the FGFR selectivity. Here we report the pharmacological profile of AZD4547, a novel and selective inhibitor of the FGFR1, 2 and 3 tyrosine kinases. AZD4547 inhibited recombinant FGFR kinase activity in vitro and suppressed FGFR signaling and growth in tumor cell lines with deregulated FGFR expression. In a representative FGFR-driven human tumor xenograft model, oral administration of AZD4547 was well tolerated and resulted in potent dose-dependent antitumor activity, consistent with plasma exposure and pharmacodynamic modulation of tumor FGFR. Importantly, at efficacious doses, no evidence of anti-KDR related effects were observed, confirming the in vivo FGFR selectivity of AZD4547. Taken together, our findings show that AZD4547 is a novel selective small molecule inhibitor of FGFR with potent antitumor activity against FGFR deregulated tumors in preclinical models. AZD4547 is under clinical investigation for the treatment of FGFR-dependent tumors.
Introduction

Fibroblast growth factors (FGFs) and their receptors perform key roles in multiple biological processes including tissue repair, hematopoiesis, angiogenesis and embryonic development. The FGF receptor (FGFR) family comprises four main members (FGFR1-4), some of which have multiple protein isoforms as a consequence of alternative splicing (1). FGFRs have a core structure containing an extracellular ligand-binding domain, a hydrophobic transmembrane domain and an intracellular kinase domain. FGF-ligand binding results in FGFR dimerization, followed by receptor autophosphorylation and activation of downstream signaling pathways. In addition to the vast complexity of a system represented by 4 receptors and 18 ligands, additional control is provided by the interaction of heparin and heparin sulphate proteoglycans (HSPGs) which modulate ligand binding to receptor (2). Cell- and tissue-specific receptor expression patterns provide a final level of control which reflects their differential roles across a variety of tissues and cell lineages.

Aside from their normal physiological roles described above, FGFs and FGFRs are emerging as oncogenes that drive proliferation of a significant proportion of human tumors and which can also mediate resistance to both cytotoxic and targeted agents. Deregulation of FGFR-signaling has been documented in clinical samples of breast (3), multiple myeloma (4), bladder (5), endometrial (6), gastric (7) and prostate cancers (8). The most frequently reported tumor-associated finding is receptor overexpression due to gene amplification or aberrant transcriptional regulation. Consequently, tumor cells acquire enhanced sensitivity to FGF ligands or become activated through...
spontaneous ligand-independent dimerization (9-13). Genetically altered forms of FGFR proteins (e.g. chimeric, truncated, mutated) have also been identified in human tumors, resulting in receptors which are either constitutively active or exhibit altered ligand-dependence (12, 14). Moreover, receptor activation through gene fusion is exemplified by the discovery of numerous FGFR1-fusion proteins in myeloproliferative disorders (15-17). Finally, FGF2 and FGFR4 have both been associated with protection against etoposide and doxorubicin-induced apoptosis in vitro (18-21), whilst increased circulating FGF2 levels show an association with tumor progression following clinical suppression of VEGF-signaling (22,23).

Blockade of FGF/FGFR signaling as a therapeutic approach to cancer is gaining momentum with a number of targeted kinase inhibitors currently in development. The most clinically advanced of these agents are mainly mixed kinase inhibitors with dominant anti-KDR pharmacology (brivanib alaninate (24), dovitinib (11), BIBF-1120 (25) and SU6668 (26). As such, their anti-FGFR activity is often relatively weak and thus, we do not believe these agents offer the opportunity to fully test the anti-FGFR hypothesis because of the confounding effects of their other pharmacological mechanisms. Accordingly, we postulate that there is a need for compounds that are more FGFR-selective, which can be used to more robustly test the FGFR tumor-drive hypothesis. In this report, we describe the preclinical profile of AZD4547, a potent and selective FGFR tyrosine kinase inhibitor currently in Phase I clinical studies.
Materials and Methods

AZD4547. N-[5-[2-(3,5-Dimethoxyphenyl)ethyl]-2H-pyrazol-3-yl]-4-(3,5-diethylpiperazin-1-yl)benzamide (AZD4547, AstraZeneca, UK; Fig.1) was synthesized according to the processes described in the International Patent Application Publication Number WO2008/075068, in particular as described in Example 80. The free base of AZD4547 (molecular weight = 463.6) was used in all preclinical studies. For in vitro studies, AZD4547 was prepared as a 10 mmol/L stock solution and diluted in the relevant assay media. For in vivo studies, AZD4547 was formulated in a 1% (v/v) solution of polyoxyethylenesorbitan monooleate (Tween 80) in deionized water. Animals were given AZD4547 (1.5mg/kg – 50mg/kg) or vehicle control once daily (qd) or twice daily (bid) by oral gavage.

Cell culture. KG1a, Sum52-PE, MCF7 and KMS11 cell lines were routinely grown in RPMI 1640 supplemented with 10% (v/v) FCS (Biochrom AG) and 2 mmol/L L-glutamine (Invitrogen). The KMS11 cell line was obtained from Jonathan Keats (Scottsdale, USA), KG1a from ATCC (Manassas, USA), MCF7 from the Imperial Cancer Research Fund (London, UK) and Sum52-PE from Asterand (Royston, UK). These cell lines were genetically tested and authenticated using the Powerplex 16 system (Promega) and were not cultured for more than 6 months prior to performing the work described here.

Inhibition of cellular receptor phosphorylation. For FGFR phosphorylation studies, FGFR1,3 or 4 transfected Cos-1 cells were cultured in DMEM growth media supplemented with 2 mM L-glutamine and 3% fetal calf serum (FCS). For FGFR2, Sum52-PE cells were cultured in RPMI 1640 (Gibco) growth
media supplemented with 2 mM L-glutamine and 10% FBS. Following 1 hour incubation with AZD4547, media was removed, cells were fixed, permeabilized and then incubated with monoclonal anti-phospho-FGFR antibody (Cell Signaling Technology) (1:1000) for 1 hour followed by incubation with anti-mouse Alexa Fluor 594 secondary antibody (1:500) and Hoechst (1:1000) for 1 hour. Fluorescence measurement was performed using Arrayscan (Cellomics).

For KDR phosphorylation studies, primary human umbilical vein endothelial cells (HUVECs) were obtained from Promocell and cultured according to the supplier’s protocol. Cells were incubated with AZD4547 for 90 minutes and then stimulated for 5 minutes with VEGF ligand (25 ng/well). Cells were lysed with standard radioimmunoprecipitation assay (RIPA) buffer containing phosphatase/protease inhibitors. Lysates were analyzed using the Human phospho-VEGF R2 (KDR) (R&D systems) ELISA protocol according to the manufacturer’s instructions.

For insulin-like growth factor-1 receptor (IGF1R) phosphorylation studies, R+ cells were derived from murine transgenic IGF1R knockouts and then stably transfected with human IGF1R. Cells cultured in DMEM (Gibco) supplemented with 1% heat inactivated FCS and 1% L-glutamine were incubated with AZD4547 and then stimulated with IGF ligand (Gropep IMOOI), followed by fixation, blocking and incubation with a rabbit anti-phospho IGF-1R/IR antibody (Biosource) (1:350) for 1 hour. Secondary detection and measurement was performed using an Acumen Explorer HTS Reader (TTP Labtech Ltd) at excitation wavelength of 488 nm and emission wavelength of 530 nm.
In vitro protein expression analysis and kinase inhibition studies. Cells were treated with AZD4547 or control for 3 hours at 37°C and then stimulated with 10 ng/ml aFGF/bFGF (Sigma) and 10 μg/ml heparin for 20 minutes. Western blotting was performed using standard SDS-PAGE procedures and antibody incubation performed overnight at 4°C. Antibodies were obtained from the following sources; FGFR1 (Epitomics), FGFR2 and FRS2 (R&D Systems), FGFR3 proteins (AbCam), α-tubulin-B512 and Bcl2 (C2) (Santa Cruz), BIM (Millipore). All other antibodies were sourced from Cell Signalling Technologies. Secondary antibodies were applied and immunoreactive proteins visualized using ‘SuperSignal West Dura’ Chemiluminescence substrate according to the manufacturer’s instructions (Pierce).

In vitro cell line proliferation studies. In vitro antiproliferative activity was measured as described previously (27). Each experiment was carried out at least in triplicate and data presented as geometric means.

In vitro cell cycle and apoptotic induction analysis. Cell lines were incubated with fixed concentrations of AZD4547 for 72 hours. For fluorescence activated cell sorting (FACS), cells were fixed using 70% ethanol and then incubated with propidium iodide/RNASe A (Sigma) labeling solution. Cell cycle profiles were assessed using a FACScalibur instrument and CellQuest analysis software (Becton Dickinson). For apoptosis analysis, cells and media were gently harvested and centrifuged, followed by washing of cell pellets. Cells were then processed for Annexin V-FITC staining and propidium iodide uptake according to the manufacturer’s instructions (Abcam). The proportion of cells staining positive for Annexin V were then assessed using a
FACScalibur instrument and quadrant sorting using CellQuest analysis software (Becton Dickinson).

**Murine plasma pharmacokinetic analyses.** Plasma samples were extracted by protein precipitation in acetonitrile, followed by LC-MS/MS detection. In short, plasma samples and standards were quenched with acetonitrile and internal standard and centrifuged. Supernatant was diluted 10-fold with deionized water and the samples were analyzed by LC-MS/MS using Masslynx and data processed using Quanlynx software (Waters).

**Immunohistochemistry.** Antigen retrieval was performed on formalin-fixed, paraffin-embedded tissues using a RHS-1 microwave vacuum processor (Milestone) at 110°C for 5 minutes in pH 6 retrieval buffer (S1699; Dako) and the following primary antibodies used: custom rabbit polyclonal antibody raised to COOH-terminal peptide of mouse CD31 (CHG-CD31-PI; AstraZeneca; 1:600), mouse monoclonal anti Ki-67 MIB-1 (Dako M7240; 1:100) and rabbit polyclonal anti-cleaved caspase 3 (Asp 175) (#9661, Cell Signaling Technology, 1:100). The appropriate Envision or biotinylated rabbit anti-goat immunoglobulin secondary antibodies were used (K4007/K4003; Dako, PK-6101; Vector Laboratories, Inc) and staining was detected using diaminobenzidine (K3468; Dako). Finally, sections were counterstained with Carazzi's hematoxylin and image analysis was performed using the Aperio Digital Pathology System (Aperio Technologies, Inc). Analysis thresholds were set and applied to all tumors within the study using the Aperio Micro Vessel Density (MVD) and nuclear software algorithms (CD31, cleaved caspase 3 and Ki67). MVD was expressed as mean number of CD31-positive vascular structures per mm² of viable tumor. Nuclear staining was expressed
as a percentage positive score (number of positive nuclei out of the total number of nuclei stained).

**In vivo tumor studies.** Swiss derived nude (nu/nu) and severe combined immunodeficient mice (Charles River) were housed in negative-pressure isolators (PFI systems Ltd.) at AstraZeneca (Alderley Park, United Kingdom). All experiments were conducted on 8–12 week old female mice in full accordance with the UK Home Office Animal (Scientific Procedures) Act 1986. Tumor xenografts were established by s.c. injection into the left flank with 0.1 ml tumor cells (1x10⁶ for LoVo, 1x10⁷ for HCT-15 and 1x10⁷ for Calu-6) or 0.2 ml (2x10⁷ for KMS11 and KG1a) mixed 1:1 with matrigel (Becton Dickenson), with the exception of LoVo and HCT-15 which did not include matrigel. Mice were randomized into control and treatment groups when tumors reached the determined size >0.2 cm³. Tumor volume (measured by caliper), animal body weight and tumor condition were recorded twice weekly for the duration of the study. Tumor volume was calculated as described previously (28).

**Pharmacodynamic studies.** Tumor samples and blood were collected from KMS11 tumor bearing mice at various time points post single dose of either AZD4547 or vehicle. Frozen tumor samples were lysed in 1x cell lysis buffer (Cell Signaling Technologies) containing phosphatase and protease inhibitors (Sigma) using a Fast Prep Homogenizer (MP Biomedicals). Tumor phospho-FGFR3 was measured by ELISA according to the manufacturer's instructions (DYC2719, R&D systems).

**In vivo cardiovascular studies.** Blood pressure, heart rate and activity patterns were collected from conscious, unrestrained rats using the
Results

**AZD4547 is a highly potent inhibitor of FGFR tyrosine kinases 1–3 and shows selectivity versus a range of additional kinases.** AZD4547 potently inhibits the tyrosine kinase activities of recombinant FGFR1,2 and 3 in vitro (IC$_{50}$ values of 0.2, 2.5 and 1.8 nmol/L, respectively; Table 1) and displays weaker activity against FGFR4 (IC$_{50}$=165 nmol/L). In vitro drug selectivity was examined against a diverse panel of representative human kinases and AZD4547 shown to inhibit recombinant VEGFR2 (KDR) kinase activity with an IC$_{50}$ of 24 nmol/L. However, when compared with FGFR1, this represents a selectivity of approximately 120-fold. Excellent selectivity for FGFR was observed across a range of unrelated tyrosine and serine/threonine kinases including IGFR (>2900-fold), CDK2 (>50,000-fold) and p38 (>50,000-fold). Broader kinase selectivity was explored using 0.1 μmol/L of AZD4547 against a range of recombinant kinases incubated with ATP at or near the appropriate enzyme K$_{m}$ concentration. No enzyme inhibition was detected against ALK, CHK1, EGFR, MAPK1, MEK1, p70S6K, PDGFR, PKB, Src, Tie2 and PI-3-kinase (data not shown).

Due to potent AZD4547 recombinant enzyme inhibition, FGFR1–4, IGFR and KDR were established as cellular phosphorylation assays and used to generate AZD4547 IC$_{50}$ values (Table 1). In cells, AZD4547 potently inhibits auto-phosphorylation of FGFR1,2 and 3 tyrosine kinases (IC$_{50}$ values of 12, 2 and 40 nmol/L, respectively; Table 1) and displays weaker inhibition of
FGFR4 cellular kinase activity (IC$_{50}$ = 142 nmol/L). Significantly weaker inhibitory activity was observed versus cellular KDR and IGFR ligand-induced phosphorylation (IC$_{50}$ values of 258 and 828 nmol/L, respectively), representing approximately 20- and 70-fold selectivity over cellular FGFR1.

**AZD4547 has potent in vitro antiproliferative effects on tumor cell lines with deregulated FGFR expression.** Three tumor cell lines were selected on the basis of known abnormalities in FGFR expression and signaling. KG1a is an acute myeloid leukemia cell line which expresses a truncated wild-type FGFR1 fusion protein (30); Sum52-PE is a breast cell line which expresses wild-type FGFR2 (31) and the KMS11 multiple myeloma line expresses a t(4;14) translocated/Y373C mutated FGFR3 protein (32). FGFR protein levels were assessed by Western blotting and overexpression was confirmed relative to a control cell line (MCF7) with no identified defects in FGFR expression or signaling (Fig. 2). The ability of AZD4547 to inhibit in vitro proliferation of these lines over a three day period was assessed using a standard metabolism-based proliferation assay. IC$_{50}$ values ranged from 18 nM in KG1a cells to 281 nM in KMS11 cells (Table 2). Notably, MCF7 cell proliferation was unaffected by incubation with AZD4547 up to a concentration of 30 μM. Moreover, AZD4547 was inactive against more than 100 additional tumor cell lines (Supplementary Table S1), demonstrating that AZD4547 has potent in vitro antiproliferative activity only against tumor cell lines expressing deregulated FGFRs.

**AZD4547 potently inhibits FGFR phosphorylation and downstream signaling in human tumor cell lines.** To assess modulation of FGFR
phosphorylation and signaling by AZD4547, cell lines were treated with drug and lysates analyzed by Western blot. All three cell-lines showed inhibition of FGFR and MAPK protein phosphorylation in a dose-dependent manner (Fig. 3A-C). With the exception of the KMS11 cell line, the concentrations of AZD4547 required to inhibit cellular FGFR phosphorylation were in good agreement with the in vitro proliferation IC\textsubscript{50} values (Table 2). The phosphorylation of FRS2 and PLC\textsubscript{γ}, downstream markers of FGFR signaling, were also inhibited (Fig. 3D-E). Notably, AKT phosphorylation (which has been described to couple often to FGFR signaling) was unaffected by AZD4547 in KG1a and KMS11 lines, but did show modulation by AZD4547 treatment in the breast cell lines, MCF7 (data not shown) and Sum52-PE. Thus, at the cellular level, AZD4547 potently inhibits FGFR phosphorylation and downstream signaling effected through FRS2, PLC\textsubscript{γ} and MAPK.

The mechanism of growth inhibition by AZD4547 is cell-line dependent.

To understand the mechanism by which AZD4547 exerts anti-proliferative activity in vitro, cellular Annexin-V apoptosis assays, flow cytometry cell cycle analysis and Western blotting were performed. Three drug concentrations were chosen (30 nmol/L, 100 nmol/L and 1 μmol/L) to ensure good coverage of the approximate IC\textsubscript{50} values for cellular proliferation and FGFR phosphorylation. Figure 4 shows the cell cycle profiles of all three lines, following three days’ treatment with AZD4547 (mean % of cells in cell cycle phase ± SEM, n=3). KG1a cells show dramatic increases in the G1 phase population upon treatment with all three doses of AZD4547 (Fig. 4A), with no increases in the sub-G1 population. The Sum52-PE breast and KMS11 multiple myeloma cell lines both displayed an acute sensitivity to induction of
cell death (sub-G1 phase) following treatment with all doses of AZD4547 (Fig. 4B and 4C). Relative to untreated control cells, annexin-V staining confirmed significant apoptotic induction in Sum52-PE and KMS11 lines, but not KG1a (Fig. 4D). Consistent with the lack of any \textit{in vitro} antiproliferative effect of AZD4547 on the MCF7 breast cell line, no significant cell cycle or apoptotic changes were observed following incubation with AZD4547 at any of the doses tested (Supplementary Figure S1). Western blotting confirmed clear induction of the apoptotic markers, cleaved caspase 3 and PARP (Fig. 4E), in treated SUM52PE and KMS11 lysates, but not in KG1a, consistent with the Annexin V and cell cycle data. Further analysis demonstrated increases in pro-apoptotic BIM protein levels in all three lines following AZD4547 treatment and high level basal expression of the anti-apoptotic protein Bcl2 in KG1a cells, but not in SUM52PE or KMS11 (Fig. 4E).

Thus, at least in the lines tested here, cell fate in response to AZD4547 treatment is likely determined by the relative levels of key pro- and anti-apoptotic proteins.

\textit{AZD4547 in vivo} antitumor activity is associated with dose-proportional pharmacodynamic modulation of phospho-FGFR3 and reduced KMS-11 tumor cell proliferation. To assess AZD4547 \textit{in vivo} efficacy, female SCID mice bearing KMS11 tumors were randomized and treated chronically with AZD4547 at a range of well-tolerated doses. Oral AZD4547 treatment resulted in dose-dependent tumor growth inhibition. Twice-daily administration of AZD4547 at 3 mg/kg gave statistically significant tumor growth inhibition of 53\% ($P<0.0005$ by one-tailed $t$ test) when compared to vehicle-treated controls, whilst doses of 12.5 mg/kg once daily and 6.25 mg/kg twice daily
resulted in complete tumor stasis \((P < 0.0001)\) (Fig. 5A). A further efficacy study in the KG1a model using 12.5mg/kd qd AZD4547 resulted in 65% TGI \((P=0.002)\) (Supplementary Figure S2).

To assess \textit{in vivo} modulation of FGFR-phosphorylation, single doses of AZD4547 were orally administered to KMS11 tumor-bearing SCID mice and tumors harvested at various time points post dose for measurement of phosphorylated FGFR3 using a sandwich ELISA assay. Total plasma concentrations of AZD4547 clearly show a direct relationship with inhibition of FGFR3 phosphorylation within KMS11 tumors \textit{in vivo} (Fig. 5B). A further study using a single 6.25 mg/kg dose of AZD4547 was conducted in order to define the duration and magnitude of the pharmacodynamic effect in tumors. Within 15 minutes of drug administration, the levels of phosphorylated FGFR3 decreased to 40–50% of control levels and inhibition was sustained for at least 6 hours. At the 16 hour timepoint, phosphorylated FGFR3 had returned to control levels (Fig. 5C). These data confirm good modulation of tumor phospho-FGFR3 levels for at least 6 hours, consistent with the plasma exposure of AZD4547.

To define the mechanism(s) through which AZD4547 elicits KMS11 tumor growth inhibition \textit{in vivo}, KMS11 tumor-bearing mice were treated with 6.25 mg/kg AZD4547 twice daily for 7 days and tumors processed for immunohistochemical analysis. Tumor sections were stained using antibodies to cleaved caspase 3, Ki67 and CD31, representing markers of cellular apoptosis, proliferation and vascularity, respectively. AZD4547 treatment caused a significant reduction in the percentage of cells staining positive for nuclear Ki67 (Fig. 5D and 5E). However, despite showing an increase in the
AZD4547-treated sections, the difference in cleaved caspase 3 staining between control versus AZD4547-treated tumor sections did not reach statistical significance. Similarly, AZD4547 treatment did not have any significant effect on CD31 positive vessel staining in KMS11 xenografts (Fig. 5D and 5E), demonstrating that at efficacious dose levels AZD4547 does not exhibit anti-angiogenic effects. In summary, AZD4547 demonstrates dose-proportional anti-tumor efficacy in an FGFR3-driven xenograft model, concurrent with pharmacodynamic target modulation, and with evidence of a predominantly antiproliferative mechanism in vivo.

**AZD4547 anti-tumor efficacy is not attributable to inhibition of KDR-driven functional effects in vivo.** It is well documented that inhibition of VEGF signaling with agents such as cediranib (KDR inhibitor) can lead to hypertension (29, 33). Therefore, the effect of AZD4547 on blood pressure was measured in conscious telemetered rats. Two separate oral doses of 10 mg/kg AZD4547 over 24 hours did not lead to any significant changes in blood pressure compared with vehicle-dosed animals (Fig. 6A represents the diastolic pressure for vehicle control and AZD4547). The free plasma exposure of AZD4547 after dosing at 10 mg/kg in rats is equivalent to the exposure in mouse which results in greater than 60% tumor growth inhibition in the KMS11 model. These data demonstrate that at equivalent efficacious doses, AZD4547 has no significant effect on blood pressure and therefore lacks in vivo anti-KDR activity.

Broad antitumor efficacy in preclinical xenograft models is a feature of agents which act predominantly through anti-angiogenic mechanisms. To examine the breadth of its activity, AZD4547 was assessed in a number of models
which have been shown to be sensitive to inhibitors of VEGF signaling, such as cediranib (28). Mice bearing Calu-6 (lung) tumors were treated chronically with either 3 mg/kg cediranib once-daily or 6.25 mg/kg AZD4547 twice-daily. Cediranib resulted in significant tumor growth inhibition (64%) which is consistent with previous reports (28), however AZD4547 lacked efficacy in this model (Fig.6B). Similarly, dosing of 6.25 mg/kg po bid AZD4547 was also inactive in two additional cediranib-sensitive xenograft models - HCT-15 and LoVo (Table 3).

Taken together, the telemetry, immunohistochemical and efficacy data suggest that the antitumor effects of AZD4547 are not likely to be attributable to inhibition of KDR activity and are a result of its potent and selective FGFR profile.
Discussion

This report provides the first pharmacological profile of the pyrazoloamide derivative, AZD4547, an orally bioavailable selective inhibitor of FGFR tyrosine kinases with potential as a targeted antitumor therapy.

AZD4547 is a potent inhibitor of FGFR tyrosine kinases 1, 2 and 3 and is also selective versus a range of other related kinases, such as KDR, IGF, PI3Ka and AKT. Potent nanomolar IC$_{50}$ values were obtained when AZD4547 was examined against recombinant FGFR kinases (FGFR1, IC$_{50}$ = 0.2 nM), whilst activity versus KDR was approximately 120-fold lower (KDR, IC$_{50}$ = 24 nM). Cellular phosphorylation data confirmed this dominant FGFR-selectivity profile with an approximate 20-fold difference over KDR (FGFR1, cellular IC$_{50}$ = 12 nM; KDR, cellular IC$_{50}$ = 258 nM).

Our data demonstrate that AZD4547 is a potent cell growth inhibitor versus tumor cell lines with known irregularities in FGFR expression. Several studies have indirectly established that elevated FGFR protein levels correlate with receptor ligand-independence and result in mitogenic growth (10-13). Qian and colleagues have previously validated FGFR3 as a proliferative driver within the KMS11 multiple myeloma cell line, which contains mutated (Y373C) and overexpressed FGFR3 (34, 35). This mutation activates FGFR3 through ligand-independent constitutive dimerization. Similarly, both KG1a and Sum52-PE cell lines exhibit a dependence on FGFR1 and FGFR2 signaling respectively for proliferation, as demonstrated using siRNA (30) and the selective commercially available FGFR-inhibitor; PD173074 (10). In cell culture, we were able to confirm deregulation of FGFR protein expression
within several ‘oncogene addicted’ human tumor cell lines and demonstrate their exquisite antiproliferative sensitivity to AZD4547. Our chosen cell lines represent both wild-type and point-mutated FGFR proteins, indicating that AZD4547 is active against the tyrosine kinase activity of both the wild-type and mutant forms of FGFR used here. Importantly, the lack of any AZD4547 antiproliferative effects in FGFR non-deregulated tumor cell lines (including MCF7), indicates a lack of non-specific cytotoxicity within the effective drug concentration range.

AZD4547 inhibits FGFR downstream signaling and induces both cytostatic and cytotoxic effects dependent upon the cellular background. In the cell lines tested here, incubation with AZD4547 resulted in potent inhibition of cellular FGFR1, FGFR2, FGFR3, FRS2 and PLCγ phosphorylation, whilst concomitant inhibition of pMAPK confirmed that FGFR signaling remains coupled to the MAPK cascade (AZD4547 is inactive versus recombinant MEK and MAPK enzymes – data not shown). This requirement for a coupled FGFR-MAPK signaling pathway suggests clear implications for an AZD4547 clinical patient selection strategy. Activating mutations within the ras gene family occur with a frequency of up to 30% across a diverse range of human tumors (36, 37) and, in the case of FGFR, circumvent the cells dependence on ligand-induced stimulation (11). However, the mutual exclusivity of FGFR and Ras mutations observed across several tumor types (including pancreatic (38), multiple myeloma (39) and urothelial carcinoma (40)) suggests that these oncogenes use common signaling pathways to maintain the tumor phenotype. Since tumor growth driven by deregulated FGFR expression (and
hence sensitive to AZD4547), is unlikely to be reliant on mutant Ras signaling, a patient selection strategy based on deregulated FGFR alone is suggested.

Sum52-PE breast cancer cells are dependent on FGFR2 signaling to activate signal transduction pathways involving FRS2, mitogen-activated protein kinase and phosphatidylinositol 3-kinase (10). Published data supports a key role for FRS2-signaling in activating both the mitogen-activated protein kinase cascade to induce cell proliferation and differentiation, and also PI3-kinase to activate cell survival pathways (41). Consistent with these data, AZD4547 shows potent inhibition of both pErk and pAKT cellular phosphorylation and concomitant induction of apoptosis in Sum52-PE cells. The lack of pAKT modulation by AZD457 in KG1a and KMS11 cells however, is likely explained by the finding that PI3K and AKT are overactivated in most myeloma and leukemia cell lines (42). Indeed, KG1a cells are known to contain a hypermethylated PTEN promoter (43), whilst KMS11 harbour constitutively active AKT (42). As neither line shows modulation of pAKT by AZD4547, we suggest that the anti-proliferative versus apoptotic response to AZD4547 is modulated by the relative balance of pro and anti-apoptotic proteins. In KG1a cells, although AZD4547 treatment induces an increase in pro-apoptotic BIM expression, the relatively high level of anti-apoptotic Bcl2 protein would appear to protect against apoptotic induction. Conversely, within SUM52PE and KMS11 cells, despite induction of BIM expression by AZD4547, basal Bcl2 levels remain low or undetectable, thus allowing apoptosis to occur in these lines. Accordingly, at least in the lines tested here, we propose that AZD4547 induces apoptosis via induction of BIM expression only in those lines where levels of the anti-apoptotic protein Bcl2, are low or undetectable.
In preclinical xenograft models, AZD4547 exposure correlated with *in vivo* antitumor efficacy and pharmacodynamic modulation of target and proliferation endpoints. Dose-related AZD4547 efficacy was demonstrated in SCID mice bearing established KMS11 tumors (99% TGI using 6.25 mg/kg twice-daily dosing). Despite observing substantial *in vivo* inhibition of phospho-FGFR3 levels in KMS11 tumors at a dose of 6.25 mg/kg, we were unable to confirm statistically significant apoptotic induction using a cleaved-caspase 3 immunohistochemical endpoint, but did observe a significant reduction in Ki67 tumor staining. The apparent phenotypic disconnect observed here between potent induction of KMS11 cell apoptosis using AZD4547 *in vitro*, compared with slowed growth in the absence of any detectable apoptosis *in vivo*, may be explained by the timepoints of tumor sampling. Immunohistochemical staining for Ki67 and CC3 was performed on tumors after 7 days of AZD4547 treatment which, based on the AZD4547 antitumor preclinical efficacy data, describes a phase of slowed tumor growth but precedes any tumor stasis effects (observed at >14 days). Indeed, this interpretation is supported by similar studies using PD173074 where low *in vitro* concentrations resulted in KMS11 cell apoptosis after 4 days incubation, whilst *in vivo* apoptotic markers only became visible within KMS11 xenograft sections after 9 days twice-daily dosing (44). Further work is currently underway to confirm this interpretation and investigate the time-dependency of the pharmacodynamic changes elicited by AZD4547.

The *in vivo* data presented here is consistent with AZD4547 being a predominantly FGFR-selective small molecule inhibitor. Treatment with AZD4547 did not result in the well-characterized blood pressure changes
associated with acute KDR inhibition in telemetered rat studies (29, 33). Furthermore, we detected no significant changes in CD31 immunohistochemical staining in KMS11 tumor xenograft sections at an efficacious dose and timepoint, nor were we able to demonstrate efficacy in KDR-sensitive xenograft tumor models. Accordingly, we believe that AZD4547 is capable of clinically testing an FGFR-tumor drive hypothesis, aiming to select patients based on deregulated tumor FGFR expression.

In summary, using a panel of tumor cell lines in vitro we have shown that endogenous FGFR protein expression levels predict for antiproliferative sensitivity to the novel, potent FGFR-selective agent, AZD4547. Our data demonstrate potent modulation of phospho-FGFR signaling within FGFR-deregulated cell lines and highlights both pro-apoptotic and antiproliferative phenotypes, dictated by the relative expressions of key pro- and anti-apoptotic proteins. AZD4547 inhibits the growth of KMS11 tumor xenografts at doses (and plasma exposure) which cause pharmacodynamic modulation of tumor phospho-FGFR. Furthermore, AZD4547 shows no in vivo evidence of anti-KDR related efficacy or physiology and is well tolerated. These data support further investigation of AZD4547 as a targeted therapeutic option for patients with tumors harboring deregulated FGFR expression. AZD4547 is currently being evaluated in Phase I clinical trials.
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References


Table 1. AZD4547 kinase activity

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Enzyme IC\textsubscript{50} (nmol/L)*</th>
<th>Cellular IC\textsubscript{50} (nmol/L)*</th>
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<tr>
<td>FGFR1</td>
<td>0.2 ± 0.06</td>
<td>12 ± 3</td>
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<tr>
<td>FGFR3</td>
<td>1.8 ± 0.33</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>FGFR2</td>
<td>2.5 ± 0.23</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>KDR</td>
<td>24 ± 0.001</td>
<td>258 ± 17</td>
</tr>
<tr>
<td>FGFR4</td>
<td>165 ± 30.3</td>
<td>142 ± 29</td>
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<tr>
<td>IGFR</td>
<td>581 ± 0.02</td>
<td>828 ± 41</td>
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<td>AXL</td>
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<tr>
<td>p38</td>
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</tr>
<tr>
<td>PI3Ka</td>
<td>&gt;100000</td>
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</table>

*The ability of AZD4547 to inhibit a range of human recombinant kinase activities was tested using ATP concentrations at, or just below, the respective K\textsubscript{m}. Data represents the mean ± SEM of at least three separate determinations. IC\textsubscript{50} values denoted as “greater than” denote the inability to reach 50% inhibition of maximal activity at the highest tested concentration.
Table 2. Summary of AZD4547 in vitro antiproliferative IC\(_{50}\) values obtained by MTS proliferation assay.

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Deregulated FGFR member</th>
<th>Proliferation IC(_{50}) (μM)</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>KG1a</td>
<td>FGFR1</td>
<td>0.018 (n=3)</td>
<td>0.0017</td>
</tr>
<tr>
<td>Sum52-PE</td>
<td>FGFR2</td>
<td>0.041 (n=4)</td>
<td>0.0185</td>
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<tr>
<td>KMS11</td>
<td>FGFR3</td>
<td>0.281 (n=5)</td>
<td>0.0294</td>
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<tr>
<td>MCF7</td>
<td>None</td>
<td>&gt;30 (n=6)</td>
<td>NA</td>
</tr>
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</table>

NA, not applicable

Table 3. Tumor growth inhibition in nude mice bearing established Calu-6 human tumor xenografts. Mice were treated orally, twice-daily with AZD4547 (6.25 mg/kg) for 14 to 17 days or cediranib (LoVo, 1.5 mg/kg; Calu-6, 6 mg/kg; HCT-15, 6 mg/kg) once-daily for up to 28 days. Percentage tumor growth inhibition was calculated as the difference between the mean change in control versus treated tumor volumes over the period of treatment.

<table>
<thead>
<tr>
<th>Inhibition of tumor growth (%)</th>
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<tr>
<td>Tumor model</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>LoVo</td>
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<tr>
<td>HCT-15</td>
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*Data previously published
**Unpublished data
**Figure legends**

**Figure 1.** Chemical structure of AZD4547.

**Figure 2.** Inhibition of *in vitro* cell proliferation by AZD4547 correlates with deregulated FGFR-expression in tumor cell lines. Western blot confirming high expression of FGFR proteins from cell lines with known deregulations.

**Figure 3.** Inhibition of FGFR signaling by AZD4547. All cell lines were incubated for 3 hours with the stated AZD4547 concentrations and then stimulated with aFGF/bFGF (10 ng/ml) and heparin (10 μg/ml) for 20 minutes. Cells were then lysed and immunoblotted for the proteins indicated. **A-C:** KMS11, Sum52-PE and KG1a were analyzed for total FGFR1-3, phospho and total FGFR, MAPK and AKT, **D:** phospho and total PLCγ and Erk, **E:** phospho and total FRS2.

**Figure 4.** The mechanism of *in vitro* growth inhibition by AZD4547 is cell line-dependent. **A–C:** Cells were exposed to AZD4547 for 72 hours and then analyzed for their cell cycle distribution using propidium iodide (PI) and a FACScalibur flow cytometry system. **D:** Quantified Annexin V flow cytometry data from cells treated *in vitro* with 100 nmol/L AZD4547 for 72 hours. *P*-values were calculated using the students *t*-test and refer to the comparison between DMSO and AZD4547 for each cell line. Data are representative of three independent experiments. NS, not significant. **E:** Cells were exposed to 100nM AZD4547 for 72 hours and then lysed and immunoblotted for the proteins indicated.
Figure 5. AZD4547 exposure correlates with in vivo antitumor activity, pharmacodynamic modulation of phospho-FGFR and reduced tumor cell proliferation. A: AZD4547 was administered by oral gavage once (qd) or twice (bid) daily to SCID mice bearing established s.c. KMS11 human tumor xenografts at the doses indicated. Tumor volumes are plotted against time. B: Pharmacokinetic/pharmacodynamic relationship in KMS11 xenograft. AZD4547 was dosed orally at a range of concentrations, and tumors and blood collected at various timepoints for ELISA analysis of phospho-FGFR3 and AZD4547 drug levels, respectively. Total plasma drug levels and the percentage inhibition of phospho-FGFR3 ("PD inhibition") compared with control are plotted. C: AZD4547 was dosed orally at 6.25 mg/kg (single dose) and plasma and tumors collected over a 16 hour time course. D: Ki67, cleaved caspase 3 (CC3), and CD31 immunohistochemical staining of KMS11 tumor sections removed after 7 days twice-daily dosing of 6.25 mg/kg AZD4547 (4 hours post-final dose). E: Image analysis data for Ki67, CC3 and CD31 immunostained tumors demonstrating mean values for each group (n=4 animals/group). Ki67 shows statistical significance with one-tailed t-test ($P=0.012^*$). NS, not significant.

Figure 6. AZD4547 antitumor efficacy is not attributable to inhibition of KDR. A: Mean diastolic blood pressure measurements of a group of unrestrained conscious telemetered rats (n=3) given 2 oral doses at 180 and 540 minutes (arrows): on day one, vehicle control (o) and the following day (day 2) AZD4547 at 10 mg/kg (●). B: Nude mice bearing established Calu-6 human tumor xenografts were treated orally, twice-daily with AZD4547 (6.25 mg/kg) or cediranib (3 mg/kg) once-daily.
Figure 2

<table>
<thead>
<tr>
<th>kDa</th>
<th>KG1a</th>
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<th>MCF7</th>
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<td>76-</td>
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</table>

GAPDH
Figure 4

A

KG1a

B

Sum52-PE

C

KMS11

D

Annexin V stained cells (%)

E

100 nM AZD4547

- - + - - + - - +

Cleaved caspase 3

PARP

EL

BIM

BCL2

α-tubulin

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Figure 5

A

![Graph showing mean tumor volume (cm³) ± SEM over treatment time (days). The x-axis represents treatment time in days ranging from 0 to 20, and the y-axis represents mean tumor volume. Several lines represent different treatment groups, including control, AZD4547 2.5 mg/kg qd, AZD4547 6.25 mg/kg qd, AZD4547 12.5 mg/kg qd, AZD4547 2.5 mg/kg bid, AZD4547 6.25 mg/kg bid, AZD4547 12.5 mg/kg bid, and AZD4547 2.5 mg/kg qd. Each line includes error bars representing SEM.](image)

B

![Scatter plot showing PD inhibition (%) vs. plasma concentration (μM). The x-axis represents plasma concentration ranging from 0.01 to 100, and the y-axis represents PD inhibition (%) ranging from 0 to 80. Each point on the graph represents a different sample.](image)

C

![Graph showing PD inhibition (%) and plasma PK over time post-dose (hours). The x-axis represents time post-dose in hours ranging from 0.25 to 16, and the y-axis represents PD inhibition (%) on the left and plasma PK (μM) on the right. Two bars represent PD inhibition, one for control and one for AZD4547 6.25 mg/kg bid. The plasma PK bar for AZD4547 6.25 mg/kg bid shows a significant decrease over time.](image)

D

![Images showing Ki67, CC3, and CD31 expression in Vehicle and AZD4547 6.25 mg/kg bid treated samples.](image)

E

![Bar charts showing mean % positive nuclear score for Ki67, cleaved caspase 3, and CD31.](image)
Figure 6

A

- Vehicle PO bid
- AZD4547 (10 mg/kg bid)

Distolic blood pressure (mm Hg)

Time (minutes)

B

- Control
- AZD2171 3 mg/kg qd
- AZD4547 0.25 mg/kg bid

Mean tumor volume (cm³) ± SEM

Treatment time (day)
**AZD4547: An orally bioavailable, potent and selective inhibitor of the Fibroblast Growth Factor Receptor tyrosine kinase family.**

Paul R Gavine, Lorraine Mooney, Elaine Kilgour, et al.

*Cancer Res* Published OnlineFirst February 27, 2012.

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