AZD4547: An Orally Bioavailable, Potent, and Selective Inhibitor of the Fibroblast Growth Factor Receptor Tyrosine Kinase Family

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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 pharmacologic 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. Cancer Res; 72(8); 2045–56. ©2012 AACR.

Introduction

Fibroblast growth factors (FGF) and their receptors carry out key roles in multiple biologic processes including tissue repair, hematopoiesis, angiogenesis, and embryonic development. The FGF receptor (FGFR) family comprises 4 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 sulfate proteoglycans, 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 physiologic roles described above, FGFs and FGFRs are emerging as oncogenes that drive proliferation of a significant proportion of human tumors and that 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, and 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), whereas 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 (kinase insert domain receptor) pharmacology (brivanib alaninate; ref. 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 that these agents offer the opportunity to fully test the anti-FGFR hypothesis because of the confounding effects of their other pharmacologic 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-driven 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

\( \text{N-}\left[5-\left[2-(3,5-\text{dimethoxyphenyl})\text{ethyl}\right]-2H-\text{pyrazol-3-yl}\right]-4\left(3,5-\text{diethyl} \text{piperazin-1-yl}\right)\text{benzamide} \) (AZD4547, AstraZeneca; 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 polyoxyethylene sorbitan monooleate (Tween-80) in deionized water. Animals were given AZD4547, a potent and selective FGFR tyrosine kinase inhibitor currently in phase I clinical studies.

Cell culture

KG1a, Sum52-PE, MCF7, and KMS11 cell lines were routinely grown in RPMI-1640 supplemented with 10% (v/v) fetal calf serum (FCS; Biochrom AG) and 2 mmol/L L-glutamine (Invitrogen). The KMS11 cell line was obtained from Jonathan Keats (Scottsdale, Arizona), KG1a from American Type Culture Collection, MCF7 from the Imperial Cancer Research Fund, and Sum52-PE from Asterand. These cell lines were genetically tested and authenticated with the Powerplex 16 System (Promega) and were not cultured for more than 6 months before conducting the work described here.

Inhibition of cellular receptor phosphorylation

For FGFR phosphorylation studies, FGFR1, 3, or 4–transfected Cos-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mmol/L L-glutamine and 3% FCS. For FGFR2, Sum52-PE cells were cultured in RPMI-1640 (Gibco), growth media supplemented with 2 mmol/L L-glutamine and 10% FBS. Following 1-hour incubation with AZD4547, media were removed; cells were fixed, permeabilized, and then incubated with monoclonal anti-phospho-FGFR antibody (Cell Signaling Technology; 1:1,000) for 1 hour followed by incubation with anti-mouse Alexa Fluor 594 secondary antibody (1:500) and Hoechst (1:1,000) for 1 hour. Fluorescence measurement was conducted with Arrayscan (Cellomics).

For KDR phosphorylation studies, primary human umbilical vein endothelial cells 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 per well). Cells were lysed with standard radioimmunoprecipitation assay buffer containing phosphatase/protease inhibitors. Lysates were analyzed with 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 (Groupe IMOO), followed by fixation, blocking, and incubation with a rabbit anti-phospho IGF1R/IR antibody (Biosource; 1:350) for 1 hour. Secondary detection and measurement was carried out with an Acumen Explorer HTS Reader (TTP Labtech Ltd.) at an 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 conducted with standard SDS-PAGE procedures and antibody incubation carried out 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), and BIM (Millipore). All other antibodies were sourced from Cell Signalling Technologies. Secondary antibodies were applied and immunoreactive proteins visualized with “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 with 70% ethanol and then incubated with propidium iodide/RNase A labeling solution. Cell-cycle profiles were assessed with a FACSCalibur instrument and CellQuest analysis software. For apoptotic analysis, cells and media were gently harvested and centrifuged, followed by washing of cell pellets. Cells were then processed for Annexin V-fluorescein isothiocyanate (FITC) staining and propidium iodide uptake according to the manufacturer’s instructions. The proportion of cells staining positive for Annexin V were then assessed with a FACSCalibur instrument and quadrant sorting was done by CellQuest analysis software.

**Murine plasma pharmacokinetic analyses**

Plasma samples were extracted by protein precipitation in acetonitrile, followed by liquid chromatography/tandem mass spectrometry (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 with Masslynx and data processed by Quanlynx software.

**Immunohistochemistry**

Antigen retrieval was carried out on formalin-fixed, paraffin-embedded tissues with 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-PL AstraZeneca: 1:600), mouse monoclonal anti Ki-67 MIB-1 (Dako M7240: 1:100), and 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-6010; Vector Laboratories, Inc.) and staining was detected with diamobenzidine (K3468; Dako). Finally, sections were counterstained with Carazzi’s hematoxylin and image analysis was conducted with the Aperio Digital Pathology System (Aperio Technologies, Carazzi’s hematoxylin and image analysis was conducted with the Aperio Digital Pathology System (Aperio Technologies, Inc.). Analysis thresholds were set and applied to all tumors within the study by the Aperio Micro Vessel Density (MVD) and staining and propidium iodide uptake according to the methodology described previously.

**Results**

**AZD4547 is a highly potent inhibitor of FGFR tyrosine kinases 1 to 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 (IC50 values of 0.2, 2.5, and 1.8 nmol/L, respectively; Table 1) and displays weaker activity against FGFR4 (IC50 = 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 IC50 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, and KDR were established as cellular phosphatase and protease inhibitors (Sigma) with 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 tumor studies**

Swiss derived nude (nu/nu) and severe combined immunodeficient mice (SCID; Charles River) were housed in negative-pressure isolators (PFI Systems Ltd.) at AstraZeneca. All experiments were carried out on 8- to 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 (1 × 106 for LoVo, 1 × 107 for HCT-15, and 1 × 107 for Calu-6) or 0.2 ml (5 × 107 for KMS11 and KG1a) mixed 1:1 with Matrigel (Becton Dickinson). 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 of more than 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.

**Pharmacodynamic studies**

Tumor samples and blood were collected from KMS11 tumor-bearing mice at various time points after single dose of either AZD4547 or vehicle. Frozen tumor samples were lysed in 1 × cell lysis buffer (Cell Signaling Technologies) containing phosphatase and protease inhibitors (Sigma) with 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 with the commercially available Data Sciences International telemetry system by the methodology described previously.

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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 that expresses a truncated wild-type FGFR1 fusion protein (30); Sum52-PE is a breast cell line that 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 3-day period was assessed with a standard metabolism-based proliferation assay. IC50 values ranged from 18 nmol/L in KG1a cells to 281 nmol/L in KMS11 cells (Table 2). Notably, MCF7 cell proliferation was unaffected by incubation with AZD4547 up to a concentration of 30 μmol/L. Moreover, AZD4547 was inactive against more than 100 additional tumor cell lines (Supplementary Table S1), showing 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 blotting. All 3 cell lines showed inhibition of FGFR and mitogen—activated protein kinase (MAPK) 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 IC50 values (Table 2). The phosphorylation of FRS2 and PLCγ, downstream markers of FGFR signaling, was also inhibited (Fig. 3D and 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 affected through FRS2, PLCγ, and MAPK.

The mechanism of growth inhibition by AZD4547 is cell line dependent

To understand the mechanism by which AZD4547 exerts antiproliferative activity in vitro, cellular Annexin V apoptosis assays, flow cytometric cell-cycle analysis, and Western blotting were conducted. Three drug concentrations were chosen (30 nmol/L, 100 nmol/L, and 1 μmol/L) to ensure good

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**Table 1. AZD4547 kinase activity**

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Enzyme</th>
<th>IC50a, nmol/L</th>
<th>Cellular IC50, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td></td>
<td>0.2 ± 0.06</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>FGFR3</td>
<td></td>
<td>1.8 ± 0.33</td>
<td>40 ± 1</td>
</tr>
<tr>
<td>FGFR2</td>
<td></td>
<td>2.5 ± 0.23</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>KDR</td>
<td></td>
<td>24 ± 0.001</td>
<td>258 ± 17</td>
</tr>
<tr>
<td>FGFR4</td>
<td></td>
<td>165 ± 30.3</td>
<td>142 ± 29</td>
</tr>
<tr>
<td>IGFR</td>
<td></td>
<td>581 ± 0.02</td>
<td>828 ± 41</td>
</tr>
<tr>
<td>AXL</td>
<td></td>
<td>&gt;2,000</td>
<td></td>
</tr>
<tr>
<td>CDK2</td>
<td></td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>FAK</td>
<td></td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>ROCK2</td>
<td></td>
<td>&gt;50,000</td>
<td></td>
</tr>
<tr>
<td>AKT1</td>
<td></td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>ALK1</td>
<td></td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td></td>
<td>&gt;100,000</td>
<td></td>
</tr>
<tr>
<td>PI3Ka</td>
<td></td>
<td>&gt;100,000</td>
<td></td>
</tr>
</tbody>
</table>

aThe ability of AZD4547 to inhibit a range of human recombinant kinase activities was tested using ATP concentrations at, or just below, the respective K_m. Data represent the mean ± SEM of at least 3 separate determinations. IC50 values denoted as “greater than” denote the inability to reach 50% inhibition of maximal activity at the highest tested concentration.
coverage of the approximate IC₅₀ values for cellular proliferation and FGFR phosphorylation. Figure 4 shows the cell-cycle profiles of the following 3 days of treatment with AZD4547 (mean percent of cells in cell-cycle phase ± SEM, n = 3). KG1a cells show dramatic increase in the G₁ phase population upon treatment with all 3 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 C). Relative to untreated control cells, Annexin V staining confirmed significant apoptotic induction in Sum52-PE and KMS11 lines, but not in KG1a (Fig. 4D). Consistent with the lack of any 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 Fig. S1). Western blotting confirmed clear induction of the apoptotic markers, cleaved caspase-3, and PARP (Fig. 4E), in treated Sum52-PE and KMS11 lysates, but not in KG1a, consistent with the Annexin V and cell-cycle data. Further analysis showed increases in proapoptotic BIM protein levels in all 3 lines following AZD4547 treatment and high-level basal expression of the antiapoptotic protein Bcl2 in KG1a cells, but not in Sum52-PE 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 ant apoptotic proteins.

**AZD4547 in vivo antitumor activity is associated with dose proportional pharmacodynamic modulation of phospho-FGFR3 and reduced KMS11 tumor cell proliferation**

To assess AZD4547 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 with vehicle-treated controls, whereas 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 with 12.5 mg/kg once daily AZD4547 resulted in 65% tumor growth inhibition (P = 0.002; Supplementary Fig. S2).

To assess 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 postdose for measurement of phosphorylated FGFR3 with a sandwich ELISA assay. Total plasma concentrations of AZD4547 clearly show a direct relationship with inhibition of FGFR3 phosphorylation within KMS11 tumors in vivo (Fig. 5B). A further study using a single 6.25 mg/kg dose of AZD4547 was conducted 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% to 50% of control levels and inhibition was sustained for at least 6 hours. At the 16-hour time point, phosphorylated FGFR3 had returned to control levels (Fig. 5C). These data confirm good modulation of tumor phosphorylation 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 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, Ki-67, 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 Ki-67 (Fig. 5D and E). However, despite showing an increase in the AZD4547-treated sections, the difference in cleaved caspase-3 staining between controls 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 E), showing that at efficacious dose levels, AZD4547 does not exhibit angiogenic effects. In summary, AZD4547 shows dose proportional antitumor efficacy in an FGFR3-driven xenograft model, concurrent with pharmacodynamic target modulation, and with evidence of a predominantly antiproliferative mechanism in vivo.

**AZD4547 antitumor 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

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**Table 2. Summary of AZD4547 in vitro antiproliferative IC₅₀ values obtained by MTS proliferation assay**

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Deregulated FGFR member</th>
<th>Proliferation IC₅₀, μmol/L</th>
<th>SEM</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>KMS11</td>
<td>FGFR3</td>
<td>0.281 (n = 5)</td>
<td>0.0294</td>
</tr>
<tr>
<td>MCF7</td>
<td>None</td>
<td>&gt;30 (n = 6)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.
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 show 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 antiangiogenic mechanisms. To examine the breadth of its activity, AZD4547 was assessed in a number of models that 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 orally twice daily AZD4547 was also inactive in 2 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 pharmacologic 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 nmol/L), whereas activity versus KDR was approximately 120-fold lower (KDR, IC_{50} = 24 nmol/L). Cellular phosphorylation data confirmed this dominant FGFR selectivity profile with an approximate 20-fold difference over KDR (FGFR1, cellular IC_{50} = 12 nmol/L; KDR, cellular IC_{50} = 258 nmol/L).

Our data show 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.
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 and a FACSCalibur flow cytometric system. D, quantified Annexin V flow cytometric data from cells treated in vitro with 100 nmol/L AZD4547 for 72 hours. P values were calculated using the Student t test and refer to the comparison between dimethyl sulfoxide (DMSO) and AZD4547 for each cell line. Data are representative of 3 independent experiments. NS, not significant.

E, cells were exposed to 100 nmol/L AZD4547 for 72 hours and then lysed and immunoblotted for the proteins indicated.
This mutation activates FGFR3 through ligand-independent constitutive dimerization. Similarly, both KG1a and Sum52-PE cell lines exhibit a dependence on FGFR1 and 2 signaling respectively for proliferation, as shown by siRNA and the selective commercially available FGFR inhibitor, PD173074. In cell culture, we were able to confirm deregulation of FGFR protein expression within several "oncogene addicted" human tumor cell lines and show their exquisite

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 (PK)/pharmacodynamic relationship in KMS11 xenograft. AZD4547 was dosed orally at a range of concentrations, and tumors and blood collected at various time points 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, Ki-67, 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 after final dose). E, image analysis data for Ki-67, CC3, and CD31 immunostained tumors showing mean values for each group (n = 4 animals per group). Ki-67 shows statistical significance with one-tailed t test. *, P = 0.012. NS, not significant.
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 nonderegulated tumor cell lines (including MCF7) indicates a lack of nonspecific 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, whereas concomitant inhibition of pMAPK confirmed that FGFR signaling remains coupled to the MAPK cascade [AZD4547 is inactive vs. recombinant MAP–extracellular signal–regulated (ERK) kinase (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 dependence of cells 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

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 1, vehicle control (○) and the following day (day 2) AZD4547 at 10 mg/kg (●), bid, twice daily; po, orally. 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.

AZD4547: A Novel, Potent, and Selective FGFR Inhibitor

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modulation of target and proliferation endpoints. Dose-related

carcinoma (40), suggests that these oncogenes use common
signaling pathways to maintain the tumor phenotype. Because
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, MAPK, and phosphoinositide 3-kinase (PI3K; ref. 10).
Published data support a key role for FRS2 signaling in
activating both the MAPK to induce cell proliferation and
differentiation and also PI3K to activate cell survival pathways
(41). Consistent with these data, AZD4547 shows potent inhi-
bition of both pERK and pAKT cellular phosphorylation and
concomitant induction of apoptosis in Sum52-PE cells. The
lack of pAKT modulation by AZD4547 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 hyper-
methylated PTEK promoter (43), whereas KMS11 harbors a
constitutively active AKT (42). As neither line shows modula-
tion of pAKT by AZD4547, we suggest that the antiproliferative
versus apoptotic response to AZD4547 is modulated by the
relative balance of pro- and antiapoptotic proteins. In KG1a
cells, although AZD4547 treatment induces an increase in
proapoptotic BIM expression, the relatively high level of anti-
apoptotic Bcl2 protein would appear to protect against apo-
ptotic induction. Conversely, within Sum52-PE 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 induc-
tion of BIM expression only in those lines where levels of the
antiapoptotic protein Bcl2 are low or undetectable.

In preclinical xenograft models, AZD4547 exposure corre-
lated with in vivo antitumor efficacy and pharmacodynamic
modulation of target and proliferation endpoints. Dose-related

AZD4547 efficacy was shown in SCID mice bearing established
KMS11 tumors (99% tumor growth inhibition with 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 by a cleaved caspase-3
immunohistochemical endpoint but did observe a significant
reduction in Ki-67 tumor staining. The apparent phenotypic
disconnect observed here between potent induction of KMS11
cell apoptosis by AZD4547 in vitro, compared with slowed
growth in the absence of any detectable apoptosis in vivo,
may be explained by the time points of tumor sampling.
Immunohistochemical staining for Ki-67 and CC3 was con-
ducted on tumors after 7 days of AZD4547 treatment, which,
shown that endogenous FGFR protein expression levels predict
antiproliferative sensitivity to the novel, potent FGFR-selective
agent, AZD4547. Our data show potent modulation of phos-
pho-FGFR signaling within FGFR-deregulated cell lines and
highlight both proapoptotic and antiproliferative phenotypes,
dictated by the relative expressions of key pro- and antiapop-
totic proteins. AZD457 inhibits the growth of KMS11 tumor
xenografts at doses (and plasma exposure), which cause phar-
macodynamic modulation of tumor phospho-FGFR. Further-
more, 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.

**Disclosure of Potential Conflicts of Interest**

P.R. Gavine and T. Klionska have employment (other than primary affilia-
tion, e.g., consulting) with AstraZeneca as the principal scientists. A.N. Brooks

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**Table 3. Tumor growth inhibition in nude mice bearing established Calu-6 human tumor xenografts**

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Inhibition of tumor growth, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AZD4547</td>
</tr>
<tr>
<td>LoVo</td>
<td>0</td>
</tr>
<tr>
<td>HCT-15</td>
<td>4.7</td>
</tr>
</tbody>
</table>

**NOTE:** 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 of tumor growth inhibition was calculated as the difference between the mean change in control versus treated tumor volumes over the period of treatment.

\(^{a}\)Data previously published.

\(^{b}\)Unpublished data.

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"In summary, using a panel of tumor cell lines in vitro, we have shown that endogenous FGFR protein expression levels predict antiproliferative sensitivity to the novel, potent FGFR-selective agent, AZD4547. Our data show potent modulation of phospho-FGFR signaling within FGFR-deregulated cell lines and highlight both proapoptotic and antiproliferative phenotypes, dictated by the relative expressions of key pro- and antiapoptotic 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."
has ownership interest (including patents) from AstraZeneca. No potential conflicts of interests were disclosed by the other authors.

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