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.

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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 sulphate 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 cancer (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 tumor-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

N-[5-[2-(3,5-Dimethoxyphenyl)ethyl]-2H-pyrazol-3-yl]-4-(3,5-dimethylpyrazolin-1-yl)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 polyoxyethylenesorbitan monooleate (Tween-80) in deionized water. Animals were given AZD4547, a potent and selective FGFR tyrosine kinase inhibitor currently in phase I clinical studies.

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 (Gropep IMOOl), 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 (Sigma) labeling solution. Cell-cycle profiles were assessed with a FACS Calibur instrument and CellQuest analysis software (Becton Dickinson). 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 (Abcam). The proportion of cells staining positive for Annexin V were then assessed with a FACS Calibur instrument and quadrant sorting was done by CellQuest analysis software (Becton Dickinson).

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 (Waters).

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-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). The following primary antibodies were used (K4007/K4003; Dako, PK-6101; Vector Laboratories) and biotinylated rabbit anti-goat immunoglobulin secondary antibodies were used (K4007/K4003; Dako, PK-6101; Vector Laboratories, Inc.) and staining was detected with diamobenidine (K3468; Dako). Finally, sections were counterstained with 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 nuclear software algorithms (CD31, cleaved caspase-3, and Ki-67). 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 of the total number of nuclei stained).

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 (2 × 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 (28).

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 (29).

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 (>2,900-fold), CDK2 (>50,000-fold), and p38 (>50,000-fold). Broader kinase selectivity was explored with 0.1 µmol/L of AZD4547 against a range of recombinant kinases incubated with ATP at or near the appropriate enzyme Kₘ concentrations. No enzyme inhibition was detected against ALK, CHK1, EGFR, MAPK1, MEK1, p70S6K, PDGFR, PKB, Src, Tie2, and PI3-kinase (data not shown).

Because of potent AZD4547 recombinant enzyme inhibition, FGFR1–4, IGFR, and KDR were established as cellular phosphorylation assays and used to generate AZD4547 IC₅₀ values (Table 1). In cells, AZD4547 potently inhibits autophosphorylation of FGFR1, 2, and 3 tyrosine kinases (IC₅₀ values of 12, 2, and 40 nmol/L, respectively; Table 1) and displays weaker inhibition of FGFR4 cellular kinase activity (IC₅₀ = 142 nmol/L). Significantly weaker inhibitory activity was observed versus cellular KDR and IGFR ligand–induced phosphorylation (IC₅₀ 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 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) pro-

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*The ability of AZD4547 to inhibit a range of human recombiant 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.

Figure 2. Inhibition of in vitro cell proliferation by AZD4547 correlates with deregulated FGFR expression in tumor cell lines. Western blot analyses confirming high expression of FGFR proteins from cell lines with known deregulations. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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...
coverage of the approximate IC₅₀ values for cellular proliferation and FGFR phosphorylation. Figure 4 shows the cell-cycle profiles of three lines, following 3 days of treatment with AZD4547 (mean percent of cells in cell-cycle phase ± SEM, n = 3). KG1a cells show a dramatic increase in the G₀ 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 a significant 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 dose 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 increased doserelated inhibition of 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 antiapoptotic 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 phosphorylated 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 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 antiangiogenic 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
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 (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 show their exquisite
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...
cancerous tissues, however, is likely explained by the concomitant induction of apoptosis in Sum52-PE cells. The inhibition of both pERK and pAKT cellular phosphorylation and differentiation and also PI3K to activate cell survival pathways activating both the MAPK to induce cell proliferation and cellular 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 conducted on tumors after 7 days of AZD4547 treatment, which, based on the AZD4547 antitumor preclinical efficacy data, describes a phase of slowed tumor growth after 4 days of incubation, while in vivo apoptotic markers only became visible within KMS11 xenograft sections after 9 days of 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.

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 antia 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.

**Disclosure of Potential Conflicts of Interest**

P.R. Gavine and T. Klinowska have employment with AstraZeneca and AstraZeneca as the principal scientists. A.N. Brooks has employment with AstraZeneca and AstraZeneca as the principal scientists. A.N. Brooks has employment with AstraZeneca and AstraZeneca as the principal scientists. A.N. Brooks has employment with AstraZeneca and AstraZeneca as the principal scientists.
has ownership interest (including patents) from AstraZeneca. No potential conflicts of interests were disclosed by the other authors.

Authors’ Contributions

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References


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