Antitumor Effects and Mechanisms of AZD4547 on FGFR2-Deregulated Endometrial Cancer Cells

Yeonui Kwak1, Hanna Cho1, Wooyoung Hur2, and Taebo Sim1,2

Abstract

Uncontrolled activation of FGFRs induces the progression of various cancers. It was recently reported that FGFR2-activating mutations are implicated in about 12% of endometrial carcinomas. AZD4547, a potent pan-FGFR inhibitor, is currently being evaluated in clinical trials for several FGFR-driven cancers. However, AZD4547 has not been examined yet against FGFR2 mutant-driven endometrial cancers. Thus, we evaluated the activity of AZD4547 against four different endometrial cancer cells, including AN3-CA, MFE296, MFE280, and HEC1A, where all but HEC1A cells express distinctive FGFR2 mutations. We found that AZD4547 exhibits potent antiproliferative activity (EC50 = 31 nmol/L) against AN3-CA cells harboring FGFR2-K310R/N550K mutant. Analysis using a phospho-kinase array revealed that AZD4547 blocks FGFR2 downstream signaling, such as p38, ERK1/2, JNK, p70S6K, and PLCγ. Moreover, oral administration of AZD4547 (30 mg/kg, every day) remarkably delayed tumor growth in a mouse xenograft model of AN3-CA cells. Unbiased reporter gene assay showed that AZD4547 antagonizes the aFGF-induced activation of several transcription factors, including EGR1, ELK-1/SRF, AP-1, and NfκB. Genome-wide transcriptome analysis revealed that AZD4547 perturbs a number of transcription factors, and EGR1 was identified as one of the major targets of AZD4547. The significance of the FGFR2–EGR1 axis in endometrial cancer progression has not been reported. In addition, using kinase-wide inhibition profiling analysis, we first identified potential new target kinases of AZD4547, including MAP4K3, MAP4K5, IRR, RET, and FLT3. Our study demonstrated that AZD4547 exhibits its therapeutic activity against endometrial cancer cells by perturbing various regulatory mechanisms related to FGFR signaling. Mol Cancer Ther; 14(10): 2292–302. ©2015 AACR.

Introduction

Fibroblast growth factor receptors (FGFR), consisting of four genes named FGFR1–FGFR4, are one of the subfamilies of receptor tyrosine kinases (RTK, ref. 1). Eighteen different FGF ligands bind to the distinct FGFRs or their alternative splicing isoforms through formation of ternary complexes with heparan sulfate proteoglycans (2). FGFRs regulate important physiologic processes, including cell growth, and their dysregulated activities are implicated in various human diseases. For example, FGFR1 overexpression resulting from 8p12 amplification induces carcinomaogenesis in the breast (3) and is implicated in 22% of squamous cell lung cancers (4). In about 15% to 20% of patients, multiple myeloma is caused by overexpression of FGFR3 within strong IgH enhancers as a result of a t(4;14)(p16.3;q32.3) chromosomal translocation (5). In 60% to 70% of non–muscle-invasive superficial bladder cancers, gain-of-function FGFR3 mutations, such as S249C/Y373C, in the extracellular region and mutations of K650 in the kinase domain are indicative of a high risk for tumor recurrence (6). In addition, a FGFR3–ETV6-fused gene generated by a t(4;12)(p16;p13) chromosomal translocation causes peripheral T-cell lymphoma (7). FGFR1–TACC1 and FGFR3–TACC3-fused genes are associated with glioblastoma multiforme (8). Overexpression of FGFR4 has been shown to be associated with 7% to 8% of rhabdomyosarcoma, which is the most common soft-tissue sarcoma in children (9, 10). Moreover, FGFR1–3 mutations found in cancers are also involved in various skeletal disorders, such as Apert syndrome and Crouzon syndrome (11–13). About 10% to 12% of endometrial cancers are associated with constitutively active FGFR2 mutations (14, 15), where S252W mutation in the extracellular domain increases the binding affinity with FGF, and N550K mutant in the kinase domain loses the autoinhibitory conformation (13, 16). Despite mutations of both the P3K pathway and FGFR2, FGFR2 inhibition alone using siRNA FGFR2 knockdown or small-molecule inhibitors blocks the growth of endometrial cancers harboring PTEN/FGFR2 mutations, while it shows no effect in HEC1A endometrial cancers expressing wild-type FGFR2 along with PTEN/KRAS mutations (17, 18).

Owing to their prominent roles in cancers, FGFRs have become significant targets for inhibitor development (19). This effort has led to the discovery of several FGFR inhibitors, including dovitinib, BGJ398, ponatinib, IY2874455, and AZD4547, all of which have entered clinical trials as potential anticancer drugs. As a frontrunner in the group of pan-FGFR inhibitors, AZD4547 was demonstrated to promote favorable therapeutic outcomes against a variety of FGFR-deregulated cancer models, including glioblastoma, non–small cell lung cancer, gastric cancer, and multiple myeloma (20–23). However, little is known about the efficacy of
AZD4547 against FGFR2 mutant-driven endometrial cancer. Here, we investigated a detailed anticancer mechanism of AZD4547 against FGFR2 mutant–expressing endometrial cancers.

Materials and Methods

Reagents

Inhibitors, including AZD4547, BGI398, sorafenib, dasatinib, and cabozantinib, were purchased from Selleck Chemicals. Recombinant human acidic FGF (aFGF) was from R&D Systems (#231-BC). Heparin sulfate (#H3149), collagen (#C7661), and iodonitrotetrazolium chloride (#I8377) were from Sigma Aldrich. Antibodies for FGFR2, actin, phospho-GSK3 (Ser9), caspase-3, and cleaved PARP were purchased from Santa Cruz Biotechnology. Other primary antibodies were purchased from Cell Signaling Technology: phospho-FGFR (Tyr653/654, #3471), phospho-S6 (S235/236; Cell Signaling Technology, #4058), phospho-JNK (Thr183/Tyr185, #4668), phospho-p38 (Thr180/Tyr182, #4511), phospho-RET (Tyr905, #3221), phospho-FLT3 (Tyr591, #3474), phospho-DDR1 (Tyr792, #11994), phospho-FMS (Tyr723, #3155), phospho-PLK1 (Thr210, #9062), phosphor PLCγ (Tyr783, #2811), phospho-STAT3 (Tyr117, #9167), phospho-STAT3 (Ser277, #1134), phospho-p70S6K (Thr421/Ser424, #9204), phospho-CREB (Ser133, #9198), phospho-IkBα (Ser32/36, #9246), FGFRI (Phospho#9740), FLT3 (Phospho#3462), RET (Phospho#3220), AKT (Phospho#4685), ERK (Phospho#4272), and EGR1 (Phospho#1435). Antibodies for immunohistochemistry were anti-Ki67 (MIB1, Dako, #M7240), AKT (#4685), ERK (#4695), HSP60 (#4870), and EGR1 (Phospho#1435). Kinases at 10^6 M were assayed by 100 μM ATP, 10 μM ATP in duplicate mode. The extent to which cell viability was measured using an MTT assay kit (Roche, #11 988 549 001). For cell-cycle analysis, cells were treated with compounds for 24 hours and harvested as above and fixed in 70% ethanol at −20°C overnight. Cells were harvested by centrifugation at 500 × g. Washed with cold DPBS, then suspended in propidium iodide/RNase solution (Cell Signaling Technology, #4087) and incubated for 30 minutes in a dark condition before flow cytometer analysis (BD Biosciences).

Apoptosis and cell-cycle analysis

For apoptosis assay, cells were treated with AZD4547 and BGI398 for 48 hours. All floating and attached cells were harvested by trypsinization and washed with PBS (DPBS pH 7.4). Cells were stained using an Annexin V-FLUOS staining Kit (Roche, #11 988 549 001). For cell-cycle analysis, cells were treated with compounds for 24 hours and harvested as above and fixed in 70% ethanol at −20°C overnight. Cells were harvested by centrifugation at 500 × g. Washed with cold DPBS, then suspended in propidium iodide/RNase solution (Cell Signaling Technology, #4087) and incubated for 30 minutes in a dark condition before flow cytometer analysis (BD Biosciences).

Soft-agar assay

On the 0.5% bottom agar, cells in the complete media containing 0.3% agar was plated at a density of 2,000 cells in 6-well plates. The plates were incubated for 3 weeks at 37°C and 5% CO2. Compounds diluted in culture media were added on the top agar. Media were refreshed twice a week. Spheroids were stained using iodonitrotetrazolium chloride (Sigma Aldrich) for 24 hours. The entire area of each well was photographed without magnification, and colonies in each well were counted using ImageJ software.

Phospho-kinase array analysis

AN3-CA cells serum-starved media (DMEM containing 0.5% FBS) overnight were treated with either DMSO or AZD4547 (300 nmol/L) for 2 hours. Cells were stimulated with 10 ng/mL aFGF and 10 μg/mL heparin sulfate for 20 minutes. Cells were then lysed and subjected to the analysis using Human-Phosphokinase Array Kit (R&D Systems) using the manufacturer’s protocol.

Kinome profiling

Kinome profiling was performed by Reaction Biology Corp. AZD4547 (1 μmol/L) was tested against 336 recombinant human kinases at 10 μmol/L ATP in duplicate mode. The extent to which kinase activity is inhibited by AZD4547 was indicated as percentage relative to DMSO-treated kinase activity (100%).

Establishment of Tel-fused tyrosine kinase–transformed Ba/F3 cell lines

Ba/F3 cells lines transformed with TEL-fused human FLT3 or RET were established as described (24).

Immunoblot analysis

Cells were incubated overnight in the base media containing 0.5% FBS, treated primarily with compound for 2 hours, and subsequently stimulated by a mixture of 10 ng/mL aFGF and 10 μg/mL heparin sulfate for 20 minutes, unless otherwise specified.

Proliferation assay

Cells were plated at 5,000 cells/well in 96-well plates. Each compound was added to wells at 10 points of 3-fold serial dilution (0–50 μmol/L). For adherent cells, cells were treated with compounds one day after cell seeding. After 72-hour exposure, cell viability was measured using an MTT assay kit (Promega). Each assay was performed in duplicate mode three independent times. Cell viability of compound-treated wells was normalized relative to 0.5% DMSO-treated wells (100%). GI50 values were calculated using Prism 5.0 software (GraphPad).
added. Luminescence values were normalized by dividing firefly emission by Renilla emission as relative luminescence units (RLU). RLU values related to DMSO controls were represented as fold changes.

Microarray analysis

AN3-CA cells were treated in three different conditions for 18 hours: (i) DMSO (0.5%) only, (ii) cotreatment of aFGF (10 ng/mL) and DMSO (0.5%), and (iii) cotreatment of aFGF (10 ng/mL) and AZD4547 (300 nM) in duplicate mode and then lysed with 1 mL of TRizol reagent (Life Technologies). Total mRNAs were isolated using RNeasy Mini Kit (Qiagen) and were subjected to GeneChip PrimeViewTM Human Gene Expression Array (Affymetrix). Biotinylated cRNA was prepared according to the standard Affymetrix protocol from 500 ng total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). After fragmentation, 12 μg of biotinylated cRNA were hybridized for 16 hours at 45°C on GeneChip Human Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed using Robust Multiarray Analysis (RMA) with Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log transformed intensity values were then analyzed using GeneSpring GX 12.6 (Agilent Technologies). Fold change filters included the requirement that the genes be present in at least 150% of controls for upregulated genes and lower than 66% of controls for downregulated genes. Hierarchical clustering data were clustered into groups that behave similarly across experiments using GeneSpring GX 12.6.1 (Agilent Technologies). This microarray data was deposited in GEO public repository (GEO access no. GSE61481).

RT-PCR

Total RNAs were extracted from cells treated with the indicated condition using TRizol reagent (Invitrogen) according to the manufacturer’s instruction. Total RNAs (2 μg) was used to synthesize cDNA with M-MLV reverse transcriptase (Promega). Equal amount of cDNA was amplified with PCR for 30 cycles. The exact size of amplified PCR products was assessed by electrophoresis in 3% agarose gel and visualized by Eco-star dye (Biofect #ES301-1000). Primer sequences are listed in Supplementary Table S1.

Gene set enrichment analysis

We utilized 509 genesets publicly available in molecular signature database (MsigDB) as described [25]. These gene sets are categorized by transcription factor–binding motif in their promoter regions. For each gene set, we calculated enrichment score to evaluate the distribution of members of a given gene set throughout total gene list. Enrichment score (ES) means the maximum distance form X axis in the enrichment plot, and high ES reflects that a gene set is overrepresented at the top of the entire list. Variation in gene set size was adjusted in normalized ES (NES). False discovery rate (FDR) reflects the probability of false positive interpretation of the gene set with NES.

Xenograft model

Animal experiments were approved by the Institutional Animal Care and Use Committee of Korea University. Tumor xenografts were established by subcutaneous injection into the right flank of 5-week-old female balb/c nude mice (Orient Bio Inc.) with AN3-CA cells (5 x 10⁶ cells/0.2 mL) mixed with Matrigel (Becton Dickinson) to 1:1. When the tumor volume reached 100 mm³ on average, the tumor-bearing mice (n = 21) were sorted randomly into 3 treatment groups: vehicle (n = 7), 10 mg/kg AZD4547 (n = 7), 30 mg/kg AZD4547 (n = 7). The final concentration of vehicle was 5% NMP, 6% solumol, 20% PEG400, 69% distilled water, and 1 N HCl. Mice were orally gavaged once daily for 15 days. Tumor volume and body weight were measured twice a week. The formula used to calculate tumor volumes is [longest length x (length vertical to the longest length)²]/2 mm³. Body weights were measured once a week.

Immunohistochemistry

Tumors were removed from subcutaneously xenografted mice 4 hours after the final oral administration. Each tumor was transferred to cassettes, followed by fixation in 10% formalin solution overnight at 4°C. Each formalin-fixed tumor was washed in flowing water for 20 minutes. The tissue cassettes were transferred to a solution of 70% ethanol. Tissues were embedded in paraffin and sliced. Paraffin-embedded tissue slices were stained using the following antibodies; Ki67 antibody (1:200), anti-phospho FGFR (1:50), anti-EGR1 (1:50), anti-phospho ERK1/2 (1:100), anti-phospho S6 (1:200), and anti-cleaved caspase 3 (1:100). The staining intensity was quantitated using the threshold function of ImageJ software.

Results

AZD4547 displays antiproliferative effects against FGFR2 mutant–expressing endometrial cancer cells

We measured the kinase-inhibitory activities of AZD4547 against WT FGFR1–4 and their mutants (Table 1). AZD4547 profoundly inhibited FGFR1–3 with the IC₅₀ values of 2, 1, and 7 nmol/L, respectively, and it showed a lower potency on FGFR4 (IC₅₀ = 56 nmol/L). The kinase-inhibitory potencies of AZD4547 were also assessed against the three gatekeeper mutants (VS61M-FGFR1, V550E/L-FGFR4) and five activating mutants, including N550H-FGFR2, K650E/M-FGFR3, G697C-FGFR3, and N535K-FGFR4. N550H-FGFR2 (IC₅₀ = 8 nmol/L), K650E-FGFR3 (IC₅₀ = 21 nmol/L), K650M-FGFR3 (IC₅₀ = 82 nmol/L), and G697C-FGFR3 (IC₅₀ = 3 nmol/L), were all potently inhibited by AZD4547, but gatekeeper mutants (VS61M-FGFR1, V550M/E-FGFR4) and N535K-FGFR4 were much less sensitive to AZD4547 (IC₅₀ > 600 nmol/L). On the basis of the potent kinase-inhibitory activity against the N550H-FGFR2 mutant (IC₅₀ = 8 nmol/L), we measured the antiproliferative activity of AZD4547 against endometrial cancer cell lines harboring FGFR2-activating mutants (Table 1). We also included HEC1A endometrial cancers expressing normal FGFR2. AZD4547 strongly suppressed the proliferation of AN3-CA cells harboring the FGFR2 double mutant K310R/N550 with the GI₅₀ value of 31 nmol/L. MFE280 (GI₅₀ = 730 nmol/L) cells, harboring N550K-FGFR2 and S252W-FGFR2, respectively, were about 7- to 20-fold less sensitive to AZD4547. On the other hand, endometrial cancer cells with other FGFR2 aberration such as Ishikawa (FGFR2 overexpression) and HEC1A (normal FGFR2) cells were resistant to AZD4547. This implicates that AZD4547 was highly effective to FGFR2-activating mutant-addicted endometrial cancers.
Biochemical kinase profiling of AZD4547 against a large panel of kinases and evaluation of its cellular activities

The 3-amidopyrazole–based structure of AZD4547 (Fig. 1A) is distinct from those of other FGFR inhibitors such as PD173074, dovitinib, NVP-BGJ398, and LY2874455. This suggests that AZD4547 is likely to display unique kinase inhibitory profile. Although the kinase inhibitory activity of AZD4547 against 24 kinases was reported previously (23), here we evaluated the in vitro biochemical activity of AZD4547 against a large panel of 336 human kinases (Fig. 1B; Supplementary Table S2). Among the large panel, the kinases, including FGFR1–4, MAP4K5, IRR, MAP4K3, DDR1, RET, MELK, FLT3, and FMS, were inhibited >90% by 1 μmol/L of AZD4547. We also found the IC50 values of AZD4547 against MAP4K5, IRR, MAP4K3, DDR1, RET, MELK, FLT3, and FMS were in a range of 0.5–84.6 nmol/L (Table 1).

We then examined antiproliferative activities using kinase-transformed Ba/F3 cell lines and human cancer cell lines (Table 1). AZD4547 inhibited the growth of Ba/F3 cell lines transformed with either FLT3-TEL or RET-TEL at moderate potency (GI50 = 4.6 μmol/L and 0.39 μmol/L, respectively). Growth of human cancer cells, including MOLM14 (FLT3-ITD/WT), MV4-11 (FLT3-ITD), and TT (RET C634W), was suppressed with the moderate GI50 values of 0.484, 0.459, and 2.9 μmol/L, respectively, consistent with the transformed Ba/F3 cellular activities of AZD4547.

Also, Western blot analysis showed that AZD4547 at 10 μmol/L exhibited a complete inhibition of FLT3 autophosphorylation in both MOLM-14 cells and FLT3-TEL–transformed Ba/F3 cells. RET autophosphorylation in TT cells was completely blocked by AZD4547 at 10 μmol/L (Fig. 1C and Supplementary Fig. S1). However, AZD4547 at 10 μmol/L did not inhibit collagen-induced DDR1 autophosphorylation in U2OS cells or DDR1-expressing HEK293T cells. These results indicate that AZD4547 has a reasonable cellular potency against FLT3 and RET, while it does not inhibit DDR1 in cell.

AZD4547 inhibits the proliferation of AN3-CA cells by mainly targeting FGFR2

We also assessed the inhibitory effects of AZD4547 against FLT3, DDR1, RET, FMS, and MELK in AN3-CA and HEC1A cells. We examined the phosphorylation of PLK1 to evaluate MELK cellular activity (26). AZD4547 at 0.1 μmol/L almost completely inhibited phosphorylation of FRS2α (Y436) in AN3-CA cells, whereas it showed no inhibition of p-FRS2α in HEC1A cells (Fig. 1D). On the contrary, phosphorylation of FLT3 was moderately attenuated by AZD4547 (1 μmol/L), while phosphorylations of DDR1, RET, FMS, and PLK1 were not inhibited at the same dosage. These results suggest that AZD4547 inhibited FGFR2 mutant–driven endometrial cancer cells mainly through inhibition of FGFR2.

We then assessed the cellular activity of AZD4547 in AN3-CA, MFE296, and HEC1A cells for inhibition of FGFR signaling. To activate the FGFR2 pathway in endometrial cancers, we used aFGF as a ligand. Meanwhile, it is of note that aFGF can activate all FGFR isotypes, and both AN3-CA and MFE296 cells reportedly contain almost equal gene copy number for each FGFR isotype (27). Time course experiments showed that AZD4547 at 1 μmol/L quickly inhibited the phosphorylations of FGFR (Y653/654) and ERK1/2 (T202/Y204) in AN3-CA and MFE296 cells, but attenuated phospho-AKT only in AN3-CA cells. In contrast, none of those signaling molecules were affected by 1 μmol/L AZD4547 in HEC1A cells (Fig. 1E). It was also observed that phospho-ERK1/2 level in AN3-CA and MFE296 cells was increased at late time due to a negative feedback mechanism. In addition, aFGF-induced phosphorylations of various FGFR downstream proteins, including PLCγ (Y783), FRS2α (Y436), AKT (T308), and ERK1/2 (T202/ Y204), were attenuated by AZD4547 in a dose-dependent manner in AN3-CA and MFE296 cells, not in HEC1A cells (Fig. 1F).

AZD4547 induces G1–S arrest and apoptosis, and inhibits colony formation of AN3-CA cells

We then examined the effects of AZD4547 on cell cycle and apoptosis using AN3-CA, MFE296, MFE280, and HEC1A cells and compared the effects of AZD4547 with those of another well-known FGFR inhibitor BGJ398. Both compounds showed similar results on all four cell lines. When treated for 24 hours, both AZD4547 and BGJ398 at 1 μmol/L induced significant G1–S arrest (P < 0.001) in AN3-CA, MFE296, and MFE280 cells, while they did not affect the cell cycle of HEC1A cells (Fig. 2A). Also, FACS

Table 1. AZD4547’s in vitro biochemical IC50 values against kinases and its antiproliferative GI50 values against human cancer cells and Ba/F3 cells

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (nmol/L)</th>
<th>Cell line</th>
<th>Genotype</th>
<th>Tissue origin</th>
<th>AZD4547</th>
<th>BGJ-398</th>
<th>Sorafenib</th>
<th>Dasatinib</th>
<th>Cabozantinib</th>
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<tr>
<td>FGFR1</td>
<td>2</td>
<td>FLT3-TEL</td>
<td>MOLM14</td>
<td>AML</td>
<td>4.6 ± 0.57</td>
<td>0.001 ± 0.16</td>
<td>0.04 ± 0.019</td>
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<tr>
<td>FGFR1 (V561M)</td>
<td>622</td>
<td>BaF3</td>
<td>FLT3-TEL</td>
<td>MTC</td>
<td>0.39 ± 0.048</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
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<td>FGFR2</td>
<td>1</td>
<td>Parental</td>
<td>RET-TEL</td>
<td>AML</td>
<td>0.98 ± 0.001</td>
<td>0.104 ± 0.015</td>
<td>0.35 ± 0.015</td>
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<tr>
<td>FGFR2 (N550H)</td>
<td>8</td>
<td>MOLM14</td>
<td>FLT3-ITD</td>
<td>AML</td>
<td>0.484 ± 0.015</td>
<td>0.0005 ± 0.002</td>
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<tr>
<td>FGFR3</td>
<td>7</td>
<td>MV4-11</td>
<td>FLT3-ITD</td>
<td>AML</td>
<td>0.459 ± 0.004</td>
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<tr>
<td>FGFR3 (K650E)</td>
<td>31</td>
<td>TT</td>
<td>RET C634W</td>
<td>MTC</td>
<td>2.9 ± 0.904</td>
<td>0.43 ± 0.105</td>
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<td>FGFR3 (K650M)</td>
<td>82</td>
<td>AN3-CA</td>
<td>FGFR2 N550K, K310R</td>
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<td>0.031 ± 0.005</td>
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<td>FGFR3 (G697C)</td>
<td>3</td>
<td>MFE296</td>
<td>FGFR2 N550K</td>
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<td>0.730 ± 0.057</td>
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<td>FGFR4</td>
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<td>Endometrium</td>
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<td>FGFR2 over exp.</td>
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<td>FGFR4 (V550L)</td>
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<td>DDRI</td>
<td>FMS</td>
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<td>RET</td>
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NOTE: Empty cells mean “not determined.”

Therapeutic Effects of AZD4547 on Endometrial Cancers

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AZD4547 inhibits multiple signalings, including JNK and ERK1/2, in endometrial cancers

We first used a phospho-kinase array to examine which signaling pathways were blocked by AZD4547 in AN3-CA cells. AZD4547 at 300 μmol/L attenuated the aFGF-induced phosphorylations of p38 (T180/Y182), ERK1/2 (T202/Y204, T185, Y187), JNK (T183/Y185, T221/Y223), CREB (S133), p70S6K (T421/S424), STAT3 (Y705), and PLCγ (Y783; Fig. 3A). We performed Western blot analysis using AN3-CA and control cells as well. AZD4547 caused similar levels of inhibition of phosphorylations in MFE280 cells and AN3-CA cells. Phosphorylations of JNK, ERK1/2, p70S6K, and p38 were blocked by AZD4547 (Fig. 3B). We repeated the same analysis using MFE296, MFE280, and HEC-1A cells as well. AZD4547 caused similar levels of inhibition of phosphorylations in MFE280 cells and AN3-CA cells. Phosphorylations of JNK, ERK1/2, p70S6K, and p38 were blocked to a lesser extent in MFE296 cells. But all phosphorylations that were blocked in AN3-CA cells were not affected in HEC1A cells. Also, AZD4547 at 3 μmol/L slightly inhibited p-phospho-GSK3β (Ser 9) of p-p70S6K and other kinases (Supplementary Fig. S2). This suggests that the activity of AZD4547 might be related mainly with its ability to block MAPK pathways.

AZD4547 perturbs various transcription factors, including SRF/Elk-1 and AP-1

Next, we performed 45 pathway dual-reporter gene assays to investigate which transcription factors were affected by
AZD4547 in AN3-CA cells. Among 45 transcription factors, four transcription factors were significantly (P < 0.05) perturbed by AZD4547 (Fig. 3C). Reporter activities (firefly/Renilla) of STAT1/2 and NFκB were observed to be downregulated by aFGF, and antagonized by AZD4547. In contrast, the transcriptional activity of AP1 and SRF/Elk-1 was upregulated by aFGF stimulation, and AZD4547 blocked the upregulation of AP1 and SRF/Elk-1. The perturbations of these four transcription factors were verified by measuring the phospho levels of the transcription factors or their upstream regulators (Fig. 3D). Western blot analysis showed that phosphorylations of JNK, ERK1/2, and IκB, which are well-known upstream regulators of AP-1, Elk-1, and NFκB, respectively, were enhanced by aFGF, and were antagonized by AZD4547. Likewise, phosphorylation of STAT1 was downregulated by aFGF stimulation, and was antagonized by AZD4547.

AZD4547 changes the transcriptions of a number of genes, including EGR1. In addition, we carried out a genome-wide transcriptome analysis using AN3-CA cells. AN3-CA cells were treated with vehicle (DMSO only), with vehicle and aFGF (DMSO+aFGF), or with AZD4547 and aFGF (AZD4547+aFGF). RNAs were harvested at 18 hours after treatment and analyzed using Affymetrix Primeview arrays (Fig. 4A). We identified that 143 probes were upregulated more than 2-fold by aFGF stimulation relative to DMSO only (P < 0.01), and that 240 probes were significantly downregulated less than 0.5-fold after cotreatment of aFGF and AZD4547 relative to aFGF+DMSO treatment (P < 0.01; Supplementary Table S3 and S4). Thus, we assumed that all 143 genes overlapped in both probe sets would be the FGFR downstream target genes (Fig. 4B; Supplementary Table S5). To validate the microarray results, reverse transcription (RT)-PCR was performed...
for six randomly selected genes, including EGR1, THBS1, SERTAD1, and SERPINE1, among the 143 genes (Fig. 4C), and GAPDH was used as a normalization control. Consistent with microarray results, all six genes were upregulated by aFGF stimulation and restored to basal levels by AZD4547 (0.3 μmol/L).

We carried out GSEA to interpret microarray data (25). We found that 95 gene sets, out of 509 gene sets from transcription factor–binding sequence elements in MSigDB (molecular signatures database), were significantly enriched at the top rank of genes downregulated by AZD4547 (FDR < 0.25, P < 0.01). A gene set with SRF-binding motif (named SRF gene set hereafter) showed the highest enrichment score, followed by EGR1–3 and PAX3 gene sets. The enrichment plot of SRF-binding genes and EGR1-binding genes showed that their gene members were highly concentrated in top tier of the entire gene list with NES value of 2.08 and 1.59, respectively (Fig. 4D). This implies that target genes of SRF and EGR1 were significantly downregulated by (aFGF+AZD4547) treatment compared with (aFGF+DMSO). EGR1 also bears SRF-binding promoter near its transcription initiation site (27). We investigated whether EGR1 plays an important role in mutant FGFR2-mediated cancer progression of endometrial cancers. Time-course stimulation of aFGF in AN3-CA cells showed that EGR1 level was rapidly increased within 1 hour, and maintained throughout the course (Fig. 4E). In addition, attenuation of FGFR2 phosphorylation through siRNA knockdown or AZD4547 treatment resulted in suppression of aFGF-mediated EGR1 phosphorylation and restored to basal levels by AZD4547 (0.3 μmol/L).
upregulation, and AZD4547 displayed more dramatic EGR1 downregulation than FGFR2 siRNA (Fig. 4F). These results indicate that EGR1 expression level is controlled by FGFR signaling.

AZD4547 causes remarkable delay of tumor growth in AN3-CA mouse xenograft model

The in vivo efficacy of AZD4547 was also examined using mouse xenograft models where AN3-CA cells are grafted through subcutaneous injection (Fig. 5A). AZD4547 displayed a dose-dependent antitumor efficacy. While the average tumor volume of the vehicle-treated group ($n = 7$) was observed to increase to around 1,000 mm$^3$, the AZD4547-treated (10 mg/kg, orally once a day) group ($n = 7$) delayed the tumor growth and shrank the tumor volume to 360 mm$^3$. Moreover, the group ($n = 7$) treated with 30 mg/kg AZD4547 further delayed the tumor growth and almost reached tumor regression within a week. In addition, AZD4547 did not reduce significantly the bodyweight of the AZD4547-treated group, which implicated that its cytotoxic effect might be negligible (Fig. 5B). We performed immunohistochemistry staining using paraffin-embedded tumor sections from a mouse administered for

Figure 4.
AZD4547 changes the transcriptions of a number of genes, including EGR1. A, heatmap representation of microarray results using an Affymetrix primeview human gene chip. AN3-CA cells were incubated in serum-free media overnight, and treated with AZD4547 (300 nmol/L) and aFGF/heparin sulfate for 18 hours. RNAs were isolated from a duplicate set of AN3-CA cells. Gene expression profiling was performed. B, schematic diagram of FGFR downstream target gene set. Analysis revealed that 143 probes were upregulated more than 2-fold by aFGF stimulation relative to DMSO only (Student's $t$ test, $P < 0.01$), and that 240 probes were significantly downregulated less than 0.5-fold after cotreatment of aFGF and AZD4547 compared with (aFGF + DMSO) treatment (Student's $t$ test, $P < 0.01$). The 143 genes overlapped in both probe sets would be the FGFR downstream target genes. Several FGFR downstream target gene set members identified included EGR1, THBS1, SERTAD1, SERPINE1, EMP1, and GDF-15/MIC-1. C, RT-PCR confirmed six genes identified from the transcriptome analysis. D, graphic representation of the enrichment of SRF target genes and EGR1 target genes in the top rank of AZD4547-regulated genes in AN3-CA cells. E, time-course stimulation of aFGF showed that EGR1 level was rapidly increased within 1 hour, and maintained throughout the course. F, FGFR2 downregulation through siRNA knockdown or AZD4547 treatment resulted in suppression of aFGF-mediated EGR1 upregulation, and AZD4547 displayed more dramatic EGR1 downregulation than FGFR2 siRNA, indicating that EGR1 level is controlled by FGFR signaling. AN3-CA cells were treated with AZD4547 for 2 hours followed by 1-h stimulation with aFGF.
14 days, and found that the levels of pERK1/2, and pS6, and Ki67 were greatly downregulated and the level of cleaved caspase-3 was enhanced by AZD4547 (30 mg/kg), which indicates that AZD4547 also effectively blocked the FGFR signalings and induced cancer cell apoptosis in vivo (Fig. 5C). We also observed that the level of EGR1 was also decreased in AZD4547-administered mouse, confirming EGR1 downregulation by AZD4547 in vivo.

**Discussion**

FGFR2-activating mutations, including S252W and N550K, identified in 12% of endometrial cancer patients provide a rational for potential therapeutic application of FGFR inhibitors for endometrial cancers (15). AZD4547 is a potent pan-FGFR inhibitor being evaluated in clinical trials for several cancers, including breast, lung, and stomach cancers, that are associated with aberrant activities of FGFRs. For the first time, we demonstrated its potential application for FGFR2-addicted endometrial cancers.

Kinome-wide in vitro profiling of AZD4547 against a panel of 336 kinases revealed that 2% (7/336) of the kinases, including FGFRs, were inhibited by AZD4547, which were MAP4K5, IRR, MAP4K3, RET, MELK, FLT3, and FMS. Investigation of its cellular activities showed that AZD4547 possesses moderate activities against FLT3 and RET in cells.

![Figure 5](image-url)

**Figure 5.**

AZD4547 caused a strong delay of tumor growth in the AN3-CA mouse xenograft model. A, in vivo antitumor efficacy of AZD4547 in the AN3-CA xenograft mouse model. AZD4547 was administered by oral gavage, once daily (per os, everyday) at the indicated doses (n = 7). Tumor size (mm³) was measured twice a week. Data was plotted as mean ± SD and analyzed using the Dunnett test. ***, P < 0.001. B, body weight was monitored during treatment with AZD4547. Data are expressed as a percentage of body weight relative to the initiation of treatment and are plotted as mean ± SEM. Dunnett test; *, P < 0.05. C, immunohistochemical staining of paraffin-embedded tumors. On the final dose day, tumors were collected from the 10-mg/kg treatment mouse group that was dosed with 30 mg/kg AZD4547 for 4 hours, while the vehicle group was dosed with vehicle as usual. Tumors were fixed, retrieved, and stained with the indicated antibodies, then photographed at 400x magnification. For each treatment group, tumors from two different mice were used for immunohistochemical analysis. Bar, 100 μm. A small fraction of each field image is presented. D, the staining intensity of each original field image (400x) was quantified using the threshold function of ImageJ software, and bar graph represents percentage (mean ± SD, n = 2) of stained area relative to the entire area of a field. Student t test; **, P < 0.05; ***, P < 0.01; ***, P < 0.001.
We found that AZD4547 was most effective to AN3-CA cells expressing FGFR2 N550K/K310R mutation. AZD4547 effectively blocked autophosphorylation of FGFRs and its subsequent downstream signaling, inducing cell-cycle arrest at G1/S transition and apoptosis in AN3-CA cells, whereas it had no significant activity on HECA1A harboring normal FGFR2. In addition, like other FGFR-targeted inhibitors such as PD173074, dovitinib, and BGJ-398, AZD4547 showed weaker activity against MFE296 (FGFR2 N550K) and MFE280 (FGFR2 S252W) cells than against AN3-CA cells (15, 18, 28).

The results from phospho-kinase array experiment using AN3-CA cells revealed that AZD4547 perturbed mainly ERK1/2 and JNK signalings. Also our reporter gene assays showed that the activities of SRF/Elk-1 and AP-1, which are effector transcription factors of ERK1/2 and JNK, respectively, were downregulated by AZD4547. This is consistent with the report that downregulation of ERK1/2 activity is associated with therapeutic effects of FGFR inhibitor (PD173074) in endometrial cancer cells harboring mutants of PTEN and FGFR2 (17). Our results also demonstrated inhibition of the MAPK pathway.

GSEA analysis from our gene transcription profiling suggested that AZD4547 suppressed cancer progression of AN3-CA cells via EGR1 downregulation. Acidic FGF is a well-known strong activator of EGR1 (29), and EGR1 gene was identified to have several functional elements, including serum response elements (SREs), AP-1, cAMP response elements (CRE), and SP-1, at the 3′ end of its promoter (30). Nonetheless, the significance of FGFR2-EGFR axis in endometrial cancer progression has not been reported yet. EGR1 expression was dependent on FGFR2 activity, because siRNA knockdown of FGFR2 perturbed aFGF-induced EGR1 upregulation. In addition, an in vivo xenograft model of AN3-CA showed that EGR1 was effectively downregulated in AZD4547-administered tumors. Our study suggests that EGR1 downregulation is related to AZD4547’s efficacy against endometrial cancers.

We also showed that AZD4547 suppressed anchorage-independent growth of AN3-CA cells and induced significant tumor regression in the AN3-CA mouse xenograft model in conjunction with a significant decrease in proliferation markers, including p-ERK1/2 and Ki67, in tumors. The efficacy of AZD4547 might be associated with the findings from unbiased reporter gene assays and transcriptome analysis using AN3-CA cells. AZD4547 perturbed the activities of a number of transcription factors, such as EGR-1 and SP1, which are related to proliferation as well as upregulating p53 activity.

We here report the detailed mechanism by which AZD4547 expresses its anticancer effect against FGFR2 mutant–dependent endometrial cancer cells. Our study demonstrates that AZD4547 exhibits its therapeutic activity against endometrial cancer cells by perturbing various regulatory mechanisms related to FGFR signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Y. Kwak, T. Sim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kwak, H. Cho
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kwak, W. Hur, T. Sim
Writing, review, and/or revision of the manuscript: Y. Kwak, W. Hur, T. Sim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kwak, T. Sim
Study supervision: T. Sim

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