AZD2171 Shows Potent Antitumor Activity Against Gastric Cancer Over-Expressing Fibroblast Growth Factor Receptor 2/Keratinocyte Growth Factor Receptor

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Abstract

Purpose: AZD2171 is an oral, highly potent, and selective vascular endothelial growth factor signaling inhibitor that inhibits all vascular endothelial growth factor receptor tyrosine kinases. The purpose of this study was to investigate the activity of AZD2171 in gastric cancer.

Experimental Design: We examined the antitumor effect of AZD2171 on the eight gastric cancer cell lines *in vitro* and *in vivo*.

Results: AZD2171 directly inhibited the growth of two gastric cancer cell lines (KATO-III and OCUM2M), with an IC_{50} of 0.15 and 0.37 μmol/L, respectively, more potently than the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib. Reverse transcription-PCR experiments and immunoblotting revealed that sensitive cell lines dominantly expressed COOH terminus–truncated fibroblast growth factor receptor 2 (FGFR2) splicing variants that were constitutively phosphorylated and spontaneously dimerized. AZD2171 completely inhibited the phosphorylation of FGFR2 and downstream signaling proteins (FRS2, AKT, and mitogen-activated protein kinase) in sensitive cell lines at a 10-fold lower concentration (0.1 μmol/L) than in the other cell lines. An *in vitro* kinase assay showed that AZD2171 inhibited kinase activity of immunoprecipitated FGFR2 with submicromolar K_i values (~0.05 μmol/L). Finally, we assessed the antitumor activity of AZD2171 in human gastric tumor xenograft models in mice. Oral administration of AZD2171 (1.5 or 6 mg/kg/d) significantly and dose-dependently inhibited tumor growth in mice bearing KATO-III and OCUM2M tumor xenografts.

Conclusions: AZD2171 exerted potent antitumor activity against gastric cancer xenografts over-expressing FGFR2. The results of these preclinical studies indicate that AZD2171 may provide clinical benefit in patients with certain types of gastric cancer.

Various anticancer therapies for gastric cancer have been investigated over the past two decades. Despite intensive studies, the prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor (1, 2), and new therapeutic modalities are needed.

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Fibroblast growth factors (FGF) and their signaling receptors have been found to be associated with multiple biological activities, including proliferation, differentiation, motility, and transforming activities (3–5). The *K-sam* gene was first identified as an amplified gene in human gastric cancer cell line KATO-III (6, 7), and its product was later found to be identical to the bacteria-expressed kinase, or keratinocyte growth factor receptor (KGFR), and FGF receptor 2 (FGFR2). FGFR2/KGFR/K-sam is preferentially amplified in poorly differentiated types of gastric cancers with a malignant phenotype, and its protein expression was detected by immunohistochemical staining from 20 of 38 cases of the undifferentiated type of advanced stomach cancer (8, 9). Thus, FGFR2 signaling may be as a promising molecular target for gastric cancer.

AZD2171 is a potent, ATP-competitive small molecule that inhibits all vascular endothelial growth factor receptors [VEGFR-1, VEGFR-2 (also known as KDR), and VEGFR-3]. *In vitro* studies have shown that recombinant VEGFR-2 tyrosine kinase activity was potently inhibited by AZD2171 (IC_{50} <1 nmol/L; ref. 10). AZD2171 also showed potent activity versus VEGFR-1 and VEGFR-3 (IC_{50}, 5 and ≤3 nmol/L, respectively). VEGF-stimulated proliferation and VEGFR-2 phosphorylation of human umbilical vascular endothelial cells

was inhibited by AZD2171 (IC_{50}, 0.4 and 0.5 nmol/L, respectively). In *in vivo* studies, inhibition of VEGFR-2 signaling by AZD2171 reduced microvessel density and dose-dependently inhibited the growth of various human tumor xenografts (colon, lung, prostate, breast, and ovary; ref. 10). These data are consistent with potent inhibition of VEGF signaling, angiogenesis, neovascular survival, and tumor growth. On the other hand, because it was known that AZD2171 also possesses additional activity against FGFR1 (IC_{50}, 26 nmol/L; ref. 10), we hypothesized that AZD2171 may exhibit the additional anticancer activity against FGFR-overexpressing gastric cancer cells.

Our previous studies showed significant activities of the dual VEGFR-2 and epidermal growth factor receptor inhibitor ZD6474 against poorly differentiated gastric cancer (11) and non–small-cell lung cancer with epidermal growth factor receptor mutations (12, 13), both *in vitro* and *in vivo*. Based on these findings, we proceeded to investigate the anticancer activity of AZD2171 in preclinical models (gastric cell lines and xenografts).

Materials and Methods

Anticancer agents. AZD2171 and gefitinib (Iressa) were provided by AstraZeneca. AZD2171 and gefitinib were dissolved in DMSO for the *in vitro* experiments, and AZD2171 was suspended in 1% (w/v) aqueous polysorbate 80 and administered in a dose of 0.1 mL/10 g per body weight in the *in vivo* experiments.

Cell culture. Human gastric cancer cell lines 44As3, 58As1, OKAJIMA, OCUM2M, KATO-III, MKN-1, MKN-28, and MKN-74 were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and penicillin-streptomycin.

Established highly tumorigenic cell line. Signet ring cell gastric carcinoma cell line KATO-III was gift from Dr. M. Sekiguchi (University of Tokyo, Tokyo, Japan). All of the presented *in vitro* experiments were done using the KATOIII cell line. We conducted a preliminary experiment to compare the cellular characteristics of TU-KATO-III cells and KATOIII cells, and the results revealed that a high expression level of FGFR2 and high sensitivity to AZD2171 were still maintained in the TU-KATO-III cells (data not shown). KATO-III did not show tumorigenicity following repeated implantation of the cultured cells into BALB/c nude mice. Following s.c. inoculation into nonobese diabetic/severe combined immunodeficient mice, 80% to 100% of the KATO-III cells caused the formation of tumor. Following this result, we cultured the cancer cells isolated from the tumor of mice that developed 2 to 3 months following the implantation of KATO-III cells and attempted s.c. injection into nude mice, in turn, of the incubated cells. This sequence of manipulations was repeated for seven cycles in an attempt to reliably isolate cell lines that would have higher potential to undergo tumor formation over short periods of time. In this way, we obtained a cell line (TU-kato-III) from KATO-III cells that possessed a high tumorigenic potential.

In vitro *growth inhibition assay.* The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the growth-inhibitory effect of AZD2171. Cell suspensions (180 μL) were seeded into each well of 96-well microculture plate and incubated in 10% fetal bovine serum medium for 24 h. The cells were exposed to AZD2171 or gefitinib at concentrations ranging from 4 nmol/L to 80 μmol/L and cultured at 37°C in a humidified atmosphere for 72 h. After the culture period, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent was added, and the plates were incubated for 4 h. After centrifugation, the culture medium was discarded, and wells were filled with DMSO. The absorbance of the cultures at 562 nmol/L was measured using Delta-soft on a Macintosh computer (Apple) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMatellics). This experiment was done in triplicate.

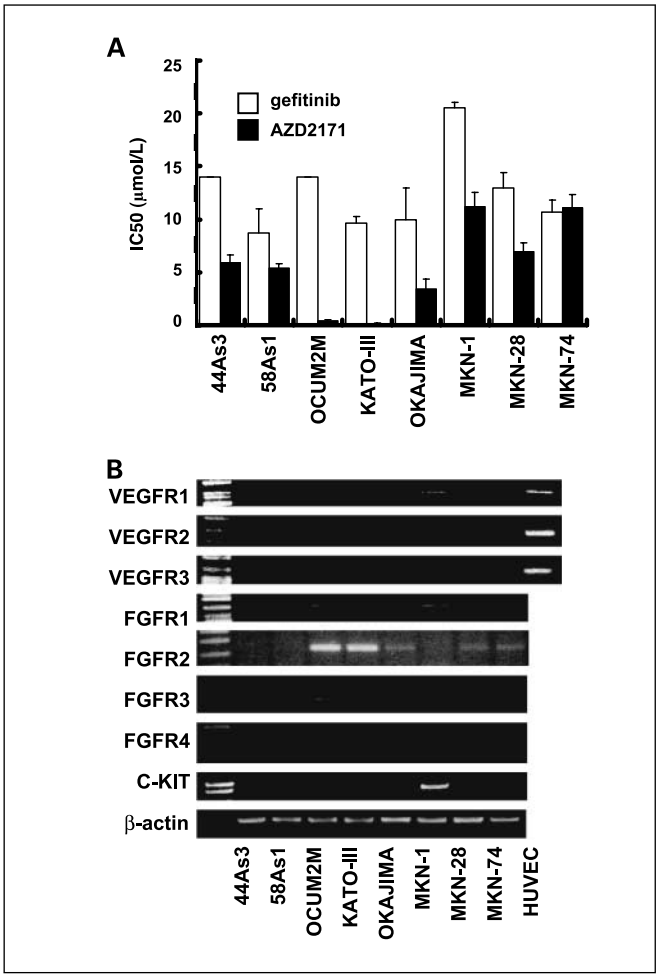

Fig. 1. *A, in vitro* growth-inhibitory effect of AZD2171 and gefitinib on eight gastric cancer cell lines. AZD2171 had a growth-inhibitory effect on KATO-III cells and OCUM2M cells (IC_{50}, 0.15 and 0.37 μmol/L, respectively). Columns, mean IC_{50} of each compound from three independent experiments; bars, SD. □, IC_{50} of gefitinib; ■, IC_{50} of AZD2171. *B,* the mRNA expression levels of VEGFRs, FGFRs, and c-KIT in gastric cancer cell lines were determined by reverse transcription-PCR. Human umbilical vascular endothelial cells were used as the positive control for the VEGFRs. No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in both sensitive cell lines, but FGFR2 was strongly detected; however, little faint or none was detected in the other cell lines.

Reverse-transcription PCR. Using a GeneAmp RNA-PCR kit (Applied Biosystems), 5 μg of total RNA from each cultured cell line was converted to cDNA. The PCR amplification procedure consisted of 28 to 35 cycles (95°C for 45 s, 62°C for 45 s, and 72°C for 60 s) followed by incubation at 72°C for 7 min, and the bands were visualized by ethidium bromide staining. The following primers were used for the PCR: human-specific β-actin, forward 5-GGAAATCGTGCGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3; VEGFR-1, forward 5-TAGCGTCACCAGCAGCGAAAGC-3 and reverse 5-CCTTTCTTTTGGGTCTCTGTGC-3; VEGFR-2, forward 5-CAGACGGACAGTGGTATGGTTC-3 and reverse 5-ACCTGCTGGTGGAAAGAACAAC-3; VEGFR-3, forward 5-AGCCATTCATCAACAAGCCT-3 and reverse 5-GGCAACAGCTGGATGTCATA-3; c-KIT, forward 5-GCCCACAATAGATTGGTATTT-3 and reverse 5-AGCATCTTTACAGCGACAGTC-3; FGFR1, forward 5-GGAGGATCGAGCTCACTCGTGG-3 and reverse

5-CGGAGAAGTAGGTGGTGTCAC-3; FGFR2, forward 5-CAGTAGGACTGTAGACAGTGAA-3 and reverse 5-CCGGTGAGGCGATCGCTCCACA-3; FGFR3, forward 5-GGTCAAGGATGGCACAGGGCTG-3 and reverse 5-AGCAGCTTCTTGTCCATCCGCT-3; and FGFR4, forward 5-CCGCCTAGAGATTGCCAGCTTC-3 and reverse 5-AGGCCTGTCCATCCTTAAGCCA-3.

Real-time reverse transcription-PCR. Real-time reverse transcription-PCR amplification was done by using a Premix Ex Taq and Smart Cycler system (Takara Bio, Inc.) according to the manufacturer's instructions. The following primers were used: FGFR2 (IIIb), forward 5-GATAAATAGTTCCAATGCAGAAGTGCT-3 and reverse 5-TGCCCTATATAATTGGAGACCTTACA-3 (7); FGFR2 (COOH-terminal), forward 5-GAATACTTGGACCTCAGCCAA-3 and reverse 5-AACACTGCCGTTTATGTGTGG-3; and human-specific β-actin, forward 5-GGAAATCGTGCGTGACATT-3 and reverse 5-CATCTGCTGGAAGGTGGACAG-3. The experiment was independently done in triplicate using β-actin as a reference to normalize the data.

Western blotting. Cells were cultured overnight in 10% serum-containing medium or serum-starved medium and exposed to 0.1 to 10 μmol/L of AZD2171 for 3 h before addition of KGF (100 ng/mL) for 15 min. Immunoblotting was done as described previously (14). In brief, after lysing the cells in radioimmunoprecipitation buffer, the lysate was electrophoresed through 10% (w/v) polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes and reacted with the following antibodies: anti-FGFR2 (H-80) and anti-FGFR2 (C-17) antibody (Santa Cruz Biotechnology, Inc.); anti-phosphotyrosine antibody PY20 (BD Transduction Laboratories); anti–phosphorylated FGFR (Tyr653/654), anti–mitogen-activated protein kinase, anti–phosphorylated mitogen-activated protein kinase antibody, anti-AKT, anti–phosphorylated AKT, and anti-rabbit horseradish peroxidase–conjugated antibody (Cell Signaling Technology); and anti-β-actin antibody (Sigma). Visualization was achieved with an enhanced chemiluminescent detection reagent (Amersham Bioscience).

FGFR2 kinase assay. FGFR2/KGFR kinase activity was quantified by using a Universal Tyrosine Kinase Assay kit (Takara) according to manufacturer's instructions. FGFR2/KGFR proteins were collected from the KATO-III, OCUM2M, and OKAJIMA cell lysates by overnight immunoprecipitation with an anti-FGFR2 antibody. The FGFR2/KGFR immune complexes were washed thrice with radioimmunoprecipitation assay buffer and diluted kinase reaction buffer. Immobilized tyrosine kinase substrate (poly[Glu-Tyr]) was incubated for 30 min at 37°C with each sample in the presence of kinase-reacting solution and ATP. Samples were washed four times, blocked with blocking solution, and incubated with anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase. The absorbance of the phosphorylated substrate was measured at 450 nm.

Chemical cross-link analysis. The chemical cross-link analysis was carried out as described previously (15). In brief, KATO-III cells and OKAJIMA cells were cultured under serum-starved conditions for 24 h, and after stimulation with KGF (100 ng/mL) for 15 min, they were collected and washed with PBS and incubated for 30 min in PBS

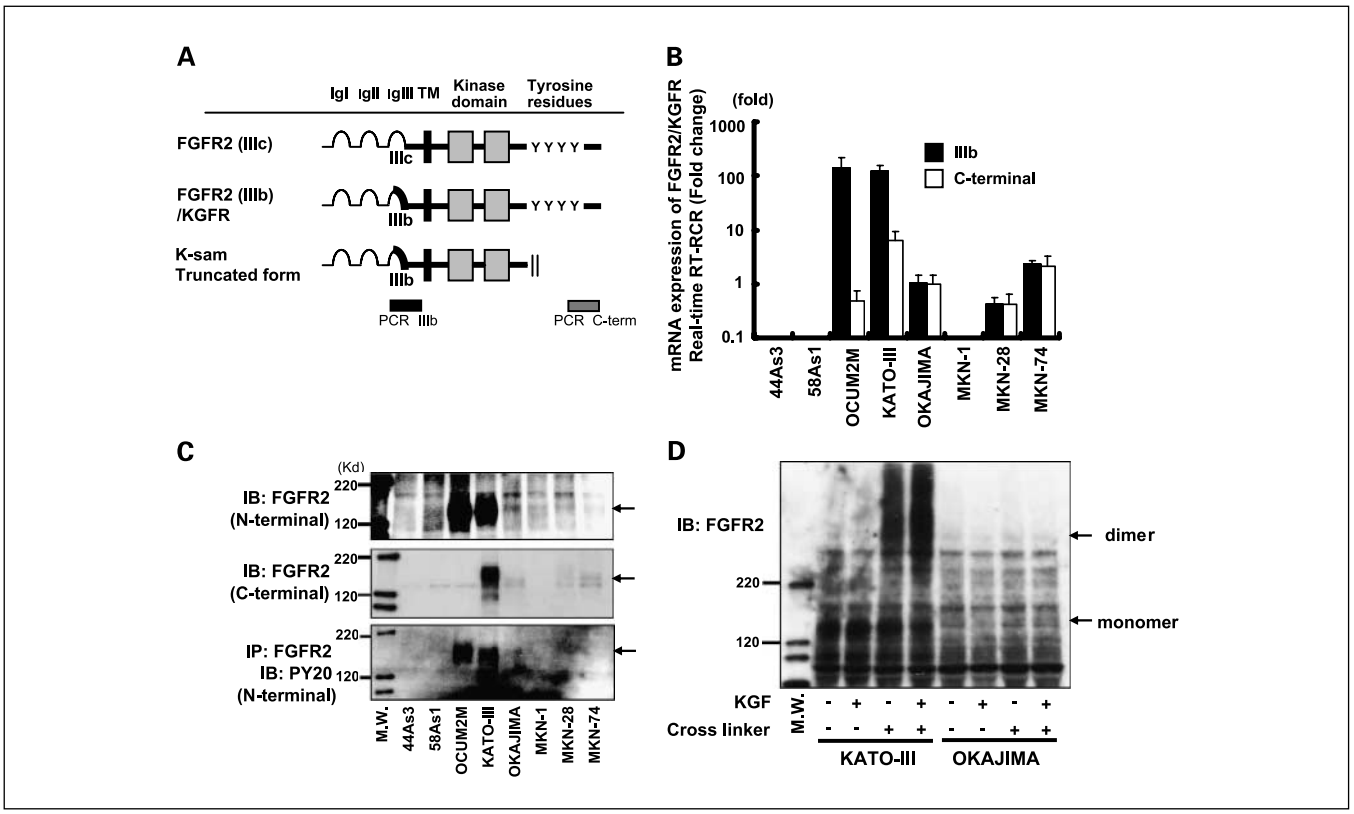

Fig. 2. *A,* schematic representation of FGFR2 and regions amplified by PCR. *B,* mRNA expression levels of FGFR2 were quantified by detecting the extracellular domain or COOH-terminal region by real-time reverse transcription-PCR. Expression in the cells is shown as a ratio to expression in OKAJIMA cells. FGFR2 was overexpressed in KATO-III cells and OCUM2M cells by about 100-fold compared with the other cell lines. The majority of the FGFR2 in the sensitive cell lines KATO-III and OCUM2M had no COOH-terminal region. *C,* protein expression levels of FGFR2 were determined by Western blotting with antibodies to the NH_2 or COOH termini. Both AZD2171-sensitive cell lines overexpressed FGFR2, and the phosphorylation levels were markedly higher. *D,* chemical cross-linking analysis. Cells were cultured under serum-starved conditions for 24 h and then stimulated with KGF (100 ng/mL) for 15 min. After collecting and washing them with PBS, they were incubated for 30 min in PBS containing cross-linker substrate. The reaction was terminated by adding 250 mmol/L glycine for 5 min. In spite of the serum-starved conditions, high levels of expression of the dimerized form were observed in KATO-III cells in the absence of ligand stimulation. This phenomenon was not observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation resulted in a mild increase in the dimerized form in KATO-III cells. Arrows indicate monomer or dimer formation.

containing 1.5 mmol/L of the non-permeable cross-linker bis-(sulfosuccinimidyl) substrate (Pierce). The reaction was terminated by adding 250 mmol/L glycine for 5 min, and the cells were analyzed by immunoblotting with FGFR2 antibody (Sigma).

FGFR2/KGFR gene silencing with small interfering RNA. Predesigned small interfering RNA (siRNA) targeting FGFR2 was purchased from Ambion. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. The cells were then transfected with the FGFR2 targeting siRNA or non-silencing siRNA using RNAiFect Transfection Reagent (Qiagen) according to the manufacturer's protocol and incubated another 72 h. Cell growth was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, 2×10^5 cells per well were plated on a six-well plate for 24 h and transfected with siRNA under the same conditions.

In vivo *experiments.* Tumorigenic TU-kato-III cells were derived from the gastric cancer cell line KATO-III. Four-week-old female BALB/c nude mice were purchased from CLEA Japan, Inc. and maintained under specific-pathogen-free conditions; 5×10^6 TU-kato-III cells or OCUM2M cells were s.c. injected into both flanks of each mouse. When the tumors had reached a volume of 0.1-0.3 cm^3, the mice were randomized into three groups (three per group) and given AZD2171, 1.5 or 6.0 mg/kg/d, or vehicle once daily by oral gavage for 3 weeks. Tumor volume was calculated using the formula: (length × width) × $\sqrt{}$(length × width) × (π/6), where length is the longest diameter across the tumor, and width is the corresponding perpendicular. All mice were sacrificed on day 21, and the tumors were collected. The protocol of the experiment was approved by the Committee for Ethics in Animal Experimentation and conducted in accordance with the Guidelines for Animal Experiments of National Cancer Center.

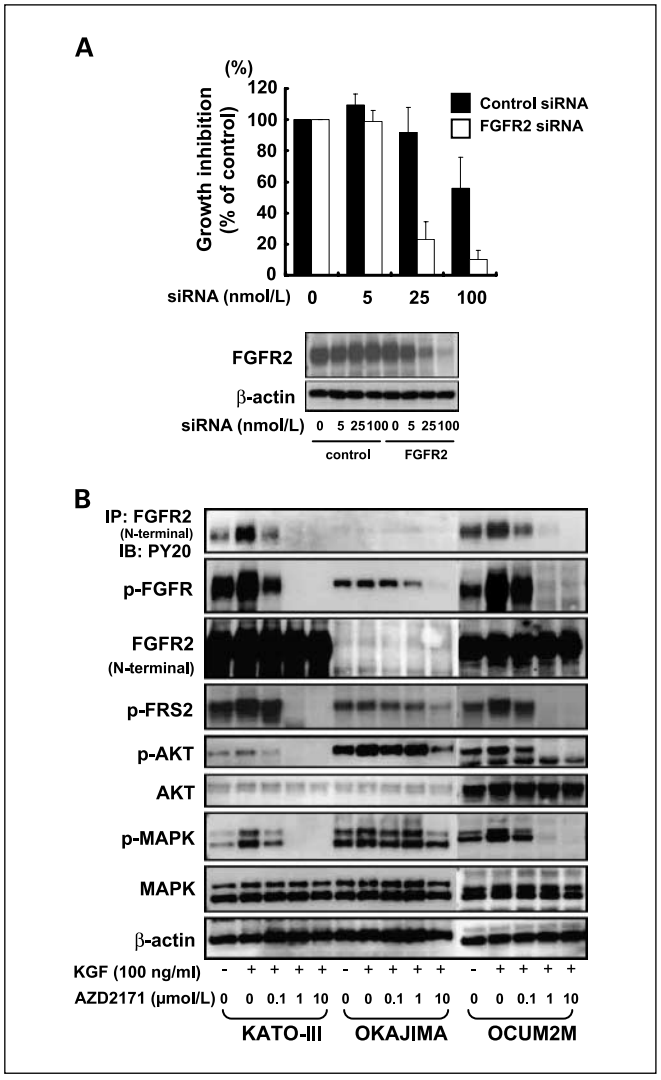

Fig. 3. *A,* FGFR2 targeting siRNA and cellular growth-inhibitory effect. KATO-III cells were plated on a 96-well plate and incubated in serum-containing medium for 24 h. After incubation, the cells were transfected with FGFR2-targeting or non-silencing siRNA and incubated for another 72 h. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For immunoblotting, 2×10^5 cells per well were plated on a six-well plate and treated similarly. Marked inhibition of cell growth (~80%) was observed by FGFR2 targeting siRNA compared with control siRNA (*top*). Reduction of FGFR2 protein expression in KATO-III cells was confirmed by immunoblotting (*bottom*). Columns, % control absorbance in three independent experiments; bars, SD. *B,* Western blotting for downstream molecules of FGFR2 signaling. Cells were cultured overnight under serum-starved conditions and exposed to 0.1 to 10 μmol/L AZD2171 for 3 h before adding 100 ng/mL KGF for 15 min. AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2 at 1 μmol/L in the sensitive cell lines, compared with 10 μmol/L in the control cell line OKAJIMA. Similar results were observed for FRS-2, AKT, and mitogen-activated protein kinase (*MAPK*).

Results

***AZD2171 showed growth-inhibitory activity* in vitro.** To evaluate the growth-inhibitory activity of AZD2171 *in vitro*, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on eight gastric cancer cell lines. The epidermal growth factor receptor–specific tyrosine kinase inhibitor gefitinib was used as a reference. The IC_{50} of gefitinib for all cell lines was between 7 and 20 μmol/L. AZD2171 inhibited the growth of KATO-III cells and OCUM2M cells (IC_{50}, 0.15 and 0.37 μmol/L, respectively) more potently than the other cell lines (Fig. 1A).

Expression levels of tyrosine kinase receptors. To elucidate the mechanism of action of AZD2171 in the two sensitive cell lines, we measured mRNA expression levels of VEGFRs, FGFRs, and c-KIT, whose kinase activity have been reported to be inhibited by AZD2171 (10). No mRNA expression of VEGFRs or c-KIT was detected by reverse transcription-PCR in either sensitive cell lines. FGFR2 transcripts, however, were strongly expressed in both sensitive cell lines but not strongly in the other cell lines (Fig. 1B). Since we previously found that FGFR2/KGFR/K-sam with a deletion of COOH-terminal exons was amplified in both sensitive cell lines (9), we speculated that amplified FGFR2/KGFR might be associated with sensitivity to AZD2171.

Sensitive cells expressed constitutively active and spontaneously dimerized FGFR2/KGFR. We quantified mRNA expression levels of FGFR2 by real-time reverse transcription-PCR with primers that detect the extracellular domain (IIIb region, see Fig. 2A) and COOH-terminal region. The results show that KATO-III cells and OCUM2M cells expressed FGFR2 100-fold higher than the other cells tested. The COOH-terminal region of FGFR2 was deleted in the KATO-III cells and OCUM2M cells (Fig. 2B). Overexpression and markedly increased phosphorylation of FGFR2 was observed in the AZD2171-sensitive cell lines (Fig. 2C).

Immunoblotting with antibodies for the COOH and NH_2 termini revealed that almost all the FGFR2 expressed by OCUM2M cells, and about half of FGFR2 expressed by KATO-III cells, were truncated (Fig. 2C). Although the KATO-III cells expressed wild-type receptor to some extent, the

Table 1. *In vitro* kinase assay of AZD2171 against FGFR2

Cell line	K_m	K_i (μmol/L)
KATO-III	8.3 ± 3.3	0.067 ± 0.017
OCUM2M	7.1 ± 1.4	0.072 ± 0.022
OKAJIMA	11.0 ± 5.0	0.049 ± 0.041

COOH-terminal truncated type was dominantly expressed in AZD2171-sensitive cell lines.

A chemical cross-linking analysis was done to evaluate the dimerization of FGFR2. High dimerization of FGFR2 was observed in the KATO-III cells even in the absence of ligand stimulation (Fig. 2D), but no such phenomenon was observed in the control undifferentiated OKAJIMA cell line. Ligand stimulation increased the level of the dimerized-form in KATO-III cells. Taken together, these findings show that the sensitive cell lines expressed high levels of FGFR2 that was highly phosphorylated and spontaneously dimerized without ligand stimulation, suggesting that FGFR2 signaling is constitutively activated in these cells. This evidence is consistent with the widely recognized findings that cancer cells sensitive to other tyrosine kinase inhibitors, such as gefitinib and imatinib, overexpress the highly phosphorylated target receptor with an increased level of dimerization in a ligand-independent manner (12, 16, 17).

FGFR2 targeting siRNA showed a potent growth-inhibitory effect on KATO-III cells. To investigate the dependency of cell growth through activated FGFR2 signaling in the AZD2171-sensitive KATO-III cell line, we evaluated the growth-inhibitory effect of siRNA targeted to FGFR2 in KATO-III cells. Targeted siRNA (5-100 nmol/L) decreased FGFR2 and inhibited cell growth (>80%) in a dose-dependent manner (Fig. 3A). The results show that most of the growth of KATO-III cells is dependent on activated FGFR2 signaling, suggesting that the FGFR signaling dependency may be responsible for the higher growth-inhibitory effect of AZD2171 on KATO-III cells.

AZD2171 inhibited FGFR2 signaling. Next, we examined the effect of AZD2171 on FGFR2 downstream phosphorylation signals (i.e., FRS-2, AKT, and mitogen-activated protein kinase). AZD2171 completely inhibited KGF-induced phosphorylation of FGFR2, FRS-2, AKT, and mitogen-activated protein kinase at 1 μmol/L in KATO-III cells, compared with 10 μmol/L in OKAJIMA cells. These results clearly show that AZD2171 possesses inhibitory activity against FGFR2 in cell-based studies and significantly inhibits the phosphorylation of FGFR2 at 1 μmol/L in sensitive cells.

FGFR2 kinase inhibition of AZD2171. To quantify the inhibitory activity of AZD2171 on FGFR2 kinase under cell-free conditions, we calculated the K_i values for immunoprecipitated FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA cells. The K_i values of AZD2171 for FGFR2 in each of these cell lines were 0.067 ± 0.017, 0.072 ± 0.022, and 0.049 ± 0.041 μmol/L, respectively (Table 1). In contrast, the K_i value of AZD2171 for recombinant VEGFR-2 was 0.0009 μmol/L (data not shown) and was consistent with previous reports (10). At the cellular level, phosphorylation of

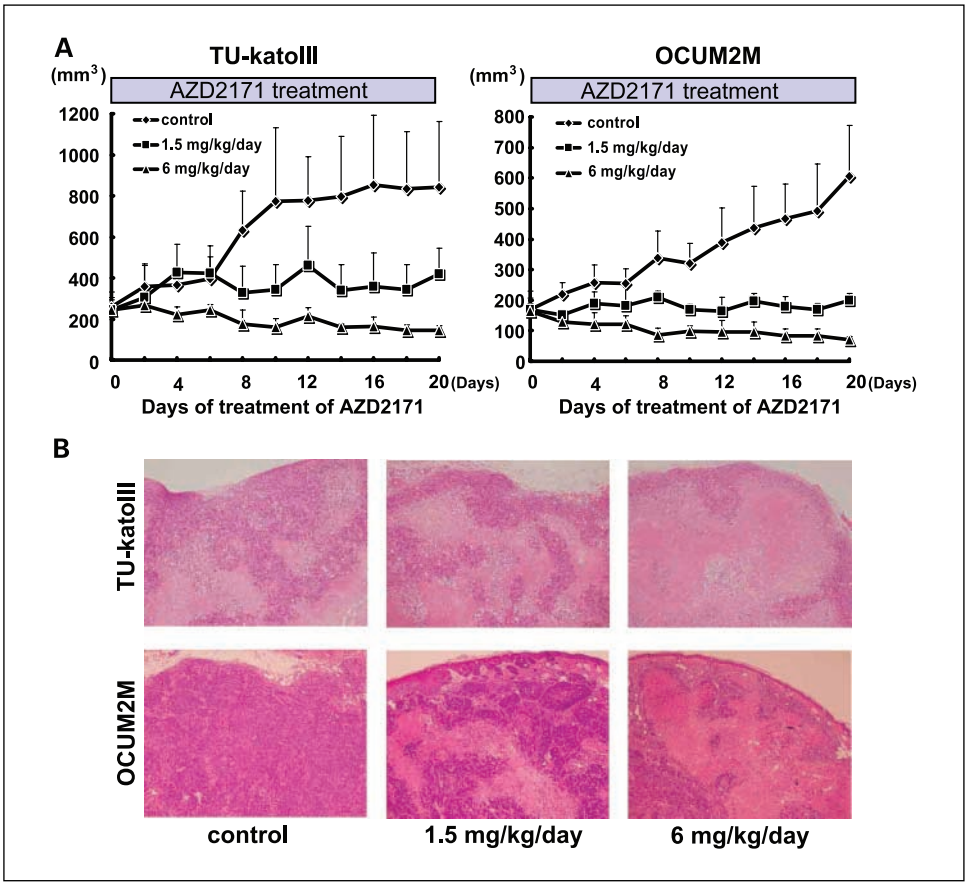

Fig. 4. *A, in vivo* growth-inhibitory effect of AZD2171 in a tumor xenograft model. After s.c. injecting 5×10^6 TU-kato-III or OCUM2M cells into both flanks of the mice, AZD2171 (1.5 or 6.0 mg/kg/d), or vehicle, was administered orally once daily for 3 wks. A marked tumor growth-inhibitory effect was observed at the low dose (1.5 mg/kg/d) of AZD2171 in both the TU-kato-III tumors and OCUM2M tumors, and the high dose (6.0 mg/kg/d) of AZD2171 completely inhibited the growth of both cell lines. *B,* representative H&E staining of tumor tissue from mice treated with AZD2171. Broad dose-dependent necrosis was observed. Original magnification, ×40.

FGFR2 was inhibited at 10-fold lower concentrations of AZD2171 in the sensitive cell lines (Fig. 3B), but there were no marked differences between the kinase-inhibitory effects among the proteins derived from the cell lines in this cell-free assay. This discrepancy is discussed in the Discussion.

In vivo *antitumor activity of AZD2171 against FGFR2-overexpressing gastric cancer.* To elucidate the *in vivo* antitumor activity of AZD2171 in mice bearing gastric cancer tumor xenografts, we used the newly established tumorigenic subline TU-kato-III (derived from KATO-III) and OCCUM2M. We attempted to perform control experiments using OKAJIMA cells *in vivo* as suggested by the reviewer. Unfortunately, however, the cell lines grew slowly in the mice, and we could not precisely evaluate the antitumor activity of AZD2171 in the model. However, the results of preliminarily experiments showed that AZD2171 seemed to be less effective against OKAJIMA cells than against KatoIII and OCUM2M cell *in vivo*. Mice implanted the TU-kato-III and OCUM2M tumors were given a low or high dose of AZD2171 (i.e., 1.5 or 6.0 mg/kg/d), or vehicle, orally for 3 weeks. AZD2171 (1.5 mg/kg/d) significantly inhibited tumor growth in the mice bearing TU-kato-III and OCUM2M tumors, and the higher dose (6.0 mg/kg/d) completely inhibited the growth of both tumor models (Fig. 4A). H&E staining showed broad dose-dependent necrosis of core tumor tissue in mice treated with AZD2171 (Fig. 4B). Thus, AZD2171 showed marked antitumor activity *in vivo* against both human gastric tumor xenografts.

Discussion

Recent studies have shown that FGFRs and their ligands are promising therapeutic target molecules for various malignant diseases, such as prostate cancer (18), breast cancer (5, 19), endometrial carcinoma (20), synovial sarcomas (21), thyroid carcinoma (22, 23), and hematopoietic malignancies (24–27). These findings are based on the biological properties of malignant cells expressing activated FGFR, like FGFR fusion tyrosine kinase, involved in chromosomal translocations, gene amplification of FGFRs, or overexpression of FGFs (5, 18–27). In the case of gastric cancer, the results of immunohistochemical analysis of clinical samples revealed that 20 of 38 cases of advanced undifferentiated type of gastric cancer were FGFR2/K-sam positive, whereas none of the 11 cases with the differentiated or intestinal type of cancer showed positive staining for K-sam (8). The results suggest that FGFR2/K-sam overexpression is associated with the undifferentiated type of stomach cancers. The results of fluorescence *in situ* hybridization analysis of the gastric cancer specimens showed gene amplification of FGFR2/K-sam in 2.9% (28). The clinical implication of FGFR2 overexpression/amplification in gastric cancers remains to be fully clarified, and further investigation is needed.

AZD2171 has the most potent kinase-inhibitory activity against VEGFR-2 (IC_{50} < 1 nmol/L); it also possesses additional activity against VEGFR-1, VEGFR-3, and c-Kit (IC_{50}, 5, ≤3, and 2 nmol/L, respectively; ref. 10). AZD2171 showed antiangiogenic activity and broad antitumor activity consistent with potent inhibition of VEGF-induced angiogenesis. We showed kinase-inhibitory activity of AZD2171 against FGFR2 in the present study. When cancer cells are dependent on FGFR2 signaling, AZD2171 can be expected to give additional therapeutic benefit in addition to its antiangiogenic effects.

A cell-based Western blotting analysis showed that phosphorylation of FGFR2 in KATO-III cells and OCUM2M cells was inhibited by AZD2171 at 10-fold lower dose than in OKAJIMA cells (Fig. 3B). However, there was no significant difference in the K_i values of AZD2171 between the FGFR2 derived from KATO-III, OCUM2M, and OKAJIMA in an *in vitro* kinase assay. This may be attributable to the different conditions between the cell-based and cell-free assays. For example, undefined intrinsic intracellular factors may influence kinase activity: (*a*) differences in baseline intracellular FGFR2 phosphatase activity in each cell line, (*b*) differences in intracellular concentration of ([transporters, such as ATP-binding cassette transporters, may be involved in this phenomenon refs. 29, 30), and (*c*) undefined intrinsic inhibitory factors that bind the compounds directly may also be involved (e.g., Brehmer D, et al. have identified various gefitinib binding proteins by affinity chromatography; ref. 31).

In conclusion, AZD2171, a potent inhibitor of all VEGFRs (VEGFR-1, VEGFR-2, and VEGFR-3), was found to have antitumor effect against gastric cancer xenografts in line with previous findings in colon, lung, prostate, breast, and ovarian tumor xenografts (10). The results of this study suggest that activation of the FGFR2 pathway may be a promising target for gastric cancer therapy. AZD2171 may provide a clinical benefit to gastric cancer patients.

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