The Tyrosine Kinase Inhibitor, AZD2171, Inhibits Vascular Endothelial Growth Factor Receptor Signaling and Growth of Anaplastic Thyroid Cancer in an Orthotopic Nude Mouse Model

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Abstract

Purpose: Anaplastic thyroid cancer (ATC) is a locally aggressive type of thyroid tumor with high rate of distant metastases. With conventional treatment, the median survival ranges from 4 to 12 months; therefore, new treatment options are needed. AZD2171 is a tyrosine kinase inhibitor of the vascular endothelial growth factor receptors (VEGFR) VEGFR-1, VEGFR-2, and VEGFR-3. The objective of the study is to determine whether AZD2171 can inhibit VEGFR-2 signaling and decrease tumor growth and prolong survival of ATC in an orthotopic nude mouse model.

Experimental Design: We examined the effects of AZD2171 on phosphorylation of VEGFR-2, mitogen-activated protein kinase, and AKT in human umbilical vascular endothelial cells. To determine the antiproliferative and antiapoptotic effects of AZD2171, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometry assays, respectively. We assessed the antitumor effects of AZD2171 in a xenograft model of ATC using control, AZD2171, paclitaxel, and combination groups by measuring tumor size and survival.

Results: Treatment with AZD2171 led to dose-dependent inhibition of VEGFR-2 phosphorylation and its downstream signaling in human umbilical vascular endothelial cells (IC50 for cell proliferation, 500 nmol/L). In the ATC cell lines DRO and ARO, IC50 was 7.5 μmol/L. AZD2171 induced apoptosis in 50% of endothelial and ATC cells at 3 and 10 μmol/L concentrations, respectively. In vivo, AZD2171 led to a significant reduction in tumor size between control and AZD2171 (P = 0.002) or AZD2171 + paclitaxel group (P = 0.002) but not the paclitaxel alone group (P = 0.11). Survival was significantly higher among AZD2171 (P < 0.001) and combination groups (P < 0.001) compared with control.

Conclusions: AZD2171 effectively inhibits tumor growth and prolongs survival of ATC-bearing mice. The main effect of AZD2171 is mediated through angiogenesis inhibition.

It is estimated that there will be 30,180 new cases of thyroid cancer in the United States in 2006. Thyroid cancer incidence is rapidly increasing and it is now the seventh most common cancer in women (1). Anaplastic thyroid cancer (ATC) accounts for a small proportion of these cancers (3%); however, each year, it is responsible for more than half of the 1,200 deaths from the disease. It is a locally aggressive type of tumor and has a high rate of distant metastases. With the conventional treatment modalities, the median survival for this disease ranges from 4 to 12 months. Current treatment modalities for ATC include surgery, radiation therapy, and chemotherapy (2, 3) The role of surgery is controversial and some authors state that an aggressive approach does not provide additional benefit in most patients (4–9).

Radiation therapy may have an effect in delaying local disease progression but it does not prolong survival (10–13), and for chemotherapy, a relatively recent phase II trial using paclitaxel showed a 53% total response rate, without an improvement in survival (14). Given our poor ability to control ATC progression with conventional modalities, new paradigms are needed for treating this disease. Targeted molecular therapy is a promising approach to evaluate in the treatment of ATC patients. Our group has shown previously that targeting epidermal growth factor receptor either with a tyrosine kinase inhibitor (15) or with a monoclonal antibody (16) is an effective approach for the treatment of ATC in an animal model. Moreover, we have shown that combined inhibition of epidermal growth factor receptor and vascular endothelial growth factor receptor (VEGFR) with a dual tyrosine kinase inhibitor alone or combined with paclitaxel also produced significant cytostatic and cytotoxic effects on ATC cell lines in vitro and reduced tumor growth of s.c. ATC xenografts in nude mice (17). The VEGF pathway is an ideal
therapeutic target because it is crucial for tumor growth and progression. Several approaches have been investigated, including agents that target either VEGF or its cell surface receptors (18) Molecules targeting VEGF include monoclonal antibodies and soluble receptor constructs. Receptor-targeted molecules include monoclonal antibodies and inhibitors of VEGFR tyrosine kinases. The small-molecule inhibitor AZD2171 is a highly potent angiogenesis inhibitor that selectively targets the VEGFRs VEGFR-1, VEGFR-2, and VEGFR-3 (19). These receptor tyrosine kinases, encoded by the FLT gene family, may fill distinct functions in regulating blood vessel growth and differentiation. VEGFR-2 exerts most of the functions of VEGF in blood vessel endothelial cells, including survival, nitric oxide production, vascular permeability, cell migration, cell proliferation, and PGI2 production (20). VEGFR-2, like many other receptors, induces proliferation through activation of the classic extracellular signal-regulated kinase pathway [p42/44 mitogen-activated protein kinase (MAPK)], leading to gene transcription. VEGFR-2 also activates phosphatidylinositol 3-kinase, which results in an increase in the lipid phosphatidylinositol (3,4,5) P3 and activation of several important intracellular molecules, such as AKT (also denoted as protein kinase B) and the small GTP-binding protein Rac (21). VEGFR-3 is more abundant in lymphatic endothelial cells and its activation induces proliferation, migration, and survival in those cells. VEGFR-1 regulates cell migration by modulating actin reorganization, which is essential for cell motility. It has also been shown to negatively influence VEGFR-2 function in cells expressing chimeric VEGFR proteins. Previous studies have shown that AZD2171 is able to reduce tumor size and prolong survival in animal models of colon, lung, prostate, and ovarian cancer (19). The objective of the present project was to determine if AZD2171 can inhibit VEGFR-2 signaling, the main effector of the functions of VEGF in endothelial and/or tumor cells in vitro, and decrease tumor growth and prolong survival in an orthotopic nude mouse model of ATC.

Materials and Methods

Cell lines and culture conditions. The ATC cell lines ARO and DRO were used. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1-glutamine, penicillin, sodium pyruvate, and nonessential amino acids (Life Technologies, Inc.). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were found to be free of Mycoplasma species. Human umbilical vein endothelial cells were also used (Cambrex) and maintained in EBM-2 medium. To split endothelial cells, Clonetics HEPES-buffered saline solution was used for rinsing followed by trypsin/EDTA. After cells were released, trypsin neutralizing solution was added to the plates.

Reagents. The VEGFR inhibitor AZD2171 was obtained from Astra-Zeneca Pharmaceuticals and the National Cancer Institute, NIH (Bethesda, MD). For in vitro testing, the drug was prepared as 20 mmol/L stock solution by adding dimethyl sulfoxide as a vehicle and storing at -20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide were purchased from Sigma-Aldrich Corp.

Materials. VEGFR-2 (KDR) antibody was purchased from Santa Cruz Biotechnology and phospho-KDR was from Biosource. Total and phosphorylated MAPK (p42 MAPK and phospho-p44/42 MAPK) and total and phospho-AKT antibodies were purchased from Cell Signaling Technology, Inc. For Western blotting, the anti-KDR antibody was used at a dilution of 1:500 and the phospho-KDR antibody at 1:1,000. The anti-MAPK and phospho-MAPK antibodies were used at a dilution of 1:4,000; the anti-AKT and phospho-AKT antibodies were used at a dilution of 1:1,000.

Measurement of cytososticy. For the thyroid cancer cell lines, 2,000 cells were plated into wells of 96-well tissue culture plates. The plates were grown in RPMI 1640 supplemented with sodium pyruvate, essential amino acids, and 2% fetal bovine serum. For human umbilical vascular endothelial cell (HUVEC), 3,200 cells were plated into the 96-well plates and the cells were grown in EGM-2. After a 24-h attachment period, the cells were refed with this medium or medium containing the VEGFR-2 inhibitor AZD2171. After incubation for 3 days, the number of metabolically active cells was determined by MTT assay: the conversion of MTT to formazan was measured by a 96-well microtiter plate reader (MR-5000, Dynatech, Inc.) at an absorbance of 570 nm. Growth inhibition was calculated with the formula: cytostasis (%)= [1 - (A / B)] × 100, where A is the absorbance of treated cells and B is that of control cells.

Measurement of apoptosis. Thyroid cancer cells were plated at a density of 2 × 10⁴ cells/mL in six-well plates (Costar) and incubated at 37°C for 24 h before treatment with AZD2171. HUVECs were also plated at a density of 5,000 cells/cm². Seventy-two hours later, the extent of cell death was determined by propidium iodide staining of hypodiploid DNA: 3 × 10⁴ cells were resuspended in Nicoletti buffer (50 μg/mL; Sigma Chemical), 0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/mL RNase (Roche) in PBS and then analyzed by fluorescence-activated cell sorting analysis (FACSscan, Becton Dickinson). The fraction of cells with sub-G₀ DNA content was assessed using the Lysis program (Becton Dickinson). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis.

Western immunoblotting. HUVECs were incubated in six-well plates in serum-free medium without growth factors for 24 h. The following day, they were incubated with AZD2171 for 90 min at concentrations varying from 0.01 to 1 μmol/L before addition of VEGF (50 ng/mL) for 7 min. For thyroid carcinoma cell lines, the concentrations of AZD2171 varied from 0.1 to 10 μmol/L. The cells were washed with PBS and lysis buffer was added (1% Triton X-100, 20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% glycerol (v/v), 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L aprotinin-leupeptin-trypsin inhibitor, 2 mmol/L sodium orthovana- date). The cells were scraped and centrifuged to remove insoluble proteins. The samples were diluted in sample buffer [10% SDS, 0.5 mmol/L Tris-HCl (pH 6.8), 1 mol/L DTT, 10% (v/v) glycerol, 1% bromophenol blue] and boiled. The proteins (40 μg) were resolved by PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 1% (w/v) bovine serum albumin in 0.1% Tween 20 (v/v) in TBS, probed with mouse monoclonal anti-VEGFR-2 antibody with 1% bovine serum albumin in 0.1% Tween 20 (v/v) in TBS, and incubated with peroxidase-conjugated sheep anti-mouse IgG antibody with 1% bovine serum albumin in 0.1% Tween 20 (v/v) in TBS. The blots were also probed with rabbit anti-phospho-VEGFR-2 antibody and phospho-MAPK antibody, diluted with 1% bovine serum albumin in 0.1% Tween 20 (v/v) in TBS, and incubated with peroxidase-conjugated sheep anti-rabbit IgG antibody. Anti-β-actin was used at a 1:1,000 concentration. The blots were also probed with anti-AKT antibody and phospho-AKT antibody in a similar fashion. Protein bands were visualized using the Enhanced Chemiluminescence Plus Western blotting detection system (Amersham Life Science).

Animals. Eight- to 12-week-old male athymic nude mice were purchased from the National Cancer Institute and housed in a specific pathogen-free animal facility. All animal procedures were done in accordance with a protocol approved by our Institutional Animal Care and Use Committee. All mice were euthanized after 6 weeks by asphyxiation with carbon dioxide.
Tumor xenograft generation for the treatment study with AZD2171 and paclitaxel. To produce tumors, DRO cells were harvested from subconfluent cultures by a brief 2-min exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum. The cells were washed once and resuspended in serum-free medium. Five hundred thousand cells were suspended in 5 μl serum-free RPMI 1640 and injected orthotopically in the thyroid gland as described previously (22). I.p. injection of a ketamine/xylazine cocktail rendered the mice fully anesthetized. Then, the mice were restrained in dorsal decubitus and a midline cervical incision was done after 70% ethanol asepsis. Using a dissecting microscope, the submandibular glands were separated in the midline until the strap muscles were found. After retracting them to the left, the right thyroid lobe was exposed and a 25-μl Hamilton syringe connected to a 30-gauge needle was used to inject the cells. The wound was closed with surgical clips. All mice were examined the day after injection. After 5 days, time when tumors have already been established, all mice were weighed and randomized into four groups (n=10 each): group one was treated with 1% (w/v) aqueous polysorbate 80; group 2 was treated with paclitaxel 200 μg i.p. on day 1 of treatment and every week thereafter until the end of the study period; group 3 was treated with AZD2171 at a dose of 3 mg/kg via gastric gavage every day; and group 4 was treated with both AZD2171 and paclitaxel. Weight was measured twice weekly and mice were sacrificed at 3 weeks or earlier if they seemed morbid (hunched over, reluctant to move, difficulty with ambulation, ruffled haircoat, or loss of body weight of >20%).

After the mice were euthanized, the xenografted tumors were obtained and measured with a caliper. The volume was calculated using the formula \(V = \frac{A \times B \times C}{6}\), where \(A\) is the length of the longest aspect of the tumor, \(B\) is the length of the tumor perpendicular to \(A\), and \(C\) is the depth of the tumor. For immunohistochemical and routine H&E staining, half of the tumor tissue was fixed in formalin and embedded in paraffin. The other half was embedded in ornithine carbamyl transferase compound (Miles, Inc.), rapidly frozen in liquid nitrogen, and stored at -70°C.

Immunohistochemical detection of VEGFR-2, proliferating cell nuclear antigen, and CD31/platelet/endothelial cell adhesion molecule 1. Immunohistochemistry was done with the following antibodies: rabbit anti-VEGFR-2 (Santa Cruz Biotechnology) at a dilution of 1:100 and mouse anti–proliferating cell nuclear antigen (DAKO Corp.) at a dilution of 1:50. For VEGFR-2 staining, paraffin-embedded sections were first dewaxed in xylene. Excess xylene was removed by washing the slides in ethanol. After treating the tissue with pepsin for 20 min at 37°C, the slides were washed thrice with PBS. Endogenous blocking was done...
with 3% H2O2 followed by protein blocking using 5% horse serum and 1% goat serum (protein-blocking solution). After washing the slides in PBS, the primary antibody (1:200 dilution) was added for 18 h at 4°C. The slides were then washed with PBS thrice, blocked again with protein-blocking solution for 1 h, and incubated with horseradish peroxidase–conjugated anti-rabbit antibody at 1:200 dilution for 1 h at room temperature. The slides were washed thrice in PBS and then incubated with 3,3′-diaminobenzidine for 10 min. After the excess 3,3′-diaminobenzidine was washed off, counterstaining was done with Gill’s no. 3 hematoxylin.

Frozen tissues were sectioned into 8- to 10-μm slices and used for detection of CD31/platelet/endothelial cell adhesion molecule 1. The slices were mounted on positively charged Plus slides (Fisher Scientific) and air-dried for 30 min; fixed in cold acetone (5 min), 1:1 acetone/chloroform (v/v; 5 min), and acetone (5 min); and then washed with PBS. Immunohistochemical procedures were done as described previously with primary antibody diluted 1:400. Peroxidase-conjugated secondary antibody was used for immunohistochemical analysis of CD31/platelet/endothelial cell adhesion molecule 1. Bleaching of fluorescence was minimized by covering the slides with 90% glycerol and 10% PBS. A positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10 to 20 min for identification of CD31/platelet/endothelial cell adhesion molecule 1. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). Control samples that had not been exposed to primary antibody showed no specific staining.

Staining for CD31/terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and phospho-VEGFR-2 double immunofluorescence assays. Frozen tissues were used for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and CD31 double immunofluorescence assays. After being mounted on slides and fixation with acetone as described above, the frozen samples were washed thrice with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with a 1:400 dilution of rat anti-mouse CD31 monoclonal antibody (human cross-reactive) overnight at 4°C. After the samples were rinsed thrice with PBS for 3 min each, the slides were incubated for 1 h at room temperature in the dark with a 1:600 dilution of secondary goat anti-rat antibody conjugated to Alexa Fluor 594 (red fluorescence). The samples were then washed thrice with PBS containing 0.1% Brij and once with PBS for 3 min. A similar protocol was used for phospho-VEGFR-2 and CD31 double immunofluorescent staining. The antibody used for this purpose was rabbit anti–phospho-VEGFR-2 (Santa Cruz Biotechnology).

TUNEL assay. A TUNEL assay was done using an apoptosis detection kit (Promega) with the following modifications: samples were fixed with 4% paraformaldehyde (methanol free) for 10 min at room temperature, washed twice with PBS for 5 min, and incubated

Table 1. Survival analysis by treatment group

<table>
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<tr>
<th>Treatment Group</th>
<th>Log-rank test</th>
<th>P</th>
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<tbody>
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<td>Control vs AZD2171</td>
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<tr>
<td>Control vs Combination</td>
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<tr>
<td>Paclitaxel vs AZD2171</td>
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<tr>
<td>AZD2171 vs Combination</td>
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with 0.2% Triton X-100 for 15 min at room temperature. After two 5-min washes with PBS, the samples were incubated with equilibration buffer for 10 min at room temperature. The equilibration buffer was drained and a reaction buffer containing 44 μL equilibration buffer, 5 μL nucleotide mix, and 1 μL terminal deoxynucleotidyl transferase (supplied in the kit) was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h, avoiding exposure to light. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were then washed thrice for 5 min to remove unincorporated fluorescein-dUTP.

**Quantification of microvessel density and apoptotic endothelial cells.** For quantification analysis, five slides were prepared for each group, and two areas were selected on each slide. To quantify the expression in the TUNEL assays, the number of positively stained cells and total cells were also counted in 10 random 0.159-mm² fields of tumor area at ×100 magnification, and the percentages of positively stained cells among the total number of cells were calculated and compared.

Immunofluorescence microscopy was done using a Leica DMLA microscope (Leica Microsystems) equipped with an HBO 100 mercury lamp and narrow band-pass filters to individually select for green, red, and blue fluorescence (Chroma Technology Corp.). Images were captured using a cooled charged coupled device Hamamatsu 5810 camera (Hamamatsu Corp.) and ImagePro Plus 6.0 software (Media Cybernetics). Stained sections were examined in the same microscope equipped with a three-chip charged coupled device color video camera (model DXC990, Sony Corp.). Photomontages were prepared using Photoshop software (Adobe Systems, Inc.). Endothelial cells were identified by red fluorescence staining, and DNA fragmentation was detected by localized green fluorescence within the nuclei of apoptotic cells. Photomontages were printed in a Sony digital color printer (model UPD7000).

To quantify microvessel density (MVD), the areas containing the highest number of tumor-associated blood vessels were identified by scanning the tumor sections at low microscopic power (40×). Vessels that were completely stained with anti-CD31 antibodies were then counted in 10 random 0.159-mm² fields at ×100 magnification. Quantification of apoptotic endothelial cells was expressed as the average of the ratios of apoptotic endothelial cells to the total number of endothelial cells in 10 random 0.011-mm² fields at ×400 magnification.

**Statistical methods.** Best-fit curves were generated for the MTT and propidium iodide assays and used to determine the concentration at which 50% of the drug effect (IC50) was exhibited. Quantified results of proliferating cell nuclear antigen, CD31, and tumor volume were compared with Kruskal-Wallis and Wilcoxon rank-sum test, as appropriate. Furthermore, a two-way ANOVA was used to test for the effect of AZD2171 and paclitaxel as single agents and for interaction between compounds, which, when positively interacting, shows drug synergy (23). Survival was analyzed with the Kaplan-Meier method. Differences between the treatment and control groups were compared with the log-rank test. A two-tailed *P* < 0.05 was considered significant. All statistical analyses were done using Stata 9 (Stata Corp.).

**Results**

**AZD2171 inhibits phosphorylation of VEGFR-2 in vitro.** We first examined the ability of AZD2171 to inhibit VEGFR-2 autophosphorylation (Fig. 1) in the endothelial cell line. HUVEC showed low levels of phospho-VEGFR-2 in serum-free conditions when examined by Western blotting. However, the addition of VEGF to the medium for 15 min resulted in the phosphorylation of both VEGFR-2 and AKT and a less

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**Fig. 5.** AZD2171 inhibits VEGFR phosphorylation. Representative tumors. In the control group, CD31-positive cells colocalized with phospho-VEGFR-2 (pVEGFR2) and were commonly seen in the periphery of the tumor. In the paclitaxel group, a similar pattern was observed. In contrast, staining for both markers was not as prominent for tumors in the AZD2171 and AZD2171 + paclitaxel groups.
prominent decrease of MAPK. AZD2171 was able to completely inhibit the VEGF-induced autophosphorylation of VEGFR-2 at 100 nmol/L (Fig. 1). At the same concentration, the phosphorylation of MAPK and AKT were partially inhibited.

AZD2171 inhibits proliferation of HUVEC but not ATC cell lines in vitro. HUVEC and ATC cell lines ARO and DRO were incubated with increasing concentrations (0-1 μmol/L for HUVEC and 0-100 μmol/L for ATC cell lines) of AZD2171 in vitro.
EGM and RPMI 1640, respectively, complemented with 2% serum. After 72 h, the MTT assay showed that the proliferation of HUVEC was inhibited by AZD2171 in a dose-dependent manner (Fig. 2A). The concentration required to cause 50% inhibition of cell growth (IC50) was determined to be 0.5 μmol/L. In contrast, the concentrations required for inhibiting proliferation of ARO and DRO cell lines were significantly higher at 7.5 μmol/L for both. The maximal inhibition of proliferation of 40% was achieved at 10 μmol/L of AZD2171 for both ATC cell lines (Fig. 2B).

AZD2171 induces apoptosis of HUVEC lines at high concentrations in vitro. To examine if AZD2171 had proapoptotic effects, HUVEC and ATC cell lines (ARO and DRO) were treated with various concentrations of AZD2171 for 48 h. Propidium iodide staining showed that AZD2171 induced apoptosis of 20% of HUVECs at a concentration of 1 μmol/L and 50% at 5 μmol/L (Fig. 2C). For ARO and DRO cell lines, 50% induction of apoptosis by AZD2171 only occurred at concentrations of 7.5 and 10 μmol/L, respectively (Fig. 2D).

AZD2171 inhibits orthotopic ATC xenograft growth in vivo. We assessed the antitumor effects of AZD2171 in a xenograft model of ATC using four randomized groups of 10 animals each: control, AZD2171, paclitaxel, and combination groups by measuring tumor size and survival. All animals tolerated both AZD2171 and paclitaxel without significant weight loss in the four groups, and all animals in the control group remained alive up to day 20 of treatment. Paclitaxel alone did not significantly inhibit the growth of orthotopic ATC xenografts generated with the DRO cell line in nude mice when compared with control. In contrast, AZD2171 alone or in combination with paclitaxel inhibited the growth of tumors when compared with the control group (Fig. 3). At the end of the 3-week treatment period, the mice treated with paclitaxel, AZD2171, and AZD2171 plus paclitaxel showed 15%, 53%, and 46% decreases, respectively, in mean tumor volume compared with the control group. AZD2171 and AZD2171 plus paclitaxel combination produced statistically significant (P = 0.002) reductions in mean tumor volume when compared with the control group. In addition, a significant difference was found between the AZD2171 alone and paclitaxel alone groups (P < 0.001). The reduction in mean tumor volume by AZD2171 alone compared with the combination with paclitaxel, however, was not statistically significant (P = 0.13). The difference in mean tumor volume between the paclitaxel group and the control group did not have statistical significance either (P = 0.43). The two-way ANOVA model revealed that only AZD2171 was significantly associated with tumor volume (P < 0.001). In contrast, no significant effect was seen with paclitaxel (P = 0.43).

AZD2171 improves the survival of athymic nude mice bearing orthotopic ATC xenografts. In the absence of any treatment, all of the control mice in the survival study succumbed to the thyroid tumors by day 20 due to obstruction of the upper aerodigestive tract (Fig. 4). The median survival periods for the control, paclitaxel, AZD2171, and combination groups

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Table 2. Quantitative analysis of immunohistochemical staining of tumors in the four groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Paclitaxel</th>
<th>AZD2171</th>
<th>Combination</th>
<th>P*</th>
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<td>190</td>
<td>51</td>
<td>57</td>
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<tr>
<td>MVD by fluorescence</td>
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<td>194</td>
<td>50</td>
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<tr>
<td>TUNEL</td>
<td>305</td>
<td>2,678</td>
<td>604</td>
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<td>0.0006</td>
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</table>

NOTE: Numbers are median values.
*Statistical analysis by Kruskal-Wallis test.
1MVD and TUNEL were measured as the number of counts of stained cells in 0.159-mm² fields at ×100 magnification.

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Fig. 7. Treatment with AZD2171 decreases MVD. A, representative sections of tumors in the four groups. MVD was measured as the count of CD31-positive cells in 0.159-mm² fields at ×100 magnification. B, quantitative analysis of MVD. Paclitaxel alone did not make a statistically significant change in the MVD. *, mean count compared with control was statistically significant for AZD2171 (P = 0.004) and combination groups (P = 0.002).
were 17, 16, 23, and 23 days, respectively. The differences in survival between the treatment groups were statistically significant by log-rank test (P < 0.001). When comparing groups, no statistically significant difference in survival was found between the control and paclitaxel-treated groups (P = 0.67). However, a difference was evident for the groups that included AZD2171 (alone or in combination) compared with the control and the paclitaxel alone groups (P < 0.001; Table 1).

AZD2171 inhibits VEGFR phosphorylation, induces endothelial cell apoptosis, and decreases MVD. A double-labeling immunohistochemical technique using antibodies against CD31/platelet/endothelial cell adhesion molecule 1 (red) and phospho-VEGFR-2 (green) showed by colocalization that endothelial cells within all the thyroid tumors expressed the activated VEGFR-2. The phosphorylation of the receptor on tumor-associated endothelial cells was inhibited by AZD2171 alone and in combination with paclitaxel but not in the control or the paclitaxel alone groups (Fig. 5).

The CD31/TUNEL fluorescent double-labeling immunohistochemical technique showed that tumors in the paclitaxel and the combination groups had the highest staining for TUNEL (Fig. 6B). However, in the paclitaxel group, it was evident that the TUNEL staining did not merge with CD31 staining and therefore represented apoptosis in the tumor cells. There was a higher percentage of tumor-associated apoptotic endothelial cells in mice treated with AZD2171 alone and in combination with paclitaxel than in the control or paclitaxel alone groups. A representative section of endothelial cells undergoing apoptosis is shown in Fig. 6A. Tumors in the AZD2171 alone group also showed neoplastic cells with positive staining for TUNEL.

To determine the effect of AZD2171 on tumor angiogenesis, MVD was quantified by staining tumor sections for CD31/platelet/endothelial cell adhesion molecule 1. The difference between the four groups was statistically significant (P < 0.001). Tumors from the mice treated with AZD2171 alone and AZD2171 in combination with paclitaxel, but not on the paclitaxel alone group, had significantly lower MVD values compared with the control group (P < 0.001; Fig. 7; Table 2). Thus, AZD2171, when given alone and in combination with paclitaxel, leads to a decrease in VEGFR-2 phosphorylation, an increase in endothelial cell apoptosis, and a concomitant decrease in tumor-associated microvessels.

Discussion

In our studies, the small-molecule tyrosine kinase inhibitor AZD2171 was found to inhibit tumor growth and prolong animal survival in an animal murine model of ATC. The main mechanism of the action of the compound was through inactivation of VEGFR-2 and downstream signaling in endothelial cells. These effects correlated with the ability of AZD2171 to inhibit proliferation of endothelial cells and induce their apoptosis both in vitro and in vivo. Interestingly, paclitaxel alone or when combined with AZD2171 did not add any significant benefit in terms of tumor growth or survival.

ATC is one of the most aggressive solid tumors with a median survival of less than 12 months. Current treatment modalities, including surgery, radiation therapy, and chemotherapy, can delay local tumor progression in some cases, but no study has shown a convincing improvement in survival. Therefore, new treatment strategies are needed. One approach is to target angiogenesis; because tumors require access to blood vessels for growth and metastasis, inhibiting vessel formation offers a way of reducing tumor size and hopefully prolonging survival for patients with ATC. In fact, MVD has been shown to relate to disease-free survival for patients with papillary thyroid cancer (24) and to the spread of follicular carcinoma (25). In addition, VEGF mRNA expression has been correlated with a lower 5-year overall survival of patients with thyroid cancers (26). VEGF plays a key role in the neovascularization of a tumor during cancer progression, as cells within the expanding mass of the tumor are frequently deprived of oxygen because of their great distance from the nearest blood vessels. As a consequence, hypoxic regions begin to form. This phenomenon induces the production of VEGF, which produces migration, proliferation, differentiation, and survival of endothelial cells (27).

AZD2171 is a potent small-molecule tyrosine kinase inhibitor of VEGFR-2 that also has activity against VEGFR-1 and VEGFR-3. A previous study by Wedge et al. showed that the drug is active against lung, prostate, colon, breast, and ovarian tumors in mouse models and that the effects are mainly mediated by its inhibition of angiogenesis. Phase II clinical trials are ongoing with this compound in patients with non–small cell lung carcinoma, squamous cell carcinoma of the head and neck, and colorectal cancer. In the present study, we tested the effect of AZD2171 in an orthotopic model of ATC. Confirming our in vitro observations, we showed that the main mechanism of action of AZD2171 in ATC is also targeting endothelial cells. This was further supported by in vivo data, in which mice treated with AZD2171 had an increased apoptosis of endothelial cells and tumor cells and a decreased MVD as measured by CD31 positive count. These findings are in contrast to the effects of the cytotoxic agent paclitaxel, which produced apoptosis in tumor but not endothelial cells.

It is known that VEGFR-2 activates phosphatidylinositol 3-kinase, which results in an increase in the lipid phosphatidylinositol (3,4,5) P3 and activation of several important intracellular molecules, such as AKT. We showed decreased phosphorylation of AKT at concentrations of 0.01 nmol/L in HUVECs. Tumor cells in the animals treated with AZD2171 also underwent apoptosis, likely through a hypoxia-induced mechanism.

Teicher et al. (28) postulated that combined administration of antiangiogenic and cytotoxic therapies would yield maximal benefit because such combinations would destroy two separate compartments of tumors: cancer cells and endothelial cells. This has proved true in the clinic for patients with colorectal cancer; bevacizumab in combination with irinotecan, fluorouracil, and leucovorin resulted in a 5-month increase in median survival (29). Therefore, we sought to determine if the combination with paclitaxel, a cytotoxic agent that produces tubulin polymerization, may have a synergistic effect with AZD2171. Interestingly, paclitaxel did not have a synergistic effect when combined with AZD2171 for inhibiting tumor size and did not affect survival. A possible explanation for these results is in the dose of AZD271; we used a dose of 3 mg/kg.
which is half of the maximum dose proposed by Wedge et al. At this dose, the authors found inhibition of tumor volume of >50% in colon, lung, and prostate tumors. However, it is possible that in our model, this dose was high enough to obscure any effect from the addition of paclitaxel. In addition, it is possible that at this dose, angiogenesis inhibition from AZD2171 compromised the delivery of paclitaxel. The results could also be explained by the lack of activity of paclitaxel on DRO cell line, and the possibility of the cell line being resistant needs to be addressed. In the present study, we were able to show that AZD2171 alone was able to slow tumor growth and increased animal survival without evidence of toxic effects in the mice. If an antiangiogenic agent like AZD2171 could be given at a dose that inhibits tumor growth and prolongs survival better than when the known cytotoxic chemotherapeutic agents are added, it would be a major improvement in targeted therapy involving angiogenesis inhibition.

We conclude that AZD2171 inhibits tumor growth and prolongs survival in a murine orthotopic model of ATC. The effects of AZD2171 seem to be mediated mainly by inhibition of angiogenesis. Therefore, AZD2171 seems to be a promising agent to evaluate in the treatment of patients with ATC (30).

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References

The Tyrosine Kinase Inhibitor, AZD2171, Inhibits Vascular Endothelial Growth Factor Receptor Signaling and Growth of Anaplastic Thyroid Cancer in an Orthotopic Nude Mouse Model

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