Vascular Endothelial Growth Factor Tyrosine Kinase Inhibitor AZD2171 and Fractionated Radiotherapy in Mouse Models of Lung Cancer

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Abstract
The vascular endothelial growth factor receptor (VEGFR) tyrosine kinases are being explored as targets for antiangiogenic cancer therapy. Radiotherapy also inhibits tumor growth and affects vasculature. We investigated the combination of the potent VEGF tyrosine kinase inhibitor AZD2171 and ionizing radiation in cell culture and mouse models of lung cancer. We show that ionizing radiation induces expression of phosphorylated VEGFR-2 (Flk-1) in endothelial cells and that this phosphorylation is inhibited by AZD2171. Human umbilical vascular endothelial cells become more sensitive to radiation after treatment with AZD2171 as determined by clonogenic assay. Matrigel assay showed a decrease in in vitro endothelial tubule formation with AZD2171/radiation combination treatment. When similar combination was applied to the H460 lung cancer xenograft model in nude mice, loss of radiation-induced phosphorylated Flk-1 was observed in the combination treatment group, which also showed a large decrease in tumor vascular density by staining of the von Willebrand factor. H460 tumor growth delay was enhanced in the combination treatment group compared with the groups treated with AZD2171 or radiation alone. Additionally, after therapy, Ki67 index showed >4-fold reduction of tumor proliferation in the combination treatment group, which also showed increased intratumoral apoptotic index by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. In conclusion, AZD2171 has the potential to improve tumor growth delay and inhibiting tumor angiogenesis (3), and inhibition of VEGF-induced angiogenesis has been established as a method of inhibiting tumor growth (4). Additionally, VEGF-mediated angiogenesis also rarely occurs in healthy adults (with the exception of wound healing and female reproductive cycling), so the inhibition of VEGF should not affect normal tissue and processes. Accordingly, VEGF has become a leading target in cancer therapy, led by the success of the humanized anti-VEGF monoclonal antibody bevacizumab (5).

Recently, the VEGF receptor tyrosine kinases (RTK) have been explored as a therapeutic target for inhibiting VEGF signaling. The VEGF RTK VEGF receptor-2 (VEGFR-2)/Flk-1/KDR is an attractive target due to its proven role in regulating VEGF-induced angiogenesis (6). Additionally, VEGFR-2 is exclusively expressed in endothelial cells, making it a selective target (7). Several tyrosine kinase inhibitors (TKI) with activity against VEGFR-2 have been developed, including PTK787 (8), ZD6474 (9), SU11248 (10), and AZD2171 (11). AZD2171 is a newly developed agent that is highly potent, with an IC50 of 0.4 to 0.5 mmol/L for inhibition of VEGF signaling. This agent has also shown significant inhibition of tumor growth in vivo at doses as low as 0.75 to 1.5 mg/kg/d (11).

Radiotherapy is known to provide tumor antiangiogenic effects in addition to its direct cytotoxic effects on tumor cells (12, 13). Broad-spectrum TKIs have been previously combined with radiation and have shown promise for improving tumor growth delay and inhibiting tumor angiogenesis (14). VEGF-2 antagonists have been shown to enhance the efficacy of radiotherapy in radioresistant tumors, further indicating their possible use as radiation enhancers (15). In the present study, we combine the VEGF-2 TKI AZD2171 with radiation in both in vitro and in vivo models. The combination of AZD2171 and radiation remains unexplored. Due to its high selectivity and potency, AZD2171 is a clinically promising agent that is also useful in the study of VEGF-2 inhibition.

Lung cancer is the second most common cause of cancer in both men and women in the United States and the leading cause of cancer deaths. Lung cancer has a dismal prognosis with 5-year relative survival rates of only 13.6% for men and 17.5% for women, so improved therapies are needed (16). Due to poor prognosis and lack of effective therapy, angiogenesis inhibitors are taking a leading role in the future of lung cancer treatment (17, 18). Angiogenesis is essential to the ability of a tumor to metastasize. Metastasis is particularly common in lung cancer, and the major cause of death from non–small cell lung cancer (NSCLC) is due to metastasis (19). Additionally, VEGF expression (20) and VEGF/VEGFR-2 pathway activation (21) are strongly correlated with poor prognosis in lung cancer patients. Therefore, we chose to investigate the combination of AZD2171 and radiotherapy in an H460 human NSCLC xenograft model.

Introduction
Tumors require the development of new blood vessels through the process of angiogenesis to match the metabolic demands necessary for tumor growth as well as facilitate metastasis (1, 2). Vascular endothelial growth factor (VEGF) is central in mediating angiogenesis (3), and inhibition of VEGF-induced angiogenesis has been established as a method of inhibiting tumor growth (4). Additionally, VEGF-mediated angiogenesis also rarely occurs in healthy adults (with the exception of wound healing and female reproductive cycling), so the inhibition of VEGF should not affect normal tissue and processes. Accordingly, VEGF has become a leading target in cancer therapy, led by the success of the humanized anti-VEGF monoclonal antibody bevacizumab (5).
Materials and Methods

Cell culture. Human umbilical vascular endothelial cells (HUVEC) were obtained from Clonetics (Walkersville, MD) and maintained in EBM-2 medium supplemented with EGM-2 MV single aliquots (BioWhittaker, Walkersville, MD). H460 lung carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated at 37°C in a 5% CO2 incubator. AZD2171 is a potent VEGFR-2 inhibitor (AstraZeneca, Inc., Macclesfield, United Kingdom). AZD2171 was dissolved in DMSO and kept as 100 μM stock solution in small aliquots at −20°C. Irradiation was given by a 137Cs irradiator (J.L. Shepherd and Associates, Glendale, CA) for in vitro experiments and by an X-ray irradiator (Therapax, Agfa NDT, Inc., Lewistown, PA) for in vivo experiments.

Western immunoblots. HUVECs were treated with AZD2171 (20 nmol/L) overnight (supplemented with 2% FBS plus 1% penicillin/streptomycin). The 2nd day, the treated cells were irradiated with either 0 or 5 Gy. Thirty minutes later, treated cells were washed with ice-cold PBS twice before the addition of lysis buffer. Protein concentration was quantified by the Bio-Rad (Hercules, CA) method. Equal amounts of protein were loaded into each well and separated by 7.5% SDS-PAGE gel followed by transfer onto nitrocellulose membrane. Membranes were blocked using 5% nonfat dry milk in PBS for 1 hour at room temperature. The blots were then incubated with antibody for phosphorylated Fk-1 (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), Fk-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (1:2,000; Sigma, St. Louis, MO) overnight at 4°C. Goat anti-rabbit IgG secondary antibodies (1:1,000; Santa Cruz Biotechnology) were incubated for 1 hour at room temperature. Immunoblots were developed using the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) according to the manufacturer’s protocol and autoradiography.

In vitro clonogenic assay. HUVECs were trypsinized and counted. Cells were diluted serially and plated out into human fibronectin cellware 60-mm dishes in triplicate per data point. Preliminary clonogenic assays were done to determine the appropriate concentration of AZD2171 to use and the appropriate number of cells to seed for each treatment group. Twenty-four hours after cells were plated, medium was replaced with 2% FBS plus 1% penicillin/streptomycin and AZD2171 was added, and 24 hours later, cells were irradiated with 0 to 6 Gy as indicated. The final AZD2171 concentration was 20 nmol/L in all treated plates. Cells were incubated for 72 hours total, medium was aspirated, and fresh medium containing penicillin/streptomycin and AZD2171 was added, and 24 hours later, cells were stained with H&E and photographs were taken via a microscope. After incubation and maintained for 6 days. Cells were then fixed for 15 minutes with methanol/acetic acid (3:1) and stained for 15 minutes with 0.5% crystal violet (Sigma) in methanol. After staining, colonies were counted with a cutoff of 50 viable cells. Calculation of dose enhancement ratio (DER) was as follows: surviving fraction = 0.25, DER = XRT / (drug + XRT).

Endothelial cell morphogenesis assay: tube formation. HUVECs were treated overnight with AZD2171 (20 nmol/L) and then treated with 3 Gy irradiation. Cells were trypsinized and counted. They were seeded at 48,000 cells per well on 24-well plates coated with 300 μL Matrigel (BD Biosciences, San Jose, CA). These cells undergo differentiation into capillary-like tube structures and were periodically observed using a microscope. After 24 hours, cells were stained with H&E and photographs were taken via a microscope. The average number of tubes for three separate microscopic fields (x 100) was counted, and representative photographs were taken.

Tumor volume assessment. Human NCI-H660 cells were used as a xenograft model in female athymic nude mice [nu/nu; 5-6 weeks old, Harlan Sprague Dawley, Inc., Indianapolis, IN]. A suspension of 2 x 10⁶ cells in 50 μL volume was injected s.c. into the left posterior flank of mice using a 1-cc syringe with 27.5-gauge needles. Tumors were grown for 6 to 8 days until average tumor volume reached 0.14 cm³. Treatment groups consisted of vehicle control [1% (w/v) aqueous polysorbate 80 in deionized water], AZD2171 alone, vehicle plus radiation, and AZD2171 plus radiation. Each treatment group contained five mice. Vehicle control and AZD2171 at doses of 0.75 mg/kg were given p.o. for 7 consecutive days. Starting on the 3rd day of AZD2171 treatment, mice in radiation groups were irradiated 1 hour after AZD2171 treatment with 2 Gy daily over 5 consecutive days. Tumors on the flanks of the mice were irradiated using an X-ray irradiator (Therapax). The nontumor parts of the mice were shielded by lead blocks. Tumors were measured twice or thrice weekly in three perpendicular dimensions by use of a Vernier caliper. Growth delay was calculated as the time for treated tumor to reach a 10-fold increase in volume minus the time for control tumors to reach a 10-fold increase in volume.

Histologic sections, von Willebrand factor staining, and immunofluorescence. Mice were implanted with H460 cells as described above in the tumor volume studies. After 6 to 8 days, mice in the drug treatment group were given with 0.75 mg/kg AZD2171 p.o. daily for 7 days. Mice in the radiation treatment group were treated with 2 Gy daily fractions for 5 days, given by use of an X-ray irradiator as described above in the tumor volume studies. After 7 days of daily treatments, mice were killed and tumors were paraffin fixed. Slides from each treatment group were then stained for von Willebrand factor (vWF) using anti-vWF polyclonal antibody (Chemicon, Temecula, CA). Blood vessels were quantified by randomly selecting 400 fields and counting the number of blood vessels per field. This was done in triplicate, and the average of the three counts was calculated. Slides from each treatment group were then stained for phosphorylated Fk-1 using anti-phosphorylated Fk-1 polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:500 in antibody buffer and incubated overnight at 4°C. Slides were washed twice in PBS and incubated with a rhodamine red-labeled goat anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:500 in antibody buffer at room temperature for 45 minutes in the dark. The slides then were washed twice in PBS. Cells were then incubated in the dark with 4,6-diamidino-2-phenylindole (DAPI) 5 μg/mL in PBS for 5 minutes and washed twice, and coverslips were mounted with glycerol/PBS (3:1) solution. Slides were examined on an Olympus (CenderValley, CA) fluorescent microscope, and color print pictures were taken.

Ki67 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. Actively replicating cells were selectively highlighted within tissue samples by immunostaining the Ki67 antigen. Sections (5 μm) were placed on charged slides and rehydrated. The sections were rehydrated and placed in heated target retrieval solution (Lab Vision, Fremont, CA) for 20 minutes. Endogenous peroxidase was neutralized with 0.03% hydrogen peroxide followed by a casein-based protein block (DakoCytomation, Carpinteria, CA) to minimize nonspecific staining. The sections were incubated with rabbit anti-human Ki67 (Vector Laboratories, Burlingame, CA) diluted 1:500 for 30 minutes. Sections without primary antibody served as negative controls. The DAKO EnVision–horseradish peroxidase (HRP)/3,3’-diaminobenzidine (DAB) System (DakoCytomation) was used to produce localized, visible staining. The slides were lightly counterstained with Mayer’s hematoxylin, dehydrated, and coverslipped. Fragmented DNA of apoptotic cells was visualized with the DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase (TdT)–Mediated dUTP Nick End Labeling (TUNEL) System (Promega Corp., Madison, WI). Tissue sections were subjected to a second fixation in 4% paraformaldehyde, rinsed, and permeabilized with proteinase K for 5 minutes. Sections then were treated with equilibration buffer (Promega) followed by biotinylated nucleotide incorporation into apoptotic cells using TdT. Endogenous peroxidase was neutralized by applying 0.3% hydrogen peroxide to the sections. Applications of streptavidin/HRP and DAB produced apoptotic-specific visible nuclear staining. Number of positive cells was scored and graphed by averaging three repeated assessments.

Statistical analysis. Analysis of study results focused on tests of the differences of the mean tumor volume among treatment groups and different time points. The data analysis was completed using the restricted residual maximum likelihood-based mixed-effect model to adjust the intracorrelation effect for the mice that had multiple measurements. The model reported in the article was selected based on the Schwarz’s Bayesian criterion. All tests of significance were two sided, and differences were considered statistically significant when P < 0.01. Statistical Analysis System version 8.2 (SAS Institute, Cary, NC) was used for all analyses.
Radiation induces phosphorylation of Flk-1 in HUVECs. Western blot analysis was used to determine the effects of radiation on Flk-1 phosphorylation in HUVECs. An increase of phosphorylated Flk-1 was detected in irradiated HUVECs 30 minutes after irradiation (Fig. 1). To establish that activation of Flk-1 could be inhibited by AZD2171, cells were treated with 20 nmol/L AZD2171. After 24 hours of treatment, cells were treated with 5 Gy. Cell lysates were then collected and analyzed. As shown in Fig. 1, treatment with AZD2171 considerably eliminated phosphorylated Flk-1 levels in both irradiated and nonirradiated HUVECs.

AZD2171 sensitizes HUVECs and vascular endothelial model to ionizing radiation. To determine whether the Flk-1 TKI AZD2171 sensitizes HUVECs to radiotherapy, clonogenic assays were done. Cells were treated with either 20 nmol/L AZD2171 or a vehicle control and incubated for 24 hours. This was followed by radiation doses of 0, 2, 4, or 6 Gy, and the drug was washed off 48 hours after irradiation. After 8 days, colonies were stained and the scored colonies were graphed. AZD2171 lowered the survival curve of irradiated HUVECs (Fig. 2A). To determine the magnitude of the radiosensitizing effect, the DER was calculated. The DER of AZD2171 was 1.24 (P = 0.01).

Because AZD2171 reduced HUVEC survival, we examined its effect on the angiogenic function of HUVECs in vitro. The formation of tubes by endothelial cells is a critical step in angiogenesis. The endothelial cell morphogenesis assay was done to examine the ability of the treated HUVECs to produce capillary-like tubular structures. Representative photographs are shown in Fig. 2B, and the mean number of counted tubes in three separate (>100) fields is shown in Fig. 2C. No treatment control had 28 (SD, 0.9) tubules per microscopic field, radiation alone had 21 (SD, 1.0) tubules, AZD2171 alone had 20 (SD, 0.9) tubules, and AZD2171/radiation combination had 12 (SD, 0.9) tubules (P < 0.002 for AZD2171/radiation versus radiation alone).

Improved tumor growth delay with combined AZD2171 and radiotherapy at well-tolerated doses. To determine whether inhibition of Flk-1 signaling enhances radiation-induced tumor growth inhibition, an H460 NSCLC xenograft model was established and tumor volumes were measured by use of calipers as described previously (22). Mice bearing H460 tumors were treated with 0.75 mg/kg p.o. for 7 days and irradiated with 2 Gy fractions following drug administration on days 3 to 7, for a total dose of 10 Gy. AZD2171 and radiotherapy combination treatment induced a tumor growth delay of 11 days compared with 1 day for AZD2171 alone and 7 days for radiation alone (P < 0.003 for AZD2171/radiation versus radiation alone; Fig. 3A).

The body weight changes were tracked in the mice to assess whether treatment with AZD2171, radiation, or combination posed systemic toxicity. As shown in Fig. 3B, the control and drug alone treatment groups showed modest weight gain (<10%), and the radiation and combination treatment groups maintained stable weight over the 18 days they were followed.

AZD2171 with radiation reduces phosphorylated Flk-1 expression and vascular density in H460 tumor models. To verify the radiation-induced expression of phosphorylated Flk-1 seen in vitro, we did a correlative study to examine phosphorylated Flk-1 staining in histologic sections from H460 tumors (Fig. 4A). No phosphorylated Flk-1 staining was appreciated in the control and AZD2171 alone tumor sections. However, there was a substantial amount of phosphorylated Flk-1 seen in the irradiated tumor section. In the combination treatment sections, the radiation-induced phosphorylated Flk-1 expression was attenuated, and only a small amount of staining could be seen.

To determine the effects of AZD2171 and radiation on tumor vasculature in vivo, mice were treated similarly to those in the tumor growth delay study, with 0.75 mg/kg AZD2171 administration for 7 days and daily 2 Gy radiation treatments on days 3 to 7. After 7 days, the tumors were resected and paraffin fixed and an antibody for vWF was used to stain for blood vessels. A representative image is shown in Fig. 4B. The number of vessels per microscopic field was then determined for each treatment group. Control tumors had an average of 15 (SD, 0.6) vessels per microscopic field, radiation alone had 11.3 (SD, 0.9) vessels, drug alone had 6 (SD, 0.6), and combination had 2.3 (SD, 0.9; P < 0.009 for AZD2171/radiation versus radiation alone; Fig. 4C).

AZD2171 with radiation reduces proliferation and increases apoptosis in H460 tumor models. To determine whether the tumor growth delay from the combined therapy results from decreased tumor proliferation and/or increased apoptosis, Ki67 and TUNEL staining was done using tissue sections from H460 tumors in all treatment groups. As shown in Fig. 5A and B, the Ki67 index was lowest in the combination treatment sections, about one half of the radiation alone group, and ∼4-fold reduction compared with the untreated group (P < 0.02 for AZD2171/radiation versus radiation alone).

As shown in Fig. 5C and D, there was an ~70% increase in apoptosis when AZD2171 was added to radiotherapy (P < 0.03 for AZD2171/radiation versus radiation alone). Additionally, the combination group has >6-fold increase in apoptosis compared with the untreated group.

Discussion

In the present study, we found that radiation induces phosphorylated Flk-1 expression in endothelial cells and tumor vasculature and that AZD2171 inhibits this expression. We show that AZD2171 sensitizes vascular endothelium to radiation both in vitro and in vivo. Combination treatment with AZD2171 and radiation also enhances tumor growth delay in an H460 xenograft model by inhibiting tumor proliferation and promoting apoptosis.

VEGF signaling has become an important target for cancer therapy due to its central role in regulating angiogenesis. Targeting VEGF signaling is proving to be a valuable therapeutic approach.
in cancer patients. Significantly, the humanized anti-VEGF monoclonal antibody bevacizumab is an effective addition to the standard treatment with carboplatin/paclitaxel in NSCLC in phase II trials (23), and phase III trials have shown promise using combination chemotherapy in various cancers, including NSCLC (24). Although bevacizumab has shown less promise as monotherapy, these results validate the mechanistic approach of combining antiangiogenesis agents with cytotoxic therapy in patients. This also provides evidence to suggest that combining VEGF signaling inhibition with cytotoxic radiotherapy may yield benefit in cancer treatment.

The present study is the first study to investigate the combination of AZD2171 and radiotherapy. Although AZD2171 is in clinical trials, the combination of this promising agent with radiation remains unexplored. Previous studies have provided the rationale for combining VEGF TKIs with radiation in tumor models (25, 26). The appeal of AZD2171 compared with previously investigated VEGFR-2 TKIs is that it is active in the subnanomolar range and shows higher selectivity for VEGFR-2 compared with other RTKs. Additionally, its pharmacokinetic properties make it appropriate for once-daily oral dosing, such as a terminal plasma half-life of 20 hours in humans (11).

Interestingly, we show that radiation induces expression of activated phosphorylated Flk-1 in HUVECs (Fig. 1). Using immunofluorescence staining of tumor sections, we also show that this radiation-induced activation of Flk-1 occurs in vivo (Fig. 4A). Addition of AZD2171 attenuates phosphorylated Flk-1 expression in both the HUVEC culture model and the tumor sections. Radiation has been shown to induce VEGF expression within tumors both in vitro and in vivo (27). Expression of VEGFR-2 (Flk-1) has also been induced in endothelial cells after irradiation in vitro (28). This suggests that there is a proangiogenic response within tumors after irradiation, antagonizing the known vascular-damaging effects of radiation. Accordingly, this implies that combining antiangiogenesis agents with radiation would provide a means of enhancing the antitumor effects of radiation.

Low doses of AZD2171 (20 nmol/L) also decrease HUVEC survival in combination with radiation as shown by clonogenic assay (Fig. 2A), suggesting that AZD2171 has the potential to sensitize existing tumor vasculature to ionizing radiation. The in vitro Matrigel assay shows that the combination treatment has an additive decrease in the ability of endothelial cells to form capillary tube-like structures compared with drug or radiation.
alone (Fig. 2B). This shows that combination treatment with AZD2171 and radiation may be an effective means of inhibiting angiogenesis in tumor models.

To further explore the clinical viability of combining these two treatment modalities, we established an H460 NSCLC xenograft model in nude mice. Mice were placed into one of four treatment groups: control, AZD2171 alone, radiation alone, or AZD2171/radiation combination. Drug treatment groups were given 0.75 mg/kg AZD2171 p.o. once daily for 7 days, and radiation groups received five 2 Gy fractions on days 3 to 7 for a total dose of 10 Gy. The drug concentration was chosen to be the smallest effective dose in previous studies (11), and radiation dosing was designed to provide clinically relevant fractionated small doses. A previous study combined VEGFR-2 blockade with radiotherapy and found that tumor growth delay was maximized when the radiation was given between days 4 and 6 after initiation of VEGFR-2 inhibition (29). Their study used an anti-VEGFR-2 monoclonal antibody and three consecutive daily fractions of 7 Gy irradiation in a human glioblastoma xenograft model in mice. To maximize clinical applicability, we chose to use five fractions of low-dose (2 Gy) irradiation in our lung cancer model. We found that the AZD2171/radiation combination treatment group showed significant tumor growth delay compared with either treatment alone. For a 10-fold increase in tumor volume, the growth delay was 1 day for low-dose AZD2171 alone, 7 days with fractionated radiotherapy alone, and 11 days for combination treatment (Fig. 3).

Figure 3. Improved tumor growth delay with combination AZD2171 treatment and radiotherapy at well-tolerated doses. H460 tumors were implanted into the posterior flank of nude mice. Mice were treated with vehicle control or 0.75 mg/kg (p.o.) AZD2171 for 7 consecutive days (five mice in each treatment group). Starting on the 3rd day of AZD2171 treatment, mice in radiation groups were irradiated 1 hour after AZD2171 treatment with 2 Gy daily for 5 days. A, tumor volume was measured with calipers and is shown for each treatment group (P < 0.003 for AZD2171/radiation versus radiation alone). B, average weight for each treatment group was also measured.

Figure 4. AZD2171 with radiation reduces phosphorylated Flk-1 expression and vascular density in H460 tumor models. A, histologic sections were obtained from the tumors of the mice in each treatment group. Slides were stained for phosphorylated Flk-1 using anti-phosphorylated Flk-1 polyclonal antibody. Slides were then incubated with a rhodamine red–labeled goat anti-mouse IgG secondary antibody and then incubated with DAPI counterstain (5 μg/mL) in PBS, and coverslips were mounted with glycerol/PBS (3:1) solution. Slides were examined on an Olympus fluorescent microscope, and representative images are shown. B, similar sections were acquired and blood vessels were stained for using an antibody for vWF. Representative sections from each treatment group are shown at ×400 magnification. C, average blood vessel density of each treatment group was determined by counting the number of blood vessels per microscopic field. This was repeated thrice. Columns, average; bars, SD. P < 0.009 for AZD2171/radiation versus radiation alone.
Our results are consistent with Winkler et al. (29) because they showed synergistic tumor growth delay when radiation doses were given on days 4 to 6 after initiation of anti-VEGFR-2 therapy. We gave lower-dose radiation on days 3 to 7 after initiation of AZD2171 and found a greatly improved tumor growth delay using this similar treatment schedule. Their data suggest that there is a critical window during which the interaction between anti-VEGFR-2 therapy and radiotherapy is maximized, as they only showed additive increases in tumor growth delay when radiation was given before, on days 1 to 3, or on days 7 to 9 after initiation of therapy. It is proposed that optimal treatment scheduling corresponds to administration of radiation during a period of vascular normalization that is induced by the anti-VEGFR-2 therapy (30). Because tumors induce high levels of angiogenesis, the antiangiogenic drug administration serves to balance this pathologic angiogenesis. Therefore, it is suggested that this period of normalization represents a balance between proangiogenic and antiangiogenic factors that transiently improves tumor blood flow and oxygenation (31). Because hypoxia is known to decrease the efficacy of radiotherapy (13), giving radiation during this normalization window could serve to enhance treatment before the tumor vessels are irreversibly damaged and tumor blood flow is diminished by radiation. In fact, it has been shown that antiangiogenic therapy can inhibit drug delivery to tumors (32) as well as affect the efficacy of radiotherapy (33). Therefore, it seems that careful treatment scheduling is crucial when combining antiangiogenic agents with conventional cytotoxic treatment modalities.

Our data show that tumor vascular density is decreased by combination therapy with AZD2171 and radiation (Fig. 4B and C). This decrease in vascular density was observed 7 days after initiation of AZD2171 treatment. This abolition of tumor vascularity not only shows the value of AZD2171 in enhancing radiation-induced vascular damage but also underscores the previously discussed importance of optimal treatment scheduling when using anti-VEGFR-2 agents. Again, decreased vascularity leads to tumor hypoxia and a decrease in the efficacy of radiotherapy and delivery of chemotherapy.

Consistent with the tumor growth delay we observed using the chosen treatment schedule, we observed decreased proliferation using Ki67 staining of histologic sections from the H460 xenograft tumors. There was a notable decrease in Ki67 staining of mice that received combination treatment compared with control or either treatment alone, with the combination treatment group showing 25% of the level of Ki67 staining of the untreated control (Fig. 5A and B). Similarly, apoptosis was increased in the combination treatment group compared with controls (Fig. 5C and D). These data suggest that the mechanism of tumor growth delay included both decreased proliferation and increased apoptosis of tumor cells.

Figure 5. AZD2171 with radiation reduces Ki67 proliferative marker and increases apoptotic index in H460 tumor models. Histologic sections were obtained from the tumors of the mice in each treatment group. Standard Ki67 and TUNEL stainings were done. Average proliferative (A and B) or apoptotic (C and D) index of each treatment group was determined by counting positive cells per microscopic field. This was repeated thrice. Columns, average; bars, SD. For Ki67 staining, comparing AZD2171/radiation combination versus radiation alone, \( P < 0.02 \). For TUNEL staining, comparing AZD2171/radiation versus radiation alone, \( P < 0.03 \).
We have shown the clinical feasibility of combining a potent, selective, orally bioavailable VEGFR-2 TKI with conventional fractionated radiotherapy. We have also shown that VEGF signaling is increased after irradiation both in vitro and in vivo, suggesting the important role that anti-VEGFR-2 therapy can play in attenuating this proangiogenic response to radiation. Additionally, tumor vascular density is decreased after combination treatment with AZD2171 and radiation, suggesting that this agent may sensitize existing tumor vasculature to radiation. Future clinical trials are needed to determine the efficacy of AZD2171 in combination with radiotherapy in lung cancer patients.

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