# Combining radiotherapy with AZD2171, a potent inhibitor of vascular endothelial growth factor signaling: pathophysiologic effects and therapeutic benefit

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## Abstract

**AZD2171 is a highly potent, orally active inhibitor of vascular endothelial growth factor receptor signaling. The potential for AZD2171 to enhance the antitumor effects of radiotherapy was investigated in lung (Calu-6) and colon (LoVo) human tumor xenograft models. Combined treatment resulted in a significantly enhanced growth delay compared with either modality alone. The enhancement was independent of whether chronic once daily AZD2171 treatment was given 2 h prior to each radiation fraction (2 Gy daily for 3 or 5 consecutive days), and daily thereafter, or commenced immediately following the course of radiotherapy. Histologic assessments revealed that 5 days of radiation (2 Gy) or AZD2171 (3 or 6 mg/kg/d) reduced vessel density and perfusion. Concomitant AZD2171 and radiation enhanced this effect and produced a significant increase in tumor hypoxia. Concomitant AZD2171 (6 mg/kg/d) was also found to reduce tumor growth significantly during the course of radiotherapy (5 × 2 Gy). However, the extent and duration of tumor regression observed postradiotherapy was similar to sequentially treated tumors, suggesting that preirradiated tumors were sensitized to AZD2171 treatment. An enhanced antivascular effect of administering AZD2171 postradiotherapy was observed in real-time in Calu-6 tumors grown in dorsal window chambers. Collectively, these data support the clinical development of AZD2171 in combination with radiotherapy. [Mol Cancer Ther 2007;6(2):599–606]**

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## Introduction

Ionizing radiation is used in the treatment of at least 50% of all cancers. As the patient population treated with radiotherapy is so large, enhancing therapeutic outcome for even a relatively small proportion has the potential to translate into highly significant clinical benefit.

One way to enhance radiotherapy outcome is to use combined treatment strategies. Over recent years, there has been a lot of interest in the use of antiangiogenic approaches in combination with radiotherapy (1). In particular, therapeutics that target vascular endothelial growth factor-A (VEGF) signaling have been investigated in preclinical models (2–12). These therapies could either directly sequester VEGF (neutralizing antibodies, e.g., bevacizumab or soluble receptors) or block VEGF-mediated signaling through VEGF receptors. The latter category includes small molecule inhibitors of VEGF receptor-2 (VEGFR-2; KDR) tyrosine kinase activity, which is predominantly responsible for transducing the angiogenic, neovascular survival, and permeability signaling responses induced by VEGF in endothelial cells (13–15). The rationale for the development of VEGF signaling inhibitors in combination with radiotherapy was originally based on the observation that VEGF could enhance endothelial cell survival (2). Consequently, a number of studies have shown that VEGF-targeting approaches can induce endothelial cell radiosensitization *in vitro* (2, 3, 5, 10, 11). One potential downside to this observation is that a substantial loss of the endothelial cell compartment has been associated with the induction of hypoxia (16, 17), which could limit the effectiveness of radiotherapy *in vivo*. Indeed, compromised perfusion as a consequence of VEGF-inhibition during radiotherapy has been associated with suboptimal efficacy (7, 8). Conversely, some studies have suggested that VEGFR-blockade can lead to a transient normalization of the vasculature, leading to a window of opportunity when perfusion, oxygenation, and consequently, radiation response may be improved (4, 18, 19). A further body of evidence suggests that targeting VEGF signaling postradiotherapy may be as critically important as its inhibition during the course of radiotherapy (7, 8, 20). Clearly, the temporal consequences of combining VEGF signaling inhibition with radiation *in vivo* can be complex and dependent on modulating a number of pathophysiologic factors.

The purpose of the present study was to investigate the efficacy of AZD2171, an orally active, highly potent, and selective VEGF signaling inhibitor of all VEGFR tyrosine kinases (VEGFR-1, -2 and -3), in combination with

radiotherapy. AZD2171 has been previously shown to inhibit VEGF-stimulated responses in endothelial cells at subnanomolar concentrations, and prevent physiologic and pathologic angiogenesis *in vivo* (21). Once daily p.o. administration of AZD2171 (≥1.5 mg/kg) significantly inhibits the growth of histologically diverse human tumor xenografts (21), an observation that has prompted the clinical development of this compound (22). In the present study, chronic administration of AZD2171 was combined with fractionated radiotherapy in lung (Calu-6) and colorectal (LoVo) human tumor xenograft models and the effect of concomitant or sequential AZD2171 administration was examined. Furthermore, the underlying basis for any interaction with radiotherapy was assessed in tumors by monitoring vessel density and perfusion, the development of tumor hypoxia, and the induction of apoptosis.

## Materials and Methods

### Cell Lines

Calu-6 human lung carcinoma cells and LoVo human colorectal cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Life Technologies (Invitrogen, Ltd., Paisley, United Kingdom) except for FCS (Biosera, East Sussex, United Kingdom). Cells were cultured in RPMI supplemented with 10% FCS and 2 mmol/L glutamine, and were routinely screened for the presence of *Mycoplasma* using a commercial assay (MycoTect Life Technologies).

### Tumor Xenografts

Cells were harvested in exponential phase growth. Calu-6 cells were prepared at a concentration of $2 \times 10^7$ cells per milliliter in a 1:1 mix of serum-free RPMI and Matrigel (phenol red–free; BD Biosciences, Erembodegem, Belgium) and LoVo cells at a concentration of $5 \times 10^7$ cells per milliliter in serum-free RPMI. Tumor xenografts were initiated in female *nu/nu* CBA mice aged 10 to 12 weeks old. Briefly, a skin flap was raised 1 cm from the tail base on the midline of the back and a 0.1 mL volume of cell suspension was implanted at an approximate depth of 1 mm. Once palpable tumors formed (5–7 days post-implantation), volume measurements were taken daily.

### Dorsal Window Chamber

The procedure followed for the introduction of dorsal window chambers was adapted from previously published methods using rats (23). Briefly, female *nu/nu* CBA mice were anesthetized by i.p. injection of Hypnorm (Janssen Animal Health, High Wycombe, Buckinghamshire, United Kingdom; 0.4 mL/kg) and midazolam 5 mg/kg. The dorsal skin was drawn up into a fold that extended along the midline of the back. A ''C'' frame bridge was then sutured in place, and a 5 mm diameter disc of skin was cut away from the middle of the fold on one side, leaving the opposing fascia and associated vasculature intact. A titanium ''saddle'' was then attached to the dorsal skin flap holding a glass coverslip in position over the exposed area. The ''C'' frame bridge was then removed. Analgesia (buprenorphine, 0.01 mg/mL) was administered pre- and postoperatively as required. The surgical site was monitored for 48 h and then $\sim 1 \times 10^6$ Calu-6 tumor cells were injected into the window chamber via the opposing side of the skin flap. From day 5 after cell implantation, tumor blood vessel development was monitored under transillumination using a NIKON Eclipse E800 microscope. Drug and/or radiation treatments were initiated ~10 days after cell implantation when substantial vascularization was apparent within the growing tumor mass.

### Drug and/or Radiation Treatment Schedules

Mice bearing established tumors of size 220 to 280 $mm^3$ ($n$ = 5–7 per group) were randomly assigned into designated treatment groups. The sample size per group was derived using archived data and was sufficient to detect a 50% change in treatment response with 80% power to a significance level of $P = 0.05$. AZD2171 or vehicle (1% polysorbate in deionized water) was administered once daily by p.o. gavage (0.1 mL/10 g body weight). Tumor-localized radiotherapy (X-ray) was administered as once daily 2 Gy fractions under ambient conditions to non-anesthetized mice restrained in lead-shielded containers. The restrained mice were held in a lead-shielded support perpendicular to the source. Irradiation was delivered at a dose rate of 0.75 Gy/min. Mice were turned around halfway through the procedure to ensure a uniform tumor dose. Tumor measurements were taken daily until the tumor volume quadrupled from that at the start of treatment (relative tumor volume × 4; RTV4), which was designated as the experimental end point. When mice received combinations of AZD2171 and radiation, two treatment regimens were used: concomitant, in which AZD2171 was given 2 h prior to each radiation fraction and daily thereafter, and sequential, in which the administration of AZD2171 was not initiated until 2 h after the radiotherapy course was completed. All procedures were carried out using protocols that had been approved by the Institutional Ethics Committee and the Home Office (project license 40/2328) and designed in accordance with the Scientific Procedures Act (1986) and UK Coordinating Committee on Cancer Research Guidelines (1999). The data calculated from the growth profile of each individual tumor were the time to RTV4, the growth delay in achieving RTV4 (time to RTV4 in treated tumors minus time to RTV4 in vehicle-treated control tumors), and tumor doubling time (the time taken for tumors to double in volume during the linear phase of tumor growth/regrowth).

### Administration of Perfusion and Hypoxic Markers

The perfusion marker Hoechst 33342 (Sigma, Poole, Dorset, United Kingdom) and the hypoxic marker pimonidazole (Chemicon Europe Ltd., Chandlers Ford, Hampshire, United Kingdom) were administered to satellite groups of tumor-bearing mice following treatment with vehicle, AZD2171 and/or radiotherapy ($n$ = 4–5 per group). Pimonidazole (60 mg/kg) was administered 2 h prior to tumor excision by i.p. injection and Hoechst 33342 (0.1 mL of a 6 mg/kg stock) administered i.v., 1 min prior to tumor excision via the tail vein. Excised tumors were rapidly excised and snap-frozen in liquid nitrogen for histologic analysis.

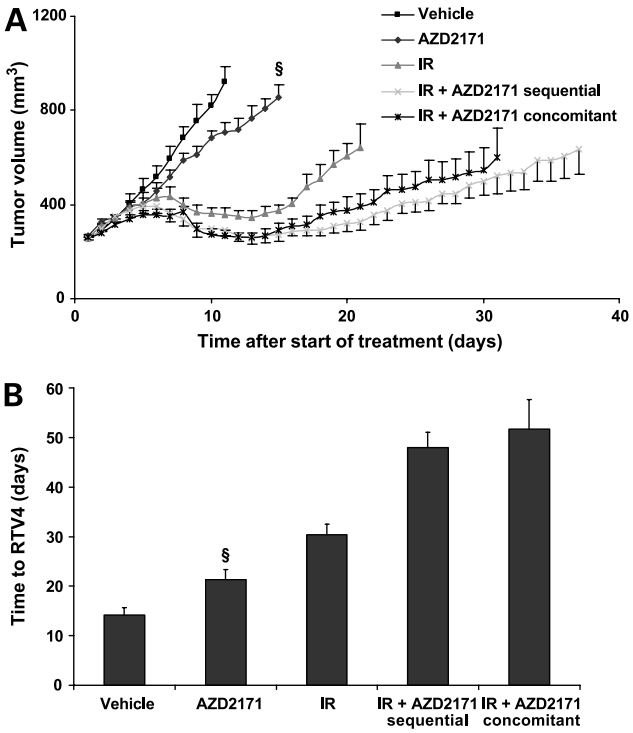


**Figure 1.** AZD2171 enhances the radiotherapy response of Calu-6 xenografts independently of whether AZD2171 is administered concomitantly or sequentially to the radiation treatment. Radiotherapy was given as 5 × 2 Gy fractions at 24-h intervals (days 1 – 5). AZD2171 (3 mg/kg/d) was given by p.o. dosing either 2 h prior to each radiation dose (concomitant; day 1 onwards) or was initiated 2 h after the completion of the radiotherapy treatment (sequential; day 5 onwards). AZD2171 was chronically dosed for the duration of the experiment. Data shown are tumor growth profiles (**A**) or time to achieve a quadrupling of tumor volume (RTV4; **B**). *Points,* average values from all the tumors in each group; *bars,* SE ($n$ = 6 for AZD2171 alone and $n$ = 7 for others). **A,** points were plotted until the time when the first tumor within the group reached the designated end-point volume (RTV4). One tumor in the AZD2171 group regressed completely and was excluded from the data analysis (§).

### Analysis of Perfusion, Vasculature, Apoptosis, and Hypoxia

Cryostat sections (5 μm) were prepared from snap-frozen tumor material. Sections were first scanned using fluorescent microscopy to determine the number of Hoechst 33342–stained (perfused) vessels. Sections were then fixed in ice-cold acetone and tumor endothelial cells stained using rat anti-CD31 (PharMingen, BD Biosciences) followed by tetramethyl rhodamine isothiocyanate–labeled goat anti-rat antibody (Molecular Probes, Invitrogen Life Technologies, Paisley, United Kingdom) as described previously (8). To detect apoptosis in the same sections, following the final wash procedures, sections were costained using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) system (Promega, Southampton, Hampshire, United Kingdom) following the manufacturer's recommended protocols. Hypoxia was detected in adjacent sections. Briefly, acetone-fixed sections were treated with 10% horse serum in PBS containing 0.1% Tween 20 (PBST) for 15 min at room temperature and washed twice using PBST. Hypoxyprobe antibody (Chemicon Europe Ltd.) was then applied (1:50 in PBST supplemented with 0.1% bovine serum albumin) and sections were incubated overnight at 4°C. Following three PBS washes, rabbit anti-mouse FITC (DAKO, Ely, Cambridgeshire, United Kingdom) was added (1:100 in PBST 0.1% bovine serum albumin) and the sections incubated for 1 h in the dark at room temperature. After three further washes with PBS, slides were mounted using fluorescent mounting medium (DAKO). To assess vessel density, apoptotic fraction, and hypoxic fraction, samples were analyzed using a NIKON Eclipse E800 with associated MetaMorph software. Vessel density was analyzed per unit area of the tumor section. Hypoxia and apoptosis were assessed as the proportion of the tumor area staining positively for the Hypoxyprobe and TUNEL labeling reagents, respectively. H&E staining was done on adjacent sections.

## Results

### AZD2171 Significantly Improves the Outcome of Radiotherapy in Human Tumor Xenografts

Initial studies were undertaken in mice bearing Calu-6 tumor xenografts. The effect of combining chronic (once daily) AZD2171 treatment with radiotherapy was assessed using concomitant and sequential scheduling regimens.

Chronic daily dosing of mice bearing Calu-6 tumor xenografts with 3 mg/kg of AZD2171 alone caused a significant decrease in tumor growth compared with vehicle-treated controls and the doubling time increased from 5 to 12 days (Fig. 1A and B; Table 1). Fractionated

**Table 1. Chronic daily dosing of AZD2171 (3 mg/kg) enhances the response of Calu-6 xenografts to fractionated radiotherapy**

| Treatment | RTV4 (d) | DT (d) | Growth delay (d) |
|---|---|---|---|
| Vehicle | 11 ± 0.5 | 5 ± 0.2 | NA |
| AZD2171* | 23 ± 2 | 12 ± 2† | 12 ± 2 |
| 3 × 2 Gy IR | | | |
| IR | 28 ± 2 | 9 ± 2† | 17 ± 2 |
| IR + AZD2171 sequential | 45 ± 5‡ | 17 ± 3‡ | 34 ± 5‡ |
| IR + AZD2171 concomitant | 46 ± 5‡ | 16 ± 1‡ | 35 ± 5‡ |
| 5 × 2 Gy IR | | | |
| IR | 32 ± 2 | 8 ± 0.5† | 21 ± 2 |
| IR + AZD2171 sequential | 48 ± 3‡ | 16 ± 2‡ | 37 ± 3‡ |
| IR + AZD2171 concomitant | 52 ± 6‡ | 17 ± 3‡ | 41 ± 6‡ |

NOTE: Tumors were treated daily with AZD2171 until the RTV4 was obtained. Growth delay ($RTV4_{treated}$ − $RTV4_{control}$). Values shown are the mean ± SE.

Abbreviations: IR, ionizing radiation; DT, tumor volume doubling time; NA, not applicable.

*One tumor treated with chronic 3 mg/kg of AZD2171 regressed.

†$P < 0.001$ versus vehicle control; Mann-Whitney $U$ test.

‡$P < 0.05$ compared with radiation or AZD2171 alone.

radiotherapy also caused a significant increase in tumor volume doubling time, although the change was not as great as that seen with AZD2171 (Table 1). When 3 mg/kg/d of AZD2171 was combined with three or five 2 Gy fractions of radiation, treatment outcome was significantly improved compared with radiotherapy alone (Fig. 1A and B; Table 1). The enhanced treatment response was independent of the scheduling regimen used and was associated with an increase in tumor volume doubling time compared with AZD2171 or radiation alone, although this only achieved statistical significance versus the latter group (Table 1). When combined with five fractions of radiotherapy, AZD2171 also seemed to improve the extent of tumor regression postradiotherapy (Fig. 1A). The overall growth delays achieved in the combined treatment groups were significantly greater than radiation or AZD2171 treatment alone, and greater than would have been anticipated from the sum of the effects of the AZD2171 and radiation treatments given independently (Table 1).

### Concomitant AZD2171 (3 mg/kg/d) Treatment Enhances the Antiendothelial Cell Effect of Radiation and Induces Tumor Hypoxia

Previous studies have suggested that doses of radiation as low as 2 Gy can inhibit endothelial cell proliferation, survival, tube formation, and invasion *in vitro* (12). VEGF is one of the critical modifiers of this response. To determine the acute effects of AZD2171, radiation, and concomitant treatment on endothelial cells *in vivo*, histologic assessments of vessel density and perfusion were done on tumors excised immediately after 5 days of continuous treatment. To relate changes in the endothelial compartment to the pathology of the tumor as a whole, the extent of tumor hypoxia and apoptosis were also assessed. Both AZD2171 (3 mg/kg) daily for 5 days and radiation alone (5 × 2 Gy) reduced vessel density in Calu-6 tumors. However, it was only when both agents were applied concomitantly that this change achieved statistical significance compared with vehicle-treated controls (Fig. 2A). Perfused vessel density was reduced significantly by both radiation and radiation with concomitant AZD2171 (Fig. 2A). AZD2171 had a more modest effect on perfusion that was not significantly different from vehicle-treated controls. Because total vessel density was reduced, AZD2171 seemed to increase the proportion of perfused tumor vessels remaining in Calu-6 tumors to ~80% compared with 60% of the total in control tumors.

The extent of tumor hypoxia was assessed by analyzing pimonidazole binding in the treated tumors. Neither AZD2171 nor radiation alone significantly affected the proportion of hypoxic tissue within treated tumors. However, concomitant AZD2171 and radiation doubled tumor hypoxia (Fig. 2B; $P = 0.05$ versus control). Analysis of TUNEL staining suggested that slight increases in apoptosis were induced by AZD2171 and the combined treatment.

### Visualizing the Effect of Radiation Combined with Sequential AZD2171 (3 mg/kg/d) Treatment

To make a direct real-time visual assessment of the effects of sequential treatment with AZD2171 on untreated versus preirradiated tumor vessels, Calu-6 xenografts were established in dorsal window chambers. Once tumors were established, mice were administered AZD2171 (3 mg/kg) daily for 3 days with or without preceding radiotherapy (3 × 2 Gy). Tumors were analyzed prior to the first drug treatment and 12 h after the third dose. Figure 3A shows

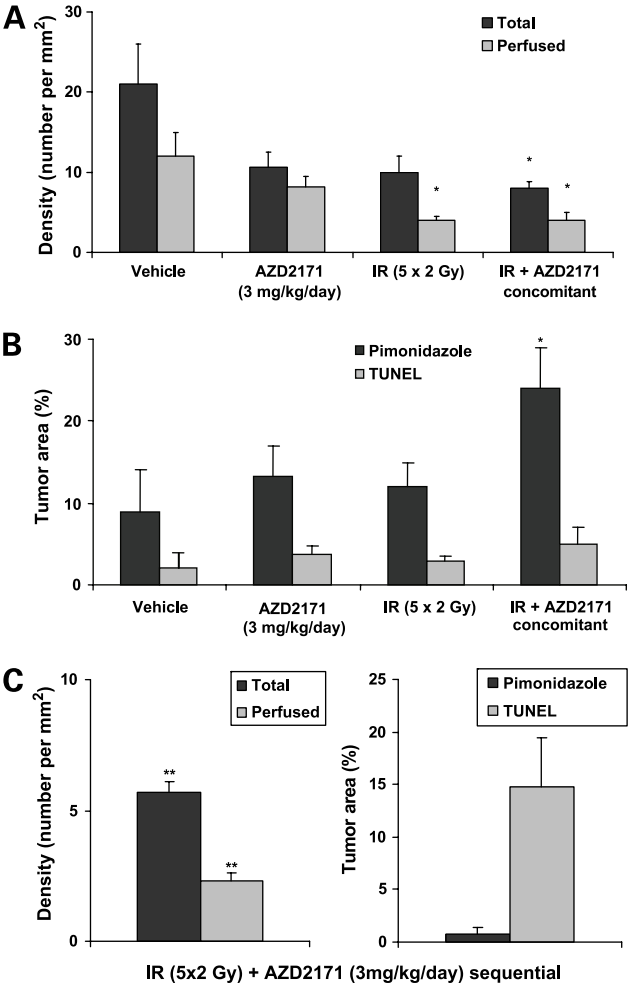


**Figure 2.** Analysis of the histologic assessment of vessel density and perfusion (**A**) and tumor hypoxia and apoptosis (**B**) in Calu-6 tumor xenografts treated with radiation, AZD2171, or concomitant treatment with both for 5 d. Total vessel density (**A**) was assessed using immunostaining for CD31 as a marker of endothelial cells. To analyze perfused vessel density, Hoechst 33342 (0.1 mL of a 6 mg/kg solution) was administered by tail-vein injection 1 min prior to sacrifice of the mice. Hypoxia was measured using pimonidazole (**B**). This was given by i.p. injection (60 mg/kg) immediately after the last dose of radiotherapy and tumors were excised 2 h later. TUNEL staining was used to analyze apoptosis. The percentage tumor area immunopositive for pimonidazole adducts or TUNEL staining (**B**). **C,** histologic analysis of tumors treated sequentially with 5 × 2 Gy radiotherapy followed by five doses of AZD2171 initiated 2 h after the last radiation dose (9-d total treatment time). Note the scales differ from those used in (**B**). *Columns,* average values; *bars,* SE. *, $P < 0.05$; **, $P < 0.005$ versus vehicle control, two-sided *t* test. Tumors treated concurrently or sequentially with radiation and AZD2171 were significantly smaller than vehicle controls at the time of excision (averages sizes, 298 ± 10 and 282 ± 27 versus 466 ± 48 $mm^3$ for controls). There was no significant difference between the sizes of the other groups.

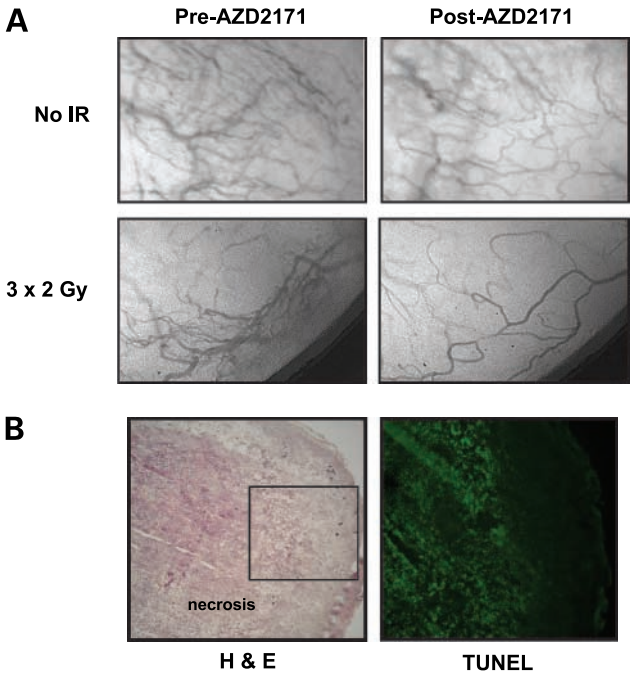


**Figure 3.** Visualizing the enhanced activity of sequential radiotherapy and AZD2171 treatment. **A,** Calu-6 tumors were initiated in dorsal window chambers. AZD2171 (3 mg/kg) was administered daily for 3 d to tumors that were previously untreated (*top right*) or that had received 3 × 2 Gy fractions of radiotherapy at daily intervals (*bottom right*). Images are representative regions of interest taken immediately before the initiation of AZD2171 (pre-AZD2171; *left*) or 12 h after the third dose (post-AZD2171; *right*). **B,** histologic examination of necrosis and apoptosis in Calu-6 tumors treated sequentially with radiotherapy (5 × 2 Gy fractions) followed by AZD2171 (3 mg/kg daily for 5 d). The tumor area analyzed by TUNEL staining is marked on the H&E-stained section.

representative regions of interest of the treated tumors. Although AZD2171 alone reduces vessel density (Fig. 3A), this is markedly enhanced in the tumors pretreated with radiation (Fig. 3A).

An additional experiment investigated histologic variables in Calu-6 tumors treated sequentially with 5 days of radiotherapy (2 Gy/d) and followed by AZD2171 (3 mg/kg/d) for 5 days (i.e., 9 days treatment duration in total; Fig. 2C). In keeping with the observations using the dorsal window chamber, sequential treatment resulted in marked vessel loss. The average vessel density observed was 6 ± 0.4 vessels per mm$^2$. This was significantly lower than that observed following radiotherapy ($P$ = 0.04) or AZD2171 alone ($P$ = 0.03). It was also a significant reduction when compared with concomitant scheduling analyzed at day 5 (vessel density 8 ± 1 per mm$^2$; $P$ = 0.02). The perfused vessel density (2 ± 0.3 per mm$^2$) was also reduced significantly when compared to treatment with AZD2171 ($P$ = 0.002) or radiation alone ($P$ = 0.04). Furthermore, the proportion of vessels that were perfused was reduced compared with controls (40% versus 60%). The remaining perfused vessels were distributed in narrow viable tissue towards the rim of the tumor (data not shown), which bordered large adjacent areas of TUNEL-positive apoptosis/necrosis (Fig. 3B). Very little hypoxia (pimonidazole binding) was evident within the viable regions (range, 0–4% in five treated tumors; Fig. 2C). The extent of TUNEL/necrosis is perhaps consistent with the tumor regression observed at day 10 of treatment in the growth delay analyses (Fig. 1A).

### Increasing the Dose of AZD2171 Controls Tumor Growth During Radiotherapy and Induces Significant Tumor Regression Postradiotherapy

The studies using 3 mg/kg/d of AZD2171 suggested that concomitant AZD2171 and radiation (5 × 2 Gy) produced a greater antivascular effect than either agent alone. However, this was not sufficient to significantly alter the tumor growth characteristics *during* the course of radiotherapy (i.e., within days 1–5). Additional studies in Calu-6 and LoVo tumor xenografts were undertaken to determine whether increasing the dose of AZD2171 (to 6 mg/kg/d) would modify the therapeutic response further, and to examine the rate of tumor regrowth following AZD2171 removal. Mice were dosed daily for a total of 28 days after which tumor regrowth was monitored until RTV4 was achieved. Combining AZD2171 with radiotherapy again improved response compared with radiotherapy alone (Fig. 4A). During the course of radiotherapy, concomitant 6 mg/kg/d AZD2171 significantly restrained tumor growth compared with radiotherapy alone in both models. In Calu-6 tumors, this equated to a 94% reduction in the change in tumor volume compared with that observed

**Table 2. Combining 28 d of AZD2171 (6 mg/kg/d) treatment with fractionated radiotherapy**

| Treatment | RTV4 (d) | DT (d) | DT after drug removal | Nadir volume, mm$^3$ (d) |
|---|---|---|---|---|
| Calu-6 | | | | |
| Vehicle | 11 ± 0.5 | 5 ± 0.2 | | |
| AZD2171* | 36 ± 2 | 17 ± 1 | | |
| IR | 32 ± 2 | 8 ± 0.5 | | 311 ± 18 (12) |
| IR + AZD2171 sequential | 50 ± 4* | NA | 7 ± 0.4 | 191 ± 23 (20)† |
| IR + AZD2171 concomitant | 48 ± 3* | NA | 7 ± 0.3 | 161 ± 7 (17)† |
| LoVo | | | | |
| Vehicle | 12 ± 1 | 6 ± 0.7 | | |
| AZD2171* | 35 ± 3 | 16 ± 2 | | |
| IR | 31 ± 1 | 9 ± 0.5 | | 275 ± 36 (10) |
| IR + AZD2171 sequential | 47 ± 2† | NA | 9 ± 1 | 204 ± 36 (13)* |
| IR + AZD2171 concomitant | 44 ± 1† | NA | 9 ± 0.5 | 164 ± 34 (12)* |

NOTE: Nadir volume, lowest recorded tumor volume after the initiation of treatment.

Abbreviations: IR, ionizing radiation; DT, tumor volume doubling time; NA, not applicable (insufficient tumor regrowth during treatment period with AZD2171 to allow an accurate determination of doubling time).

*$P$ < 0.05 compared with radiation alone.

†$P$ < 0.001 compared with radiation alone, Mann-Whitney $U$ test. Values shown are the mean ± SE.

during the 5-day course of radiotherapy alone (volume changes, day 5 versus day 1 of treatment: radiotherapy alone +125 ± 25 $mm^3$; concomitant treatment +8 ± 7 $mm^3$; $P = 0.003$). For LoVo tumors, concomitant AZD2171 caused regression during the radiation treatment such that the inhibition of growth was 164% versus irradiated tumors (volume change −38 ± 20 $mm^3$ versus +60 ± 21 $mm^3$ for radiotherapy alone; $P = 0.04$). Tumor regression was observed postradiotherapy in both models, which, when compared with the effects of radiation alone, was more marked in Calu-6 tumors. Nadir tumor volumes (i.e., smallest volume following start of treatment) in combination treatment groups were significantly less than those observed with radiation alone in both tumor types (Table 2). Interestingly, in spite of the fact that concomitant AZD2171 seemed to control tumor growth during the course of radiotherapy, the volumes of concomitant and sequentially treated tumors were effectively matched within the first 4 to 8 days postradiotherapy in both tumor models. Upon cessation of AZD2171 treatment, tumor regrowth mimicked that of tumors treated with radiation alone after a short delay of 1 to 2 days (Fig. 4A).

Histologic analysis of Calu-6 tumors excised after concomitant treatment with 5 × 2 Gy and 6 mg/kg/d of AZD2171 revealed total and perfused vessel densities of 8 ± 2 and 5 ± 2 $mm^2$, respectively, and a tumor hypoxic fraction of 23 ± 6%. These data were similar to that observed with the lower dose combination (Fig. 2A and B). However, apoptosis was more prevalent at this dose compared with that observed in the 3 mg/kg/d concomitant group (8 ± 2% versus 5 ± 2%) and four times that observed in vehicle-treated controls (2 ± 2%). The TUNEL-positive regions observed were coincident with hypoxic regions that were distal to perfused vessels (Fig. 4B).

## Discussion

In the present study, the effect of combining radiation and AZD2171, a highly potent, orally active inhibitor of VEGF signaling, was investigated *in vivo*. Chronic administration of AZD2171 caused a dose-dependent reduction of tumor growth, consistent with previous studies (21). When combined with fractionated radiotherapy, a significant increase in growth delay was observed compared with either radiation or AZD2171 alone. This was observed whether AZD2171 was administered concomitantly or sequentially to radiation treatment. Tumor regrowth following combined treatment with AZD2171 and radiation was slowed when compared with tumors treated with either agent alone. In addition, the extent of tumor

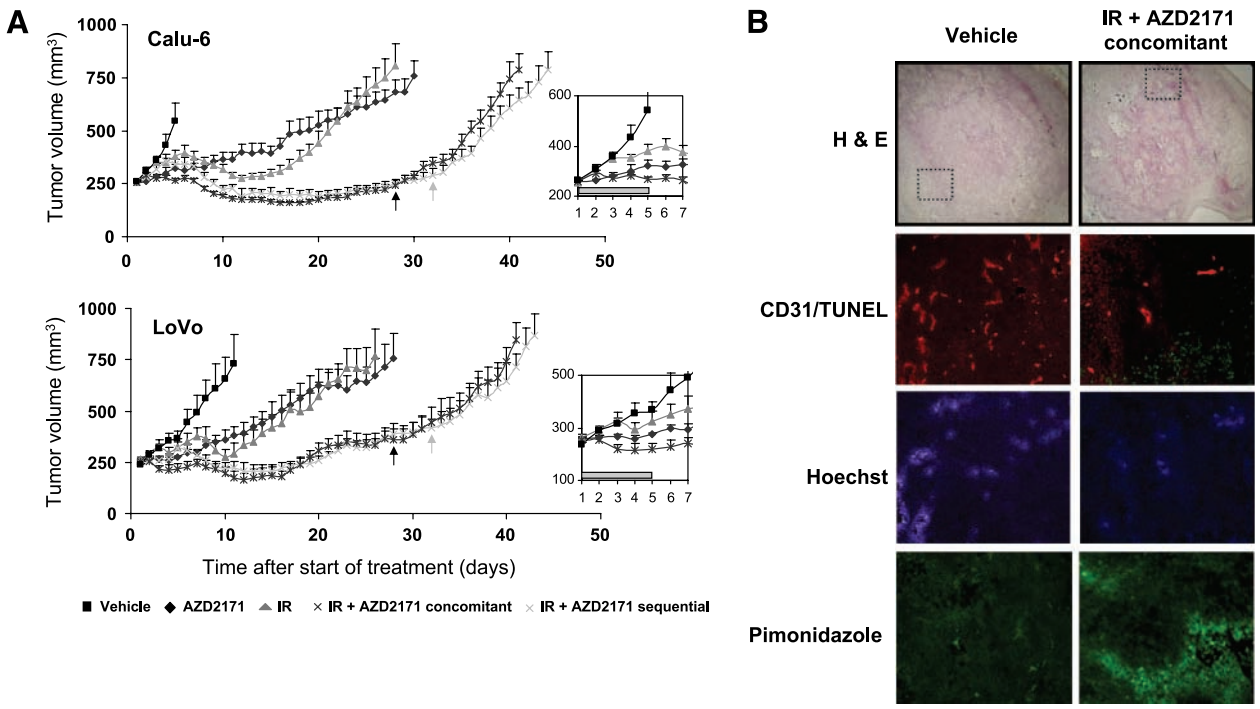


**Figure 4.** Combining 6 mg/kg/d AZD2171 with radiotherapy (5 × 2 Gy) induces significant tumor regression in Calu-6 and LoVo tumor xenografts (**A**). AZD2171 was administered for a total of 28 d (concomitant days 1 – 28 or sequential days 5 – 32). *Black* and *gray arrows,* the cessation of AZD2171 treatment for the concomitant and sequential treatment groups, respectively. Tumor regrowth after removal of the AZD2171 mirrored that of irradiated tumors. *Points,* average values; *bars,* SE ($n = 7$ Calu-6; $n = 5$ LoVo) plotted until the time when the first tumor within the group reached RTV4. *Insets,* expansions of the tumor growth during the course of radiation treatment (given on days 1 – 5; *shaded bars*). **B,** representative images of vessel distribution, perfusion, hypoxia, and apoptosis in a vascular ''hot-spot'' of a vehicle-treated Calu-6 tumor compared with that of a tumor treated with concomitant AZD2171 (6 mg/kg/d) and radiation for 5 d. Overlays of vessels (CD31; *red*) and apoptosis (TUNEL; *green*), or perfusion (Hoechst 33342; *blue*) and hypoxia (pimonidazole binding; *green*). *Boxes,* relative positions of the regions of interest on the H&E-stained tumor sections.

regression postradiotherapy was enhanced by the combined treatment, particularly when a dose of 6 mg/kg/d AZD2171 was used.

Several mechanisms have been postulated by which inhibitors of VEGF signaling could improve the therapeutic effects of radiation. These include a direct enhancement of the radiosensitivity of tumor endothelial cells (2) and a normalization of tumor vasculature leading to a therapeutic window whereby tumor oxygenation is increased (19). Exploitation of both of these effects would require concomitant treatment with an inhibitor of VEGF signaling. Alternatively, a crucial role for VEGF in tumor remodeling and growth postradiotherapy has been suggested from studies demonstrating a positive interaction between radiotherapy and VEGF-targeting when the agent is given as an adjuvant to radiation (7, 8, 20). These data have lead to the suggestion that preirradiated tumor vasculature is more sensitive to inhibition of VEGF signaling (20).

To investigate the underlying biology behind the interaction between AZD2171 and radiotherapy in the present study, vessel density and function, tumor hypoxia, and apoptosis were assessed in tumors treated with five doses of AZD2171 administered concomitantly with or sequentially to 5 × 2 Gy radiotherapy and compared with either agent alone. Potent antivascular effects were observed in tumors treated with radiation or AZD2171 alone (vessel densities 10 ± 2 and 11 ± 2 per $mm^2$, respectively, compared with 21 ± 5 per $mm^2$ in controls; $P < 0.05$, one-sided $t$ test). Concomitant AZD2171 further reduced vessel density (to 8 ± 1 per $mm^2$; $P = 0.01$ versus controls, two-sided $t$ test).

Despite a relatively small change in vessel density, a marked increase in hypoxia was observed in the group receiving concomitant treatment. In contrast, five daily doses of AZD2171 or radiation alone had little or no effect on this variable, although chronic administration of AZD2171 (for 28 days) to Calu-6 tumors has been shown to increase tumor hypoxia significantly (24). In tumors treated with radiotherapy alone, reduced oxygen consumption by tumor cells damaged with radiation treatment could account for an unchanged hypoxic fraction in spite of a marked reduction in vessel density. With AZD2171 treatment alone, the lack of correlation between hypoxia and the antivascular effect observed may have been associated with the improved perfused fraction associated with treatment over this time frame. Although this may be interpreted as a potential normalization of the vessels (25), a more stringent assessment of the oxygenation profile during the treatment course would be required to substantiate this suggestion.

In contrast with these data, a recent study using another VEGFR tyrosine kinase inhibitor (PTK787/ZK222584) in a murine breast tumor model, suggested that the inhibitor alone could induce hypoxia; however, concomitant treatment with radiotherapy ablated this effect (17). The increased hypoxia observed with PTK787/ZK222584 was predominantly associated with a shift in already hypoxic regions to a lower oxygen tension. This would not have been revealed in the current study that focused on changes in hypoxic area. The discrepancy with respect to the combined treatment may reflect differences in the magnitude of VEGF signaling inhibition between AZD2171 versus PTK787/ZK222584. Rapid elimination of endothelial cells (observed with AZD2171) could potentially reduce the effectiveness of radiation, whereas a more protracted effect would allow radiation to first constrain the tumor cell population, reducing oxygen consumption and hypoxia.

Although concomitant radiotherapy and AZD2171 increased the hypoxic fraction, this did not have a detrimental effect on therapeutic outcome, at least when AZD2171 treatment was maintained postradiotherapy as in the current study. This suggests that the hypoxic cells were not all viable, and hence, were unable to contribute to radioresistance. This is supported by the coincidence of TUNEL-positive regions (reflecting cell death by apoptosis and necrosis) and hypoxia revealed by immunofluorescence (Fig. 4B). Concomitant scheduling with 6 mg/kg/d of AZD2171 did have some added benefit in that it produced a greater control of tumor growth during the course of radiotherapy. This, however, did not improve the extent of tumor regression observed postradiotherapy compared with that in the sequentially treated group. Nor did it significantly affect the time at which the nadir tumor volume was achieved, suggesting a more rapid regression rate in the sequential treatment group. This highlights the importance of VEGF-mediated cell signaling in the processes underlying tumor regrowth after radiotherapy. Furthermore, it may suggest that there is a finite vessel population that is sensitive to treatment with AZD2171 and radiotherapy, and that AZD2171 does not have to be present during the course of radiotherapy to control this population. Previous studies have suggested that the preirradiation of tumor endothelial cells enhances the efficacy of VEGFR-2–targeted agents *in vivo* (20). It could be that there is a general sensitization of the vasculature as a whole or that the vessels remaining after radiation treatment were more sensitive to VEGF signaling inhibition. Interestingly, upon removal of chronic AZD2171 therapy, the treated tumors resumed a growth rate comparable with radiation treatment alone (Table 2; Fig. 4A), suggesting that during the dosing period, AZD2171 constrains tumor growth through a mechanism independent of the effects of radiation alone.

The data presented here are consistent with a more potent antitumor effect of AZD2171 after radiotherapy than when administered to previously untreated tumors. When five doses of AZD2171 were administered immediately after radiotherapy, the distribution of the remaining vessels was localized to a thin band of aerobic viable tissue surrounding large regions of TUNEL-stained apoptosis/necrosis. The extent of tumor cell loss was consistent with the tumor regression observed in combined treatment groups. Following the regression period, tumor regrowth was slowed compared to that observed with AZD2171 alone. These data are consistent with the observations of Zips et al. (20), although they noted that the effect of

PTK787/ZK222584 was reduced as tumor volumes increased. This apparent acquired resistance to VEGFR inhibition, attributed to a reduced reliance on VEGFR-mediated angiogenesis in larger tumors, was not observed in the present study.

In summary, the data presented support the use of the VEGFR signaling inhibitor AZD2171 in combination with radiotherapy. Both sequential and concomitant treatment schedules produced an enhanced response versus radiation alone, and similar effects in terms of overall growth delay. Although this suggests that having AZD2171 present during the course of radiotherapy does not provide further benefit to commencing AZD2171 treatment postradiotherapy, the ability of concomitant AZD2171 to reduce tumor growth during the course of irradiation may be beneficial in some settings.

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