

ZD6474, a Potent Inhibitor of Vascular Endothelial Growth Factor Signaling, Combined With Radiotherapy: Schedule-Dependent Enhancement of Antitumor Activity

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ABSTRACT

Purpose: Vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis and acts as a radiation survival factor for endothelial cells. ZD6474 (*N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine) is a potent VEGF receptor 2 (KDR) tyrosine kinase inhibitor (TKI) that has additional activity *versus* the epidermal growth factor receptor. This study was designed to determine the efficacy of combining ZD6474 and radiotherapy *in vivo*.

Experimental Design: The Calu-6 (non-small-cell lung cancer) tumor model was selected because it was found to be unresponsive to treatment with a selective epidermal growth factor receptor TKI but responds significantly to treatment with selective VEGF receptor TKIs. Tumor-bearing mice received either vehicle or ZD6474 (50 mg/kg, by mouth, once daily) for the duration of the experiment, with or without radiotherapy (3 × 2 Gy, days 1–3). Two combination schedules were examined: (a) ZD6474 given before each dose of radiation (concurrent schedule); and (b) ZD6474 given 30 minutes after the last dose of radiotherapy (sequential schedule).

Results: The growth delay induced using the concurrent schedule was greater than that induced by ZD6474 or radiation treatment alone (22 ± 1 *versus* 9 ± 1 and 17 ± 2 days, respectively; *P* = 0.03 *versus* radiation alone). When administered sequentially, the growth delay was markedly enhanced (36 ± 1 days; *P* < 0.001 *versus* radiation alone or the concurrent schedule). Intravenous administration of Hoechst 33342 showed a trend toward reduced tumor perfusion

after ZD6474 treatment, and a pairwise comparison (*versus* control) was significant after three doses of ZD6474 (*P* = 0.05 by one-tailed *t* test). Thus, impaired reoxygenation between fractions in the concurrent protocol may be the causal basis for the schedule dependency of the radiopotential observed.

Conclusions: ZD6474 may be a successful adjuvant to clinical radiotherapy, and scheduling of the treatments could be important to ensure optimal efficacy.

INTRODUCTION

Vascular endothelial growth factor A (VEGF-A) has a pivotal role in pathological angiogenesis including sustained neovascularization that is required for all solid tumor growth. The signaling response is transmitted via the tyrosine kinase activity of the VEGF family of transmembrane receptors. Tyrosine kinase activation is stimulated after VEGF ligand binding and receptor dimerization. VEGF is a potent stimulator of endothelial cell proliferation, migration, and survival. In addition VEGF acts as an important endothelial survival factor in newly formed vessels and stimulates vessel hyperpermeability, which may contribute to the high interstitial pressure commonly observed in solid tumors.

The critical importance of VEGF in the growth of experimental tumors has been demonstrated when stasis or regression was observed after treatment with neutralizing antibodies (1–3) or soluble forms of VEGF receptors (VEGFRs) that effectively sequester the protein (4). Consequently, the development of clinically applicable inhibitors of VEGF signaling has been an area of avid research (5). These can target either the VEGF protein directly or inhibit the activation of the cognate receptors. Of the three major receptors for VEGF family ligands [Flt-1 (VEGFR1), KDR (Flk-1; VEGFR2), and Flt-4 (VEGFR3)], activation of KDR alone is sufficient to promote all of the phenotypic characteristics associated with VEGF-mediated signaling (6–8).

In addition to the effect of VEGF on tumor angiogenesis and growth, VEGF can also play an important role in the response of tumors to radiotherapy. This appears to be predominantly achieved through the ability of VEGF to enhance endothelial cell survival (9–11) recently suggested to be the critical factor determining tumor radiation response (12). These observations raise the possibility of combined therapeutic strategies, although there are complexities surrounding the application of antiangiogenic agents in a radiotherapy context. In particular, inhibition of VEGF signaling has the potential to impact on tumor oxygenation and proliferation kinetics, which could have profound effects on the response to radiation.

ZD6474 is a potent (IC₅₀, 40 nmol/L), orally active, low molecular weight inhibitor of KDR tyrosine kinase activity (13) that is currently undergoing clinical evaluation (14). ZD6474

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also has additional activity *versus* the epidermal growth factor receptor (EGFR) tyrosine kinase (IC_{50} , 500 nmol/L; ref. 15). Chronic oral dosing of mice bearing human tumor xenografts of diverse tissue origin with ZD6474 has been previously shown to induce a dose-dependent inhibition of tumor growth (16). The present study was designed to determine the efficacy of a combination of ZD6474 and radiotherapy in a tumor model *in vivo*.

The Calu-6 (human non-small-cell lung carcinoma) tumor model was selected for these studies because when Calu-6 tumors were grown as xenografts in nude mice, these tumors were found to be unresponsive to treatment with a selective EGFR tyrosine kinase inhibitor (TKI) but have been previously shown to be sensitive to treatment with a VEGFR TKI that has no activity *versus* EGFR (17). Use of Calu-6 would therefore be expected to avoid any radiopotentiative contribution from inhibition of EGFR signaling by ZD6474, which has been observed with the selective EGFR TKI gefitinib (Iressa) in tumors that are growth responsive to EGFR tyrosine kinase inhibition (18–20).

The aim of this study was to evaluate the effects of VEGF signaling inhibition on the response to radiotherapy, to determine whether scheduling of ZD6474 affected antitumor efficacy, and to investigate whether the effects of the treatments were related to changes in tumor perfusion and vascularity.

MATERIALS AND METHODS

Cell Line Details. Calu-6 human non-small-cell lung carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). All cell culture reagents were obtained from Invitrogen (Paisley, UK). Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 2 mmol/L glutamine and routinely screened for the presence of *Mycoplasma* (Mycotect assay; Invitrogen).

Initiation of Calu-6 Tumors. Calu-6 cells were harvested in exponential phase growth and prepared at a concentration of 2×10^7 cells/mL in a 1:1 mix of serum-free RPMI 1640 and Matrigel (phenol red-free; BD Biosciences, Oxford, UK). Tumor xenografts were initiated by the intradermal injection of a 0.1-mL volume of the prepared cell stock on the midline of the back of 8- to 10-week-old female athymic (nu/nu) Swiss mice bred at Alderley Park (Macclesfield, United Kingdom). Tumor implantations and all experimental procedures were carried out at the University of Manchester, where mice were maintained in negative pressure isolators and provided with sterilized food and water *ad libitum*. All procedures were carried out following institutional guidelines that were in accordance with the Scientific Procedures Act 1986 (project license numbers 40/1770 and 40/2328) and in line with the United Kingdom Coordinating Committee on Cancer Research Guidelines 1999.

Drug and/or Radiation Treatment Schedules. Mice ($n = 8$ mice per group) bearing Calu-6 tumors with a volume of 110 to 240 mm³ were randomly assigned to receive gefitinib (100 mg/kg) or vehicle (0.5% polysorbate in deionized water). Treatment was administered once daily by oral gavage for the duration of the experiment. For the ZD6474 and radiation studies, mice bearing established tumors of 220 to 300 mm³ were randomized into groups of eight to receive either vehicle (1%

polysorbate in deionized water) or ZD6474 (25 or 50 mg/kg) by mouth once daily at 0.1 mL/10 g body weight for the duration of the experiment. ZD6474 or vehicle was administered with or without radiotherapy [3×2 Gy or 5×2 Gy (vehicle only), at 24-h intervals]. Localized radiotherapy was administered at a dose rate of 2 Gy/min on unanesthetized mice that were restrained in lead-shielded holders (21). The experimental end point was taken as the time for the Calu-6 tumor to reach a relative tumor volume of $4 \times$ that at the initiation of therapy (RTV₄). Where mice received 50 mg/kg ZD6474, two combined treatment schedules were examined: (a) ZD6474 dosing given 2 hours before the first dose of radiation (concurrent schedule); and (b) ZD6474 dosing given 30 minutes after the last dose of radiotherapy (sequential schedule). Combined modality treatment with radiation and 25 mg/kg ZD6474 was assessed only with sequential scheduling.

Evaluation of the Effect of Radiotherapy on Vascular Endothelial Growth Factor Protein Levels. Calu-6 xenografts were exposed to 3×2 Gy fractions according to the schedule given above or exposed to a single dose of 5 Gy. Tumors were excised 72 hours after the completion of the radiotherapy treatment. The average sizes of the tumors on excision were $1,062 \pm 30$, 918 ± 33 , and 836 ± 18 mm³ for control, 3×2 Gy-treated tumors, and 5 Gy-treated tumors, respectively. Tumor samples were freeze-dried and then lysed in ice-cold modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4) containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, and aprotinin, leupeptin, and pepstatin at 1 μg mL⁻¹ each]. To remove debris, samples were centrifuged at 3,200 rpm for 10 minutes at 4°C, and supernatants were removed and centrifuged at 13,000 rpm for 20 minutes at 4°C. Protein concentration of the final cleared samples was determined by Pierce assay. Human and mouse VEGF were determined by enzyme-linked immunosorbent assay using Quantikine Human and Murine VEGF Immunoassays (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's recommended protocols.

Influence of ZD6474 on Vascular Perfusion. For the perfusion studies, tumor-bearing mice were given either a single dose or three doses of ZD6474 (50 mg/kg) at 24-hour intervals. Two hours after the final dose, 0.1 mL of Hoechst 33342 (6 mg/mL in PBS) was administered via tail vein injection, and the mice were humanely killed 1 minute later. The tumors were rapidly excised and snap frozen. Cryostat sections were prepared at 5-μm thickness. Using fluorescent microscopy, the number of Hoechst-stained (perfused) vessels was determined for each section. Sections were then fixed in ice-cold acetone, and nonspecific antibody binding sites were blocked using 10% horse serum in PBS containing 0.1% Tween 20 (PBST). Rat antimouse CD31 (PharMingen, BD Biosciences) was applied at a concentration of 1:250 in PBST supplemented with 0.1% bovine serum albumin and left overnight at 4°C. Primary antibody binding was disclosed using a tetramethylrhodamine isothiocyanate-labeled goat antirat antibody (1:150 in PBST supplemented with 0.1% bovine serum albumin; Molecular Probes, Invitrogen). The total number of vessels and the proportion of the section area staining positively with the CD31 antibody was

then determined for each section and related to the total section area.

Statistical Analyses. Log-rank P analysis was used to evaluate the statistical significance of the growth delay data obtained. The interaction between radiation and ZD6474 when using concurrent *versus* sequential scheduling was explored using a two-way analysis of variance (ANOVA) with two between-group factors. A one-tailed t test, assuming unequal variance, was used to examine differences in tumor perfusion after 1 day or 3 days of ZD6474 treatment. A Jonckheere-Terpstra one-sided nonparametric test for ordered alternatives was also used to examine the trend toward reduced tumor perfusion with ZD6474 treatment. Two-tailed t tests assuming unequal variance were applied to all other data sets. P values of ≤ 0.05 were considered significant, and data cited within the text are mean values \pm SE.

RESULTS

Calu-6 Tumor Xenografts Are Unresponsive to Chronic Oral Dosing with the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Gefitinib. Mice bearing Calu-6 tumors were randomized to receive once-daily treatment with gefitinib (100 mg/kg by mouth) or polysorbate vehicle. Gefitinib had no effect on the subsequent growth of the tumors (Fig. 1). In responsive tumor models (including those derived from non-small-cell lung cancer), substantial growth delays are achieved using markedly lower doses of gefitinib (22).

ZD6474-Mediated Tumor Growth Inhibition. Chronic oral dosing of tumor-bearing mice with ZD6474 has been previously shown to produce a dose-dependent inhibition of Calu-6 xenograft growth (16). In the present study, Calu-6 xenografts exhibited an aggressive growth profile. The time taken for

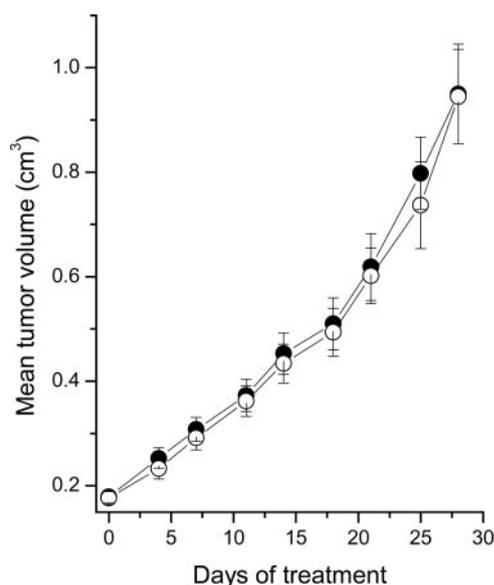


Fig. 1 Calu-6 tumor xenografts are unresponsive to the EGFR TKI gefitinib. Gefitinib (100 mg/kg; \circ) or 0.5% polysorbate vehicle (\bullet) was administered by mouth once daily to mice with established tumors. Data presented are mean values ($n = 8$ per group); error bars indicate SE.

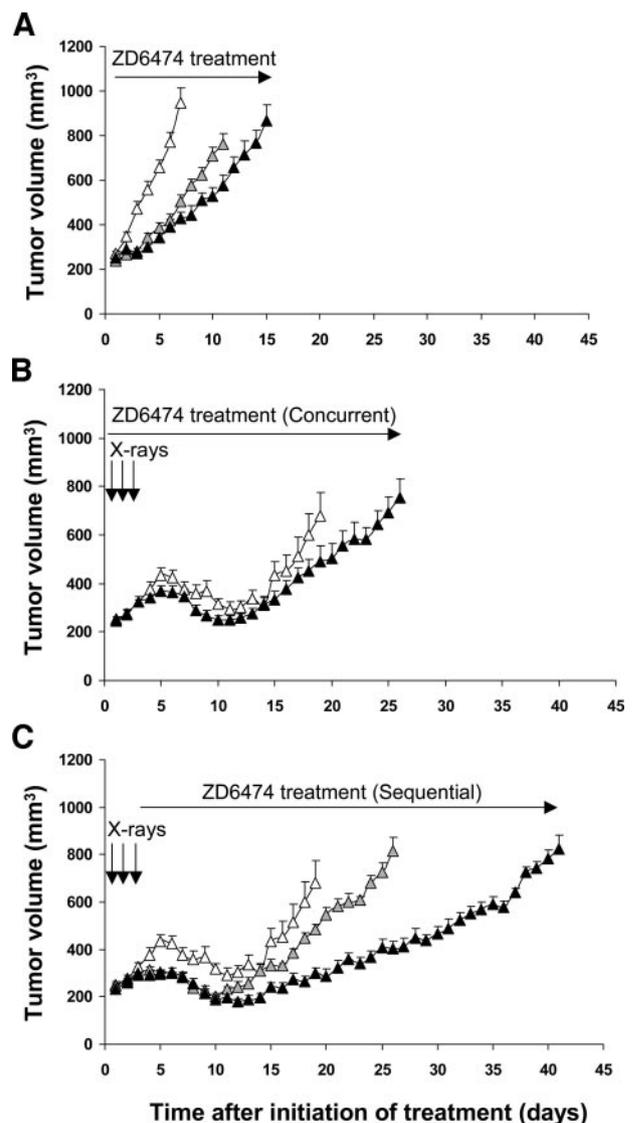


Fig. 2 A. ZD6474 exerts a dose-dependent inhibitory effect on the growth of Calu-6 tumor xenografts *in vivo*. B. Concurrent administration of ZD6474 two hours before each fraction of radiotherapy yields a greater growth delay than that induced by radiotherapy or ZD6474 alone. C. Sequential treatment with ZD6474 after radiotherapy increases the efficacy of the combined treatment. \triangle , tumors treated with vehicle (1% polysorbate in deionized water); \bullet , tumors treated with 25 mg/kg ZD6474; \blacktriangle , tumors treated with 50 mg/kg ZD6474 (each administered daily by mouth for the duration of the experiment). Radiation treatment was given as three 2-Gy fractions administered at 24-hour intervals, as indicated by the arrows. Data presented are mean values ($n = 8$ per group); error bars indicate SE.

control tumors treated with 1% polysorbate vehicle to achieve a relative tumor volume equivalent to $4\times$ that at the start of treatment (RTV_4) was 8 ± 0.5 days compared with approximately 18 days in the previous study (16). However, the ability of ZD6474 treatment to induce a highly significant dose-dependent inhibition of Calu-6 xenograft growth was recapitulated in the present study (Fig. 2A; Table 1). The tumor doubling time increased from 4 ± 0.3 days (vehicle) to 6 ± 0.3 and 8 ± 0.6

Table 1 Influence of ZD6474 and/or radiotherapy (Rx) on Calu-6 tumor growth

Treatment		Tumor volume doubling time (days ± SE) *	RTV ₄ (days ± SE)	Growth delay (days ± SE)
Rx	ZD6474			
—	Vehicle	4 ± 0.3	8 ± 0.5	NA
—	25 mg/kg	6 ± 0.3	13 ± 0.4	5 ± 0.6
—	50 mg/kg	8 ± 0.6	17 ± 1.0	9 ± 1.1
3 × 2 Gy	Vehicle	6 ± 0.6	25 ± 1.7	17 ± 1.8
3 × 2 Gy	50 mg/kg concurrent	10 ± 0.5	30 ± 1.0	22 ± 1.1
3 × 2 Gy	25 mg/kg sequential	10 ± 0.5	28 ± 0.8 †	20 ± 0.9
3 × 2 Gy	50 mg/kg sequential	13 ± 1	44 ± 0.9	36 ± 1.0
5 × 2 Gy	Vehicle	9 ± 0.6	46 ± 4.0 †	38 ± 4.0

* Doubling times were calculated from the linear growth phase of each individual tumor within the treatment group.

† Based upon $n = 7$; one tumor per group did not achieve RTV₄ within 100 days after treatment.

days for tumors treated with 25 or 50 mg/kg/d ZD6474, respectively. Furthermore, the percentage of growth inhibition, calculated as the difference between the change in growth in vehicle- and ZD6474-treated tumors up to the point of excision of the first vehicle-treated tumor (day 7), was highly consistent, yielding values of 61% and 73% (with 25 and 50 mg/kg/d, respectively). Previously published values of 56% and 78% have been reported in Calu-6 tumor-bearing mice treated with 25 and 50 mg/kg ZD6474 for 28 days (16).

Concurrent ZD6474 Produces an Enhancement of Fractionated Radiotherapy. The potential of ZD6474 to enhance the outcome of radiotherapy in Calu-6 xenografts was first investigated in a concurrent schedule whereby 50 mg/kg ZD6474 was given by mouth 2 hours before each 2-Gy radiation fraction for a period of 3 days and thereafter on a daily basis. Tumor doubling time in the combined treatment group was greater than that observed in mice treated with 50 mg/kg/d ZD6474 or radiotherapy alone (Fig. 2B; Table 1). The time to achieve a relative tumor volume 4× that at the start of treatment (RTV₄) in the combined treatment group was significantly greater ($P = 0.03$) than that seen in tumors treated only with radiotherapy (Figs. 3 and 4; Table 1). Analysis of these data using two-way ANOVA with two between-group factors indicated that there was no apparent interaction between ZD6474 and radiotherapy ($P = 0.29$) when combined concurrently.

The Impact of ZD6474 Treatment on Radiotherapy Outcome Is Markedly Enhanced using a Sequential Protocol. Initiation of 50 mg/kg ZD6474 treatment after completion of the radiotherapy schedule produced a highly significant increase in both the subsequent doubling time of the treated tumors ($P = 0.005$ versus 50 mg/kg ZD6474 alone) and the time to RTV₄ compared with radiation or ZD6474 alone (Figs. 2C, 3, and 4). The growth delay induced by the combined treatment was greater than the sum of the growth delays induced by the two treatment modalities in isolation (36 days versus 26 days) and mimicked that induced by 5 × 2 Gy of radiotherapy (Fig. 4; Table 1). Using this sequential scheduling, a highly significant interaction was revealed between ZD6474 and radiation when

assessed by a two-way ANOVA [$F_{1,28}(0.05) = 23.60$; $P < 0.0001$]. The combination was also well tolerated. Sequential scheduling of radiation, followed by 50 mg/kg ZD6474 for 15 days, induced a mean body weight loss of 6.5%. This was not significantly different from that induced by 15 days of treatment with 50 mg/kg ZD6474 alone ($P > 0.8$). After continued ZD6474 therapy for 38 days after irradiation, the mean body weight loss was unchanged compared with that measured after 15 days of treatment with drug.

Halving the dose of ZD6474 used to 25 mg/kg in the sequential protocol produced a similar response to that seen with the 50 mg/kg dose given concurrently (Figs. 2C and 3; Table 1). Interestingly, one tumor within this treatment group did not achieve a RTV₄ within 100 days of treatment (Fig. 3). Tumor volume doubling time was increased significantly compared with either ZD6474 treatment alone ($P < 0.001$) or radiation alone ($P < 0.005$). However, sequential ZD6474 treatment using a 25 mg/kg dose did not significantly increase the time to RTV₄ compared with radiation alone ($P = 0.353$).

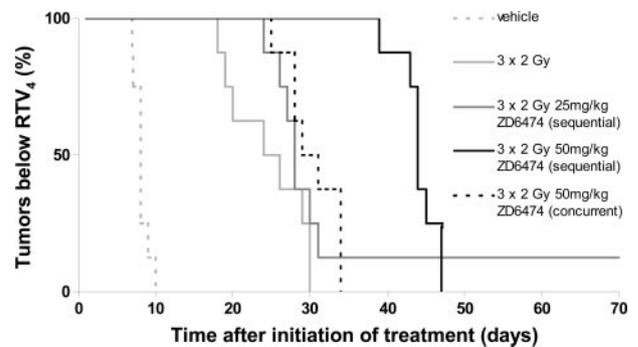


Fig. 3 Diagrammatic representation of the influence of combining ZD6474 with radiation treatment on the time taken for each individual tumor within the treatment groups to achieve RTV₄.

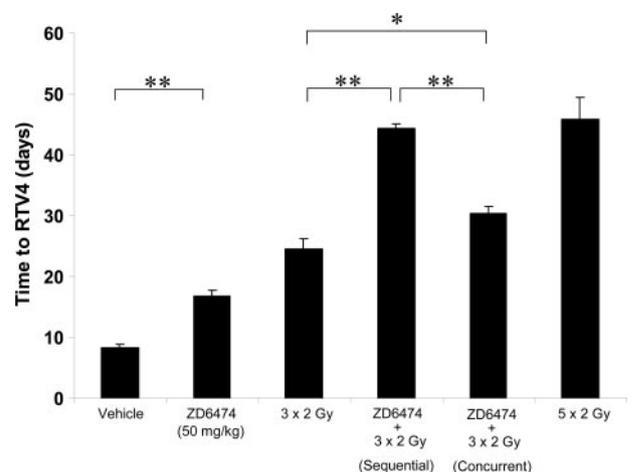


Fig. 4 Influence of ZD6474 (50 mg/kg, daily, by mouth) and/or radiation treatment on time to achieve RTV₄. Data shown are mean values (±SE) with $n = 8$ per group (except for 5 × 2 Gy radiotherapy, where $n = 7$ per group because one tumor did not achieve RTV₄ 100 days after initiation of treatment. *, $P = 0.03$; **, $P < 0.001$ (log-rank P test).

The Effect of Radiation Treatment on Vascular Endothelial Growth Factor Protein Levels in Calu-6 Tumor Xenografts. Human tumor-derived VEGF after the fractionated radiation protocol (202 ± 28 pg mg⁻¹ protein; $n = 5$) was not significantly different from that obtained in control tumors (214 ± 18 pg mg⁻¹ protein; $n = 4$). In contrast, the single dose of 5 Gy induced a significant increase in tumor VEGF [314 ± 29 pg mg⁻¹ protein; $n = 4$ ($P = 0.04$)]. Interestingly, the 3×2 Gy treatment schedule did result in an almost 3-fold increase in host-derived murine VEGF levels (11 ± 4 versus 27 ± 6 pg mg⁻¹ protein), although the induction did not achieve statistical significance ($P = 0.07$).

ZD6474 Treatment Is Associated with Decreased Tumor Vessel Perfusion. The number of Hoechst-labeled perfused vessels was evaluated 2 hours after one or three doses of

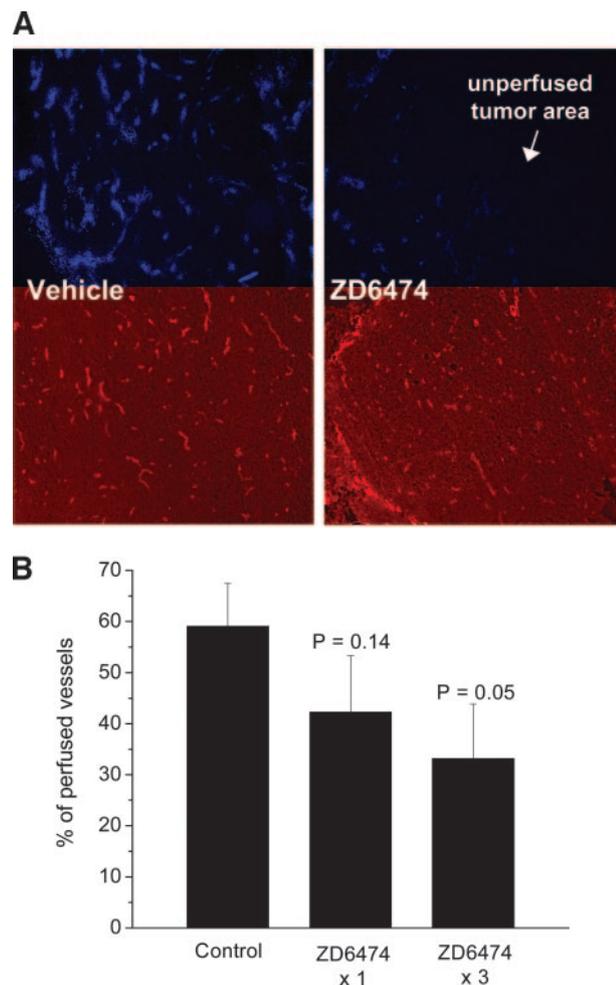


Fig. 5 ZD6474 induces a significant trend toward reduced tumor perfusion with increasing period of dosing. **A**, Hoechst staining of perfused vessels in a tumor treated with three daily fractions of polysorbate vehicle or ZD6474 (50 mg/kg/d). Perfused vessels are only apparent in the periphery of the ZD6474-treated tumor. *Bottom panels* show CD31 staining, revealing that the vessel distribution is identical in the two tumors. **B**, quantification of perfused vessels in tumor. Data presented are average values (\pm SE; $n = 6$ –10 per group). Treatment with ZD6474 (50 mg/kg/d) produced a significant reduction in mean tumor perfusion ($P = 0.05$ by one-tailed t test).

Table 2 Influence of ZD6474 on vessel density and perfusion

Treatment *	Vessel density per mm ² (\pm SE)	Perfused vessels (%) (\pm SE)
Vehicle \times 1	23 \pm 2	57 \pm 14
ZD6474 \times 1	19 \pm 2	42 \pm 12
Vehicle \times 3	22 \pm 4	61 \pm 11
ZD6474 \times 3	20 \pm 1	33 \pm 12

* ZD6474 (50 mg/kg) or vehicle was administered either once or three times with a 24-hour interval between doses.

ZD6474 (50 mg/kg) administered at 24-hour intervals. This was related to the total number of vessels disclosed by CD31 staining (Fig. 5A). To analyze the influence of ZD6474 on tumor perfusion, control groups from 1-day and 3-day vehicle treatment were pooled because these were not significantly different (Table 2: $P > 0.8$ by two-tailed t test), indicating that vehicle treatment did not affect perfusion. The percentage of perfused vessels reduced in a stepwise fashion, dependent on the number of doses of ZD6474 given (Fig. 5B), and reached statistical significance after 3 days of ZD6474 administration ($P = 0.05$, one-tailed t test). A Jonckheere-Terpstra nonparametric test confirmed a trend toward greater reductions in perfusion with increased duration of ZD6474 treatment ($P = 0.03$). ZD6474 treatment did not significantly affect the total number of vessels detected per unit area of tumor over the time periods investigated (Table 2). However, analysis of the extent of CD31-positive staining did reveal a 38% reduction in vessel area after three administrations of ZD6474 compared with vehicle-treated controls (positive area, $2.9 \pm 0.3\%$ versus $1.8 \pm 0.1\%$) that approached statistical significance ($P = 0.06$). No significant change in vessel area was apparent after a single dose of ZD6474.

DISCUSSION

The purpose of the present study was to evaluate the influence of VEGF signaling inhibition on radiation response in human tumor xenografts by examining radiotherapy combination regimens with the KDR tyrosine kinase inhibitor ZD6474. Although ZD6474 is also known to have additional activity versus EGFR tyrosine kinase, this tumor xenograft was selected to study KDR inhibition because it was found to be unresponsive to treatment with the selective EGFR tyrosine kinase inhibitor gefitinib (100 mg/kg/d, by mouth). The mechanism of Calu-6 resistance to EGFR inhibitors is likely to be attributable to signaling responses that are independent of EGFR status because sequencing of the EGFR cytoplasmic domain from these cells has confirmed it to be wild-type, and gefitinib treatment has been found to efficiently block EGFR activation in Calu-6 tumor cells (data not shown).

Consistent with previous observations (16) and inhibition of KDR, chronic administration of ZD6474 produced a dose-dependent reduction in the growth rate of Calu-6 tumor xenografts. When combined with 3×2 Gy radiation treatment, 50 mg/kg/d ZD6474 resulted in a significantly increased growth delay compared with radiotherapy alone. However, scheduling of ZD6474 relative to radiotherapy had a profound effect on the enhancement. Sequential, chronic administration of ZD6474

after the radiation treatment significantly enhanced growth delay. Subsequent analyses revealed a significant interaction between the two modalities, but with the radiopotentiating influence of ZD6474 being dependent on the dose of ZD6474 administered. Sequential combination treatment with 50 mg/kg/d ZD6474 proved more efficacious than would have been predicted from simply adding the RTV₄ responses generated by ZD6474 (50 mg/kg/d) and radiation alone. In contrast, the response after concurrent treatment with ZD6474 and radiotherapy revealed no significant interaction between the two modalities.

The positive interaction observed when ZD6474 is used as an adjuvant to radiotherapy supports an important role for VEGF-mediated signaling in the tumor response to radiotherapy *in vivo*. Previous studies have indicated that VEGF is induced in tumors after irradiation (9). *In vitro* studies have implied a role for the mitogen-activated protein kinase pathway in this response (23). An additional *in vivo* mechanism could be a consequence of increased tumor hypoxia, which can occur transiently after radiation treatment. This would lead to elevated VEGF gene expression via the transcription factor hypoxia-inducible factor-1 (24). Enhanced VEGF levels after radiotherapy would be anticipated to play a role in overall radiation response by stimulating the neovascularization required to support tumor remodeling and subsequent regrowth after a radiation insult. The fractionated dose protocol used in the present study (3 × 2 Gy at 24-hour intervals) was insufficient to induce tumor-derived VEGF. However, some induction of host-derived VEGF was detected. Despite being unable to detect an increase in tumor VEGF levels after irradiation, abrogation of VEGF-dependent survival signaling in endothelial cells may be the mechanistic basis for the enhanced effect of ZD6474 and radiotherapy in the sequential schedule. This conclusion is supported by *in vitro* studies that report radiosensitization of endothelial cells as a consequence of treatment with specific or broad spectrum inhibitors of VEGF signaling (9–11, 25).

The radiopotentiating effect of ZD6474 was reduced when ZD6474 was administered before each fraction of radiation. Studies undertaken in an attempt to define the basis of this observation suggested that, consistent with an inhibition of VEGF signaling, ZD6474 may inhibit tumor perfusion. After 3 days of ZD6474 administration (50 mg/kg/d), the proportion of perfused tumor vessels was less than that in vehicle-treated controls ($P = 0.05$), and a reduction in CD31-positive vessel area approached statistical significance ($P = 0.06$). These observations are consistent with previous reports of reduced tumor vascular permeability/perfusion after acute ZD6474 treatment (assessed by dynamic contrast-enhanced magnetic resonance imaging; ref. 26) and reduced CD31 staining in Calu-6 xenografts after chronic long-term (24-day) exposure to ZD6474 (16).

One possible consequence of reduced perfusion could be an increase in hypoxic fraction within the treated tumors that could potentially impact on tumor response to fractionated radiotherapy. It has been shown that pretreatment with VEGF targeting antibodies causes sensitization to treatment with single doses of radiation (20, 30, or 40 Gy), irrespective of tumor oxygenation status at the time of radiotherapy (27). However, in the context of clinically relevant fractionated 2 Gy treatments, as

examined here, the impact of ZD6474 on reoxygenation between fractions may be important. Studies in murine tumor models have demonstrated that a 2-Gy radiation dose can induce transient increases in tumor blood flow using power Doppler sonography (10). The reduced perfusion observed as a consequence of ZD6474 administration may inhibit this effect, thereby limiting the potential for reoxygenation and the efficacy of the fractionated radiotherapy used.

Although a number of studies have investigated the interplay between antiangiogenic treatment and radiation response, very few have considered the precise scheduling required for optimal radiation enhancement using clinically relevant fractionated protocols. The study of Ning *et al.* (28) investigated the influence of both SU5416 (a specific KDR TKI; ref. 29) and SU6668 on outcome of fractionated radiotherapy in a murine squamous cell carcinoma model, SCCVII (27). Drugs were administered either 30 minutes before or 30 minutes after each 2-Gy fraction of radiation given daily for 5 days. In the case of SU5416, radioenhancement was greater when the drug was administered after each radiation dose, whereas with SU6668, the growth delay was identical for both protocols (28). However, neither drug was evaluated by a regimen involving sequential treatment. Recently, PTK787/ZK222584, a potent inhibitor of KDR and VEGFR1 (30), was observed as having no beneficial effect on radiotherapy outcome if given before or during the course of fractionated radiation (31). Consistent with the results reported here, sequential treatment yielded a marked improvement on radiotherapy outcome in the squamous cell carcinoma models used (31). In contrast with the present study, PTK787/ZK222584 was only administered on the days of radiation treatment in the concurrent schedule and not continued after radiotherapy. Interestingly, concomitant scheduling of PTK787/ZK222584 has been previously reported to enhance radiotherapy outcome in SW480 colorectal xenografts (11), suggesting that model-dependent effects, in addition to differences in selectivity and/or pharmacokinetic profile of the specific agents used, may also need to be taken into account.

Collectively, these data clearly demonstrate the complexities with combined strategies using radiation and inhibition of VEGF signaling and reveal that scheduling could play an important role determining optimal radiotherapeutic benefit. The present study focused on the use of Calu-6 xenografts and specifically demonstrates that inhibition of VEGFR tyrosine kinase activity by ZD6474 has the potential to enhance the effect of radiotherapy. The reduced efficacy of concurrent scheduling may not be as prevalent in other tumor types that are responsive to inhibition of both VEGFR and EGFR tyrosine kinase activity because EGFR inhibition may prevent repopulation between fractions and confer a benefit to radiation response (18). However, in the absence of definitive data assessing the relative importance of these two opposing effectors of radiation response, the present study would support the development of ZD6474 as an adjuvant to radiotherapy in the clinical setting.

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