Antitumor activity of ZD6126, a novel vascular-targeting agent, is enhanced when combined with ZD1839, an epidermal growth factor receptor tyrosine kinase inhibitor, and potentiates the effects of radiation in a human non-small cell lung cancer xenograft model

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Abstract

Objective: Targeting the tumor vasculature may offer an alternative or complementary therapeutic approach to targeting growth factor signaling in lung cancer. The aim of these studies was to evaluate the antitumor effects *in vivo* of the combination of ZD6126, a tumor-selective vascular-targeting agent; ZD1839 (gefitinib, Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor; and ionizing radiation in the treatment of non-small cell lung cancer xenograft model. Methods: Athymic nude mice with established flank A549 human non-small cell lung cancer xenograft model xenografts were treated with fractionated radiation therapy, ZD6126, ZD1839, or combinations of each treatment. ZD6126 (150 mg/kg) was given i.p. the day after each course of radiation. Animals treated with ZD1839 received 100 mg/kg per dose per animal, 5 or 7 days/wk for 2 weeks. Immunohistochemistry was done to evaluate the effects on tumor growth using an anti-Ki67 monoclonal antibody. Effects on tumor-induced vascularization were quantified using an anti–factor VIII–related antigen monoclonal antibody. Results: ZD6126 attenuated the growth of human A549 flank xenografts compared with untreated animals. Marked antitumor effects were observed when animals were treated with a combination of ZD6126 and fractionated radiation therapy with protracted tumor regression. ZD6126 + ZD1839 resulted in a greater tumor growth delay than either agent alone. Similar additive effects were seen with ZD1839 + fractionated radiation. Finally, the addition of ZD6126 to ZD1839 and radiation therapy seemed to further improve tumor growth control, with a significant tumor growth delay compared with animals treated with single agent or with double combinations. Immunohistochemistry showed that ZD1839 induced a marked reduction in A549 tumor cell proliferation. Both ZD1839 and ZD6126 treatment substantially reduced tumor-induced angiogenesis. ZD6126 caused marked vessel destruction through loss of endothelial cells and thrombosis, substantially increasing the level of necrosis seen when combined with radiation therapy. The combination of radiation therapy, ZD6126, and ZD1839 induced the greatest effects on tumor growth and angiogenesis. Conclusion: This first report shows that a selective vascular-targeting agent (ZD6126) + an anti–epidermal growth factor receptor agent (ZD1839) and radiation have additive *in vivo* effects in a human cancer model. Targeting the tumor vasculature offers an excellent strategy to enhance radiation cytotoxicity. Polytargeted therapy with agents that interfere with both growth factor and angiogenic signaling warrants further investigation. [Mol Cancer Ther 2004;3(8):977–83]

Introduction

The survival of a tumor is dependent on the development of an intratumoral vascular network necessary to ensure continued expansion, invasion, and metastasis. Interfering with the vascular network active within a particular tumor represents an attractive alternative for complementary targeting strategies in tumors. ZD6126 is a novel vascular-targeting agent developed to selectively disrupt intratumoral immature vasculature. It is a prodrug of the tubulin-binding agent ZD6126 phenol (*N*-acetylcolchinol) that binds β-tubulin, inhibiting tubulin polymerization and thereby disrupting the microtubular network, which is responsible for maintaining the shape of the immature endothelial cells. In proliferating tumor neoendothelium, these microtubule changes result in a rapid alteration in cell

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shape that leads to tumor vessel occlusion. Within normal vasculature, however, the presence of a supporting actin cytoskeleton in these mature endothelial cells prevents tubulin disruption and conformational changes that occur in the tumor vasculature. Rapid tumor endothelial retraction is seen following ZD6126 treatment, resulting in loss of endothelial cells and exposure of the basal lamina (1). Ensuing fibrin deposition and platelet accumulation is associated with vessel congestion and marked necrosis of surrounding tumor cells. Normal endothelial cells of nontumor tissue are unaffected by ZD6126.

ZD6126 treatment has been shown to induce extensive central necrosis in a range of tumor models (including human xenografts derived from colon, lung, breast, and ovarian tumors), leaving only a thin rim of viable tumor tissue at the periphery (1). This observation is likely to arise from the fact that the outermost tumor cells are able to obtain blood supply from the vessels in the surrounding normal tissues. These proliferating cells may be more sensitive to conventional therapy such as ionizing radiation, rationalizing the therapeutic potential of a combined approach using a vascular-targeting agent such as ZD6126 and radiation therapy. Recent investigations have shown enhancement of radiation therapy in a KHT sarcoma xenograft model with once-weekly administration of ZD6126 (2).

There may be multiple and redundant intracellular pathways cancer cells use to overcome blockade of a particular receptor or protein signal. Thus, it would seem rational to explore ways to block several complementary signaling pathways. In addition to angiogenic processes, the epidermal growth factor signaling pathway is critical to tumor cell proliferation in many tumor types including lung cancer. A direct relationship between epidermal growth factor receptor (EGFR) phosphorylation and cell cycle initiation has been shown (3). Inhibition of downstream signaling with agents that perturb this pathway produces tumor regressions in preclinical models and lung cancer patients. Preclinical data also show that inhibition of the EGFR pathway can potentiate the cytotoxic effects of radiation (4, 5). ZD1839 (gefitinib, Iressa) is an EGFR tyrosine kinase inhibitor that has been approved in Japan, Australia, and the United States for the treatment of advanced, chemorefractory non-small cell lung cancer xenograft model (NSCLC). ZD1839 is also being investigated in several other solid tumors, including head and neck, colorectal cancer, and breast cancer. Potent antitumor effects with ZD1839 monotherapy have been shown in a range of human tumor xenografts that express EGFR, although response is not clearly correlated with the degree of EGFR cell surface receptor expression (6–10). Importantly, EGFR expression is up-regulated in cancer cells after exposure to ionizing radiation and in turn has been associated with reduced sensitivity or resistance to ionizing radiation (11, 12). Thus, combining ZD1839 with radiation would seem logical, and in fact, ZD1839 has improved the effects of radiation therapy in studies in several preclinical cancer types (8–10, 13). This may not be enough, however, to counteract signaling from other EGFR family members or block, for example, phosphoinositide 3-kinase-Akt signaling despite affecting mitogen-activated protein kinase signaling. Continued proliferation and survival through alternative stimuli such as angiogenesis may continue to drive the cancer cell.

The potential for acquired resistance to growth factor signaling inhibition through EGFR alone suggests that attacking other pathways that contribute to cancer cell survival, such as angiogenesis, might improve tumor control further. In light of the recent promising preclinical studies with ZD6126, the present report investigated the therapeutic potential of combining ZD6126 and/or ZD1839 with radiation therapy to inhibit human NSCLC xenografts. We also investigated the effects of combining ZD1839, clinically approved for the treatment of advanced NSCLC, and ZD6126 in a similar NSCLC xenograft model. Finally, the potential benefit of triple therapy with ZD6126, ZD1839, and fractionated radiation therapy was evaluated.

Materials and Methods

Animals and Tumor Models

Five- to 6-week-old female BALB/cAnNCrlBR athymic (*nu+/nu+*) were purchased from Charles River Laboratories (Milan, Italy). Animal experiments were conducted at the University of Naples ''Federico II'' (Naples, Italy). The research protocol was approved, and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized to the University of Naples Medical School Animal Facility for 1 week before they received injections of cancer cells: 5×10^6 A549 human NSCLC cells were resuspended in 100 μL Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected s.c. in mice. Tumors were allowed to develop for ~7 to 10 days until they reached a ~200 mm^3, when treatment was initiated. Animals were given food and water *ad libitum*. All animal procedures and maintenance were conducted in accordance with institutional guidelines of the University of Naples. Mice from each group were sacrificed at the conclusion of treatment with ZD1839, ZD6126, and radiation to perform immunohistochemical analysis.

Compounds

ZD6126 and ZD1839 were kindly supplied by AstraZeneca (Macclesfield, United Kingdom). ZD6126 was dissolved in 0.9% saline and injected i.p. into a volume of 0.1 mL to deliver a dose of 150 mg/kg per animal, 1 day/wk for 2 weeks (in groups treated with radiation, ZD6126 was always given after radiation was completed each week). ZD1839 was supplied in lyophilized format and prepared as reported previously (7). ZD1839 was given at a daily dose of 100 mg/kg per animal i.p., 5 or 7 days for 2 weeks.

Cell Lines and Cell Culture

Low-passage human A549 NSCLC cells were obtained from the American Type Culture Collection (Rockville, MD). A549 cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) in a humidified incubator with 5% CO_2.

Treatment Regimens

Irradiation was done on anesthetized mice using a linear accelerator delivering 9 MeV electrons for a prescribed dose of 4 Gy per fraction. Irradiation was delivered only to the flanks bearing the A549 xenografts with lead shielding applied to the remainder of the animal body. Tumor response was assessed in two studies. In the first series of experiments, four groups of eight animals were treated with either 4 Gy of radiation therapy as described above for 2 consecutive days per week for 2 weeks or ZD6126 150 mg/kg i.p. once a week for 2 weeks. The third group received a combination of the two treatments at the same dose and regimen with the ZD6126 delivered 24 hours after the second radiation dose each week for a total of 2 weeks. Control animals received no treatment. Treatment was followed by a 4-week follow-up period.

In the second study, eight treatment groups were assessed (five animals per group). In addition to the three regimens described above, groups received ZD1839 (100 mg/kg/d i.p.) for 5 days/wk. This dose was established as safe for the animals in previous experiments (with and without radiation therapy or ZD6126) for a total of 2 weeks. One group received triple combination of ZD6126, ZD1839, and radiation therapy. Control animals received no treatment. In this particular study, two animals per group were sacrificed after the 2-week treatment concluded for immunohistochemical analysis of the effects of the different treatments on the xenografts.

Tumors were examined from each treatment group at the end of the 2-week treatment period. Other animals were followed up for ~105 days in the second animal experiment evaluating ZD6126, ZD1839, and radiation therapy after completion of the treatment period, and tumor volume was determined weekly. Tumor volume measurements were evaluated weekly in all experiments by caliper and calculated by the formula: $V = \pi$ (smaller diameter)^2 (larger diameter) / 6.

Immunohistochemical Analysis for Tumor Necrosis and Effects on Tumor Vasculature

Histologic assessment was done to evaluate the effects of ZD6126, ZD1839, radiation therapy and combinations of all three treatments on tumor vasculature. Two additional animals added at the outset within each of the treatment groups from the second animal experiment were euthanized after the 2-week treatment course was concluded (day 28 after implantation). Tumors were excised and fixed immediately in formalin and embedded in paraffin wax blocks. Subsequently, sections of the blocks were stained with H&E. Tumor necrosis was assessed by light microscopy. Tumor proliferation was assessed using an anti-Ki67 monoclonal antibody (1:100 dilution, clone MIB1, DBA, Milan, Italy), and the percentage of specifically stained cancer cells for Ki67 was recorded. Vascularization was determined by staining sections using a monoclonal antibody against the human factor VIII–related antigen (1:50 dilution, DAKO, Milan, Italy). The number of microvessels per field was scored by averaging five field counts of two individual tumors for each group.

Statistical Analysis

The Student's t test was used to evaluate the statistical significance of the results. All P values represent two-sided tests of statistical significance. All analyses were done with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

Results

Antitumor Activity of ZD6126 in Combination with Radiation Therapy

As shown in Fig. 1, we first evaluated the effects of a 2-week radiation therapy treatment (16 Gy total dose per mouse) alone and/or in combination with ZD6126 (150 mg/kg i.p. per mouse on the day following the second dose of radiation therapy for 2 weeks) on the growth of established A549 lung adenocarcinoma xenografts. Both ZD6126 and radiation therapy alone modestly inhibited tumor growth over untreated controls (not statistically significant for radiation). However, a significant and prolonged tumor growth inhibition was observed with the combination of ZD6126 and radiation therapy ($P = 0.01$ as compared with untreated control mice or to single agent treated mice). In this particular experiment, at day 40, for example, the average tumor volume after tumor cell injection was ~0.2 cm^3 in the ZD6126 + radiation therapy group compared with tumors treated with radiation therapy (~2.5 cm^3) or ZD6126 alone (~1.6 cm^3) as seen in Fig. 1.

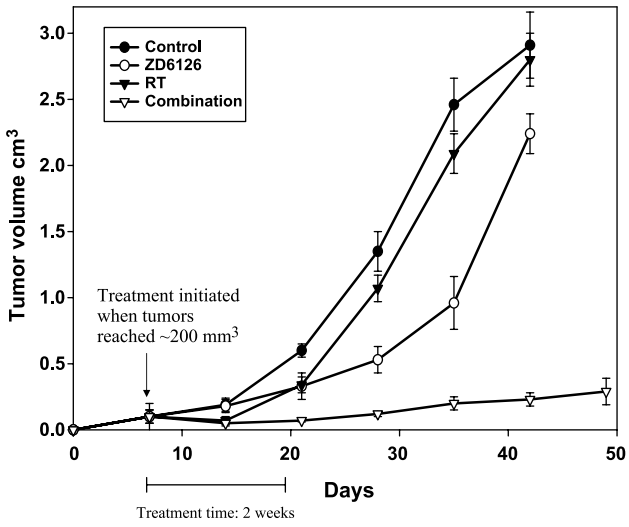

Figure 1. Effect of radiation therapy (*RT*) in combination with ZD6126 on A549 xenografts. Animals were followed for a 4-week period after the end of the treatment protocol (see Materials and Methods for details). Eight mice per treatment group. Student's t test was used to compare tumor sizes among different treatment groups at day 42 following A549 cell injection. Statistically significant differences: ZD6126 + radiation therapy versus control (two-sided $P < 0.01$), ZD6126 + radiation therapy versus ZD6126 alone (two-sided $P < 0.01$), and ZD6126 + radiation therapy versus radiation therapy alone (two-sided $P < 0.01$).

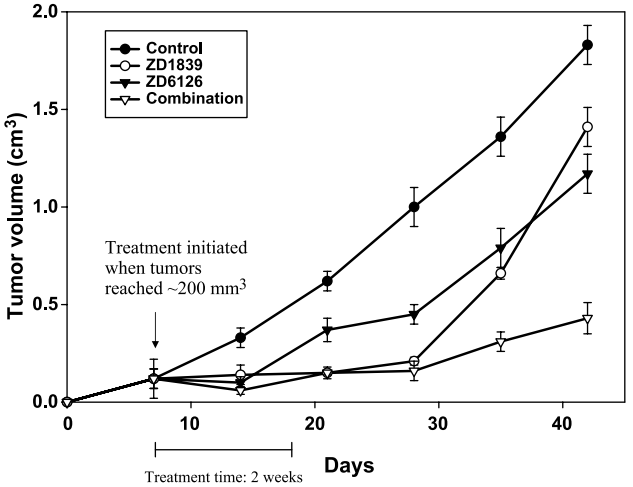

Figure 2. Effect of ZD1839 in combination with ZD6126 on A549 xenografts. Animals were followed for a 4-week period after the end of the treatment protocol (see Materials and Methods for details). Five mice per treatment group. Student's *t* test was used to compare tumor sizes among different treatment groups at day 42 following A549 cell injection. Statistically significant differences: ZD6126 + ZD1839 versus control (two-sided $P < 0.01$), ZD6126 + ZD1839 versus ZD6126 alone (two-sided $P < 0.01$), and ZD6126 + ZD1839 versus ZD1839 alone (two-sided $P < 0.01$).

Antitumor Activity of the Combination of ZD1839 and ZD6126

We investigated the antitumor activity of ZD6126 in combination with ZD1839 and compared the effects with either agent alone and with untreated controls. As shown in Fig. 2, modest tumor growth inhibition was observed with 2 weeks of ZD1839 or two administrations of ZD6126 over 2 weeks in contrast to untreated controls. Combination treatment with ZD6126 + ZD1839 as shown in Table 1, however, resulted in a significantly greater tumor growth inhibition [0.69 cm^3 at day 49 from tumor cell injection as compared with ZD6126 (1.1 cm^3) or ZD1839 (1.89 cm^3) alone ($P = 0.01$ as compared with untreated control mice or to single agent treated mice)].

Antitumor Activity of Combination Treatments with ZD6126, ZD1839, and Radiation Therapy

We next did an experiment to evaluate the effects of a triple combination of radiation therapy, ZD6126, and ZD1839 on the growth of established A549 xenografts. As illustrated in Fig. 3, a similar tumor growth inhibition was obtained when mice were treated with ZD6126 + radiation therapy, ZD1839 + radiation therapy, or ZD6126 + ZD1839. However, a trend toward greater tumor growth inhibition was observed in animals treated with the triple combination of ZD6126, ZD1839, and radiation therapy. This was reflected by a significant improvement in A549 tumor growth delay. For example, A549 tumors in mice treated with the triple combination of radiation therapy, ZD6126, and ZD1839 reached a mean tumor volume of ~2.5 cm^3 (that was considered not compatible with normal mouse life) within an average period of 105 ± 5 days as compared with ZD6126 + radiation therapy treated mice (77 ± 4 days),

Table1. Antitumor activity of ZD6126 in combination with ZD1839 and/or ionizing radiation on A549 human NSCLC xenografts

Treatment	Average Tumor Volume on Day 49 after Tumor Cell Injection (cm^3)	Average Time (d) to Reach a Tumor Volume of ~2.5 cm^3
Control	2.57 ± 0.2	49 ± 3
Radiation therapy	1.1 ± 0.1	60 ± 4
ZD6126	1.56 ± 0.1	61 ± 3
ZD1839	1.89 ± 0.15	54 ± 5
Radiation therapy + ZD6126	0.76 ± 0.07	77 ± 4
Radiation therapy + ZD1839	0.42 ± 0.05	82 ± 3
ZD6126 + ZD1839	0.69 ± 0.04	80 ± 4
Triple combination	0.23 ± 0.03	105 ± 5

NOTE: Each group consisted of five mice. The treatment protocol was described in Materials and Methods. The average tumor volume on day 49 following tumor cell injection in control mice (2.57 ± 0.2 cm^3) was considered not compatible with mice normal life (~10–15% of a nude mouse body weight). Therefore, when tumors in each treatment group reached a comparable tumor volume, mice were sacrificed.

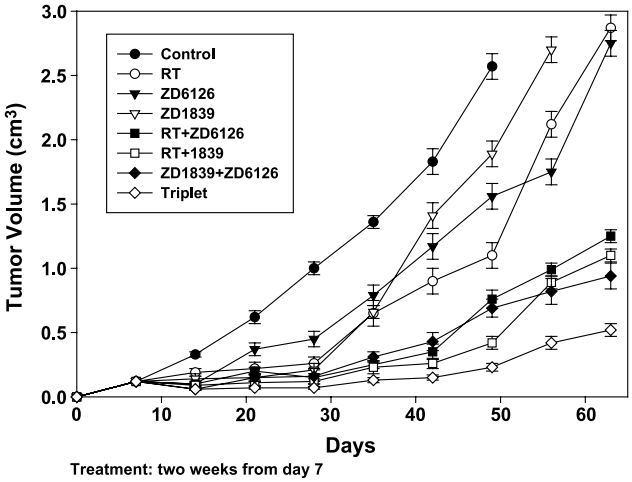

Figure 3. Effects of triple therapy with ZD6126, ZD1839, and radiation therapy (*RT*) on A549 tumor xenografts. Although time points are out to ~4 weeks, which were relevant to show the differences in treatment effects among the different groups, animals were in fact followed out much farther to measure time for tumors to reach 2.5 cm (~105 days from the end of treatment for the triple combination group; see Materials and Methods for details). Five mice per treatment group. Student's *t* test was used to compare tumor sizes among different treatment groups at day 42 following A549 cell injection. Relevant statistically significant differences with regard to comparing doublet combinations with single treatment and comparing doublet treatments with triple combination therapy: ZD6126 + ZD1839 versus ZD6126 alone (two-sided $P < 0.01$), ZD6126 + ZD1839 versus ZD1839 alone (two-sided $P < 0.01$), ZD6126 + ZD1839 versus radiation therapy alone (two-sided $P < 0.01$), ZD6126 + radiation therapy versus ZD6126 alone (two-sided $P < 0.01$), ZD6126 + radiation therapy versus ZD1839 alone (two-sided $P < 0.01$), ZD6126 + radiation therapy versus radiation therapy alone (two-sided $P < 0.01$), radiation therapy + ZD1839 versus ZD6126 alone (two-sided $P < 0.01$), radiation therapy + ZD1839 versus ZD1839 alone (two-sided $P < 0.01$), and radiation therapy + ZD1839 versus radiation therapy alone (two-sided $P < 0.01$). All treatment modalities were statistically effective (ZD6126, ZD1839, and radiation therapy) over the control animals.

Table 2. Immunohistochemical evaluation of A549 tumor xenografts

Treatment	Tumor Size (cm^3)	Proliferative Activity (% Ki67)	Tumor Necrosis	Microvessel Counts
Control	1.0 ± 0.1	70 ± 5	Absent	28 ± 3
Radiation therapy	0.26 ± 0.05	50 ± 5	Rare, focally present within tumor mass	25 ± 2
ZD1839	0.21 ± 0.05	40 ± 5	Absent	19 ± 1
ZD6126	0.45 ± 0.06	60 ± 5	Widely present within tumor mass	12 ± 2
ZD1839 + ZD6126	0.16 ± 0.06	35 ± 5	Widely present within tumor mass	8 ± 2
Radiation therapy + ZD6126	0.15 ± 0.03	45 ± 5	Widely present within tumor mass	8 ± 2
Radiation therapy + ZD1838	0.12 ± 0.02	15 ± 5	Diffuse within tumor mass	10 ± 2
Triple combination	0.05 ± 0.01	2 ± 1	Almost completely necrotic tumor mass	1 ± 1

NOTE: Histology and immunohistochemical analysis were done on tumors obtained from mice on day 28 following A549 tumor cell injection. The percentage ± SD of specifically stained cancer cells for Ki67 was recorded. The number ± SD of microvessels for field was measured using a monoclonal antibody raised against the human factor VIII–related antigen and scored by averaging five field counts of two individual tumors for each group.

radiation therapy + ZD1839 treated mice (82 ± 3 days), or ZD6126 + ZD1839 treated mice (80 ± 4 days; Table 1). ZD6126 administration resulted in no undue toxicity when delivered i.p. alone or in combination with radiation therapy or ZD1839. Combined treatments with ZD6126, ZD1839, and/or radiation therapy at the dose and schedule tested were well tolerated by mice because no weight loss or other signs of acute or delayed toxicity were observed.

Effects on Tumor Proliferation, Angiogenesis, Vasculature, and Necrosis

To assess for immunohistochemical effects of ZD6126 and/or ZD1839 and/or radiation on growth and angiogenesis, we have quantified the tumor-induced vascularization as microvessel density count (MVD) using an anti–factor VIII–related antigen monoclonal antibody. Table 2 shows the reduction in Ki67 staining in tumors treated with either ZD6126 or ZD1839, consistent with a reduced proliferative activity within the cancer cells. Ki67 staining was further reduced when ZD6126 and ZD1839 were combined. The greatest reduction was observed in tumors treated with all three modalities. Table 2 also shows that tumor necrosis was absent in the untreated tumors and in tumors treated with ZD1839 only. Rare, focal necrosis was seen within the ZD1839-treated tumors. In contrast, necrosis was widely prevalent throughout the tumors treated with ZD6126 monotherapy, tumors treated with ZD6126 and ZD1839, and tumors treated with radiation therapy and ZD6126. Diffuse but less extensive necrosis was observed in tumors treated with radiation therapy and ZD1839. Complete necrosis was seen in tumors treated with triple therapy. Both ZD1839 (MVD count 19 ± 1) and ZD6126 (MVD count 12 ± 2) treatment substantially reduced MVD compared with control (MVD count 28 ± 3) as measured by factor VIII–related antigen staining of microvessels shown in Table 2 and Fig. 4. Dual combinations produced even greater inhibition of angiogenesis (range 8–10 MVD counts with ZD6126 + ZD1839, ZD1839 + radiation therapy, and ZD6126 + radiation therapy), and the triple combination (MVD count 1 ± 1) resulted in a marked reduction in microvessel development (Table 2 and Fig. 4).

Discussion

Tumor hypoxia has been a major focus of radiation oncology investigations for many years (14), and considerable evidence has accumulated to indicate that hypoxia results in radioresistance. Various approaches to overcome this impediment to treatment success have been sought. Through direct interference with intratumoral vasculature, ZD6126 may counter this problem by eliminating the survival potential of the central hypoxic regions of tumors via the induction of central necrosis. In this regard, ZD6126 has shown rapid tumor necrosis in a variety of human tumor models in both single and multiple dosing regimens (15). Importantly, ZD6126 i.p. administration resulted in significant antivascular effects at doses well below the maximum tolerated dose with minimal toxicity (16). When combined with fractionated radiation therapy in a KHT sarcoma model, significant cooperative effects were observed with ZD6126 given with either single or fractionated radiation therapy. The results seemed additive. With regard to sequencing, ZD6126 provided similar radiation therapy enhancement when given 24 hours before, immediately after, and 4 hours after radiation therapy, with the optimal time being 30 minutes to 1 hour after radiation (2).

The application of the combined therapy to the treatment of locally advanced lung cancer seems reasonable. Local control in patients with locally advanced NSCLC remains a major problem despite treatment with concurrent chemoradiation. This is due in part on the fact that many patients present with large unresectable lesions. Additionally, these large tumors often have hypoxic, necrotic areas within the central aspects of the tumor that may be more resistant to the effects of radiation. Several strategies have evolved to tackle this problem including altered fraction radiation, although, in conjunction with chemotherapy, this approach can be quite toxic with regard to esophagitis and respiratory compromise (15).

The present study was undertaken to evaluate novel strategies with ZD6126 in the treatment of NSCLC and to evaluate its effects with radiation or ZD1839. The rationale behind the latter approach was to determine if

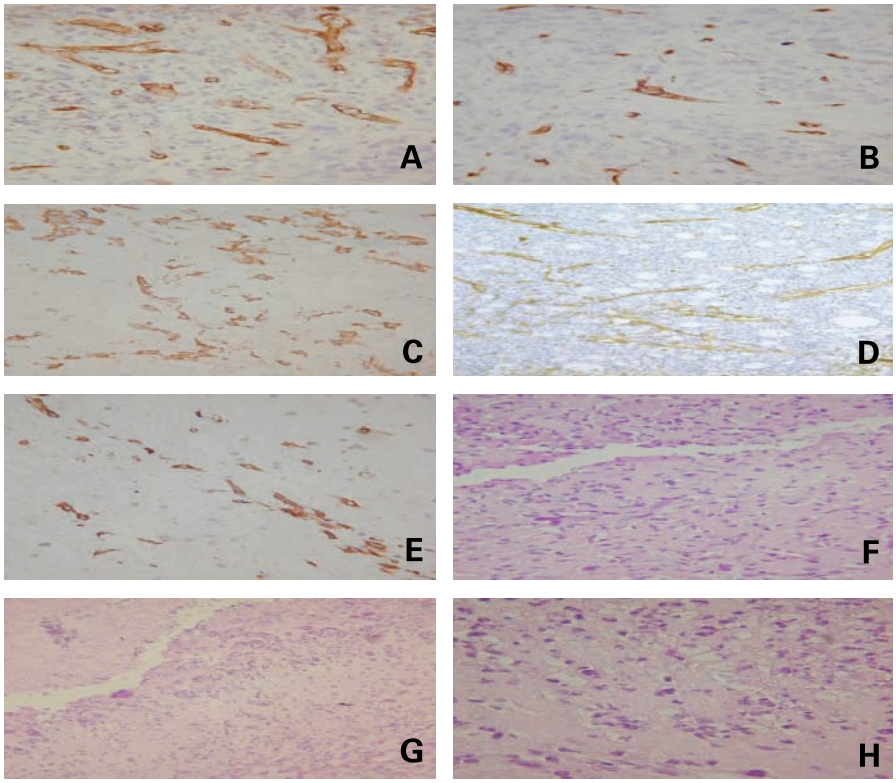

Figure 4. Immunohistochemistry of A549 xenografts for evaluation of neoangiogenesis. Mice bearing A549 tumor xenografts were treated as described in Materials and Methods. **A,** factor VIII – related antigen staining in A549 control xenografts. **B,** factor VIII – related antigen staining in gefitinib-treated A549 xenografts. **C,** factor VIII – related antigen staining in ZD6126-treated A549 xenografts. **D,** factor VIII – related antigen staining in radiation therapy treated A549 xenografts. **E,** factor VIII – related antigen staining in radiation therapy + gefitinib treated A549 xenografts. **F,** factor VIII – related antigen staining in radiation therapy + ZD6126 treated A549 xenografts. **G,** factor VIII – related antigen staining in ZD6126 + gefitinib treated A549 xenografts. **H,** factor VIII – related antigen staining in radiation therapy + gefitinib and ZD6126 treated A549 xenografts. Magnification, ×25.

perturbing both EGFR and angiogenic signaling might produce greater tumor growth inhibition than either biological therapy alone. In our xenograft model, ZD6126 treatment was seen to result in endothelial cell loss and pronounced disruption of tumor microvessels. These findings are consistent with previous reports (2, 17). As a result, areas of tumor necrosis were common in the ZD6126-treated tumors. The cooperative tumor growth inhibitory effects observed with ZD6126 and radiation therapy in this NSCLC model were consistent with the findings by Siemann and Rojiani (2) in a sarcoma model. Similar to Blakey et al., effective tumor growth delay was seen with ZD6126 doses well below the reported maximum tolerated dose of 400 mg/kg (2). With respect to sequencing, we elected to administer ZD6126 after fractionated radiation; however, administering ZD6126 prior to radiation therapy would have had similar effects in this NSCLC model. Further work is ongoing to evaluate the issue of sequencing and the optimal dosing of ZD6126 in NSCLC tumors with different histology.

Because intense interest has also developed around strategies that interfere with EGFR signaling in NSCLC, it would seem logical to evaluate whether antagonizing a different component of the cancer cell (immature tumor vasculature) would strengthen response to EGFR inhibitors and radiation. ZD1839, an p.o. bioavailable anilinoquinazoline with anti-EGFR tyrosine kinase activity, showed encouraging response rates in heavily pretreated patients with advanced NSCLC (17, 18). ZD1839 was shown to enhance the antitumor activity of radiation in ZD1839 responsive NSCLC xenografts (7, 8). The mechanistic determinants of ZD1839 responsiveness in a variety of tumor types remains uncertain, and it is known that the level of EGFR expression does not correlate with treatment outcome (19). The baseline levels of activated downstream signaling pathways such as Akt or mitogen-activated protein kinase may explain responsiveness to various anti-EGFR molecules. In the present study, combinations of either ZD1839 or ZD6126 with fractionated radiation therapy showed similar levels of efficacy in the A549 NSCLC xenograft model, which expresses moderate levels of EGFR. ZD1839 is currently undergoing clinical investigation in patients with lung cancer in combination with radiation.

Combining new targeted therapies with different modes of action within the cancer cell is based in part on the rationale that cancer cells may override EGFR blockade by up-regulating signaling through angiogenic pathways. To this end, human A431 cancer cells xenografts can acquire resistance to anti-EGFR monoclonal antibodies such as cetuximab and hR3 by altered tumor-induced angiogenesis due to the constitutive overexpression of proangiogenic growth factors (vascular endothelial growth factor) by cancer cells (20). It has been also suggested that activation of the insulin-like growth factor receptor 1 may impact on activation of the antiapoptotic phosphoinositide 3-kinase signaling pathway that counteracts the growth inhibitory actions of EGFR inhibitors in human glioblastoma cells (21). In this context, preclinical experimental models have

shown that the combination of anti-EGFR agents with other antisignaling agents, such as inhibitors of the cyclic AMP–dependent protein kinase (type I protein kinase A; ref. 22), a vascular endothelial growth factor antisense oligonucleotide (23), or the anti-ErbB-2 monoclonal antibody trastuzumab (24), can lead to sustained antitumor activity. In pancreatic tumor models, combination treatment with an EGFR tyrosine kinase inhibitor and a vascular endothelial growth factor receptor tyrosine kinase inhibitor produced superior local control of xenografts than either agent alone (25). When ZD6126 and ZD1839 were combined in our NSCLC model, we also observed cooperative effects with a greater tumor growth delay than with either agent alone. Apparently, our studies suggest that biologically active agents that target specific aspects of cancer growth and angiogenesis could be combined safely with radiation therapy. The theory would be that polytargeted therapy would provide similar or improved control rates to concurrent chemoradiation, the current standard in advanced lung cancer management, with reduced toxicity. It was encouraging that the triple combination of radiation therapy, ZD6126, and ZD1839 resulted in the greatest effects on tumor growth and angiogenesis, and this tripe combination remained well tolerated.

ZD6126 provides an alternative strategy to radiosensitization in tumors that are unresponsive or resistant to EGFR inhibitors. Future studies will compare effects of ZD6126 and radiation therapy in both EGFR tyrosine kinase inhibitor responsive and unresponsive tumors as well as compare the cooperative effects to chemoradiation using agents' standard to NSCLC treatment such as the taxanes and cisplatin. Finally, studies are planned to evaluate further the triple combination effects of ZD6126, ZD1839, and radiation in comparison with chemoradiation with the end points of tolerability and survival in mouse xenograft NSCLC models. The therapeutic potential of ZD6126, alone and in combination with other anticancer agents, in the treatment of solid tumors is currently being assessed in a series of phase II clinical trials.

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