

ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor with additional activity against epidermal growth factor receptor tyrosine kinase, inhibits orthotopic growth and angiogenesis of gastric cancer

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Abstract

Vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) have been strongly implicated in the growth and metastasis of gastric cancer. The purpose of this study was to examine the effects of ZD6474, an inhibitor of VEGF receptor (VEGFR) tyrosine kinase with additional activity against EGF receptor (EGFR), on tumor growth and angiogenesis in an orthotopic model of gastric cancer. *In vitro*, ZD6474 inhibited human umbilical vascular endothelial cell and TMK-1 human gastric tumor cell proliferation in a dose-dependent fashion. EGF-mediated activation of EGFR and Erk-1/2 was decreased in tumor cells after ZD6474 treatment. In addition, VEGF-mediated activation of VEGFR2 and Erk-1/2 was decreased in human umbilical vascular endothelial cells. TMK-1 human gastric adenocarcinoma cells were injected into the gastric wall of nude mice. ZD6474 therapy was initiated on day 10. Mice ($n = 14$ per group) were treated p.o. with (a) 1% Tween 80 (control), (b) 50 mg/kg/d ZD6474, or (c) 100 mg/kg/d ZD6474. Mice were sacrificed on day 33. Tumors from each group were stained for markers of blood vessels, pericytes, proliferation, and apoptosis. ZD6474 at both 50 and 100 mg/kg/d led to marked inhibition of tumor growth ($P < 0.05$). ZD6474 reduced tumor cell proliferation by 48% in the 50 mg/kg/d group and 65% in the 100 mg/kg/d group ($P < 0.03$) and increased tumor cell apoptosis ($P < 0.001$) *in vivo*. ZD6474 led to a 69% decrease in microvessel

density in the 50 mg/kg/d group ($P < 0.001$) and a 62% decrease in the 100 mg/kg/d group ($P < 0.001$). Although microvessel density was decreased by ZD6474, the remaining vessels showed a relatively higher percentage of pericyte coverage (3-fold increase; $P < 0.001$), perhaps reflecting selective loss of uncovered vessels in the ZD6474 group. In conclusion, therapies such as ZD6474 that target two distinct aspects of tumor growth, angiogenesis and tumor cell proliferation, warrant further investigation. [Mol Cancer Ther 2004;3(9):1041–8]

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide, with ~21,600 people diagnosed in the United States every year (1). Unfortunately, the 5-year survival rate in the United States has remained at ~20% for the past 20 years (1), indicating that new therapies to combat this disease are urgently needed (2).

One target for new therapies is angiogenesis, the dynamic process by which the blood supply of a tumor is created from preexisting blood vessels and endothelial precursor cells (3, 4). The roles of vascular endothelial growth factor (VEGF) and its receptors as important mediators in endothelial cell proliferation, migration, invasion, and differentiation during angiogenesis have been well established (4). The expression of VEGF is strongly correlated with tumor progression and poor prognosis in gastrointestinal malignancies, including gastric cancer (5–7), and previous studies have established an association among VEGF expression, increased microvessel density, and decreased survival in gastric cancer (6). In addition, preclinical studies of agents that selectively target VEGF and its receptors in gastric cancer have shown significant antitumor effects, confirming that this ligand/receptor system is a valid target for gastric cancer therapy (8–10).

The proliferation and growth of gastric cancer is mediated by a variety of growth factors and cytokines. An important growth factor receptor/ligand system implicated in gastric cancer progression is the epidermal growth factor (EGF) receptor (EGFR) and its ligands, EGF, transforming growth factor- α , and heparin binding EGF (11, 12). Ligand binding stimulates receptor dimerization and phosphorylation of specific tyrosine residues within the carboxyl terminus of EGFR (13). This leads to the activation and phosphorylation of downstream signaling intermediates and finally to enhancement of cellular proliferation and survival (13).

Therapies that target angiogenesis or EGFR are showing promise in clinical trials for gastrointestinal cancers (14, 15).

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A variety of therapeutic modalities have been proposed to inhibit the activities of the VEGF receptor (VEGFR) and EGFR. These include monoclonal antibodies, tyrosine kinase inhibitors, ribozyme constructs, and soluble decoy receptors. Small-molecule tyrosine kinase inhibitors act by competitive binding at the receptor ATP binding site, thereby inhibiting receptor kinase activity and activation of downstream signaling pathways (16). ZD6474, a hetero-aromatic-substituted anilinoquinazoline, acts as a tyrosine kinase inhibitor and can inhibit VEGFR2 and EGFR at nanomolar concentrations (16). It has shown efficacy *in vivo* against s.c. xenografts of several tumor types (16) but has not been tested against orthotopic tumors from human gastric tumor cell lines.

Previous studies have highlighted the potential importance of targeting multiple compartments of a tumor, namely, the proliferating tumor cells and tumor-associated endothelial cells (17). This has been accomplished by administering agents such as VEGFR inhibitors in combination with chemotherapy and radiation or EGFR inhibitors plus antiangiogenic agents (18–21). One study, using therapeutic antibodies, compared EGFR inhibition, VEGFR2 inhibition, and the combination in an orthotopic model of gastric cancer (8). Data from this study suggested that inactivation of both pathways was most effective at inhibiting tumor growth. Therefore, we hypothesized that a p.o. agent, ZD6474, targeting both tumor cell population via EGFR tyrosine kinase inhibition and the endothelial cell population via VEGFR2 inhibition, would be an effective means of inhibiting growth of gastric cancers *in vivo*.

Materials and Methods

Cell Cultures

TMK-1, a poorly differentiated human gastric adenocarcinoma cell line, was the generous gift of Dr. Eiichi Tahara (Hiroshima University, Hiroshima, Japan) and was cultured and maintained in DMEM supplemented with 10% fetal bovine serum as described previously (8). Human umbilical vascular endothelial cells (HUVEC) were obtained from American Type Culture Collection (Manassas, VA) and cultured according to the provider's guidelines. The cultures were free of *Mycoplasma* and the following murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by MA Bioproducts, Walkersville, MD). Cells for injection were harvested from subconfluent cultures. Trypan blue exclusion was used to ensure cell viability of >90%.

Formulation of ZD6474

ZD6474 [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine] was obtained from AstraZeneca (Macclesfield, United Kingdom). This compound was milled overnight on a blood

rotator in 1% Tween 80 (Sigma Chemical Co., St. Louis, MO) in PBS and given as a homogeneous suspension by p.o. gavage. Fresh ZD6474 was prepared every 5 days during the treatment period.

In vitro Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M2128) was purchased from Sigma Chemical and a stock solution was prepared by dissolving 5 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 1 mL PBS and filtering the solution to remove particulates. The solution was protected from light, stored at 4°C, and used within 1 month. To determine proliferation, cells were seeded into 96-well plates in triplicate and allowed to adhere overnight in 10% complete DMEM. The cultures were washed, the medium was changed, and cells were exposed to 0.1, 1, or 10 $\mu\text{mol/L}$ ZD6474 alone; 40 ng/mL EGF alone or with ZD6474; or 20 ng/mL VEGF alone or with ZD6474 in the presence of 10% complete MEM. After 72 hours, the number of metabolically active cells was determined. After a 2-hour incubation in medium containing 0.42 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the cells were lysed in DMSO. A MR-5000 96-well microtiter plate reader at 570 nm (Dynatech, Chantilly, VA) monitored the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by metabolically viable cells.

Western Blot Hybridization

Cells were rinsed twice with ice-cold PBS and lysed with protein lysis buffer [20 mmol/L sodium phosphate (pH 7.4), 150 mmol/L sodium chloride, 1% Triton X-100, 5 mmol/L EDTA, 5 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, 1 $\mu\text{g/mL}$ leupeptin, 500 $\mu\text{mol/L}$ Na_3VO_4]. Protein was quantified spectrophotometrically. Aliquots (50 μg) of the protein were subjected to electrophoresis on 10% polyacrylamide gels and the protein was transferred to a nitrocellulose membrane (Millipore, Bedford, MA) by electrotransfer. Following blocking with 5% milk in 0.5% Tween 20 in PBS, the membrane was probed with the primary antibody (monoclonal mouse anti-EGFR antibody, Zymed, South San Francisco, CA), rabbit anti-EGFR Tyr¹¹⁷³ phospho-specific antibody (Biosource, Camarillo, CA), mouse monoclonal anti-p44/42 mitogen-activated protein kinase Thr²⁰²/Tyr²⁰⁴ phospho-specific antibody (Erk-1/2, Cell Signaling, Beverly, MA), rabbit anti-mitogen-activated protein kinase antibody (tErk-1/2, Oncogene, San Diego, CA), rabbit anti-VEGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-VEGFR2 Tyr¹⁰⁵⁴/Tyr¹⁰⁵⁹ phospho-specific antibody (Biosource). The membranes were washed and treated with the secondary antibody [sheep antimouse (Amersham, Little Chalfont, United Kingdom) or goat anti-rabbit (Bio-Rad, Hercules, CA)] labeled with horseradish peroxidase. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham).

Animal Studies

Eight-week-old male BALB/c athymic nude mice (obtained from the National Cancer Institute Animal Production Area, Frederick, MD) were acclimated for

1 week and caged in groups of five. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center (Houston, TX).

Mice were anesthetized by methoxyflurane inhalation (Metofane, Medical Developments, Springvale, Victoria, Australia) and, under sterile conditions, subjected to an upper midline laparotomy. TMK-1 cells (10^6) in HBSS were injected into the wall of the mid-stomach. Ten days later, when tumors were ~1 to 2 mm in diameter (as determined by previous studies (22)), mice were randomly assigned to one of three groups: (a) daily dose of 1% Tween 80 in PBS (control; $n = 14$), (b) 50 mg/kg/d ZD6474 ($n = 14$), or (c) 100 mg/kg/d ZD6474 ($n = 14$). Body weight at randomization was similar among groups. Two mice in the 100 mg/kg/d ZD6474 group died because of esophageal perforation that occurred during administration of ZD6474 during the first week of therapy. One additional mouse in the 100 mg/kg/d ZD6474 group died of unknown causes on day 18. ZD6474 therapy at both 50 and 100 mg/kg/d led to a nonserious skin rash (dermatitis) that did not require early termination of the experiment in 71% and 82% of mice, respectively, versus 7% of the control group. On day 33, when the control mice exhibited decreased activity and weight loss, all mice were weighed and killed by CO₂ asphyxiation. Their tumors were excised and weighed and tumor tissue was placed in either 10% buffered formalin for paraffin fixation or optimal cutting temperature compound (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen for frozen tissue sections.

Immunostaining

Five frozen tumors from each group were sectioned (8–10 μ m thick) and stained by immunohistochemistry as described previously (23). The antibodies used were rat antimouse CD31 (PharMingen, San Diego, CA), mouse antihuman α -smooth muscle actin (DAKO, Carpinteria, CA), mouse anti-BrdUrd (Becton Dickinson, Franklin Lakes, NJ), goat antirat Texas red secondary antibody (The Jackson Laboratory, West Grove, CA), and goat antimouse Alexa Fluor 488 secondary antibody (Molecular Probes, Eugene, OR). Terminal deoxynucleotidyl transferase-mediated nick end labeling was done using a commercially available apoptosis detection kit (Promega, Madison, WI).

Image Analysis

Sections were examined using a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip charge-coupled color camera (DXC-960 MD, Sony, Tokyo, Japan). The images were analyzed using Optimas image analysis software version 5.2 (Bothell, WA). Positive cells were counted using Scion software based on the NIH image program for Macintosh (Scion Corporation, Frederick, MD). The numbers of positive cells reported were the means of the number of cells in five random 0.05 mm² high-power fields from each tumor. Five tumors from each group were assessed in this means.

Statistical Analysis

Statistical differences among groups were examined using the two-tailed Student's *t* test with InStat statistical

software (GraphPad Software, San Diego, CA). The results of the *in vivo* experiments were tested for outliers using Grubb's test (<http://www.graphpad.com>). $P \leq 0.05$ was considered statistically significant.

Results

In vitro Studies of Proliferation and Apoptosis

To determine whether ZD6474 has direct inhibitory effects on endothelial cells and tumor cells *in vitro*, HUVECs and TMK-1 cells were cultured with 0.1, 1, or 10 μ mol/L ZD6474 with or without ligand stimulation (EGF or VEGF). After incubation for 72 hours with 1 μ mol/L ZD6474, there was 26% fewer TMK-1 cells following EGF stimulation, whereas, in the control cells, there was only 7% fewer ($P < 0.05$; Fig. 1A). Similar results were obtained with endothelial cells, wherein VEGF-stimulated HUVECs incubated with 0.1 μ mol/L ZD6474 resulted in 14% fewer cells after 72 hours compared with no change in cell number for non-VEGF-stimulated HUVECs ($P < 0.01$; Fig. 1B). The addition of the ligands (VEGF and EGF) increased the total cell number for both cell lines and this increased proliferation was inhibited by the addition of 0.1 and 1.0 μ mol/L ZD6474. The highest dose of ZD6474 (10 μ mol/L) resulted in cell death in both treated and untreated groups. These data show that ZD6474 selectively inhibits EGF-stimulated or VEGF-stimulated cell growth and survival in TMK-1 and HUVECs, respectively.

To further explore the spectrum of inhibition of tyrosine kinase activity *in vitro*, TMK-1 cells were pretreated with

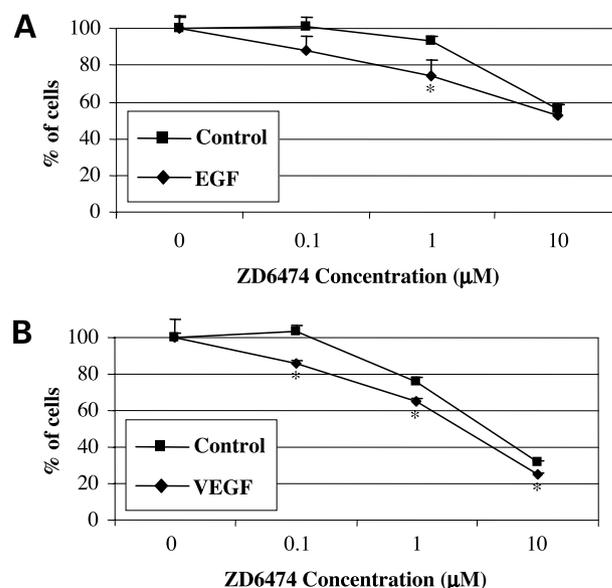


Figure 1. Effect of ZD6474 on tumor and endothelial cell proliferation. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay was done to determine the sensitivity of stimulated and unstimulated gastric cancer cells and endothelial cells to various doses of ZD6474. Results show that ZD6474 is able to selectively inhibit growth factor-dependent proliferation of both TMK-1 gastric cancer cells (A) and HUVECs (B) in a dose-dependent fashion. *, $P < 0.03$.

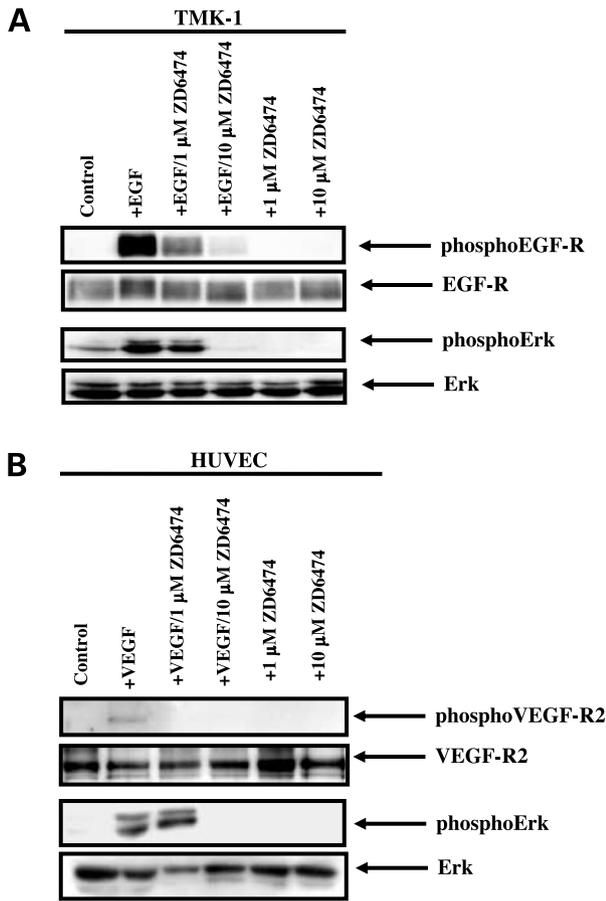


Figure 2. Effect of ZD6474 on cytokine-mediated phosphorylation of tyrosine kinase receptors *in vitro*. TMK-1 cells (A) or HUVECs (B) were serum starved (1% media) overnight and treated with ZD6474 at the indicated doses for 3 hours. Cells were stimulated for 5 minutes with the appropriate growth factor, either 40 ng/mL EGF (A) or 20 ng/mL VEGF (B). Phosphorylated and total protein levels were determined by Western blot analysis. Data show that ZD6474 significantly inhibited EGF-mediated stimulation of phospho-EGFR and phospho-Erk-1/2. ZD6474 also significantly inhibited VEGF-mediated stimulation of phospho-VEGFR2 and phospho-Erk-1/2.

ZD6474 at 1 or 10 μmol/L and briefly stimulated with EGF for 5 minutes. Western blotting showed that ZD6474 potently inhibited EGFR phosphorylation in TMK-1 cells in a dose-dependent fashion (Fig. 2A). This corresponded

Table 1. Antitumor effects of ZD6474 in the TMK-1 orthotopic gastric cancer model

	Control	ZD6474 50 mg/kg/d	ZD6474 100 mg/kg/d
Tumor volume, mm ³ (mean ± SEM)	966 ± 354	183 ± 37*	157 ± 53*
Tumor mass, g (mean ± SEM)	0.8 ± 0.2	0.2 ± 0.1*	0.1 ± 0.04*
Peritoneal carcinomatosis	11/15 (73%)	7/14 (50%)	6/11 (55%)

*Student's *t* test, *P* < 0.05.

with a decrease in the phosphorylation of the downstream signaling intermediate Erk-1/2. Furthermore, ZD6474 at 1 μmol/L completely inhibited VEGF-mediated VEGFR2 phosphorylation in HUVECs (Fig. 2B). Again, Erk-1/2 phosphorylation was also decreased. There was no significant change in Akt phosphorylation for either cell line (data not shown).

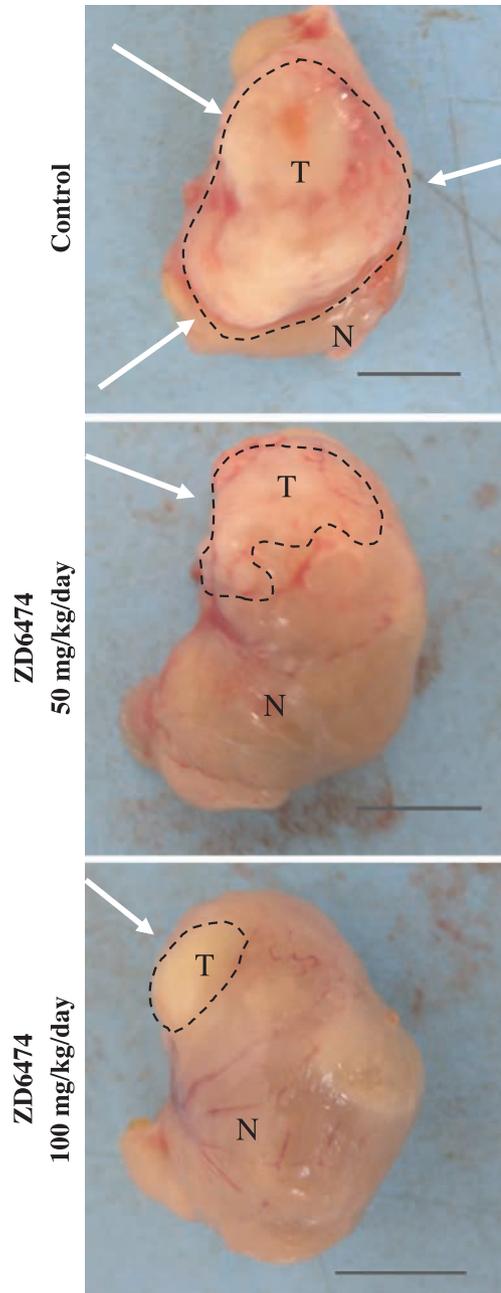


Figure 3. Effect of ZD6474 on the growth of gastric tumors. TMK-1 cells (10⁶) were injected into the stomach walls of nude mice and ZD6474 therapy was begun on day 10. Administration of 50 or 100 mg/kg/d ZD6474 was effective at inhibiting tumor growth on day 33 after 23 days of therapy at the termination of the experiment. Scale bar, 0.5 cm.

Table 2. Histologic analysis of ZD6474-treated tumors

	Control	ZD6474 50 mg/kg/d	ZD6474 100 mg/kg/d
Microvessel density (mean \pm SEM)	8.7 \pm 0.9	2.7 \pm 0.4*	3.3 \pm 0.6*
Proliferative index of tumor cells (mean \pm SEM)	8.0 \pm 1.8	4.2 \pm 0.7*	2.8 \pm 0.9*
Tumor apoptotic index (mean \pm SEM)	0.2 \pm 0.2	4.7 \pm 1.7*	9.7 \pm 2.3*

*Student's *t* test, $P < 0.05$.

Effect of ZD6474 on Growth of Primary Gastric Tumors

Therapy with 50 mg/kg/d ZD6474 decreased tumor volume by 81% ($P < 0.03$) and tumor mass by 75% ($P < 0.02$) compared with the control group (Table 1; Fig. 3). Therapy with 100 mg/kg/d ZD6474 also led to significant decreases in tumor volume (84%; $P < 0.05$) and tumor mass (88%; $P < 0.03$). There was a trend toward a decrease in the incidence of peritoneal carcinomatosis in both groups receiving ZD6474 therapy, but this did not reach statistical significance.

Decreased Microvessel Density in ZD6474-Treated Tumors

Mice treated with 50 mg/kg/d ZD6474 had a 69% decrease in tumor microvessel density ($P < 0.001$; Table 2;

Fig. 4). Similarly, in the group treated with 100 mg/kg/d ZD6474, there was a 62% decrease in microvessel density ($P < 0.001$). Tumors were stained at the end of the experiment on day 33 after 23 days of therapy.

Tumor Cell Proliferation and Apoptosis *In vivo*

ZD6474 treatment of TMK-1 gastric tumors in an orthotopic environment led to decreased tumor cell proliferation and increased tumor cell apoptosis (Table 2; Fig. 4). There was a 48% decrease in BrdUrd-positive cells within tumors treated with 50 mg/kg/d ZD6474 ($P < 0.03$) and a 65% decrease with 100 mg/kg/d ZD6474 ($P < 0.03$). Terminal deoxynucleotidyl transferase-mediated nick end labeling staining revealed a 96% to 98% increase in the ZD6474-treated groups ($P < 0.001$), indicating an increase in tumor cell apoptosis. Tumors were stained at the end of the experiment on day 33 after 23 days of therapy.

ZD6474 Selectively Targets Endothelial Cells with Poor Pericyte Coverage

It has been hypothesized that pericytes protect endothelial cells from the apoptotic effects of antiangiogenic therapy (24, 25). Therefore, dual immunofluorescent staining was done using antibodies that detect pericytes (α -smooth muscle actin) and endothelial cells (CD31). After 23 days of therapy, the proportion of endothelial cells associated with pericytes increased from 22% in the control group to 63% in the 50 mg/kg/d ZD6474 group and 68% in the 100 mg/kg/d ZD6474 group ($P < 0.001$; Fig. 5). This apparent increase in pericyte coverage in the ZD6474-treated

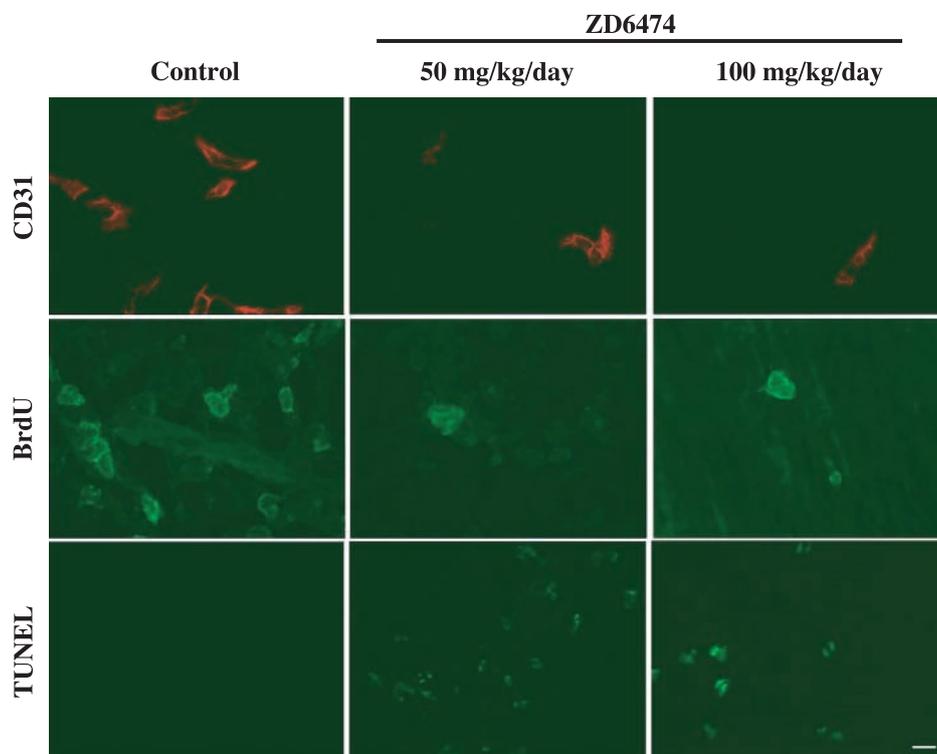


Figure 4. Effect of ZD6474 on microvessel density, cell proliferation, and apoptosis in tumors. After tumor harvest on day 33, tissue sections were stained with antibodies against CD31 (microvessel density), BrdUrd (cell proliferation, *BrdU*), or terminal deoxynucleotidyl transferase-mediated nick end labeling (cell apoptosis, *TUNEL*). *Red*, CD31-positive vessels; *green*, either BrdUrd-positive or terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells. Scale bar, 10 μ m.

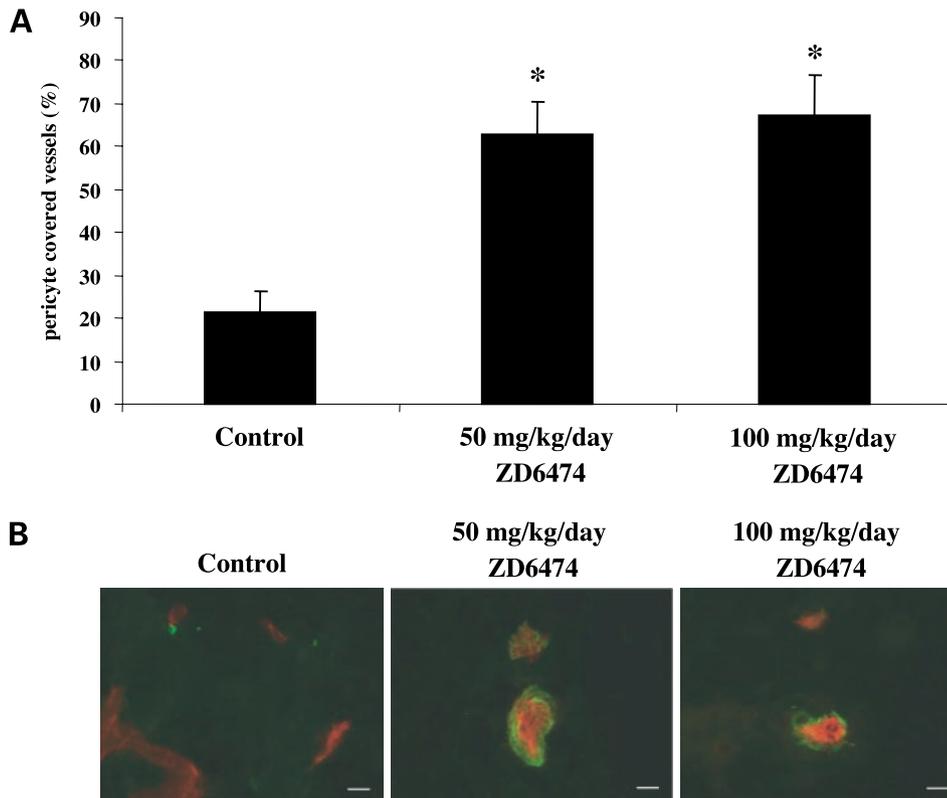


Figure 5. Pericyte coverage of endothelial cells in ZD6474-treated tumors. Mice were treated with ZD6474 and tumors were harvested at sacrifice. Tissues were stained for pericyte coverage using antibodies against endothelial cells (CD31, red) and pericytes (α -smooth muscle actin, green). Pericyte coverage was determined by the percentage of vessels that had at least 50% coverage of associated α -smooth muscle actin-positive cells. Although the microvessel density decreased after ZD6474 treatment, the percentage of endothelial cells covered by pericytes increased, suggesting that endothelial cells without pericyte coverage were targeted by this therapy. **A**, increased percentage of pericyte-covered vessels in the ZD6474 treated tumors compared with control. The percentage was determined by counting positive vessels in five high-powered fields within each individual tumor and five tumors per group. **B**, representative photomicrographs of pericyte coverage from each group of mice (left to right: control, 50 mg/kg/d ZD6474, and 100 mg/kg/d ZD6474). Scale bar, 10 μ m. *, $P < 0.001$.

groups occurred simultaneously with a 62% to 69% decrease in the tissue density of tumor endothelial cells ($P < 0.05$; Table 2), suggesting that ZD6474 selectively targeted endothelial cells with poor pericyte coverage.

Discussion

Two critical processes for the growth of solid tumors are tumor cell proliferation and angiogenesis. Increased expression of growth factors and their receptors, particularly EGFR, have been implicated in the progression of gastric cancer (11, 12). Gastric cancer progression is also associated with increased angiogenesis mediated by VEGF/VEGFR (5, 6). This study was undertaken to determine whether targeted therapy directed at these two pathways would have a significant effect on tumor growth in an orthotopic model of gastric cancer. A previous study showed that combining anti-VEGFR and anti-EGFR therapies was more effective than either therapy alone. Therefore, we investigated the ability of ZD6474, a small tyrosine kinase inhibitor with activity against both VEGFR and EGFR, to inhibit gastric cancer growth. *In vitro* studies confirmed that ZD6474 inhibited both EGFR and VEGFR2 phosphorylation in the presence of EGF or VEGF, respectively. In addition, ZD6474 showed selective inhibition of EGF-induced and VEGF-induced proliferation of TMK-1 human tumor cells and human endothelial cells, respectively. We also found that ZD6474 significantly decreases

orthotopic TMK-1 tumor growth at both 50 and 100 mg/kg/d. Finally, histologic studies suggested that ZD6474 affects both tumor cells and endothelial cells *in vivo*, resulting in decreased microvessel density and decreased tumor cell proliferation.

One major target of ZD6474 is the inhibition of VEGFR2 phosphorylation, resulting in inhibition of downstream signaling. Previous studies have determined inhibition of this receptor to be at nanomolar concentrations of ZD6474 (16). Indeed, our *in vitro* studies confirmed ZD6474 inhibition of VEGF-stimulated VEGFR2 phosphorylation, Erk-1/2 phosphorylation, and endothelial cell proliferation at relatively low doses (<100 nmol/L). The tumor vasculature is composed of multiple cell types, including endothelial cells and pericytes. Pericytes are derived from the smooth muscle cells that surround blood vessels and have many functions in the vasculature such as vessel contraction, regulation of blood flow, permeability, and protection and stabilization of the endothelium (26, 27). Previous studies suggested the importance of VEGF/VEGFR in endothelial cell survival, as withdrawal of VEGF led to selective apoptosis of endothelial cells not protected by pericytes (24). Based on the above findings, we can hypothesize that the anti-VEGFR2 activity of ZD6474 could act selectively on "unprotected" endothelial cells, leading to a selective loss of endothelial cells not associated with pericytes in the residual tumors. Such an effect might be expected to lead to a relative increase in the percentage

of endothelial cells covered by pericytes. A recent study using interleukin-12 as an antiangiogenic agent clearly showed that the reduced microvessel density seen after therapy was specifically attributed to a decrease in angiogenic sprouts that were not associated with pericytes (25). Additionally, in a previously published study by our group, a specific inhibitor of human EGFR (C225, cetuximab) was used in this orthotopic model of gastric cancer (8). In that study, treatment of gastric tumors with C225 did not affect angiogenesis as determined by microvessel density. Our data support the hypothesis that inhibition of VEGFR, but not EGFR, by ZD6474 leads to a significant relative increase in the percentage of endothelial cells covered by pericytes owing to loss of endothelial cells without pericyte coverage.

Evaluation of pathologic specimens has revealed a strong association between activation of the EGF/EGFR axis and growth of gastric cancer (11, 12). Preclinical studies using agents that target EGFR have provided further confirmation of its importance in gastric cancer growth (8, 28). Treatment of orthotopically implanted tumors with a monoclonal antibody targeting the EGFR (C225) as a single agent showed a trend toward decreased tumor growth (8), which was enhanced by the addition of anti-VEGFR2 therapy. In addition, the use of an adenoviral vector encoding antisense EGFR inhibited gastric tumor growth by 93% in a s.c. model of MKN28 gastric cancer (28). Previous studies using ZD6474 have consistently showed its anti-VEGF activity *in vivo* (16), and a recent publication also showed its potent anti-EGFR activity in fibroblasts and colon cancer xenografts (29). However, we wanted to confirm the activation of EGFR and determine the direct effects of this agent on the TMK-1 gastric cancer cell line both *in vivo* and *in vitro*. Our *in vitro* studies determined that EGFR is present and functional on these tumor cells. Our results confirmed that activation of EGFR and Erk-1/2 was significantly decreased by treatment with ZD6474 *in vitro*. This signaling inhibition was also associated with a decrease in tumor cell proliferation *in vitro*.

In conclusion, our data show that ZD6474 is effective at inhibiting the growth of gastric cancer growing orthotopically in nude mice and drug therapy is associated with a decrease in microvessel density and tumor cell proliferation, consistent with significant effects against both vascular and tumor cell compartments of the tumor. In addition, in this model, the effects of ZD6474 on tumor vessels seem to be more selective against vessels with poor pericyte coverage. These data confirm the importance of EGFR and VEGFR2 signaling in the growth and progression of gastric cancer and support the concept that simultaneous inhibition of both of these signaling pathways may have the potential to provide therapeutic benefit.

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