Antitumor Effects of ZD6474, a Small Molecule Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor, with Additional Activity against Epidermal Growth Factor Receptor Tyrosine Kinase[1]

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ABSTRACT

Purpose: Vascular endothelial growth factor (VEGF) is a major mitogen for endothelial cells and enhances vascular permeability. Enhanced VEGF secretion is found in human cancers and correlates with increased tumor neovascularization. ZD6474 is a p.o. bioavailable, VEGF flk-1/KDR receptor (VEGFR-2) tyrosine kinase inhibitor with antitumor activity in many human cancer xenografts and is currently in Phase I clinical development.

Experimental Design: We tested the effects of ZD6474 on EGFR phosphorylation in cell expressing functional epidermal growth factor receptor (EGFR) and the antiproliferative and the proapoptotic activity of ZD6474 alone or in combination taxanes in human cancer cell lines with functional EGFR but lacking VEGFR-2. The antitumor activity of this drug was also tested in nude mice bearing established GEO colon cancer xenografts.

Results: ZD6474 causes a dose-dependent inhibition of EGFR phosphorylation in mouse NIH-EGFR fibroblasts and human MCF-10A ras breast cancer cells, two cell lines that overexpress the human EGFR. ZD6474 treatment resulted in a dose-dependent inhibition of soft agar growth in seven human cell lines (breast, colon, gastric, and ovarian) with functional EGFR but lacking VEGFR-2. A dose-dependent supra-additive effect in growth inhibition and in apoptosis *in vitro* was observed by the combined treatment with ZD6474 and paclitaxel or docetaxel. ZD6474 treatment of nude mice bearing palpable GEO colon cancer xenografts (which are sensitive to inhibition of EGFR signaling) induced dose-dependent tumor growth inhibition. Immunohistochemical analysis revealed a significant dose-dependent reduction of neoangiogenesis. The antitumor activity of ZD6474 in GEO tumor xenografts was also found to be enhanced when combined with paclitaxel. Tumor regression was observed in all mice after treatment with ZD6474 plus paclitaxel, and it was accompanied by a significant potentiation in inhibition of angiogenesis. Six of 20 mice had no histological evidence of tumors after treatment with ZD6474 plus paclitaxel.

Conclusions: This study suggests that in addition to inhibiting endothelial cell proliferation by blocking VEGF-induced signaling, ZD6474 may also be able to inhibit cancer cell growth by blocking EGFR autocrine signaling. These results provide also a rationale for the clinical evaluation of ZD6474 combined with taxanes in cancer patients.

INTRODUCTION

Tumor angiogenesis is the process leading to the formation of blood vessels within a tumor and plays a key role in cancer cell survival in local tumor growth and in the development of distant metastases (1, 2). New blood vessels are required to provide an adequate oxygen and nutrient supply to the growing tumor mass and for initiating invasion and metastatic spread. Growth factors and growth inhibitors are known to regulate blood vessel development (1, 2). Certain growth factors and cytokines, including bFGF[3], VEGF, interleukin 8, and TGF-α,
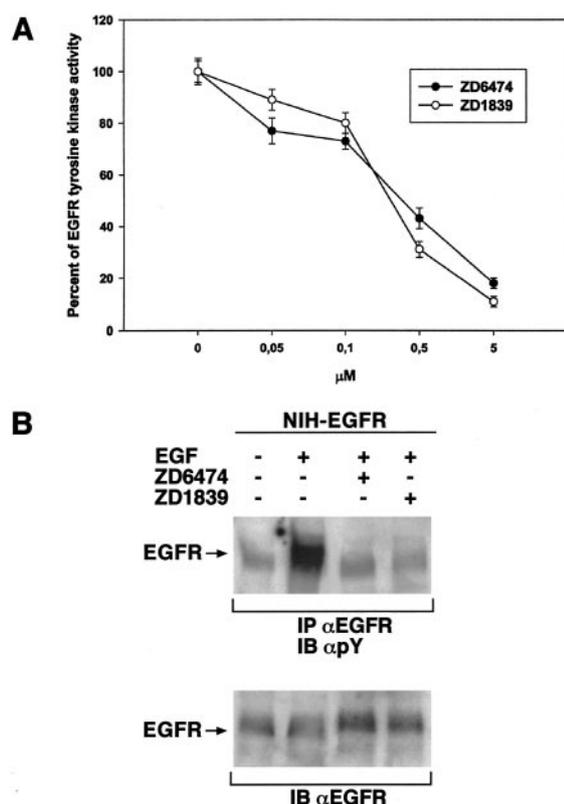
[3] The abbreviations used are: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; MAb, monoclonal antibody; TKI, tyrosine kinase inhibitor; MVC, microvessel count.

A, dose-dependent inhibition of EGFR tyrosine kinase activity by ZD6474 or by ZD1938 in mouse NIH-EGFR fibroblasts.

B

Fig. 1 A, dose-dependent inhibition of EGFR tyrosine kinase activity by ZD6474 or by ZD1938 in mouse NIH-EGFR fibroblasts. Data represent the average (±SD) of triplicate determinations. B, dose-dependent inhibition of EGF-induced EGFR autophosphorylation by ZD6474 or by ZD1839 in mouse NIH-EGFR fibroblasts. Mouse NIH-EGFR fibroblasts were treated for 6 h with ZD6474 (1 μM) or with ZD1839 (1 μM) in presence or in the absence of EGF (20 ng/ml). Protein extracts were immunoprecipitated with the MAb 528 anti-EGFR MAb, resolved by a 7.5% SDS-PAGE, and probed with either the PY20 anti-P-tyr MAb (*top panel*) or an antihuman EGFR MAb (*bottom panel*). Immunoreactive proteins were visualized by enhanced chemiluminescence.

Fig. 2 Dose-dependent inhibition of EGF-induced EGFR autophosphorylation by ZD6474 or by ZD1839 in human MCF-10A ras cells. Serum-starved MCF-10A ras cells were treated for 3 h with ZD6474 or with ZD1839 (0.1, 0.5, or 1 μM), followed by addition of complete medium containing EGF (20 ng/ml) for 15 min. Protein extracts were immunoprecipitated with the MAb 528 anti-EGFR MAb, resolved by a 7.5% SDS-PAGE, and probed with either the PY20 anti-P-tyr MAb (*A*) or an antihuman EGFR MAb (*B*). Immunoreactive proteins were visualized by enhanced chemiluminescence. *C*, Control untreated cells: *Lane 1*, cells treated with ZD1839 (0.1 μM); *Lane 2*, cells treated with ZD6474 (0.1 μM); *Lane 3*, cells treated with ZD1839 (0.5 μM); *Lane 4*, cells treated with ZD6474 (0.5 μM); *Lane 5*, cells treated with ZD1839 (1 μM); and *Lane 6*, cells treated with ZD6474 (1 μM).

have been identified as positive regulators of angiogenesis and are generally secreted by cancer cells to stimulate endothelial cell growth through paracrine mechanisms (1, 2). VEGF is a potent and specific mitogen for endothelial cells that activates the angiogenic switch *in vivo* and enhances vascular permeability (3, 4). VEGF binds to two distinct receptors on endothelial cells, flt-1 (VEGFR-1) and flk-1/KDR receptor (VEGFR-2; Refs. 3, 4). VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell permeability, proliferation, and differentiation (3, 4). Enhanced expression of VEGF is generally correlated with increased neovascularization as measured by MVC within the tumor (5). Moreover, the density of microvessels in the areas of most intense neovascularization has been demonstrated to be an independent prognostic factor in breast and non-small cell lung cancer patients (6–8). VEGF expression can be increased in cancer cells by different mechanisms, most notably hypoxia (2–4). Additionally, activation of EGFR signaling by EGF or by TGF-α can up-regulate the production of VEGF in human cancer cells (9, 10). In this

respect, we and others have provided evidence that EGFR blockade causes inhibition of the secretion of VEGF and of other angiogenic growth factors, including bFGF, interleukin 8, and TGF-α (11–16).

The increasing understanding of the molecular mechanisms that control angiogenesis has allowed the development of drugs that could interfere with this process (2, 17). Among the approaches that have been proposed for blocking VEGF-induced endothelial cell proliferation and subsequent tumor angiogenesis, a neutralizing anti-VEGF MAb, blocking MAbs against the VEGFR-2 or selective inhibitors of the VEGFR-2 tyrosine kinase, are currently in preclinical and clinical development (18–27). ZD6474 is a recently described p.o. bioavailable, small molecule VEGFR-2 TKI, with an IC_{50} of ~0.04 μM against the isolated enzyme activity *in vitro* (28, 29). ZD6474 has no effect on other isolated kinases, including platelet-derived growth factor receptor b, FGFR1, c-erbB-2, c-kit, type 1 insulin-like growth factor receptor (29). This compound significantly inhibits tumor growth in a broad range of established human cancer xenografts in nude mice and is currently in Phase I evaluation in cancer patients (28–31). ZD6474 is an anilinoquinazoline. Quinazolines are one of the most promising classes of small molecule inhibitors of different growth factor receptor tyrosine kinases (32, 33). In this respect, another anilinoquinazoline derivative, ZD1839 (Iressa), is a potent and selective EGFR-TKI, which is currently in advanced clinical development (34, 35).

ZD6474 has also been shown to inhibit isolated EGFR tyrosine kinase *in vitro* and EGFR signaling in endothelial cells: the IC_{50} for inhibition of EGF-induced human umbilical vascular endothelial cell proliferation is ~3-fold greater than that required to inhibit VEGF-induced proliferation (170 and 60 nM, respectively; Ref. 29). These data prompted us to evaluate whether ZD6474 could also demonstrate significant EGFR-TKI activity in tumor cells. In this study, we show that ZD6474: (*a*) inhibits EGFR tyrosine kinase activity in intact tumor cells; (*b*) inhibits the *in vitro* growth of several human cancer cell lines that express functional EGFR but do not express VEGFR-2; (*c*) potentiates the antitumor activity of the cytotoxic drugs do-
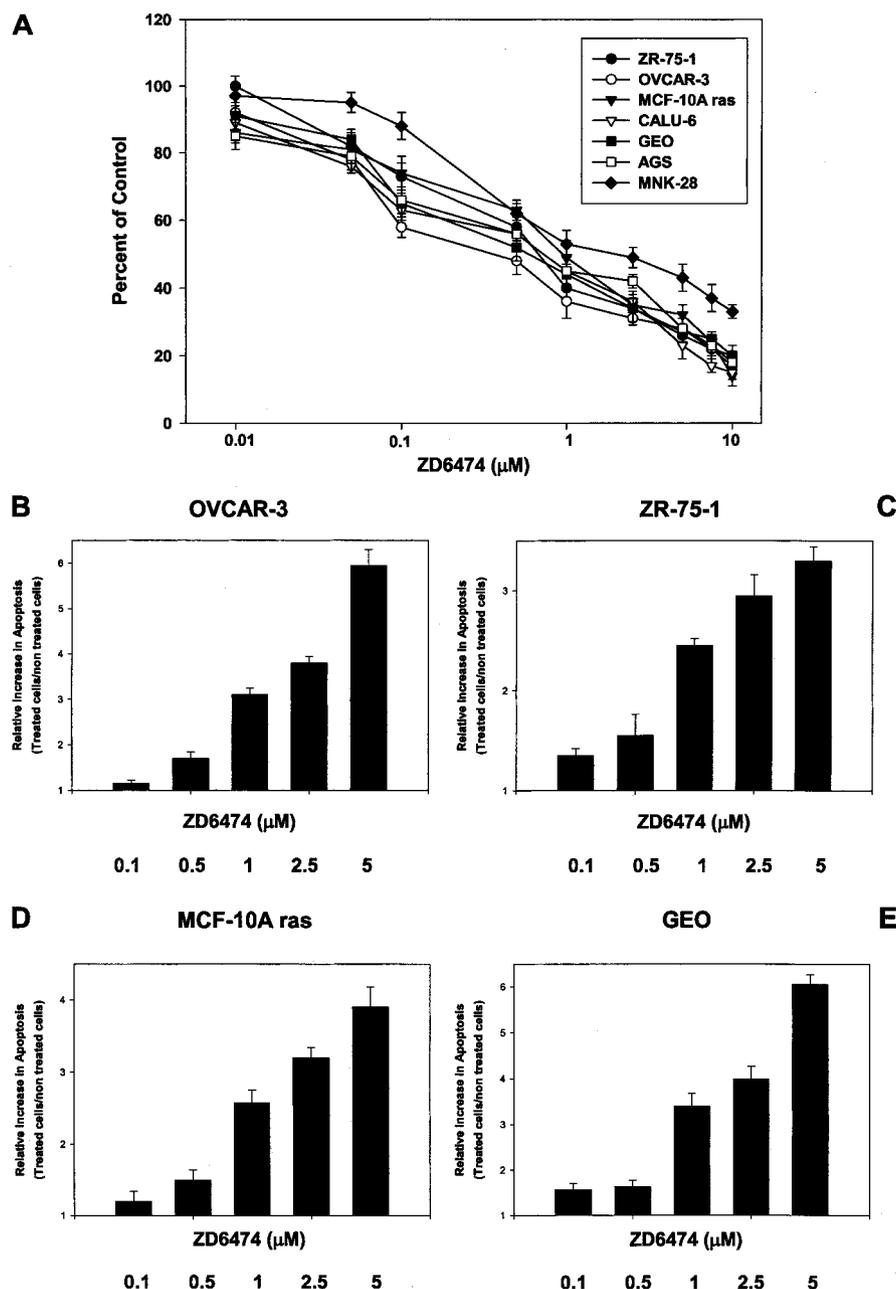
Fig. 3 A, dose-dependent growth inhibitory effects of ZD6474 on the soft agar growth of human ZR-75-1, MCF-10A ras, OVCAR-3, CALU-6, AGS, MNK-28, and GEO cells. Cells were treated with the indicated concentrations of ZD6474 each day for 5 consecutive days. Colonies were counted after 10–14 days. Data represent the average (±SD) of three different experiments, each performed in triplicate. *B–E*, dose-dependent induction of programmed cell death by treatment with ZD6474 in human OVCAR-3, ZR-75-1, MCF-10A ras, and GEO cells, respectively. Cells were treated each day for 3 days with the indicated doses of ZD6474. Data represent the average (±SD) of quadruplicate determinations.

cetaxel and paclitaxel *in vitro* in several human cancer cell lines that express functional EGFR but do not express VEGFR-2; and (*d*) potentiates the antitumor activity of paclitaxel *in vivo*. These results provide experimental evidence that ZD6474 is a small molecule TKI that can block both angiogenesis by inhibiting the VEGF signaling pathway in endothelial cells and also tumor cell growth by inhibiting the EGFR autocrine pathway.

MATERIALS AND METHODS

Drugs. ZD6474 and ZD1839 were kindly provided by AstraZeneca Pharmaceuticals, Macclesfield (United Kingdom). Paclitaxel was purchased from Bristol Myers Squibb Italia

(Rome, Italy). Docetaxel was purchased from Aventis Italia (Milan, Italy).

Cell Lines. All human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF-10A ras cells have been obtained by cotransfection of human nontransformed MCF-10A cells with an expression vector plasmid containing the human activated c-Ha-*ras* proto-oncogene and an expression vector plasmid containing the neomycin-resistance gene (36). NIH-EGFR cells were kindly provided by Dr. Pier Paolo P. Di Fiore (European Institute of Oncology, Milan, Italy). NIH-EGFR cells were obtained after transfection of mouse NIH-3T3 fibroblasts with an expression

Table 1 Effects of ZD6474 treatment on cell cycle distribution

	Treatment	G_0-G_1 (%)	S (%)	G_2-M (%)
GEO colon cancer cells	Control	61	32	7
	ZD6474 (0.1 μM)	72	20	8
	ZD6474 (1 μM)	78	15	7
	ZD6474 (2.5 μM)	84	10	6
OVCAR-3 ovarian cancer cells	Control	56	34	10
	ZD6474 (0.1 μM)	62	28	10
	ZD6474 (1 μM)	73	18	9
	ZD6474 (2.5 μM)	82	10	8

vector plasmid containing the full-length human EGFR cDNA (37). NIH-EGFR, GEO, OVCAR-3, ZR-75-1, CALU-6, AGS, and MNK-28 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), penicillin (100 UI/ml), streptomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. MCF-10A Ha-_ras_ cells were grown in a 1:1 (v/v) DMEM and Ham's F-12 mixture, supplemented with 5% heat-inactivated horse serum, 20 mM HEPES (pH 7.4), 4 mM glutamine, 0.5 μg/ml hydrocortisone (Sigma), 10 ng/ml EGF, 10 μg/ml insulin (Collaborative Research Products, Bedford, MA), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. All cancer cell lines expressed both EGFR and TGF-α but not VEGFR-1 or VEGFR-2 (Ref. 38 and our unpublished data).

In Vitro **EGFR Tyrosine Kinase Assay.** Subconfluent NIH-EGFR was stimulated with EGF, 100 ng/ml for 10 min, before it was harvested and solubilized in lysis buffer as described previously (37). Two hundred μg of total protein extracts were immunoprecipitated with MAb 528 anti-EGFR monoclonal antibody (kindly provided by Dr. John Mendelsohn, M. D. Anderson Cancer Center, Houston, TX). Immunocomplexes were recovered with protein A Sepharose beads and incubated for 20 min at room temperature in a buffer containing 200 μM poly[L-glutamic acid-L-tyrosine (Sigma)], 2.5 μCi (γ-^{32}P)ATP, and unlabelled ATP to a final concentration of 20 μM in the presence or the absence of different concentrations of ZD6474 or of ZD1839. Samples were spotted on Whatman 3MM paper (Springfield Mill, Whatman, United Kingdom), and ^{32}P incorporation was measured with a beta counter scintillator (Beckman).

Immunoprecipitation and Western Blot Analysis. Total cell protein extracts were obtained as previously described (39) from serum-starved NIH-3T3 EGFR cells or MCF-10A ras cells that were treated with complete medium containing EGF (20 ng/ml) in the presence or in the absence of the indicated concentrations of ZD6474 or of ZD1839. Proteins were immunoprecipitated with MAb 528 as reported previously (39). For Western blot analysis, immunoprecipitates were resolved by a 7.5% SDS-PAGE and probed with either an antihuman EGFR monoclonal antibody (Transduction Laboratories, Lexington, KY) or the PY20 anti-P-tyrosine monoclonal antibody (Transduction Laboratories). For VEGF expression, total cell lysates were obtained from homogenated GEO tumor specimens, and protein extracts were resolved by a 7.5% SDS-PAGE and probed either with an antihuman VEGF monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or with an antihuman actin monoclonal antibody (Transduction Laboratories). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, London, United Kingdom), as described previously (39).

Growth in Soft Agar. Cells (10^4 cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated with different concentrations of ZD6474 alone and/or in combination with the indicated concentrations of docetaxel or paclitaxel. After 10 to 14 days, cells were stained with nitro blue tetrazolium (Sigma), and colonies > 0.05 mm were counted as described previously (39).

Evaluation of Apoptosis. The induction of programmed cell death was determined as previously reported (39) by the Cell Death Detection ELISA Plus Kit (Boeheringher Mannheim, Indianapolis, IN). Briefly, 5×10^4 cells/well were seeded into 6-multiwell cluster dishes. After appropriate treatment with different concentrations of ZD6474 alone or in combination with docetaxel or paclitaxel, cells were washed with PBS, and 0.5 ml of lysis buffer was added. After a 30-min incubation, the supernatant was recovered and assayed for DNA fragments as recommended by the manufacturer at 405 nm using a Microplate Reader Model 3550-UV (Bio-Rad, Milan, Italy). Each treatment was performed in quadruplicate. Additional plates identically treated were analyzed for cell number with a hemocytometer to normalize the values for cell numbers. The results are expressed as relative to untreated control samples.

Flow Cytometric Analysis of Cell Cycle Distribution and of Induction of Apoptosis. To additionally evaluate the induction of apoptosis, 10^5 cells (GEO and OVCAR-3) were plated in complete medium in 60-mm tissue culture dishes (Becton Dickinson) and treated every day for a total of 3 days with different concentrations of ZD6474. After 4 days, both adherent and detached cells were harvested. Flow cytometric analysis of apoptotic cell death was performed on cell pellet fixed in 70% ethanol, washed in PBS, and mixed with RNase (Sigma) and propidium iodide (Sigma) solution as reported previously (40). DNA content was analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA) coupled with a Hewlett Packard computer, and the percentage of apoptotic cells was calculated by gating the hypodiploid region on the DNA content histogram using the Lysys software (Becton Dickinson) as reported previously (40). Cell cycle data analysis was performed using the CELL-FIT software (Becton Dickinson) as reported previously (40).

GEO Xenografts in Nude Mice. Four to 6-week-old female BALB/c athymic (nu+/nu+) mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with the institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized at the University of Naples Medical School Animal Facility for 1 week before they were injected with cancer cells. Mice received s.c. injections of 10^7 GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA).
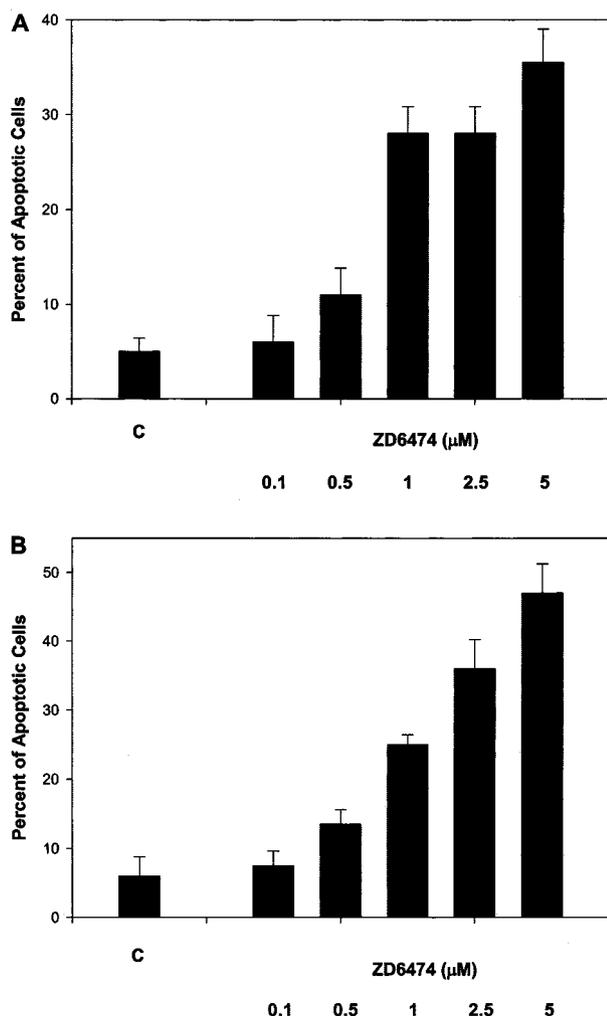
Fig. 4 Flow cytometric analysis of induction of programmed cell death by treatment with ZD6474 in human OVCAR-3 (*A*) and GEO (*B*) cells, respectively. Cells were treated each day for 3 days with the indicated doses of ZD6474. Data represent the average (±SD) of triplicate determinations.

Fig. 5 Growth inhibitory effects of treatment with ZD6474 (0.01, 0.05, 0.1 μM) in combination with docetaxel (*A*) or with paclitaxel (*B*) on the soft agar growth of human GEO cells. Cells were treated with the indicated concentrations of cytotoxic drug on day 1 followed by the indicated concentrations of ZD6474 on each day from day 2 to day 6. Colonies were counted after 10–14 days. Data represent the average (±SD) of three different experiments, each performed in triplicate.

After 7 days, when established tumors of ~0.2–0.3 cm³ in diameter were detected, 10 mice/group were treated i.p. on days 1–5 of each week for the indicated times with ZD6474 at the indicated daily doses. To determine the effects of the combination of ZD6474 and paclitaxel, additional groups of 10 mice were treated i.p. on days 1–5 of each week for 4 weeks with different doses of ZD6474 in combination with paclitaxel (20 mg/kg) on day 1 of each week for 4 weeks. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

Immunohistochemical Analysis. Immunocytochemistry was performed on formalin-fixed, paraffin-embedded tissue sections (5 μm) of GEO xenografts as reported previously (11, 16). After overnight incubation with the appropriate primary antibody at 4°C, sections were washed and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit, Vector Labo-
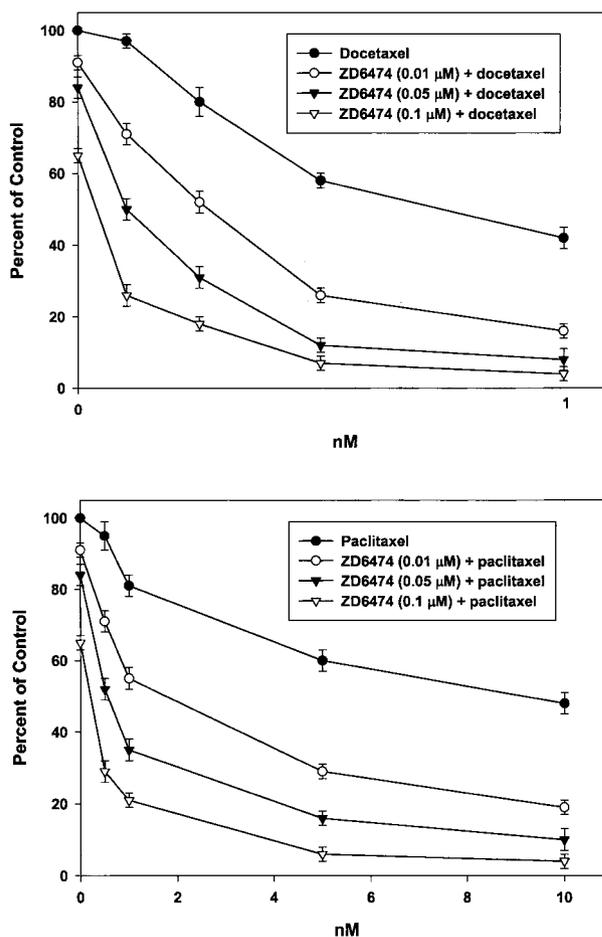
ratory, Burlingame, CA), washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as described previously (16). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. The following antibodies were used: an anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) used at 1:100 dilution; an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology) used at 1:50 dilution; an anti-bFGF rabbit polyclonal antibody (Santa Cruz Biotechnology) used at 1:200 dilution; and an antihuman TGF-α mouse monoclonal antibody (Ab-2; Oncogene Science, Manhasset, NY) used at 1:100 dilution. To determine the percentage of positive cells, at least 1000 cancer cells/slide were counted and scored (11, 16). Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. New blood vessels were detected using a monoclonal antibody raised against the human factor VIII-related antigen (Dako, Milan, Italy) at the dilution
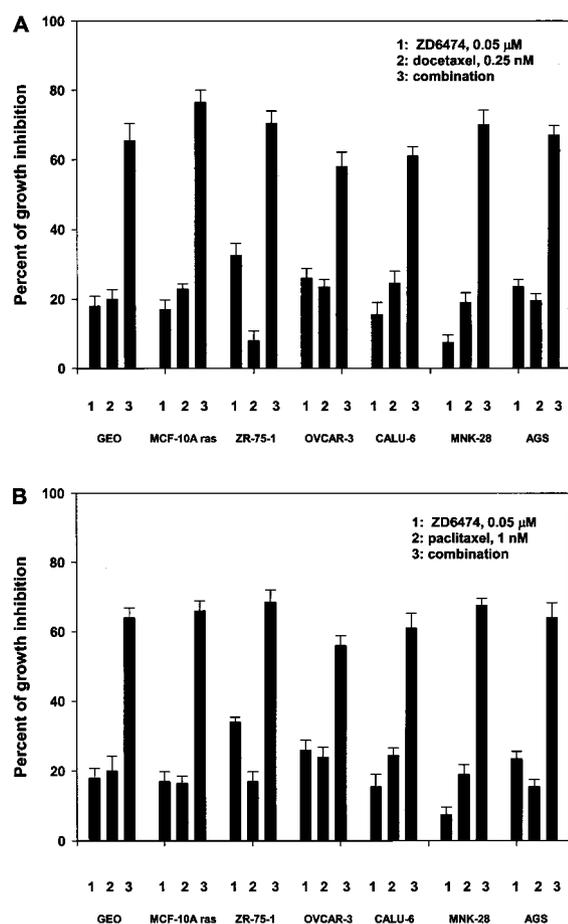
Fig. 6 Growth inhibitory effects of the combined treatment with ZD6474 and docetaxel (*A*) or paclitaxel (*B*) on the soft agar growth of GEO, MCF-10A ras, ZR-75-1, OVCAR-3, CALU-6, MNK-28, and AGS cancer cell lines. Cells were plated in soft agar and treated with the indicated concentration of cytotoxic drug on day 1 and with ZD6474 (0.05 μM) on days 2–6 or with each agent alone. Colonies were counted after 10–14 days. Data are expressed as percentage growth inhibition as compared with the growth of untreated control cells. For each cell line, *Lane 1*, docetaxel (or paclitaxel); *Lane 2*, ZD6474; *Lane 3*, docetaxel (or paclitaxel) plus ZD6474. Data represent the average ($\pm$SD) of three different experiments, each performed in duplicate.

of 1:50 and stained with a standard immunoperoxidase method (Vectastain ABC kit). Each slide was scanned at low power ($\times$10–100 magnification), and the area with the higher number of new vessels was identified (hot spot). This region was then scanned at $\times$250 microscope magnification (0.37 mm^2). Five fields were analyzed, and for each of them, the number of stained blood vessels was counted. For individual tumors, MVC was scored by averaging the five field counts (11, 16).

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

RESULTS

To determine whether ZD6474 treatment could interfere with EGFR activation in whole cells, we evaluated EGFR tyrosine kinase activation in mouse NIH-3T3 fibroblasts that overexpress the human EGFR after transfection with a plasmid containing the human EGFR cDNA (NIH-EGFR cells; Ref. 37). As illustrated in Fig. 1*A*, a dose-dependent inhibition of EGFR tyrosine kinase activity was observed after treatment with ZD6474 (IC$_{50}$ of ~0.25 μM). This effect was comparable with that observed by treatment of NIH-EGFR cells with ZD1839, a potent and selective EGFR-TKI included as a positive control in the experiments (Fig. 1*A*). Inhibition of EGFR autophosphorylation was also detected after immunoprecipitation with an anti-EGFR MAb and subsequent Western blotting with an antiphosphotyrosine antibody of NIH-EGFR cell extracts treated with 1 μM ZD6474 or with 1 μM ZD1839 (Fig. 1*B*). A similar dose-dependent inhibition of EGFR phosphorylation was also demonstrated in human MCF-10A ras-transformed mammary epithelial cells after treatment with equivalent doses of ZD6474 or of ZD1839 (0.1, 0.5, or 1 μM; Fig. 2).

To determine whether ZD6474 could have a direct antiproliferative effect on cancer cell growth, seven human cancer cell lines, including breast (ZR-75–1, MCF-10A ras), ovarian (OVCAR-3), non-small cell lung (CALU-6), colon (GEO), gastric (AGS, MNK-28) cancer cells, that have functional EGFRs and secrete high levels of TGF-α, the EGFR-specific ligand, but that lack both VEGFR-1 and VEGFR-2 (Ref. 38 and our unpublished data), were treated with different doses of ZD6474 in an anchorage-independent growth assay. As shown in Fig. 3*A*, ZD6474 treatment resulted in a dose-dependent inhibition of colony formation in soft agar with an IC$_{50}$ ranging between 0.5 and 1 μM. Generally, treatment with a selective EGFR inhibitor such as ZD1839 or the blocking anti-EGFR MAb C225 has a cytostatic effect with cell cycle arrest in the G$_1$ phase (35, 40). However, in some cancer cell lines, EGFR inhibitors may also induce apoptosis, particularly at higher doses (35, 41). To evaluate whether ZD6474 treatment could cause any specific perturbation of the cell cycle, analysis of GEO and OVCAR-3 cells treated with different doses of ZD6474 was performed (Table 1). Compared with control untreated cells, ZD6474 treatment caused an accumulation of cells in the G$_0$-G$_1$ phases in both cell lines. For example, in GEO cells, the percentage of G$_0$-G$_1$ cells increased from 61 to 84% after treatment with ZD6474, 1 μM (Table 1). Next, we evaluated if the ZD6474-induced antiproliferative effect was accompanied by induction of programmed cell death. Fig. 3, *B–E*, shows that a dose-dependent increase in apoptosis was observed in the four human cancer cell lines that were treated with ZD6474 (up to a 6-fold enhancement with 5 μM ZD6474). The induction of apoptosis by ZD6474 treatment was confirmed by assessing the percentage of fragmented DNA by flow cytometric evaluation of propidium iodide staining of OVCAR-3 and GEO cancer cells (Fig. 4, *A* and *B*).

We and others have demonstrated that the inhibition of EGFR signaling potentiates the antitumor activity of different cytotoxic drugs and that this effect is particularly strong with taxanes (15, 16, 39–45). To examine this effect with ZD6474, GEO cancer cells were used because we have shown previously that this cancer cell line is sensitive to inhibition of EGFR
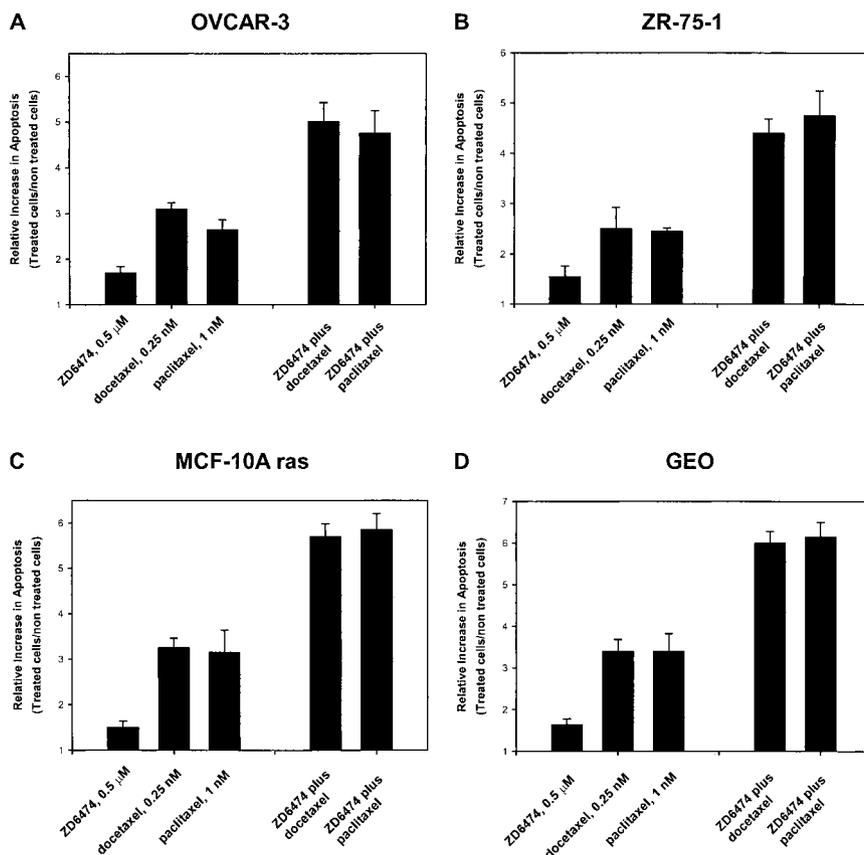
A OVCAR-3

B ZR-75-1

C MCF-10A ras

D GEO

Fig. 7 Induction of apoptosis by treatment with ZD6474 in combination with taxanes in OVCAR-3 (*A*), ZR-75-1 (*B*), MCF-10A ras (*C*), and GEO (*D*). Cells were treated with ZD6474 (0.5 μM) each day for 3 days; docetaxel (0.25 nM) on day 1; paclitaxel (1 nM) on day 1; or with the combination of ZD6474 and each taxane. On day 4, the cells were collected. Data are presented as relative increase in apoptosis of treated cells compared with untreated control cells and represent the average (SD) of quadruplicate determinations.

signaling with EGFR blocking antibodies, EGFR antisense oligonucleotides, and EGFR tyrosine kinase inhibition (11, 35, 39). As illustrated in Fig. 5, a supra-additive growth inhibition of colony formation in soft agar was observed in GEO colon cancer cells treated with the combination of different concentrations of ZD6474 and docetaxel or paclitaxel. A similar potentiation of the antiproliferative activity of docetaxel or paclitaxel was observed also when the other six human cancer cell lines were treated with ZD6474 (Fig. 6 and data not shown).

We next examined whether the cooperative growth inhibitory effect of taxanes and ZD6474 could involve induction of programmed cell death in cancer cells. OVCAR-3, ZR-75–1, MCF-10A ras, and GEO cells were treated with docetaxel, 0.25 nM or with paclitaxel, 1 nM, alone or in combination with ZD6474, 0.5 μM, a dose that induced only a small increase in apoptosis. In these experiments, ZD6474-potentiated apoptosis induced by both taxanes by ~2–3-fold in each of the four cancer cell lines tested (Fig. 7).

GEO tumor growth was also inhibited by ZD6474 *in vivo*. ZD6474 treatment (25–150 mg/kg/day) of nude mice bearing established GEO tumor xenografts (0.25 cm^3 in volume) produced a dose-dependent inhibition of tumor growth (Fig. 8*A*). This effect was cytostatic rather than cytotoxic. In fact, GEO tumors resumed a growth rate comparable with controls within 1–2 weeks of the termination of treatment (data not shown). ZD6474 treatment was well tolerated by mice with no signs of acute or delayed toxicity. Because GEO cells form moderately

differentiated adenocarcinomas that express the EGFR and various autocrine and paracrine growth factors, including TGF-α, bFGF and VEGF, when injected s.c. in immunodeficient mice (11), we have evaluated the effect of ZD6474 treatment on the production of these growth factors *in vivo*. Immunohistochemical evaluation of the expression of TGF-α, bFGF, and VEGF, which was performed on GEO tumors at the end of the 2 weeks of treatment, revealed a marked and dose-dependent reduction in the percentage of positive GEO cells for all three growth factors that paralleled a dose-dependent reduction in GEO tumor cell proliferation, as assessed by Ki67 nuclear staining (Table 2). On the same tumor samples, we examined VEGF expression by Western blotting (Fig. 9). We observed a marked inhibition of VEGF expression in GEO tumors from mice treated with ZD6474 (25 mg/kg/dose or 50 mg/kg/dose, Fig. 9, *Lanes 3* and *4*, respectively). Furthermore, to assess a direct effect on neo-angiogenesis of ZD6474 treatment, tumor-induced vascularization was quantified by immunohistochemistry as MVC in the areas of most intense neovascularization, using an antifactor VIII-related antigen MAb (11, 16). ZD6474 treatment substantially reduced MVC in a dose-dependent manner. In fact, a 50% reduction in MVC was detected with the lowest dose tested (25 mg/kg/dose), whereas an almost complete suppression in MVC was observed at 100 or 150 mg/kg/dose.

We have previously demonstrated that anti-EGFR-selective drugs such as ZD1839 significantly enhance the antitumor activity of the cytotoxic drug paclitaxel in nude mice bearing
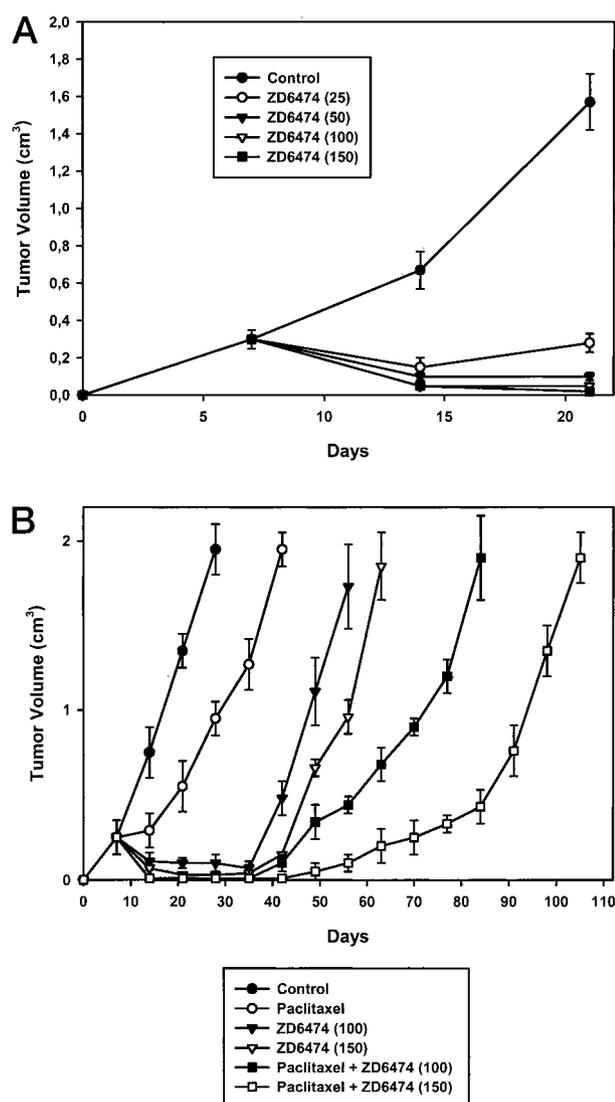
Fig. 8 A, antitumor activity of ZD6474 treatment on established GEO human colon carcinoma xenografts. Mice were injected s.c. into the dorsal flank with 10^7 GEO cells. After 7 days (average tumor size, 0.25 cm^3), the mice were treated i.p. on days 1–5 of each week for 2 weeks with ZD6474 at the indicated daily doses. Each group consisted of 10 mice. Data represent the average ($\pm$ SD). Student's *t* test was used to compare tumor sizes among different treatment groups at day 21 after GEO cell injection. ZD6474 (25 mg/kg/dose) *versus* control (two-sided $P < 0.01$); ZD6474 (50 mg/kg/dose) *versus* control (two-sided $P < 0.01$); ZD6474 (100 mg/kg/dose) *versus* control (two-sided $P < 0.01$); and ZD6474 (150 mg/kg/dose) *versus* control (two-sided $P < 0.01$). B, antitumor activity of ZD6474 treatment in combination with paclitaxel on established GEO human colon carcinoma xenografts. Mice were injected s.c. in the dorsal flank with 10^7 GEO cells. Data represent the average ($\pm$SD). After 7 days (average tumor size, 0.2–0.3 cm^3), mice were treated i.p. on days 1–5 of each week for 4 weeks with ZD6474 (100 or 150 mg/kg/dose), alone or in combination with paclitaxel (20 mg/kg/dose) on day 1 of each week for 4 weeks.

GEO xenografts (39). Previous studies have also suggested that, at least in some settings, paclitaxel treatment may affect tumor neovascularization *in vivo* (46). We therefore tested the effect of the combined treatment of paclitaxel and ZD6474. Nude mice

bearing established GEO xenografts (0.25 cm^3 in volume) were treated with paclitaxel (20 mg/kg/dose) on day 1 of each week for 4 weeks and/or with ZD6474 (range, 25–150 mg/kg/dose) on days 1–5 of each week for 4 weeks. As shown in Fig. 8*B*, the antitumor effect of ZD6474 in combination with paclitaxel was greater than for either agent alone, with greater suppression of tumor growth at the end of the 4 weeks in both combination treatment groups compared with the control group or compared with groups treated with a single agent (Table 3). For example, GEO tumors in mice treated with paclitaxel plus ZD6474 (100 or 150 mg/kg/dose) reached a mean tumor volume of ~2 cm^3 within an average period of 86 ($\pm$4) or 108 ($\pm$6) days, respectively, as compared with control untreated mice (28 $\pm$ 3 days; Table 3). Furthermore, the combined treatment was highly effective also in terms of cure with tumor eradication because no histological evidence of GEO tumors was observed in 2 of 10 mice and in 4 of 10 mice in these two groups of mice, respectively (Table 3). Combined treatments with ZD6474 and paclitaxel at the doses and schedule tested were well tolerated by mice, with no significant weight loss or other signs of acute or delayed toxicity. Paclitaxel treatment only slightly affected the expression of TGF-α, bFGF, and VEGF and induced a reduction in MVC from 18 to 15 MVC/field compared with control mice. In contrast, the greatest suppression of TGF-α, bFGF, and VEGF expression and of MVC was generally observed after combined treatment with ZD6474 (25 or 50 mg/kg/dose) and paclitaxel (Table 2). Finally, Western blot analysis on GEO tumor extracts revealed a reduction in VEGF expression to almost undetectable levels after the combined treatment with ZD6474 plus paclitaxel (Fig. 9).

DISCUSSION

Inhibition of growth factor receptor activation and/or intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of selective anticancer therapies (47). A direct approach for the therapeutic blockade of growth factor receptor signals in human cancer has been recently developed with the discovery of low molecular weight compounds that inhibit ligand-induced activation of growth factor receptor tyrosine kinase enzymatic activity (32, 33). Growth factor receptors of the EGFR family are considered a valuable target for cancer therapy. In this respect, various selective EGFR-TKIs have been successfully tested as anticancer drugs *in vitro* and in preclinical models and a number are now in late stage clinical development (34, 35).

Another family of growth factor receptors that have been proposed as targets for cancer therapy is represented by the cell membrane receptors for angiogenic growth factors on endothelial cells. In this respect, various small molecules TKIs with antiangiogenic properties are currently in preclinical as well as in early clinical development (2). ZD6474 is an anilinoquinazoline derivative with a potent inhibitory activity on the VEGFR-2 tyrosine kinase and is currently in Phase I clinical trials in cancer patients (28–31).

In this study, we also report that ZD6474 is an inhibitor of EGFR tyrosine kinase. We demonstrate that ZD6474 blocks EGFR tyrosine kinase activation in cells that are devoid of VEGFRs and which overexpress the EGFR (NIH-EGFR mouse

Table 2 Immunohistochemical analysis of GEO colon cancer xenografts after treatment with ZD6474 and paclitaxel

| Treatment	Tumor volume (cm^3)	Ki67 (% positive cells)	TGF-α (% positive cells)	bFGF (% positive cells)	VEGF (% positive cells)	Factor VIII-related antigen (MVCs)
Control	1.45 (±0.2)	70 (±5)	70 (±5)	65 (±4)	70 (±8)	18 (±2)
ZD6474 (25 mg/kg)	0.38 (±0.05)	50 (±8)	55 (±6)	45 (±5)	50 (±7)	9 (±2)
ZD6474 (50 mg/kg)	0.24 (±0.05)	40 (±6)	40 (±6)	30 (±5)	35 (±3)	6 (±2)
ZD6474 (100 mg/kg)	0.15 (±0.03)	25 (±5)	35 (±6)	25 (±3)	20 (±4)	1 (±1)
ZD6474 (150 mg/kg)	0.05 (±0.01)	20 (±5)	25 (±4)	15 (±5)	10 (±3)	1 (±1)
Paclitaxel (400 μg)	0.65 (±0.1)	50 (±7)	65 (±5)	60 (±5)	60 (±6)	15 (±3)
Paclitaxel (400 μg) + ZD6474 (25 mg/kg)	0.10 (±0.02)	30 (±5)	20 (±6)	10 (±4)	10 (±5)	3 (±1)
Paclitaxel (400 μg) + ZD6474 (50 mg/kg)	0.05 (±0.02)	25 (±4)	15 (±2)	10 (±3)	5 (±1)	1 (±1)
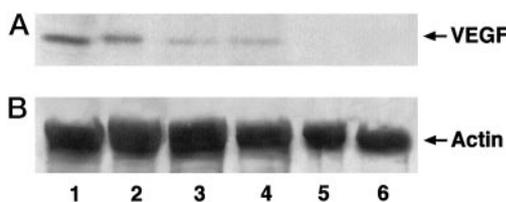
Fig. 9 Western blot analysis of VEGF expression in human GEO colon cancer xenografts. Mice were injected with GEO cells and were treated with paclitaxel, ZD6474, or with a combination of both drugs as reported in Fig. 8. *Lane 1*, control, untreated tumors; *Lane 2*, tumors from mice treated with paclitaxel (20 mg/kg/dose) on day 1 of each week for 2 weeks; *Lane 3*, tumors from mice treated on days 1–5 of each week for 2 weeks with ZD6474 (50 mg/kg/dose); *Lane 4*, tumors from mice treated on days 1–5 of each week for 2 weeks with ZD6474 (100 mg/kg/dose); *Lane 5*, tumors from mice treated with paclitaxel (20 mg/kg/dose) on day 1 of each week for 2 weeks plus ZD6474 (50 mg/kg/dose) on days 1–5 of each week for 2 weeks; *Lane 6*, tumors from mice treated with paclitaxel (20 mg/kg/dose) on day 1 of each week for 2 weeks plus ZD6474 (100 mg/kg/dose) on days 1–5 of each week for 2 weeks. Fifty μg of total cell proteins were fractionated through 12% SDS-PAGE, transferred to nitrocellulose filters, incubated with (*A*) a specific anti-human VEGF monoclonal antibody or (*B*) with an antihuman actin monoclonal antibody.

Table 3 Antitumor activity of ZD6474 alone or in combination with paclitaxel on GEO human colon cancer xenografts

Treatment	Average tumor volume on day 28 after tumor cell injection (cm^3)	Average time (days) to reach a tumor volume of ~2 cm^3
Control	1.95 (±0.15)	28 (±3)
Paclitaxel	0.95 (±0.1)	42 (±3)
ZD6474 (25 mg/kg)	0.48 (±0.1)	44 (±2)
ZD6474 (50 mg/kg)	0.26 (±0.1)	50 (±4)
ZD6474 (100 mg/kg)	0.1 (±0.05)	57 (±2)
ZD6474 (150 mg/kg)	0.03 (±0.01)	66 (±4)
Paclitaxel + ZD6474 (25 mg/kg)	0.22 (±0.1)	65 (±3)
Paclitaxel + ZD6474 (50 mg/kg)	0.03 (±0.01)	76 (±5)
Paclitaxel + ZD6474 (100 mg/kg)	0.01 (±0.01)	86 (±4)[a]
Paclitaxel + ZD6474 (150 mg/kg)	0.01 (±0.01)	108 (±6)[b]

[a] Two of 10 mice were without histologic evidence of GEO tumors at this time point.

[b] Four of 10 mice were without histologic evidence of GEO tumors at this time point.

fibroblasts and Ha-ras-transformed MCF-10A human mammary epithelial cells) in a dose-dependent manner. These effects occur in a dose range that is comparable with that of the selective EGFR-TKI, ZD1839. ZD6474 treatment also causes a dose-dependent inhibition of cell growth and induction of apoptosis in seven human cancer cell lines that express EGFR, secrete TGF-α, but lack both VEGFR-1 and VEGFR-2, suggesting that the antiproliferative effect of ZD6474 on these cells is probably attributable to the inhibition of the EGFR mitogenic signaling. Furthermore, similar to the results obtained with ZD1839 or with other anti-EGFR agents such as C225 (35, 41), we have found a significant potentiation of induction of programmed cell death, of cytotoxicity *in vitro*, and of antitumor activity *in vivo* with the combined treatment with paclitaxel or docetaxel and ZD6474.

The antitumor and antiangiogenic activity of ZD6474 *in vivo* can be enhanced by its use in combination with a taxane such as paclitaxel. In fact, treatment with the two drugs produced a complete regression of established palpable GEO tumors in mice with no histological evidence of GEO tumors in ~30% of mice. Paclitaxel antitumor activity on GEO tumor

xenografts *in vivo* has been previously shown to be enhanced when combined with anti-EGFR agent ZD1839 (16, 39). In addition, paclitaxel has also been found to enhance the antitumor efficacy of a VEGFR-2 blocking antibody *in vivo*, by potentiating the antiangiogenic response and inducing tumor and endothelial cell apoptosis (48). Paclitaxel may therefore potentiate the inhibition of VEGF signaling and EGF signaling by ZD6474.

The results of this study provide experimental evidence that a small molecule TKI that has a potent antitumor activity by a direct antiangiogenic mechanism (*i.e.,* the blockade of VEGFR-2 signaling in endothelial cells) can also directly inhibit cancer cell growth by interfering with the EGFR autocrine pathway. In this regard, the greater selectivity of ZD6474 for VEGFR-2 compared with EGFR both *in vitro* (using isolated enzymes) and in cellular assays (using growth factor-stimulated endothelial cell proliferation) suggests that the contribution of EGFR tyrosine kinase inhibition to the antitumor activity of ZD6474 *in vivo* may be more pronounced at the higher doses of ZD6474 and most apparent in EGFR-dependent tumors. We suggest that treatment with ZD6474 could block neoangiogenesis more efficiently than treatment with a selective anti-VEGFR agent, because in addition to a direct inhibitory effect

on VEGFR-2 signaling, it will also have an indirect effect on angiogenesis via blockade of EGFR-induced paracrine production of angiogenic growth factors such as VEGF, bFGF and TGF-α by cancer cells.

These results may have future clinical relevance. Ongoing Phase I studies will determine a well-tolerated dose of ZD6474 that can be administered to cancer patients (31). If the plasma levels of ZD6474 are in the effective range for both inhibition of neoangiogenesis through an anti-VEGFR-2 mechanism and inhibition of cancer cell growth through an anti-EGFR mechanism, ZD6474 treatment could be explored in a clinical setting as a novel approach for the simultaneous inhibition of both the endothelial cell and the cancer cell compartments. Chronic oral administration of such an agent may have the potential to produce long-term control of local cancer cell growth, neoangiogenesis, and metastatic spread. Finally, this study provides an experimental basis for the clinical development of a therapeutic approach based on the combination of ZD6474 with paclitaxel or docetaxel in cancer patients.

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Antitumor Effects of ZD6474, a Small Molecule Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor, with Additional Activity against Epidermal Growth Factor Receptor Tyrosine Kinase

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