

Antitumor Activity of ZD6474, a Vascular Endothelial Growth Factor-2 and Epidermal Growth Factor Receptor Small Molecule Tyrosine Kinase Inhibitor, in Combination with SC-236, a Cyclooxygenase-2 Inhibitor

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ABSTRACT

Purpose: The epidermal growth factor receptor (EGFR) autocrine pathway plays an important role in cancer cell growth. Vascular endothelial growth factor (VEGF) is a key regulator of tumor-induced endothelial cell proliferation and vascular permeability. Enhanced cyclooxygenase-2 (COX-2) expression has been linked to cancer cell proliferation, EGFR activation, VEGF secretion, and tumor-induced angiogenesis. ZD6474 is an orally available, small molecule, dual VEGF receptor-2 (VEGFR-2) and EGFR tyrosine kinase inhibitor. We investigated the activity of ZD6474 in combination with SC-236, a selective COX-2 inhibitor, to determine the antitumor activity of the simultaneous blockade of EGFR, COX-2, and VEGF functions.

Experimental Design: The antitumor activity *in vitro* and *in vivo* of ZD6474 and/or SC-236 was tested in human cancer cell lines with a functional EGFR autocrine pathway.

Results: The combination of ZD6474 and SC-236 determined supra-additive growth inhibition in all cancer cell lines tested. In nude mice bearing established human colon (GEO) or lung adenocarcinoma (A549) cancer xenografts and treated with ZD6474 and/or SC-236 for 3 weeks, a reversible tumor growth inhibition was seen with each agent, whereas a more prolonged growth inhibition that

lasted for 3 to 5 weeks following the end of treatment resulted from the combination of the two agents. A long-term, 10-week treatment with ZD6474 plus SC-236 resulted in sustained tumor growth inhibition in all mice with tumor eradication in 3 of 10 GEO tumor-bearing mice and in 4 of 10 A549 tumor-bearing mice.

Conclusions: This study provides a rationale for evaluating the simultaneous blockade of EGFR, COX-2, and VEGF signaling as cancer therapy in a clinical setting.

INTRODUCTION

The transforming growth factor α (TGF α) epidermal growth factor receptor (EGFR) autocrine pathway plays a key role in the development and progression of human epithelial cancers (1). Overexpression of TGF α and/or EGFR has been detected in the majority of human carcinomas, has been associated with resistance to cytotoxic drugs and to hormone therapy, and is generally an indicator of poor prognosis (1). For these reasons, the blockade of the EGFR-driven autocrine pathway has been proposed as a target for anticancer therapy (2). Several pharmacologic approaches have been developed for blocking EGFR. The two most successful approaches for the treatment of cancer patients have been thus far the anti-EGFR-blocking monoclonal antibodies (MAB), such as Cetuximab, and the selective EGFR small molecule tyrosine kinase inhibitors (TKI), such as Gefitinib and Erlotinib (3–5).

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 (6). 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 (6). Vascular endothelial growth factor (VEGF) is a potent and specific mitogen for endothelial cells that activates the angiogenic switch *in vivo* and enhances vascular permeability (7). VEGF binds to two distinct receptors on endothelial cells, flt-1 (VEGFR-1) and flk-1/KDR receptor (VEGFR-2; ref. 7). VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell permeability, proliferation, and differentiation (7). Enhanced production of VEGF is generally correlated with increased neovascularization within the tumor (6, 8). VEGF expression can be increased in cancer cells by different mechanisms, most notably by hypoxia (6, 7). Experimental evidence has been provided for a link between EGFR signaling and angiogenic mechanisms (9). In fact, it has been shown that EGF and TGF α can up-regulate the production of VEGF in human cancer cells (10, 11). Furthermore, treatment with anti-EGFR agents determines an antitumor effect *in vivo* that

Received 7/8/04; revised 10/30/04; accepted 11/4/04.

Grant support: Associazione Italiana per la Ricerca sul Cancro.

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is due both to the direct blockade of the EGFR-dependent mitogenic pathway and, at least in part, to the inhibition of the secretion of various paracrine growth factors, including TGF α , VEGF, basic fibroblast growth factor, and interleukin-8 (IL-8), which stimulate the migration, proliferation, and functional differentiation of intratumor endothelial cells (12–19). Various small molecule TKIs with anti-angiogenic properties are currently in clinical development (20–22). Among these, TKIs that block both the VEGFR tyrosine kinase activity and the EGFR tyrosine kinase activity have been recently described. AEE-788 is a small molecule TKI that efficiently blocks EGFR, erbB-2, and also VEGFR-1 and VEGFR-2 (23). ZD6474 is an orally bioavailable, small molecule, anilinoquinazoline derivative that is a potent inhibitor of the VEGFR-2 tyrosine kinase with additional anti-EGFR tyrosine kinase activity (24, 25). ZD6474 significantly inhibits tumor growth in a broad range of established human cancer xenografts in nude mice and is currently in phase II development in cancer patients. ZD6474 has potent antitumor activity by a direct anti-angiogenic mechanism (i.e., the blockade of VEGFR-2 signaling in endothelial cells) but can also directly inhibit cancer cell growth by interfering with the EGFR autocrine pathway. Furthermore, 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, basic fibroblast growth factor, and TGF α by cancer cells (24, 25).

Cyclooxygenase-2 (COX-2) is an inducible enzyme that is involved in inflammation; however, increasing evidence suggests a key role in promoting tumor cell growth and angiogenesis, probably through the activity of COX-2-derived prostaglandins, such as prostaglandin E₂ (26, 27). COX-2 overexpression has been observed in a variety of human cancers, including colon, cervix, lung, prostate, and breast and has been associated with poor prognosis (28–30). It has been reported that newly formed blood vessels in tumors overexpress COX-2, whereas quiescent vasculature expresses only the COX-1 enzyme (26, 30, 31). COX-2 stimulation of neoangiogenesis has been associated with the induction of basic fibroblast growth factor and VEGF in cancer cells (32, 33). Recent studies have shown a functional cross-talk between EGFR intracellular signaling and the COX-2 pathway (34). EGFR activation induces COX-2 expression and prostaglandin E₂ production in cancer cells. COX-2-derived prostaglandins, such as prostaglandin E₂, synergistically enhance EGFR-dependent signaling in gastrointestinal cancer cells by several mechanisms, including increase in the production of the EGFR-specific ligands amphiregulin and TGF α , and the src-dependent intracellular transactivation of the EGFR that also involves the activation of the anti-apoptotic phosphatidylinositol 3-kinase–Akt pathway (35–39). Sulindac, a nonsteroid, anti-inflammatory drug that inhibits both COX-1 and COX-2, when given in combination with a selective EGFR inhibitor, is able to confer protection from development of intestinal neoplasia in a murine model of familial adenomatous polyposis (40). Furthermore, we have recently shown that the combined treatment with Gefitinib, a selective EGFR TKI, and SC-236, a selective COX-2 inhibitor, has a potent cooperative antitumor and anti-angiogenic activity in

human colon cancer xenografts (41). Therefore, a combined blockade of different relevant cancer signaling pathways may represent a suitable and potentially more effective anticancer strategy, although it should be carefully evaluated if this approach might also determine an increased toxicity.

On the basis of the functional interactions between EGFR, COX-2, and VEGF signaling, in the present study we have investigated the antitumor activity of ZD6474 in combination with SC-236 to determine whether the simultaneous blockade of EGFR, COX-2, and VEGFR functions could obtain a better anticancer efficacy.

MATERIALS AND METHODS

Drugs. Clinical grade ZD6474 was kindly provided by Dr. Anderson Ryan (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). SC-236 was obtained from Pharmacia (St. Louis, MO).

Cell Lines. GEO human colon cancer, MKN-28 human gastric cancer, A549 human non-small cell lung cancer, and MiaPaca2 human pancreatic cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mmol/L HEPES (pH 7.4), penicillin (100 IU/mL), streptomycin (100 μ g/mL), and 4 mmol/L glutamine (ICN, Irvine, CA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Growth in Soft Agar. Cells (10⁴ cells/well) were suspended in 0.5 mL 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 mL 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and treated with different concentrations of ZD6474 and/or SC-236. After 10 to 14 days, cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO) and colonies larger than 0.05 mm were counted as previously described (25). The results of the combined treatment with ZD6474 and SC-236 were analyzed according to the method of Chou and Talalay (42, 43) by using the CalcuSyn software program (Biosoft, Cambridge, United Kingdom). The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. CI = 1 denotes additivity; CI > 1, antagonism; CI = 1 to 0.7, slight synergism; CI = 0.7 to 0.3, synergism; CI < 0.3, strong synergism.

Evaluation of Vascular Endothelial Growth Factor Secretion. The concentration of VEGF in the conditioned medium obtained from GEO, A549, MKN-28, and MiaPaca2 cells was measured using a commercially available sandwich ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Assays were done in quadruplicate using 24-hour collected serum-free conditioned medium. Results were normalized for the number of producing cells and reported as picograms of VEGF per 10⁶ cells per 24 hours.

Immunoprecipitation and Western Blot Analysis. Total cell protein extracts were obtained, as previously described (25), from A549 cells, which were cultured for 24 hours in the presence or absence of 1 μ mol/L ZD6474 and/or 2.5 μ mol/L SC-236. For the assessment of EGFR expression and evaluation of EGFR phosphorylation, proteins were first immunoprecipitated

with the anti-EGFR MAb 528, kindly provided by Dr. John Mendelsohn (University of Texas M. D. Anderson Cancer Center, Houston, TX) as previously reported (25). Subsequently, immunoprecipitates were resolved by a 7.5% SDS-PAGE and probed with either an anti-human EGFR MAb (Transduction Laboratories, Lexington, KY) or the PY20 anti-P-tyrosine MAb (Transduction Laboratories). Furthermore, total cell protein extracts from A549 cells were resolved by a 4% to 20% SDS-PAGE and probed with one of the following antibodies: anti-human VEGF mouse MAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt mouse MAb (Cell Signaling, Beverly, MA), anti-phospho (Ser⁴⁷³)-Akt mouse MAb (Cell Signaling), anti-human COX-2 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-extracellular signal-regulated kinase 1/2 mouse MAb (Santa Cruz Biotechnology), or anti-phospho-extracellular signal-regulated kinase 1/2 mouse MAb (Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham International, Little Chalfont, United Kingdom) as described previously (25).

Tumor Xenografts in Nude Mice. Four- to six-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 Federico II Animal Care and Use Committee. Mice were acclimatized at University of Naples Federico II Medical School Animal Facility for 1 week before being injected with cancer cells. Mice were injected s.c. with 10⁷ GEO or A549 cells that had been resuspended in 200 μ L Matrigel (Becton Dickinson). After 7 days, when established tumors of ~0.2 to 0.3 cm³ diameter were detected, mice were treated i.p. with ZD6474 (75 mg/kg/d on days 1-5 of each week) and/or with p.o. administration of SC-236 (6 mg/kg on days 1-5 of each week) for the indicated time periods. For each experiment, treatment groups comprised 10 mice. Tumor volume was measured using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Immunohistochemical Analysis. Immunocytochemistry was done on formalin-fixed, paraffin-embedded tissue sections (5 μ m) of GEO and A549 xenografts as previously reported (25). 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 avidin-biotin complex kit, Vector Laboratory, Burlingame, CA), washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as previously described (25). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. To determine tumor cell proliferative activity, an anti-Ki67 MAb (clone MIB1, DBA, Milan, Italy) was used at 1:100 dilution. To determine the percentage of positive cells, at least 1,000 cancer cells per slide were counted and scored. New blood vessels were detected using a MAb raised against the human factor VIII-related antigen (DAKO, Milan, Italy) at the dilution of 1:50 and stained with a standard immunoperoxidase method (Vectastain avidin-biotin complex kit). Each slide was scanned at low power ($\times 10$ -100 magnification) and the area with the higher number of new vessels was identified (hotspot). This region

Table 1 Effect of the combination of ZD6474 and SC-236 on human cancer cell lines soft agar growth

Cell line	ZD6474 (IC ₅₀ , μ mol/L)	SC-236 (IC ₅₀ , μ mol/L)	CI at IC ₅₀ *
MKN-28	0.75	4	0.34
MiaPaca2	0.5	1.2	0.43
GEO	0.5	1.2	0.47
A549	0.6	2	0.36

NOTE. IC₅₀ values for ZD6474 or SC-236 treatment were calculated by performing dose-response experiments. Cancer cells were treated with different concentrations of ZD6474 (range, 0.05-2.5 μ mol/L) each day, for a total of 5 days or of SC-236 (range, 0.05-10 μ mol/L) each day, for a total of 5 days. Colonies were counted after 10-14 days.

*Cancer cells were treated with six different concentrations of ZD6474 (range, 0.01-2.5 μ mol/L) each day, for a total of 5 days and of SC-236 (range, 0.05-12.5 μ mol/L) each day, for a total of 5 days at a fixed drug ratio ZD6474:SC-236 1:5. Colonies were counted after 10-14 days. CI values were calculated according to the Chou and Talalay mathematical model for drug interactions using the CalcuSyn software.

was then scanned at $\times 250$ microscope magnification (0.37 mm²). Five fields were analyzed; for each of them, the number of stained blood vessels was counted. For individual tumors, microvessel count was scored by averaging the five field counts (25).

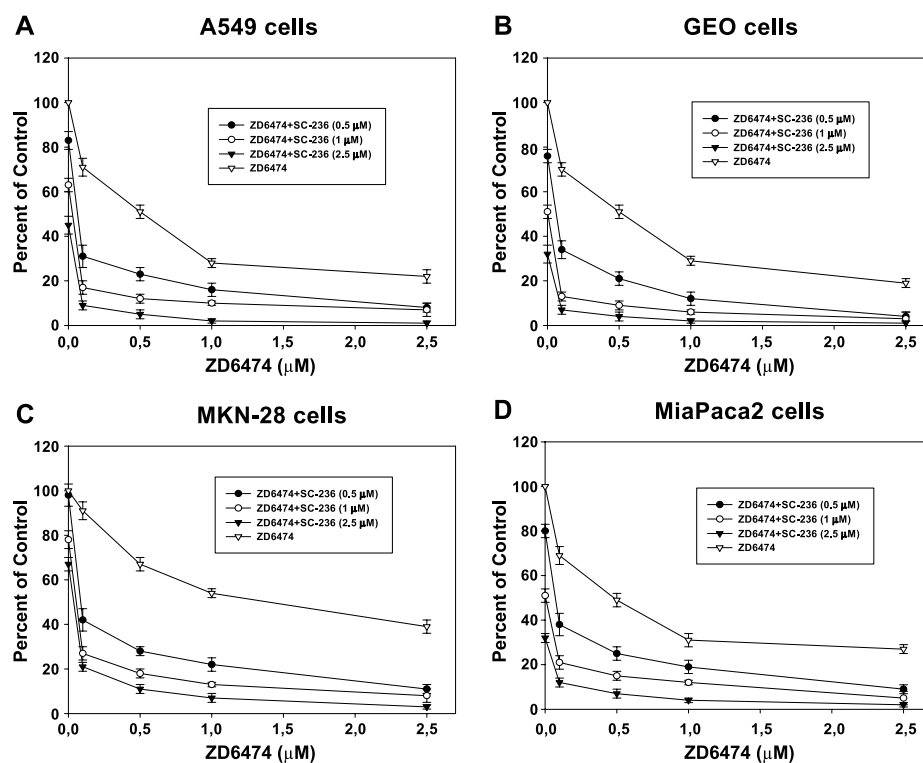
RESULTS

We first evaluated the effect of ZD6474 treatment on the growth of various human epithelial cancer cell lines in soft agar. For this purpose, we selected GEO colon adenocarcinoma, A549 non-small cell lung adenocarcinoma, MiaPaca2 pancreatic adenocarcinoma, and MKN-28 gastric cancer cell lines. These four cell lines express functional EGFR, ranging from ~40,000 (GEO) to 80,000 (MiaPaca2 and MKN-28) to 150,000 (A549) EGF binding sites per cell, and secrete high levels of TGF α (17).⁴ However, these cancer cell lines lack both VEGFR-1 and VEGFR-2 (17).⁴ As shown in Table 1, ZD6474 treatment resulted in a dose-dependent inhibition of colony formation in soft agar with an IC₅₀ value ranging between 0.5 and 0.75 μ mol/L in all cancer cell lines tested. Similarly, these cell lines were treated with different concentrations of the COX-2-selective inhibitor SC-236. A dose-dependent inhibition of anchorage-independent growth in semisolid medium was also observed in all four cancer cell lines (IC₅₀, range, 1.2-4 μ mol/L; Table 1).

A series of experiments was next done to evaluate the antiproliferative effects of the combination of ZD6474 and SC-236 treatment on the anchorage-independent growth of all four cancer cell lines. A supra-additive and/or frankly synergistic growth inhibitory effect was observed with all doses of ZD6474 and SC-236 tested (Fig. 1). Subsequently, the antiproliferative effects of ZD6474 and SC-236 combinations were evaluated by using the method of Chou and Talalay (42, 43) for the determination of the type of interaction (antagonistic, additive, or synergistic cell growth inhibition) between the two drugs. As shown in Table 1, a clear synergistic growth inhibitory effect was found in the four human cancer cell lines with CI values at IC₅₀ ranging from 0.34 to 0.47.

⁴ Unpublished data.

Fig. 1 Growth inhibitory effects of treatment with ZD6474 in combination with SC236 on the soft agar growth of human A549 (A), GEO (B), MKN-28 (C), and MiaPaca2 (D) cancer cells. Cells were treated with the indicated concentrations of each drug alone (SC-236, 0.5, 1, and 2.5 $\mu\text{mol/L}$; ZD6474, 0.25, 0.5, 1, and 2.5 $\mu\text{mol/L}$) or in combination for 5 consecutive days starting on day 1. Colonies were counted after 10-14 days. Three different experiments were each done in triplicate: points, mean; bars, SD.



To determine the effects on EGFR-dependent intracellular signaling, human lung adenocarcinoma A549 cells were treated with 2.5 $\mu\text{mol/L}$ SC-236 and/or 1 $\mu\text{mol/L}$ ZD6474 for 24 hours. Inhibition of EGFR autophosphorylation was detected following immunoprecipitation with an anti-EGFR MAb and subsequent Western blotting with an antiphosphotyrosine antibody of A549 cell extracts treated with ZD6474 and with ZD6474 plus SC-236, whereas only a slight reduction in EGFR tyrosine phosphorylation was found following treatment with the COX-2-selective inhibitor alone (Fig. 2). Mitogen-activated protein kinase (MAPK) and Akt are two major intracellular downstream signaling pathways that are directly or indirectly activated in response to ligand-dependent EGFR functional activation (4). SC-236 treatment did not significantly affect the levels of activated, phosphorylated MAPK and Akt (Fig. 2). In contrast, an almost complete suppression of phosphorylated Akt was observed following treatment with ZD6474 and ZD6474 plus SC-236, whereas phosphorylated MAPK levels were significantly reduced by ZD6474 (~50%) and by ZD6474 plus SC-236 (~70-80%; Fig. 2). Finally, COX-2 expression was inhibited by both SC-236 and ZD6474 treatment (~50% reduction by each treatment alone) and completely suppressed by the combined treatment with the two agents (Fig. 2). It has been shown that VEGF production is controlled by several positive stimuli in cancer cells, including EGFR and COX-2 activation. As shown in Fig. 2, SC-236 or ZD6474 treatment determined a significant inhibition in VEGF protein levels in total cell extracts (~30% and 70%, respectively), whereas the combined treatment almost completely suppressed VEGF expression. Similarly, both agents alone resulted in inhibition in the secretion of VEGF into the

conditioned medium of all four human cancer cell lines that were tested, with a minor effect of SC-236 single-agent treatment on A549 lung adenocarcinoma cells (Table 2). An additive inhibition in VEGF secretion into the conditioned medium was observed with the SC-236 plus ZD6474 combination in all four human cancer cell lines (Table 2).

Table 2 Vascular endothelial growth factor secretion by human cancer cells following treatment with ZD6474 and/or SC-236

	VEGF (pg/10 ⁶ cells/24 h)
A549	
Control	176 ± 20 (100%)
SC-236 ($\mu\text{mol/L}$)	146 ± 15 (83%)
ZD6474 ($\mu\text{mol/L}$)	141 ± 10 (80%)
Combination	60 ± 15 (34%)
GEO	
Control	175 ± 20 (100%)
SC-236 ($\mu\text{mol/L}$)	140 ± 8 (80%)
ZD6474 ($\mu\text{mol/L}$)	105 ± 10 (60%)
Combination	30 ± 4 (17%)
MiaPaca2	
Control	190 ± 16 (100%)
SC-236 ($\mu\text{mol/L}$)	145 ± 10 (76%)
ZD6474 ($\mu\text{mol/L}$)	128 ± 12 (67%)
Combination	75 ± 6 (39%)
MKN-28	
Control	86 ± 10 (100%)
SC-236 ($\mu\text{mol/L}$)	50 ± 4 (58%)
ZD6474 ($\mu\text{mol/L}$)	43 ± 3 (50%)
Combination	14 ± 2 (16%)

NOTE. Percent of VEGF secreted into the conditioned medium compared with control, untreated cells are given. The results are average ± SD of two independent experiments each done in triplicate.

We next investigated the *in vivo* antitumor activity of SC-236 and/or ZD6474 in nude mice bearing GEO colon cancer or A549 lung adenocarcinoma xenografts. After 7 days, when established GEO or A549 tumors of ~ 0.2 to 0.3 cm^3 were detectable, groups of 10 mice were treated for 3 weeks with each agent alone or in combination. As shown in Fig. 3, a significant 60% to 80% inhibition of tumor growth was obtained with ZD6474 or SC-236 single-agent treatment at the end of the 3 weeks of therapy. However, this effect was reversible upon cessation of treatment because both GEO and A549 tumors resumed a growth rate similar to that of control untreated mice within 1 to 2 weeks. In contrast, a prolonged growth inhibition resulted from the combination of ZD6474 and SC-236 for 3 weeks with $\sim 90\%$ growth inhibition that lasted for 3 to 5 weeks following the end of treatment (Fig. 3).

To determine the effects of ZD6474 and/or SC-236 treatment on cancer cell proliferation *in vivo* and on tumor-induced neovascularization, two mice per group were sacrificed after 2 weeks of treatment. Tumors were then

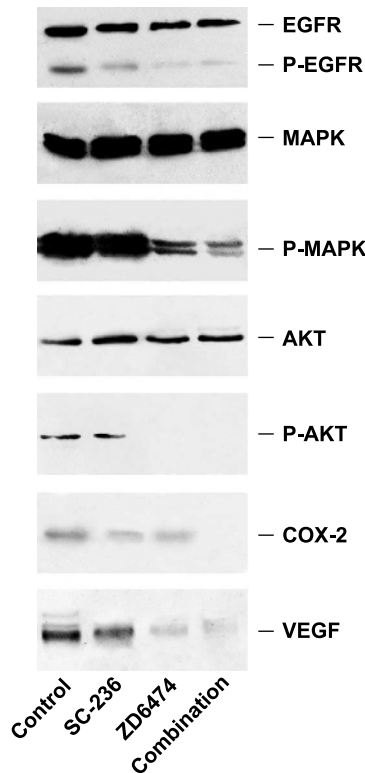


Fig. 2 Western blot analysis of protein expression following SC-236 and/or ZD6474 treatment in human A549 cancer cells. A549 cells were treated with SC-236 ($2.5 \mu\text{mol/L}$) and/or ZD6474 ($1 \mu\text{mol/L}$) for 24 hours. For the evaluation of EGFR and P-EGFR expression, total cell protein extracts 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 (*P-EGFR*) or an anti-human EGFR MAb (*EGFR*). For the evaluation of MAPK, phosphorylated MAPK, Akt, phosphorylated Akt, COX-2, and VEGF expression, total cell proteins were fractionated through 4-20% SDS-PAGE, transferred to nitrocellulose filters, and incubated with the appropriate antibodies as described in Materials and Methods. Immunoreactive proteins were visualized by enhanced chemiluminescence.

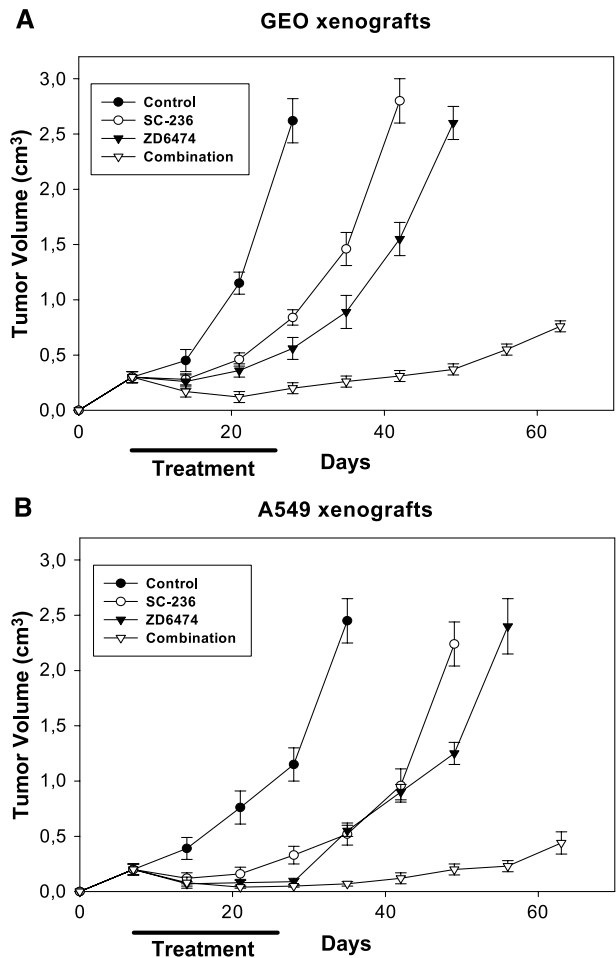


Fig. 3 Antitumor activity of ZD6474 and/or SC-236 treatment on established GEO (A) and A549 (B) human cancer xenografts. Mice were injected s.c. into the dorsal flank with either 10^7 GEO or A549 cancer cells. After 7 days (average tumor size, 0.2 – 0.3 cm^3), mice were treated as follows: \square , control untreated mice; \blacktriangledown , ZD6474 (75 mg/kg/d i.p.), days 1–5 of each week for 3 weeks; \circ , SC-236 (6 mg/kg/d p.o.), days 1–5 of each week for 3 weeks; ∇ , combination of ZD6474 and SC-236 for 3 weeks. Each group consisted of 10 mice. Points, mean; bars, SD.

collected and analyzed by immunohistochemistry for Ki-67 staining in the nuclei of cancer cells as a marker of tumor proliferation and by a MAb against factor VIII-related antigen to assess microvessel counts in the most intense areas of neovascularization. Table 3 illustrates that SC-236 and ZD6474 treatment as single agent caused a 40% to 60% reduction in the percentage of Ki-67-stained cancer cells in both GEO and A549 xenografts. A supra-additive effect was observed with the combined treatment (Table 3). SC-236 resulted in a small decrease in microvessel counts compared with control untreated mice, whereas ZD6474 treatment significantly inhibited neovessel formation within the tumor: 8 ± 1 versus 18 ± 2 microvessels/field in GEO xenografts and 7 ± 3 versus 24 ± 3 microvessels/field in A549 xenografts (Table 3). An almost complete suppression in angiogenesis was observed following the combined treatment with the two agents in both tumor xenograft models (Table 3).

Table 3 Immunohistochemical evaluation of tumor cell proliferation and angiogenesis in GEO and A549 xenografts

Treatment	Tumor size (cm ³)	Proliferative activity (%)	Microvessel count
GEO			
Control	1.15 ± 0.1	70 ± 5	18 ± 2
SC-236	0.46 ± 0.06	45 ± 5	15 ± 2
ZD6474	0.36 ± 0.05	35 ± 5	8 ± 1
Combination	0.12 ± 0.05	10 ± 3	2 ± 1
A549			
Control	0.76 ± 0.15	75 ± 5	24 ± 3
SC-236	0.16 ± 0.06	40 ± 5	19 ± 2
ZD6474	0.08 ± 0.03	25 ± 5	7 ± 3
Combination	0.04 ± 0.01	10 ± 5	1 ± 1

NOTE. Mice bearing GEO or A549 tumor xenografts were treated as described in Materials and Methods. Analysis was done on day 21 following tumor cell injection (at the end of 2 weeks of treatment). As measure of proliferative activity, the percent ± SD of Ki67-specific nuclear staining in cancer cells was recorded. To determine the percentage of positive cells, ~1,000 cancer cells were scored. As measure of neoangiogenesis, the number of microvessels for field ± SD was measured using a MAb raised against the human factor VIII-related antigen and was scored by averaging five field counts of two individual tumors for each group.

Because the antitumor activity of SC-236 plus ZD6474 combination was highly effective but was reversible within 3 to 5 weeks in a short term, 3-week treatment protocol, we evaluated the effects of a long term, 10-week treatment in nude mice bearing established GEO or A549 xenografts. Mice were injected s.c. with GEO or with A549 cancer cells and treated for 10 weeks using the same experimental protocol described above (Fig. 3) for the 3-week treatment. A delayed tumor growth was observed in the ZD6474 plus SC-236 groups, which was accompanied by a prolonged life span of mice that was significantly different from that of untreated controls or single-agent-treated groups. As shown in Table 4, GEO tumors in mice treated with SC-236 plus ZD6474 reached a mean tumor volume of ~2.5 cm³ (volume that is considered not compatible with the normal life of a nude mouse because it determines an undue stress because of excessive tumor burden) within an average period of 182 ± 6 days compared with control untreated mice (28 ± 3 days), to SC-236-treated mice (77 ± 5 days), or to ZD6474-treated mice (91 ± 4 days). Similarly, in A549 xenograft-bearing mice, tumors reached a mean tumor volume of ~2.5 cm³ in mice treated with the combination of SC-236 and ZD6474 within an average period of 189 ± 7 days compared with control untreated mice (35 ± 2 days), to SC-236 treated mice (84 ± 4 days), or to ZD6474 treated mice (98 ± 5 days; Table 5). Furthermore, the combined treatment was highly effective also in terms of cure with tumor eradication in 3 of 10 mice and in 4 of 10 mice in the GEO and in the A549 groups, respectively (Tables 4 and 5). Combined treatments with ZD6474 and SC-236 at the dose and schedule tested for the 10-week treatment protocol were well tolerated by mice, with no weight loss or other signs of acute or delayed toxicity.

DISCUSSION

The blockade of the functional activity of cell membrane growth factor receptors, which play a key role in cancer cell proliferation and in tumor-induced angiogenesis, is being developed as a valuable strategy for cancer therapy. However, recent experimental data suggest that cancer cells may escape from growth inhibition by using alternative growth pathways or

by constitutive activation of downstream signaling effectors (44–48). In fact, it is conceivable that multiple growth-controlling pathways are intrinsically altered or can be activated in cancer cells following treatment with specific inhibitors that are selective for a single growth factor receptor, contributing to the development of acquired resistance to these drugs. Therefore, simultaneous inhibition of multiple molecular targets may be required to obtain an optimal therapeutic effect in human epithelial cancers (49).

In this study, we have evaluated the possibility of obtaining a better and more sustained control of tumor growth and of tumor-induced angiogenesis by the combined blockade of three distinct yet related signaling pathways in cancer and in endothelial cells, such as EGFR, VEGFR, and COX-2. In this respect, a functional cross-talk between EGFR intracellular signaling, COX-2, and VEGF production has been recently proposed (34). EGFR activation induces COX-2 expression and prostaglandin E₂ production in cancer cells. Moreover, prostaglandin E₂ may synergistically enhance EGFR-dependent

Table 4 Antitumor activity of ZD6474 in combination with SC-236 on GEO human cancer xenografts

Treatment	Average tumor volume day on 28 after tumor cell injection (cm ³)	Average time (days) to reach a tumor volume of ~2.5 cm ³
Control	2.45 ± 0.2	28 ± 3
SC-236	0.31 ± 0.07	77 ± 5
ZD6474	0.25 ± 0.01	91 ± 4
SC-236 + ZD6474	0.15 ± 0.04	182 ± 6*

NOTE. Each treatment (SC-236, ZD6474, or SC-236 + ZD6474) was started on day 7 following GEO tumor cell s.c. injection when the average tumor volume was 0.30 ± 0.05 cm³. SC-236 treatment: 6 mg/kg/dose p.o. 5 days/wk starting on day 7 following tumor cell injection. ZD6474 treatment: 75 mg/kg/dose p.o. 5 days/wk starting on day 7 following tumor cell injection. Treatment was maintained for 10 weeks. Each group consisted of 10 mice. The average tumor volume on day 28 following tumor cell injection in control mice, 2.45 ± 0.2 cm³, was considered not compatible with mice normal life (~10% of a nude mouse body weight). Therefore, when tumors in each treatment group reached a comparable tumor volume, mice were sacrificed.

*Three of ten mice were without histologic evidence of GEO tumors at the end of 10 weeks of treatment.

Table 5 Antitumor activity of ZD6474 in combination with SC-236 on A549 human cancer xenografts

Treatment	Average tumor volume day on 35 after tumor cell injection (cm ³)	Average time (days) to reach a tumor volume of ~2.5 cm ³
Control	2.40 ± 0.3	35 ± 2
SC-236	0.23 ± 0.03	84 ± 4
ZD6474	0.16 ± 0.02	98 ± 5
SC-236 + ZD6474	0.05 ± 0.01	189 ± 7*

NOTE. Each treatment (SC-236, ZD6474, or SC-236 + ZD6474) was started on day 7 following A549 tumor cell s.c. injection when the average tumor volume was $0.20 \pm 0.05 \text{ cm}^3$. SC-236 treatment: 6 mg/kg/dose p.o. 5 days/wk starting on day 7 following tumor cell injection. ZD6474 treatment: 75 mg/kg/dose p.o. 5 days/wk starting on day 7 following tumor cell injection. Treatment was done for 10 weeks. Each group consisted of 10 mice. The average tumor volume on day 35 following tumor cell injection in control mice, $2.40 \pm 0.3 \text{ cm}^3$, was considered not compatible with mice normal life (~10% of a nude mouse body weight). Therefore, when tumors in each treatment group reached a comparable tumor volume, mice were sacrificed.

*Four of ten mice were without histologic evidence of A549 tumors at the end of the 10 weeks of treatment.

signaling in gastrointestinal cancer cells by several mechanisms, including increase in the production of the EGFR-specific ligands amphiregulin and TGF α and the src-dependent intracellular transactivation of the EGFR that involves also the activation of the anti-apoptotic phosphatidylinositol 3-kinase-Akt pathway (35–39).

We have used the dual VEGFR-2 TKI and EGFR TKI, ZD6474, and SC-236, a celecoxib analogue with selective anti-COX-2 activity. Here we report that the combined treatment with ZD6474 and SC-236 has a significant cooperative growth inhibitory effect *in vitro* in four different human cancer cell lines that express functional EGFRs. This activity is clearly synergistic with a CI of ~0.3 to 0.5. Cancer cell growth inhibition is accompanied by down-regulation in the expression of COX-2, reduction in VEGF secretion, and blockade of the activation of two key intracellular downstream signaling molecules, such as MAPK and Akt. Moreover, *in vivo* experiments in nude mice bearing established human GEO colon carcinoma or A549 lung adenocarcinoma xenografts have shown a significant cooperative antitumor activity when ZD6474 and SC-236 were used in combination. In this respect, a 10-week, long-term treatment with well-tolerated doses of the two agents determined a sustained delay of tumor growth with an high proportion (30–40%) of mice that were cured and had no histologic evidence of tumor at the end of the experiment. Immunohistochemical analysis of tumor samples obtained from mice treated with the two drugs in combination showed a cooperative inhibition of cancer cell proliferation and an almost complete suppression of neovessel formation. This may be due to the combined inhibitory effects of both SC-236 and ZD6474 on cancer cell production of pro-angiogenic factors, such as VEGF, which may result in a significant reduction in endothelial cell migration and growth. Furthermore, ZD6474 anti-angiogenic activity is probably due to the direct effect of ZD6474 on the VEGFR-2 on endothelial cells with endothelial cell growth inhibition and to the indirect inhibition in cancer

cells of the production of several pro-angiogenic factors, including VEGF (25).

The potent *in vivo* antitumor and anti-angiogenic activity of ZD6474 and SC-236 in combination may be explained by an effective multisignaling blockade. It may be hypothesized that alternative growth factor signaling pathways may lead to cancer cell proliferation when a single pathway, such as the EGFR, is blocked by a selective EGFR inhibitor. In this respect, we have recently shown that acquired resistance to pure EGFR inhibitors, such as Cetuximab and Gefitinib, may develop in mice bearing GEO colon cancer xenografts and chronically treated with one of these agents (50). EGFR inhibitor-resistant GEO cells exhibit a significant increase in VEGF production, in COX-2 expression, and in activated MAPK, suggesting that these molecular changes can contribute to the EGFR-independent growth of these cancer cells. In this context, experimental evidence has been provided that simultaneous targeting of different pathways is an effective cancer treatment and may prevent the development of treatment-resistant cancer cells. As an example, sustained antitumor activity can be obtained through the combination of selective anti-EGFR compounds with other anti-signal transduction agents. These include inhibitors of the type I cyclic AMP-dependent protein kinase, VEGF antisense oligonucleotides, endostatin, and the anti-c-erbB-2 MAb trastuzumab (15, 51–55). Similarly, a cooperative antitumor activity has been reported by the combination of trastuzumab with the selective COX-2 inhibitor celecoxib (56). We have recently shown that the combined treatment of three different signal transduction inhibitors targeting EGFR, type I cyclic AMP-dependent protein kinase and bcl-2/bcl-xL (57) or EGFR, type I cyclic AMP-dependent protein kinase, and COX-2 (41) results in a significant antitumor activity in a human colon cancer xenograft model. The combined ZD6474 plus SC-236 treatment, providing simultaneous inhibition of neoangiogenesis through anti-VEGFR-2 and anti-COX-2 mechanisms and inhibition of cancer cell growth through anti-EGFR and anti-COX-2 mechanisms, could, therefore, be an effective anticancer approach by blocking both endothelial cell and cancer cell proliferation. Because both ZD6474 and various selective COX-2 inhibitors are in advanced-stage clinical development, these results provide a rationale for translating this therapeutic strategy into a clinical setting in cancer patients.

ACKNOWLEDGMENTS

We thank Dr. Anderson Ryan for the generous gift of ZD6474 and for helpful discussions.

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