Antitumor Activity of ZD6474, a Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor, in Human Cancer Cells with Acquired Resistance to Anti-epidermal Growth Factor Receptor Therapy

Fortunato Ciardiello,1 Roberto Bianco,2 Roberta Caputo,2 Rosa Caputo,2 Vincenzo Damiano,2 Teresa Troiani,2 Davide Melisi,2 Ferdinando De Vita,1 Sabino De Placido,3 A. Raffaele Bianco,2 and Giampaolo Tortora2

1Cattedra di Oncologia Medica, Dipartimento Medico-Chirurgico di Interististica Clinica e Sperimentale “F Magrassi e A Lanzara,” Seconda Università degli Studi di Napoli, Naples, Italy, and 2Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Università degli Studi di Napoli Federico II, Naples, Italy

ABSTRACT

Purpose: The epidermal growth factor receptor (EGFR) autocrine signaling pathway is involved in cancer development and progression. EGFR inhibitors such as C225 (cetuximab), a chimeric human-mouse anti-EGFR monoclonal antibody, and ZD1839 (gefitinib), a small molecule EGFR-selective tyrosine kinase inhibitor, are in advanced clinical development. The potential emergence of cancer cell resistance to EGFR-expressing cancers treated with EGFR inhibitors could determine lack of activity of these drugs in some cancer patients. Vascular endothelial growth factor (VEGF) is secreted by cancer cells and plays a key role in the regulation of tumor-induced endothelial cell proliferation and permeability. ZD6474 is a small molecule VEGF flk-1/KDR (VEGFR-2) tyrosine kinase inhibitor that also demonstrates inhibitory activity against EGFR tyrosine kinase.

Experimental Design: The antitumor activity of ZD1839, C225, and ZD6474 was tested in athymic mice bearing human GEO colon cancer xenografts. GEO cell lines resistant to EGFR inhibitors were established from GEO xenografts growing in mice treated chronically with ZD1839 or C225. Expression of EGFR was evaluated by flow cytometry. Expression of various proteins involved in intracellular cell signaling was assessed by Western blotting. Tumor growth data were evaluated for statistical significance using the Student’s t test. All Ps were two-sided.

Results: Although chronic administration of optimal doses of C225 or ZD1839 efficiently blocked GEO tumor growth in the majority of mice, tumors slowly started to grow within 80–90 days, despite continuous treatment. In contrast, continuous treatment of mice bearing established GEO xenografts with ZD6474 resulted in efficient tumor growth inhibition for the entire duration of dosing (up to 150 days). ZD6474 activity was also determined in mice pretreated with ZD1839 or C225. When GEO growth was apparent after 4 weeks of treatment with EGFR inhibitors, mice were either re-treated with EGFR inhibitors or treated with ZD6474. GEO tumor growth was blocked only in mice treated with ZD6474, whereas tumor progression was observed in mice re-treated with C225 or ZD1839. GEO tumors growing during treatment with C225 or with ZD1839 were established as cell lines (GEO-C225-RES and GEO-ZD1839-RES, respectively). Cell membrane-associated EGFR expression was only slightly reduced in these cell lines compared with parental GEO cells. Western blotting revealed no major change in the expression of the EGFR ligand transforming growth factor α of bcl-2, bcl-xL, p53, p27, MDM-2, akt, activated phospho-akt, or mitogen-activated protein kinase. However, both GEO-C225-RES and GEO-ZD1839-RES cell lines exhibited a 5–10-fold increase in activated phospho-mitogen-activated protein kinase and in the expression of cyclooxygenase-2 and of VEGF compared with GEO cells. GEO-C225-RES and GEO-ZD1839-RES growth as xenografts in nude mice was not significantly affected by treatment with either C225 or ZD1839 but was efficiently inhibited by ZD6474.

Conclusions: Long-term treatment of GEO xenografts with selective EGFR inhibitors results in the development of EGFR inhibitor-resistant cancer cells. Growth of EGFR inhibitor-resistant tumors can be inhibited by ZD6474. These data indicate that inhibition of VEGF signaling has potential as an anticancer strategy, even in tumors that are resistant to EGFR inhibitors.

INTRODUCTION

Growth factors regulate cancer development through several mechanisms. These include uncontrolled cell growth caused...
by the autocrine production of growth factors by cancer cells and stimulation of tumor neovascularization through paracrine activation of normal endothelial cells by angiogenic growth factors secreted by cancer cells (1). 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. This overexpression 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 (2–4). Several approaches have been developed for blocking EGFR activation and/or function in cancer cells. Various anti-EGFR-blocking monoclonal antibodies (MAbs) have been generated and characterized for their biological and potentially therapeutic properties (2–4). One of these agents, C225 (cetuximab), a chimeric human–mouse IgG1 MAb, is in Phase II–III clinical evaluation in cancer patients (5). Several compounds that block the ligand-induced activation of the EGFR tyrosine kinase have been developed (6). Among these, ZD1839 (gefitinib), an anilinoquinazoline, is an orally active EGFR tyrosine kinase inhibitor, which has shown clinical activity in non-small cell lung cancer, as well as other tumor types. ZD1839 is currently under clinical evaluation in Phase II and III trials in cancer patients (7).

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 (8, 9). VEGF binds to two distinct receptors on endothelial cells: flt-1 (VEGFR-1) and flk-1/KDR receptor (VEGFR-2; Refs. 8, 9). VEGFR-2 is considered the key signaling receptor for endothelial cell permeability, proliferation, and differentiation (8, 9). Enhanced expression of VEGF is generally correlated with increased neovascularization within the tumor (9, 10). VEGF expression can be increased in cancer cells by different mechanisms, most notably hypoxia (9, 11). In addition, activation of EGFR signaling can up-regulate the production of VEGF in human cancer cells (12, 13). 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 basic fibroblast growth factor, interleukin 8, and TGF-α (14–19). Among the approaches that have been proposed for blocking VEGF-induced endothelial cell proliferation and subsequent tumor angiogenesis, a promising one is the blockade of VEGFR-2 activation in endothelial cells (11). ZD6474 is an orally bioavailable, small molecule VEGFR tyrosine kinase inhibitor that also has activity against EGFR tyrosine kinase (20, 21). Therefore, ZD6474, in addition to inhibiting endothelial cell proliferation by blocking VEGF-induced signaling, could inhibit cancer cell growth by blocking EGFR autocrine signaling. This compound is currently in early clinical evaluation in cancer patients (22).

Results from Phase I–II trials in advanced cancer demonstrate that both C225 and ZD1839 have an acceptable tolerability profile and an interesting clinical activity in patients with a variety of tumor types (23–26). However, clinical responses have been observed only in a subgroup of cancer patients. Several key clinical questions need to be addressed, which patients are most likely to have a therapeutic benefit, what are the potential predictive factors of response or of resistance that could be useful in a clinical setting, and what are the best strategies for combining anti-EGFR drugs with other anticancer treatments. The integrity of the EGFR-activated downstream intracellular signal transduction machinery could influence the response to anti-EGFR drugs. Recent experimental evidence suggests that cancer cells may escape from growth inhibition by using alternative growth pathways or by constitutive activation of downstream signaling effectors (27–29). In fact, it is conceivable that multiple growth-controlling pathways are intrinsically altered or can be activated in cancer cells after treatment with selective signal transduction inhibitors such as anti-EGFR agents, contributing to the development of acquired resistance to these drugs.

In the present study, we have evaluated the effects of chronic treatment with selective anti-EGFR agents such as C225 and ZD1839 in a xenograft model of human GEO colon cancer that is initially sensitive to the antitumor activity of these drugs. Long-term treatment of GEO xenografts with selective EGFR inhibitors results in the development of EGFR inhibitor-resistant cancer cells. In contrast, chronic treatment with ZD6474 results in efficient long-term control of GEO tumor growth. Furthermore, growth of EGFR inhibitor-resistant tumors could be inhibited by ZD6474. This drug may therefore determine a more efficient in vivo tumor growth inhibition by blocking VEGF-induced signaling in endothelial cells and by preventing or delaying the emergence of EGFR-independent cancer cells.

MATERIALS AND METHODS

Drugs. Clinical grade ZD6474 and ZD1839 were kindly provided by Dr. Anderson Ryan (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). C225 anti-EGFR human–mouse chimeric anti-EGFR IgG1 MAb was supplied by ImClone Systems (New York, NY).

Tumor 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 Federico II Animal Care and Use Committee. Mice were acclimatized at the University of Naples Federico II Medical School Animal Facility for 1 week before receiving injections of cancer cells. Mice received s.c. injected with 10^7 cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when established tumors of ~0.2–0.3 cm³ diameter were detected, mice were treated i.p. with ZD1839 (150 mg/kg/day, days 1–5 of each week), ZD6474 (75 mg/kg/day on days 1–5 of each week), or C225 (1 mg daily on days 2 and 5 of each week) for the indicated time periods. For each experiment, treatment groups comprised eight mice. Tumor volume was measured using the formula \( \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2 \).

Cell Lines. Human GEO colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). GEO-ZD1839-RES and GEO-C225-RES cells were established as in vitro cell lines after cancer cell recovery and enzymatic treatment (28) from in vivo GEO tumor xenografts. Results from Phase I–II trials in advanced cancer demonstrate that both C225 and ZD1839 have an acceptable tolerability profile and an interesting clinical activity in patients with a variety of tumor types (23–26). However, clinical responses have been observed only in a subgroup of cancer patients. Several key clinical questions need to be addressed, which patients are most likely to have a therapeutic benefit, what are the potential predictive factors of response or of resistance that could be useful in a clinical setting, and what are the best strategies for combining anti-EGFR drugs with other anticancer treatments. The integrity of the EGFR-activated downstream intracellular signal transduction machinery could influence the response to anti-EGFR drugs. Recent experimental evidence suggests that cancer cells may escape from growth inhibition by using alternative growth pathways or by constitutive activation of downstream signaling effectors (27–29). In fact, it is conceivable that multiple growth-controlling pathways are intrinsically altered or can be activated in cancer cells after treatment with selective signal transduction inhibitors such as anti-EGFR agents, contributing to the development of acquired resistance to these drugs.

In the present study, we have evaluated the effects of chronic treatment with selective anti-EGFR agents such as C225 and ZD1839 in a xenograft model of human GEO colon cancer that is initially sensitive to the antitumor activity of these drugs. Long-term treatment of GEO xenografts with selective EGFR inhibitors results in the development of EGFR inhibitor-resistant cancer cells. In contrast, chronic treatment with ZD6474 results in efficient long-term control of GEO tumor growth. Furthermore, growth of EGFR inhibitor-resistant tumors could be inhibited by ZD6474. This drug may therefore determine a more efficient in vivo tumor growth inhibition by blocking VEGF-induced signaling in endothelial cells and by preventing or delaying the emergence of EGFR-independent cancer cells.
nografts in mice that were treated i.p. for 14 weeks with either 
C225 (1 mg daily on days 2 and 5 of each week) or with 
ZD1839 (150 mg/kg/day on days 1–5 of each week). GEO, 
GEO-ZD1839-RES, and GEO-C225-RES cells were maintained 
in DMEM supplemented with 10% heat-inactivated fetal bovine 
serum, 20 mM HEPES (pH 7.4), penicillin (100 U/ml), strep-
tomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United 
Kingdom) in a humidified atmosphere of 95% air and 5% CO2 
at 37°C.

Flow Cytometric Evaluation of EGFR Expression on 
the Cell Membrane. Cells were detached with a nonenzym-
ic cell dissociation solution (Sigma Chemical Co., St. Louis, 
MO) and incubated for 1 h at 4°C with either C225 or with an 
isotype matched control MAb (human myeloma IgG1; Sigma 
Chemical Co.). The cells were counterstained with FITC-
labeled goat antihuman IgG antiserum (Southern Biotechnol-
ogy, Birmingham, AL), as reported previously (30). Flow 
cytometric analysis was performed with a FACSscan fluores-
cence-activated cell analyzer (Becton Dickinson, San Jose, CA). 
Forward- and side-scattered cell gating was performed to detect 
fluorescence only on intact living cells. Histogram showing the 
specific immunofluorescent labeling were generated using Con-
sort 30 software (Becton Dickinson). Approximately 10,000 
cells were evaluated for each cell line.

Immunoprecipitation and Western Blot Analysis. For 
the assessment of EGFR expression and evaluation of EGFR 
phosphorylation, total cell protein extracts were obtained, as 
previously described (31), from GEO, GEO-ZD1839-RES, and 
GEO-C225-RES cells, which were cultured for 1 h in the 
presence or absence of 1 μM ZD1839, 1 μM ZD6474, or 2.5 
μg/ml C225. Proteins were immunoprecipitated with MAb 528, 
kindly provided by Dr. John Mendelsohn (University of Texas 
M.D. Anderson Cancer Center, Houston, TX), as reported 
previously (31). For Western blot analysis, immunoprecipitates 
were resolved by a 7.5% SDS-PAGE and probed with either an 
antihuman EGFR MAb (Transduction Laboratories, Lexington, 
KY) or the PY20 anti-P-tyrosine MAb (Transduction Laborato-
ries). For evaluation of the expression of different proteins, total 
cell protein extracts from GEO, GEO-ZD1839-RES, and GEO-
C225-RES cells were resolved by a 4–20% SDS-PAGE and 
probed with one of the following antibodies: antihuman VEGF 
mouse MAb (Santa Cruz Biotechnology, Santa Cruz, CA); 
anti-MDM-2 mouse MAb (Oncogene); anti-akt mouse MAb 
(Cell Signaling); anti-phospho(Ser73)-akt mouse MAb (Cell 
Signaling); antihuman cyclooxygenase-2 (COX-2) rabbit polycy-
clonal antibody (Santa Cruz Biotechnology); anti-p53 mouse 
MAb (Santa Cruz Biotechnology); anti-27 mouse MAb (Santa 
Cruz Biotechnology); anti-bcl-2 mouse MAb (Santa Cruz Bio-
technology); anti-bcl-xL rabbit polyclonal antibody (Transdu-
cion Laboratories); anti-ERK 1/2 mouse MAb (Santa Cruz Bio-
technology); or anti-phospho-ERK1/2 mouse MAb (Santa Cruz 
Biotechnology). Immunoreactive proteins were visualized by 
enhanced chemiluminescence (Amersham International, Lon-
don, United Kingdom), as described previously (31).

Evaluation of VEGF Secretion. The concentration of 
VEGF in the conditioned medium obtained from GEO, GEO-
ZD1839-RES, and GEO-C225-RES cells was measured using a 
commercially available sandwich ELISA kit (R&D Systems, 
Inc., Minneapolis, MN) according to the manufacturer’s instruc-
tions. Assays were performed in quadruplicate using 24-h col-
lected serum-free conditioned medium. Results were normalized 
for the number of producing cells and reported as pg of growth 
factor/10^6 cells/24 h.

Growth in Soft Agar. Cells (10^4 cells/well) were sus-
pended 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-multwell cluster dishes (Becton Dickinson, Lincoln Park, 
NJ) and treated with different concentrations of ZD1839, C225, 
or ZD6474. After 10–14 days, cells were stained with nitro blue 
tetrazolium (Sigma Chemical Co.), and colonies > 0.05 mm 
were counted as described previously (31).

Statistical Analysis. The Student’s t test was used to 
evaluate the statistical significance of the results. All Ps repre-
ted 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

We have previously demonstrated that short-term treatment 
with anti-EGFR drugs such as ZD1839 and C225 causes a 
dose-dependent antitumor activity in athymic mice bearing es-
hablished human GEO colon cancer xenografts (15, 19, 31). This 
effect is mainly cytostatic. We therefore evaluated whether a 
continuous treatment with anti-EGFR agents could determine a 
more sustained and prolonged tumor growth control. GEO cells 
(10^7) were injected s.c. into the dorsal flank of athymic, nude 
mice. After 1 week, when established GEO xenografts were 
palpable with a tumor size of ~0.2–0.3 cm^3, different groups of 
mice were treated i.p. on days 1–5 of each week with ZD1839 
(150 mg/kg/day) or on days 2 and 5 of each week with C225 
(1 mg/dose) either for 4 weeks or continuously. These doses 
were chosen as the optimal doses that were previously shown to 
determine a complete GEO tumor growth inhibition after 4 
weeks of treatment (15, 19, 31). As illustrated in Fig. 1, 4 
weeks’ treatment with ZD1839 or with C225 caused an almost 
complete suppression of tumor growth. However, 3–5 weeks 
after cessation of treatment, the GEO tumor growth rate was 
comparable with the growth rate of GEO tumors in untreated 
control mice. Continuous treatment with both anti-EGFR drugs 
causc a more prolonged GEO tumor inhibition compared with 
mice treated for only 4 weeks with each agent. However, after 
11–12 weeks of continuous treatment with either ZD1839 or 
with C225, GEO tumors began to grow, eventually reaching a 
growth rate comparable with untreated control tumors after 
20–22 weeks. As shown in Fig. 1, similar groups of mice were 
treated with an optimal dose of ZD6474 (75 mg/kg/day) on days 
1–5 of each week (21) for 4 weeks or continuously. As with the 
results observed with ZD1839 and C225 treatment, a 4-week 
treatment with ZD6474 produced GEO tumor growth inhibition 
that was reversible upon cessation of treatment. In contrast, 
GEO tumor growth was effectively blocked in mice treated 
continuously with ZD6474 for 23 weeks. This antitumor effect 
was significantly different to the GEO tumor growth in mice 
treated chronically with either C225 or ZD1839 (Fig. 1).

We next evaluated whether treatment with ZD6474 could
have antitumor activity in GEO tumors after treatment with either anti-EGFR agent. Mice bearing palpable GEO tumors were treated for 4 weeks with ZD1839 (Fig. 2A) or with C225 (Fig. 2B). After an interval of 4 weeks, when GEO tumors were starting to regrow, mice were randomly assigned to receive continuous treatment with either the EGFR inhibitor used as initial treatment and control untreated mice; □, C225 (1 mg daily) days 2 and 5 of each week as continuous treatment; ▼, ZD1839 (150 mg/kg/day) days 1–5 of each week as continuous treatment; ▾, ZD6474 (75 mg/kg/day) days 1–5 of each week as continuous treatment; ◐, C225 (1 mg daily) days 2 and 5 of each week for 4 weeks; ■, ZD1839 (150 mg/kg/day) days 1–5 of each week for 4 weeks; and ●, ZD6474 (75 mg/kg/day) days 1–5 of each week for 4 weeks. Each group comprised 8 mice. Data represent the average (± SD). The Student’s t test was used to compare tumor volumes among different treatment groups and control untreated mice. For the groups of mice treated for 4 weeks with C225, ZD1839, or ZD6474, tumor volumes in all treatment groups were significantly different compared with controls ($P < 0.001$ for each comparison) on day 35 after GEO cell injection (end of the treatment period). For the groups of mice that received continuous treatment with C225, ZD1839, or ZD6474, evaluation of differences in tumor volumes was performed on day 170 after GEO cell injection. ZD6474 continuously treated mice versus ZD1839 continuously treated mice ($P < 0.001$); ZD6474 continuously treated mice versus C225 continuously treated mice ($P < 0.001$); C225 continuously treated mice versus ZD1839 continuously treated mice ($P = 0.85$).

Fig. 1  Antitumor activity of C225, ZD1839, or ZD6474 treatment on established GEO human colon carcinoma xenografts. Mice were injected s.c. into the dorsal flank with $10^7$ GEO cells as described. After 7 days (average tumor size, 0.2–0.3 cm$^3$), mice were treated i.p. as follows: ●, control untreated mice; □, C225 (1 mg daily) days 2 and 5 of each week as continuous treatment; ▼, ZD1839 (150 mg/kg/day) days 1–5 of each week as continuous treatment; ▾, ZD6474 (75 mg/kg/day) days 1–5 of each week as continuous treatment; ◐, C225 (1 mg daily) days 2 and 5 of each week for 4 weeks; ■, ZD1839 (150 mg/kg/day) days 1–5 of each week for 4 weeks; and ●, ZD6474 (75 mg/kg/day) days 1–5 of each week for 4 weeks. Each group comprised 8 mice. Data represent the average (± SD). The Student’s t test was used to compare tumor volumes among different treatment groups and control untreated mice. For the groups of mice treated for 4 weeks with C225, ZD1839, or ZD6474, tumor volumes in all treatment groups were significantly different compared with controls ($P < 0.001$ for each comparison) on day 35 after GEO cell injection (end of the treatment period). For the groups of mice that received continuous treatment with C225, ZD1839, or ZD6474, evaluation of differences in tumor volumes was performed on day 170 after GEO cell injection. ZD6474 continuously treated mice versus ZD1839 continuously treated mice ($P < 0.001$); ZD6474 continuously treated mice versus C225 continuously treated mice ($P < 0.001$); C225 continuously treated mice versus ZD1839 continuously treated mice ($P = 0.85$).

To study the potential mechanisms of EGFR inhibitor-acquired GEO cancer cell resistance, mice bearing GEO tumors were sacrificed after ~14 weeks of continuous treatment with either ZD1839 or C225. Tumors were excised and two cell lines established in vitro, GEO-ZD1839-RES and GEO-C225-RES. Both GEO-ZD1839-RES and GEO-C225-RES cells had a morphological appearance, in vitro growth rate, and soft-agar cloning efficiency similar to that of parental GEO cells (data not shown). To determine whether the lack of sensitivity to the growth inhibitory effects of ZD1839 or of C225 was due to loss of EGFR expression or to an alteration in EGFR-driven intracellular signaling, cell membrane-associated EGFR expression was determined by flow cytometry. As shown in Fig. 3A, EGFR was detected on the cell membrane of both GEO-ZD1839-RES and GEO-C225-RES cells at levels that were only partially reduced compared with parental GEO cells. Furthermore, treatment with 1 μM ZD1839 for 1 h efficiently blocked EGFR autophosphorylation in all three cell lines (Fig. 3B). A similar inhibition in EGFR phosphorylation was observed after treatment with 2.5 μg/ml C225 for 1 h or with 1 μM ZD6474 for 1 h (data not shown). We next investigated which downstream signal pathways were changed in the GEO cell lines that were...
derived from EGFR inhibitor-resistant GEO tumors. No difference in the expression of the EGFR ligand TGF-α was detected by Western blot analysis of proteins extracted from GEO-ZD1839-RES and GEO-C225-RES cells compared with GEO cells (Fig. 4). Similarly, little or no changes in the expression of bcl-2, of bcl-xL, of total mitogen-activated protein kinase (MAPK), of total Akt, and of activated, phosphorylated akt, of p53, of p27, or MDM-2 were observed in these cancer cell lines. Expression of VEGFR-2 was not detectable in GEO, GEO-ZD1839-RES, and GEO-C225-RES cells (data not shown). In contrast, a 5–10-fold increase in the expression of COX-2, of activated, phosphorylated MAPK, and of VEGF was found in GEO-ZD1839-RES and GEO-C225-RES cells compared with GEO cells (Fig. 4). Furthermore, analysis of VEGF secretion into the conditioned medium revealed an increase in GEO-ZD1839-RES cells (870 ± 15 pg/10⁶ cells/24 h) and in GEO-C225-RES cells (1150 ± 35 pg/10⁶ cells/24 h) compared with GEO cells (160 ± 15 pg/10⁶ cells/24 h).

The in vitro susceptibility of EGFR inhibitor-resistant GEO cancer cell lines to the growth inhibitory effects of anti-EGFR drugs was evaluated in an anchorage-independent growth assay. As shown in Fig. 5, treatment with C225, ZD1839, or ZD6474 resulted in a dose-dependent inhibition of parental GEO cell colony formation in soft agar, whereas growth of GEO-ZD1839-RES and GEO-C225-RES cells was not significantly affected. Finally, the in vivo effects of these agents were tested on GEO-ZD1839-RES and GEO-C225-RES xenografts in athymic mice. Whereas C225 or ZD1839 treatment did not significantly

Fig. 2 Antitumor activity of ZD6474 treatment on established GEO human colon carcinoma xenografts after treatment with ZD1839 or with C225. Mice were injected s.c. into the dorsal flank with 10⁷ GEO cells as described in “Materials and Methods.” After 7 days (average tumor size, 0.2–0.3 cm³), 16 mice were treated i.p. with ZD1839 (150 mg/kg/day) days 1–5 of each week for 4 weeks (A), and 16 mice were treated i.p. with C225 (1 mg daily) days 2 and 5 of each week for 4 weeks (B). A group of 8 mice served as untreated control. After additional 4 weeks, when GEO tumors started to regrow, the 16 mice pretreated with ZD1839 were randomly assigned to receive continuous treatment with either the same dose and schedule of ZD1839 or with ZD6474 (75 mg/kg/day on days 1–5 of each week; A), and the 16 mice pretreated with C225 were randomly assigned to receive continuous treatment with either the same dose and schedule of C225 or with ZD6474 (75 mg/kg/day on days 1–5 of each week; B). Data represent the average (±SD). The Student’s t test was used to compare tumor volumes among different treatment groups and control untreated mice. For the groups of mice treated for 4 weeks with ZD1839 (A) or ZD1839 (B), tumor volumes in the treated groups were significantly different compared with controls (P < 0.001 for each comparison) on day 35 after GEO cell injection (end of the treatment period). For the groups of mice that received a second period of treatment with ZD1839 or ZD6474 (A) or with C225 or ZD6474 (B), evaluation of differences in tumor volumes was performed on day 110 after GEO cell injection. A, ZD6474-treated mice versus ZD1839-treated mice (P < 0.001). B, ZD6474-treated mice versus C225-treated mice (P < 0.001).
affect the growth of either tumor type, ZD6474 treatment resulted in significant growth inhibition in GEO-ZD1839-RES and GEO-C225-RES xenografts (Fig. 6).

**DISCUSSION**

Interference with the activation of growth factor receptors and/or with the intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anticancer therapies. A large body of experimental evidence supports a key role for EGFR activation in a wide variety of human epithelial cancers and blockade of EGFR is one of the most promising approaches in this area (1–4). EGFR-driven intracellular signaling controls not only cancer cell proliferation but also several processes that are important for tumor progression, including invasion, angiogenesis, and metastasis (4). In this respect, EGF and TGF-α can up-regulate the production of VEGF by human cancer cells (12, 13). A direct approach for the therapeutic blockade of EGFR-driven signals in human cancer has been recently obtained with the development of anti-EGFR-blocking antibodies such as C225 and with the development of low molecular weight compounds that inhibit ligand-induced activation of EGFR tyrosine kinase enzymatic activity, such as ZD1839 (2–6). Both C225 and ZD1839 have been successfully tested as anticancer drugs in preclinical models, with C225 in advanced clinical development and ZD1839 having been launched for the treatment of...

---

### Fig. 3

**A**, flow cytometric analysis of specific immunofluorescence labeling of cell membrane-associated EGFR expression in GEO, GEO-ZD1839-RES, and GEO-C225-RES cells. GEO (positive), epidermal growth factor receptor (EGFR)-specific staining; GEO (negative), nonimmune human IgG1 staining; GEO-ZD1839-RES, EGFR-specific staining; and GEO-C225-RES EGFR-specific staining. Nonimmune human IgG1 staining for GEO-ZD1839-RES and GEO-C225-RES cells was very similar to that of GEO cells (data not shown). The histogram to the right of the vertical bar represents the proportion of cells that are specifically stained with the anti-EGFR antibody. A total of 97% of GEO cells are positive for cell membrane-associated EGFR expression; 85% of GEO-ZD1839-RES cells are positive for cell membrane-associated EGFR expression. 68% of GEO-C225-RES cells are positive for cell membrane associated EGFR expression. *B*, inhibition of EGFR autophosphorylation by ZD1839 treatment in human GEO, GEO-ZD1839-RES, and GEO-C225-RES cells. GEO, GEO-ZD1839-RES, and GEO-C225-RES cells were treated for 1 h with ZD1839. Protein extracts were immunoprecipitated with the monoclonal antibody (Mab) 528 anti-EGFR MAb, resolved by a 7.5% SDS-PAGE, and probed with either the PY20 anti-P-tyr Mab (pEGFR) or an anti-human EGFR Mab. Immunoreactive proteins were visualized by enhanced chemiluminescence. −, control, untreated cells; +, cells treated with 1 μM ZD1839.

---

Downloaded from clincancerres.aacrjournals.org on October 31, 2017. © 2004 American Association for Cancer Research.
lung cancer. However, relatively few preclinical studies have been undertaken to evaluate how cancer cell resistance against EGFR inhibitors may develop (28). This is a potentially relevant clinical issue because it is conceivable that cancer cells may possess both intrinsic and acquired resistance not only to conventional cytotoxic treatments but also to molecularly targeted drugs.

In this study, we report that chronic continuous treatment with selective anti-EGFR drugs such as C225 and ZD1839, of human GEO colon cancer cells propagated as s.c. xenografts in athymic mice, results in the development of EGFR inhibitor-resistant tumors. This acquired resistance does not seem to be due to a loss in the expression or to a functional alteration of EGFR. In particular, both GEO-ZD1839-RES and GEO-C225-RES cells have only a modest reduction in the expression of cell membrane-associated EGFR compared with parental GEO cells (~15 and 30% reduction, respectively). Moreover, EGFR auto-phosphorylation could be efficiently inhibited by treatment with either C225 or ZD1839 in both EGFR inhibitor-resistant GEO cell lines, suggesting that a functional EGFR is expressed in these cells. EGFR inhibitor-resistant GEO cells exhibit a 5–10-fold increase in VEGF production and secretion compared with GEO cells, suggesting that a contributing mechanism to GEO tumor growth escape from chronic EGFR inhibition could be an increased angiogenic potential through enhanced endothelial cell proliferation and permeabilization. These results are in agreement and extend those of Viloria-Petit et al. (28), which have demonstrated, using human epidermoid A431 cells, that the in vivo development of cancer cells constitutively overexpress VEGF and become resistant to the antitumor activity of two EGFR blocking MAbs (C225 and hR3). The results of our study also show that certain intracellular signaling pathways are up-regulated in EGFR inhibitor-resistant GEO cells. Although no significant differences in the expression of bcl-2, bcl-xl, total MAPK, total Akt, and activated, phosphorylated akt, p53, p27, or MDM-2 were observed in these cancer cell lines compared with EGFR inhibitor-sensitive parental GEO cells, both GEO-ZD1839-RES and GEO-C225-RES cells exhibit a 5–10-fold increase in COX-2 expression and in phosphorylated, activated MAPK. In this respect, it has recently been suggested that up-regulation of the insulin-like growth factor I receptor may determine continuous activation of the antiapoptotic phosphoinositide 3-kinase/akt signaling pathway that could counteract the antiproliferative effects of the EGFR inhibitor AG1478 in human glioblastoma cells in vitro (29). Moreover, in human breast cancer cell lines that overexpress c-erbB-2, increased activation of the insulin-like growth factor I receptor pathway has been shown to interfere with the growth inhibitory effects of the anti-c-erbB-2 blocking MAb trastuzumab (27). The constitutive activation of two intracellular signal transduction pathways such as COX-2 and MAPK that are downstream to the activated EGFR as well as to other mitogenic stimuli (32, 33)

![Fig. 4 Western blot analysis of protein expression in GEO, GEO-ZD1839-RES, and in GEO-C225-RES cells. Fifty μg of 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.](clincancerres.aacrjournals.org)
could contribute to the EGFR-independent in vivo growth of GEO cancer cells. In particular, increasing evidence supports the role of COX-2 in promoting tumor cell growth and angiogenesis, probably through the activity of COX-2-derived prostaglandins (PGE2; Ref. 32). COX-2 overexpression has been shown in human colon cancer and has been associated with neovascularization and with poor prognosis (34). Moreover, COX-2-dependent promotion of neoangiogenesis has been associated with induction of basic fibroblast growth factor and VEGF (32, 35). On the other hand, activation of the MAPK cascade is associated with up-regulation of VEGF expression in cancer cells (36, 37). Therefore, an increase in COX-2 and MAPK pathways could contribute to the enhanced production of VEGF in EGFR inhibitor-resistant GEO cells.

The present study has shown that unlike C225 or ZD1839, chronic continuous treatment with ZD6474, a small molecule tyrosine kinase inhibitor with unique characteristics, being both a potent antiangiogenic agent (through VEGR-2 signaling

Fig. 5 Effects of C225 (A), of ZD1839 (B), or of ZD6474 (C) on the soft agar growth of GEO, GEO-ZD1839-RES, and GEO-C225-RES cells. Cells were treated with the indicated concentrations of drug 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.

Fig. 6 Antitumor activity of ZD6474 treatment on established GEO-C225-RES (A) or GEO-ZD1839-RES (B) human colon carcinoma xenografts. Mice were injected s.c. into the dorsal flank with 10^7 GEO cells. After 7 days (average tumor size, 0.1 cm^3), mice were treated i.p. for 3 weeks with ZD6474 (75 mg/kg/day) days 1–5 of each week, ZD1839 (150 mg/kg/day) days 1–5 of each week, or with C225 (1 mg daily) days 2 and 5 of each week. Each group consisted of 8 mice. Data represent the average (±SD). The Student’s t test was used to compare tumor volumes among different treatment groups and control untreated mice on day 28 after GEO cell injection (end of the treatment period). A, ZD6474-treated mice versus ZD1839-treated mice (P < 0.001); ZD6474-treated mice versus C225-treated mice (P < 0.001); ZD6474-treated mice versus control-treated mice (P < 0.001). No statistically significant difference was observed between C225-treated mice or ZD1839-treated mice and control-untreated mice. B, ZD6474-treated mice versus ZD1839-treated mice (P < 0.001); ZD6474-treated mice versus C225-treated mice (P < 0.001); ZD6474-treated mice versus control-treated mice (P < 0.001). No statistically significant difference was observed between C225-treated mice or ZD1839-treated mice and control-untreated mice.
inhibition) and also an efficient inhibitor of the EGFR tyrosine kinase (20, 21), does not result in the emergence of GEO tumor resistance during the time frame studied. Furthermore, sequential ZD6474 treatment of GEO tumor xenografts after C225 or ZD1839 results in antitumor activity in contrast to re-treatment with either selective anti-EGFR agent. Although GEO-C225-RES and GEO-ZD1839-RES xenografts are resistant to treatment with C225 or with ZD1839, the in vivo growth of these tumors is blocked by ZD6474 treatment. This activity is most probably due to the inhibitory effect of ZD6474 on VEGF signaling in endothelial cells. This property, in comparison with agents such as ZD1839 and C225 that selectively inhibit only the EGFR, may have relevance in the clinical setting. ZD6474 has broad-spectrum activity, including activity against tumors that are not responsive to EGFR inhibition (20). The data reported here therefore also suggest potential benefit in both acquired and intrinsic EGFR resistance.

There is now a large body of preclinical evidence showing that simultaneous targeting of multiple pathways is a suitable strategy in the treatment of cancer (38). In this context, experimental models have been used to demonstrate that significant and sustained antitumor activity can be obtained through the combination of selective anti-EGFR agents with other antisignal transduction agents. These may include inhibitors of the type I cyclic AMP-dependent protein kinase (PKA) (15, 39), VEGF antisense oligonucleotides (17), endostatin (40), and the anti-c-erbB-2 MAb trastuzumab (41–44). We have also recently demonstrated that the combined treatment of three different signal transduction inhibitors targeting EGFR, inhibitors of the type I cyclic AMP-dependent protein kinase, and bel-2/bcl-XL (45) or EGFR, inhibitors of the type I cyclic AMP-dependent protein kinase, and COX-2 (46) results in a sustained GEO tumor growth inhibition. ZD6474 therapy, providing simultaneous inhibition of neoangiogenesis through an anti-VEGF-2 mechanism and inhibition of cancer cell growth through an anti-EGFR mechanism, could, therefore, be an effective anticancer approach, inhibiting both endothelial cell and cancer cell proliferation.

ACKNOWLEDGMENTS

We thank Drs. Anderson Ryan, Steve Wedge, and Alan Barge (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom) for the generous gift of ZD6474 and ZD1839 and for helpful discussions. We also thank Gaetano Borriello for his excellent technical assistance.

REFERENCES


5. Mendelsohn, J. Blockade of receptors for growth factors: an antican-


9. Ferrara, N. The role of vascular endothelial growth factor in patho-


13. Gille, J., Swerlick, R. A., and Caughman, S. W. Transforming growth factor α-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP2-dependent DNA bind-


21. Ciardiello, F., Caputo, R., Damiano, V., Caputo, R., Troiani, T., Vitagliano, D., Carlonagno, F., Veneziani, B. M., Fontanini, G., Bia-
anco, A. R., and Tortora, G. Antitumor effects of ZD6474, a small