

Combined Targeting of Endothelin A Receptor and Epidermal Growth Factor Receptor in Ovarian Cancer Shows Enhanced Antitumor Activity

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Abstract

Ovarian carcinomas overexpress endothelin A receptors (ET_AR) and epidermal growth factor (EGF) receptor (EGFR). In these cells, endothelin-1 (ET-1) triggers mitogenic and invasive signaling pathways that are in part mediated by EGFR transactivation. Combined targeting of ET_AR, by the specific ET_AR antagonist ZD4054, and of EGFR by the EGFR inhibitor gefitinib (IRESSA), may offer improvements in ovarian carcinoma treatment. In HEY and OVCA 433 ovarian carcinoma cells, ET-1 or EGF induced rapid activation of EGFR, p42/44 mitogen-activated protein kinase (MAPK), and AKT. ZD4054 was able to reduce the ET-1-induced EGFR transactivation. Gefitinib significantly inhibited EGF- and ET-1-induced EGFR phosphorylation, but incompletely reduced the ET-1-induced activation of downstream targets. ZD4054 plus gefitinib resulted in a greater inhibition of EGFR, MAPK, and AKT phosphorylation, indicating the critical role of these interconnected signaling proteins. ZD4054 effectively inhibited cell proliferation, invasiveness, and vascular endothelial growth factor (VEGF) secretion. Concomitantly, ZD4054 enhanced apoptosis and E-cadherin promoter activity and expression. In both cell lines, the drug combination resulted in a significant decrease in cell proliferation (65%), invasion (52%), and VEGF production (50%), accompanied by a 2-fold increase in apoptosis. The coadministration of ZD4054 enhanced the efficacy of gefitinib leading to partial (82%) or complete tumor regression on HEY ovarian carcinoma xenografts. Antitumor effects were paralleled by biochemical and immunohistologic evidence of decreased vascularization, Ki-67, matrix metalloproteinase-2 (MMP-2), VEGF, MAPK and EGFR, and enhanced E-cadherin expression. The cross-signaling between the EGFR/ET_AR pathways provides a rationale to combine EGFR inhibitors with ET_AR antagonists, identifying new effective therapeutic opportunities for ovarian cancer. [Cancer Res 2007;67(13):6351-9]

Introduction

The lack of specific marker of early diagnosis makes epithelial ovarian cancer the fifth leading cause of cancer-related mortality in Western world women (1). Although during the last decades,

overall survival has improved due to the use of new chemotherapy regimens, the majority of patients will relapse and develop resistant disease. Therefore, new therapeutic strategies for ovarian cancer treatment are continuously explored (2). The endothelin (ET) family is composed of three isopeptides, ET-1, ET-2, and ET-3, which act through two distinct subtypes of G-protein coupled receptors (i.e., ET_A and ET_B). The ET_A receptor (ET_AR) is highly specific for ET-1, whereas ET_BR binds ET-1, ET-2, and ET-3 with the same affinity (3). ET-1 has been implicated in the pathophysiology of a wide range of human tumors, including ovarian carcinoma (4-6). ET-1 and the ET_AR are expressed in 85% of ovarian carcinoma and their expression correlates with advanced stages (6, 7). Moreover, ET-1 is present at high levels in ovarian tumor ascites (8). In this tumor cells, ET-1 acts as an autocrine growth factor selectively through the ET_AR (5-10), and triggers an array of multiple signaling pathways including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase-dependent AKT and integrin-linked-kinase (7, 11), src-mediated epidermal growth factor (EGF) receptor (EGFR) transactivation (12), and, finally, p125 focal adhesion kinase and paxillin activation, which are thought to transduce signals involved in tumor cell invasion. ET-1, acting through ET_AR, consistently induces also the activity of multiple metastasis-related proteinases, such as matrix metalloproteinases (MMP) and the urokinase-type plasminogen activator system (10). Moreover, ET-1 inhibits gap junction intercellular communication by inducing phosphorylation of connexin 43, allowing tumor cells to escape growth control and invasion (13). Upon being activated, the ET_AR mediates pleiotropic activities, including enhanced cell proliferation, escape from apoptosis, angiogenesis, epithelial-mesenchymal transition (EMT), and increased motility and invasiveness (14). These findings indicate that activation of ET_AR by ET-1 is a key mechanism in the cellular signaling network promoting ovarian cancer growth and progression. These findings complement and extend the recent analysis of gene expression profile of late-stage ovarian cancer whereby ET_AR has been identified as a metastasis-associated gene (14-16). Moreover, gene expression profile associated with response to chemotherapy in ovarian cancer identified ET_AR as one of the genes highly expressed in postchemotherapy samples compared with untreated primary tumors (17). Besides ET_AR, EGFR is amplified and/or overexpressed in many human cancers, including ovarian cancer, where it is frequently associated with poor prognosis and has been correlated with tumor resistance to chemotherapy, thus representing a critical therapeutic target (18-22). Gefitinib (ZD1839, Iressa) is a low molecular weight quinazoline derivative that specifically inhibits the activation of EGFR tyrosine kinase through competitive binding of the ATP-binding domain of the receptor (21). Preclinical activity was shown

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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in vivo by the inhibition of tumor growth in mice bearing HX62 ovarian carcinoma xenografts (22). Rapid regrowth was observed in the majority of mice when the drug was discontinued, suggesting that chronic administration is required to maintain control of tumor proliferation. In spite of the early encouraging results obtained with gefitinib alone or in combination with chemotherapy in phase I/II studies conducted in platinum-resistant ovarian cancer patients (23–25), recent studies suggest that gefitinib is well tolerated but has minimal activity in patients with recurrent ovarian carcinoma. Response to gefitinib associated with activating mutation of EGFR catalytic domain was observed only in 3.5% of these patients (26); therefore, other factors besides mutations could account for this modest response. Cooperation and cross-talk between redundant biochemical pathways appear to be the main reasons for the failure of a therapy aimed at interfering with a single specific molecular target. The lack of response to EGFR inhibition may therefore suggest that the tumor can maintain critical survival pathways through either constitutively activated downstream mediators or alternative receptor signaling. ET_A R has been implicated in ligand-independent activation of EGFR (12). Activation of redundant downstream signaling through ET_A R may override the effects of EGFR inhibitors, thus representing a possible mechanism of resistance to anti-EGFR therapy.

Recently, a molecular signature of lung cancer cells resistant to EGFR inhibitors has been described, characterized by numerous genes related to EMT, such as loss of E-cadherin expression. Interestingly, restoring E-cadherin expression increases sensitivity to EGFR inhibitor (27–29). Understanding the regulatory loops that allow close coordination of EMT program and EGFR inhibitor sensitivity may provide a novel strategy capable of overcoming the occurrence of resistance and to increase the magnitude and duration of response. Our previous results demonstrating that ET_A R blockade reverses EMT and restores E-cadherin expression (7) provide the basis to test whether the addition of a specific ET_A R antagonist to gefitinib may increase the therapeutic activity of the treatment. The predominant role played by ET_A R in cancer has led to the development of small molecules that antagonize the binding of ET-1 to ET_A R (30). Among various ET_A R antagonists, ZD4054 is a small molecule that potently and selectively binds to the ET_A R, blocking signal transduction pathways implicated in cancer cell proliferation (31, 32).

We hypothesize that by blocking both the ET_A R and EGFR pathways, we can enhance antitumor effects achieved by either single agent. In the present study, we tested *in vitro* and *in vivo* the antiproliferative, antiangiogenic, anti-invasive, and proapoptotic activities of ZD4054. The evidence of cross-talk between the EGFR and ET_A R pathways, along with the emerging role of endothelin axis in ovarian tumorigenesis and progression, provides a rationale for evaluating drugs that target these two pathways for ovarian cancer treatment. Thus, *in vitro* and *in vivo*, the combination of ZD4054 and gefitinib in ovarian carcinoma can maximally inhibit not only cell proliferation and survival but also neovascularization and downstream signaling pathways leading to cancer invasion, EMT, and metastatic spread.

Materials and Methods

Materials. Clinical grade ZD4054, *trans, trans*-2-(4-methoxyphenyl)-4-(1-3-benzodiazol-5-yl)-1-(dibutylaminocarbonylmethyl)-pyrrolidine-3-carboxylic acid, and ZD1839, gefitinib, 4-quinazolinamine, *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-4-morpholin propoxy] were kindly provided by AstraZeneca.

ET-1 (Peninsula Laboratories) was used at 100 nmol/L and incubated with the cells for the indicated times. EGF (Upstate) was used at 10 ng/mL. Pretreatment of cells with 1 μ mol/L ZD4054 or gefitinib was done for 30 min before the addition of ET-1 or EGF.

Cell lines. Human ovarian carcinoma cell lines, HEY and OVCA 433, generously provided by Prof. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy), previously characterized for ET-1 receptor expression and for ET-1 production (5, 6), were cultured in DMEM containing 10% FCS and 1% penicillin-streptomycin at 37°C under 5% CO₂-95% air. The cells were serum starved by incubation for 24 h in serum-free DMEM. All culture reagents were from Invitrogen.

Cell proliferation assay. [³H]thymidine incorporation was measured as previously described (5). Cells were seeded in 96-well plates at ~80% confluence (2 × 10⁶ per well) and incubated in serum-free medium for 24 h to induce quiescence and 100 nmol/L ET-1 was added. Responses to all treatments were assayed in sextuplicate, and results were expressed as the means of three separate experiments.

Apoptosis assay. The induction of programmed cell death was determined as previously described (9) by the cell death detection ELISA Plus kit (Boehringer Mannheim). Briefly, 5 × 10⁴ cell/well were seeded into 12-well plates. After a 48-h treatment, cells were lysed and the supernatant was recovered and assayed for histone-associated DNA fragments, as recommended by the manufacturer, at 405 nm by the use of a microplate reader. The experiments were done in quadruplicate and repeated twice.

For detection of early apoptotic events, floating and adherent cells were collected. Cells (1 × 10⁶) were double stained with FITC-conjugated Annexin V and propidium iodide using the Vybrant Apoptosis Kit according to the manufacturer's instructions (Molecular Probes) and were immediately analyzed by cytofluorometric analysis.

Vascular endothelial growth factor secretion. Serum-starved HEY cells were plated at 1 × 10⁶ per dish and treated for 24 h with 1 μ mol/L ZD4054 or 1 μ mol/L Iressa, alone and in combination. The vascular endothelial growth factor (VEGF) protein levels in the cell conditioned medium were determined in triplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems). The sensitivity of the assay is <5.0 pg/mL. Intra-assay variation is 5.4% and interassay variation is 7.3%.

Western blot analysis. Cell lysates obtained from cell cultures or homogenized HEY tumor specimens were subjected to SDS-PAGE (7.5–12.5%) and revealed by Western blotting using antibody to VEGF (Sigma), or EGFR, or phospho-845-EGFR, or phospho-AKT, or AKT, and phospho-p42/44 MAPK and p42/44 MAPK, caspase-3 (Cell Signalling), or to E-cadherin (Santa Cruz Biotechnology). Blots were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The membranes were reprobed with anti- β -actin to assure the equal amount of protein (Oncogene, CN Biosciences, Inc.). The proteins were visualized by enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and quantified using NIH image program (Scion).

Luciferase reporter gene assay. To measure the transcriptional activity of E-cadherin promoter, 3 × 10⁵ cells per well were transiently transfected using LipofectAMINE reagent (Invitrogen) with 0.5 μ g pGL2 Ecad3/luc construct (kindly provided by Dr. E.R. Fearon, University of Michigan, Ann Arbor, MI), or with empty control vectors (Promega). All constructs were cotransfected with pCMV- β -galactosidase plasmid (Promega). After 24 h of transfection, serum-starved cells were treated with ET-1 and/or inhibitors for additional 24 h. Luciferase activity was determined in the cell lysates using the Luciferase assay system (Promega) and normalized to β -galactosidase activity. The mean of three independent experiments done in sextuplicate was reported.

Small interfering RNA treatment. HEY cells were transfected with 100 nmol/L small interfering RNA (siRNA) duplexes against ET_A R mRNA (SMART pool) or scrambled mock siRNA obtained commercially (Dharmacon). siRNA transfection using LipofectAMINE reagent (Invitrogen) was done according to manufacturer's protocol. Cells were harvested 48 h later and ET_A R mRNA and protein levels were determined. In the luciferase assay, cells were transfected with 0.5 μ g pGL2 Ecad3/luc construct 24 h after being transfected with 100 nmol/L ET_A R siRNA. Cells were then treated with ET-1 (100 nmol/L) and harvested after 24 h.

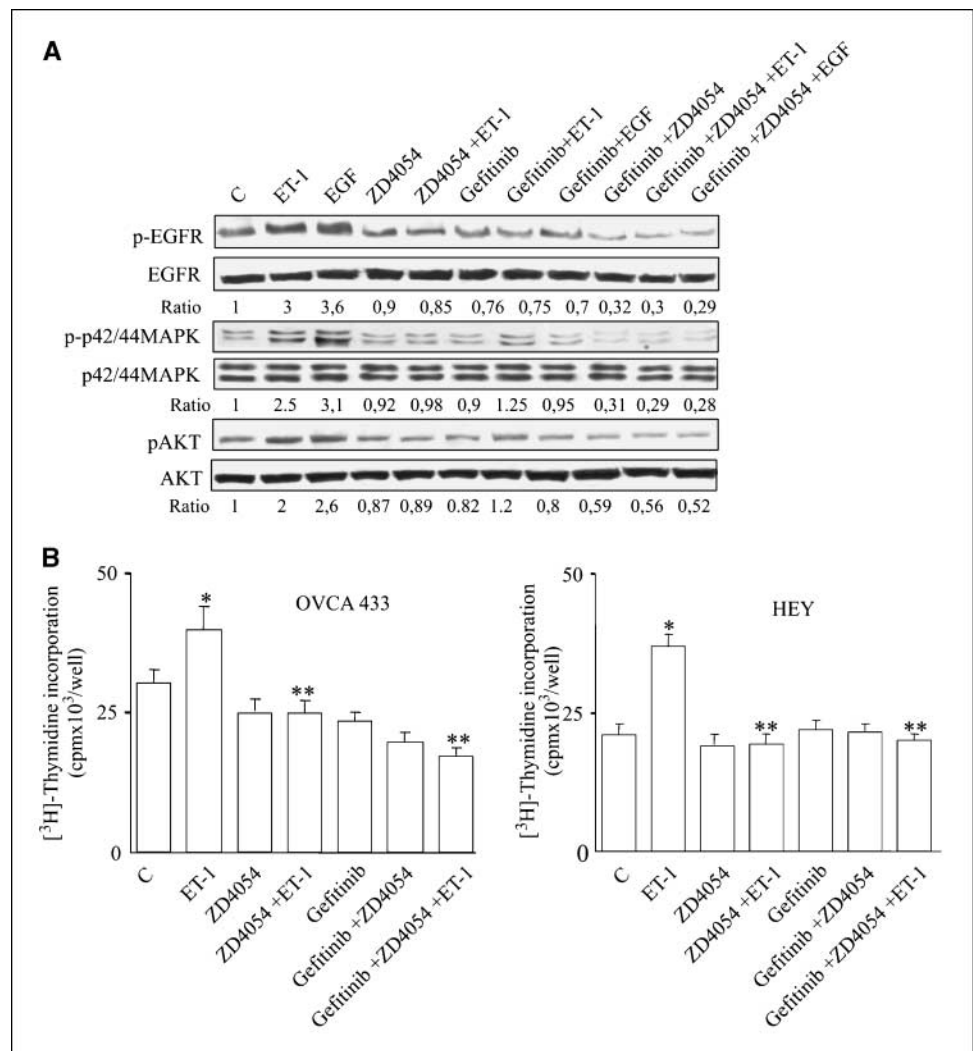
Chemoinvasion assay. Chemoinvasion assay was carried out with a 48-well modified Boyden chamber (NeuroProbe) and 8 μm pore size polyvinyl pyrrolidone-free polycarbonate Nucleopore filters (Costar) as previously described (10). The filters were coated with an even layer of 0.5 mg/mL Matrigel (BD Transduction Laboratories). The lower compartment of the chamber was filled with ET-1 (100 nmol/L) and/or ZD4054 (1 $\mu\text{mol/L}$). Serum-starved HEY cells ($5 \times 10^5/\text{mL}$) were placed in the upper compartment (55 $\mu\text{L}/\text{well}$). ZD4054 was previously added to the cells and preincubated for 15 min at 37°C. In the chemoinvasion assay done using ET_AR siRNA, HEY cells were transfected for 48 h and then incubated in the upper compartment. After 24 h of incubation at 37°C, the filters were removed, stained with Diff-Quick (Merz-Dade), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

Xenografts in nude mice. Female athymic (nu^+/nu^+) mice, 4 to 6 weeks of age (Charles River Laboratories), were treated following the guidelines for animal experimentation of the Italian Ministry of Health. Mice were injected s.c. into one flank with 1.5×10^6 viable HEY cells. After 7 days, when tumor reached ~0.2 to 0.3 cm in diameter, mice were randomized in groups ($n = 10$) to receive different treatments. One group was treated i.p. for 21 days with ZD4054 (diluted in PBS) at the daily dose of 10 mg/kg/d. Control mice were injected with drug vehicle. For combination treatment study, one group was treated i.p. for 21 days with ZD4054 (10 mg/kg/d), one group was treated for 21 days p.o. with gefitinib (125 mg/kg/d, diluted in polyethylene glycol 400), and one group was treated with ZD4054 in

combination with gefitinib. Control animals received equal volume of polyethylene glycol 400 p.o. as animal receiving gefitinib and were injected with equal volume of saline solution as animal receiving ZD4054. On day 40 after tumor injection, tumors were removed from control and treated mice, and snap frozen for immunohistochemical and immunoblot analysis. Tumor size was measured with calipers and was calculated using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Immunohistochemistry. Indirect immunoperoxidase stain of tumor xenografts was done on acetone-fixed 4- μm tissue sections as previously described (30). Briefly, sections were incubated with monoclonal rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1; generously donated by Dr. A. Mantovani, Mario Negri Institute, Milan, Italy), anti-Ki-67 monoclonal antibody (clone MIB1; Ylem), and anti-MMP-2 monoclonal antibody (Oncogene Research Products). The avidin-biotin assays were done using the Vectastain Elite kit (for nonmurine primary antibodies) and the Vector MOM immunodetection kit (for murine primary antibodies) obtained from Vector Laboratories. Mayer's hematoxylin was used as nuclear counterstain. Negative control stain was represented by sections in which the incubation with the primary antibody was either omitted or substituted by isotype-matched immunoglobulins, respectively. The evaluation of MVD was done by two independent observers on a $\times 200$ field according to the criteria of Weidner et al. (33). Ki-67 positivities were expressed as the percentage of tumor cells with nuclear staining counted in five separate $\times 40$ microscopic fields (at least 200 cells per field were counted).

Figure 1. Effects of ZD4054 and/or gefitinib on ET-1-induced EGFR, AKT, and p42/44 MAPK activation and cell proliferation in ovarian cancer cells. **A**, serum-starved HEY cells were treated with 100 nmol/L ET-1 or 10 ng/mL EGF alone or in combination with 1 $\mu\text{mol/L}$ ZD4054 or 1 $\mu\text{mol/L}$ gefitinib for 10 min. Whole-cell lysates were blotted for detection of phosphorylated and total EGFR, AKT, and p42/44 MAPK using specific antibodies. The ratio of phospho-EGFR, phospho-p42/44 MAPK, and phospho-AKT to each total protein was assessed by densitometric analysis. **B**, serum-starved OVCA 433 and HEY cells were treated with 100 nmol/L ET-1 alone or in combination with 1 $\mu\text{mol/L}$ ZD4054 and/or 1 $\mu\text{mol/L}$ gefitinib and cell proliferation was analyzed after 24 h. Columns, averages of sextuplicate determinations of three separate experiments; bars, SD; *, $P < 0.0001$ compared with control; **, $P < 0.005$ compared with ET-1.



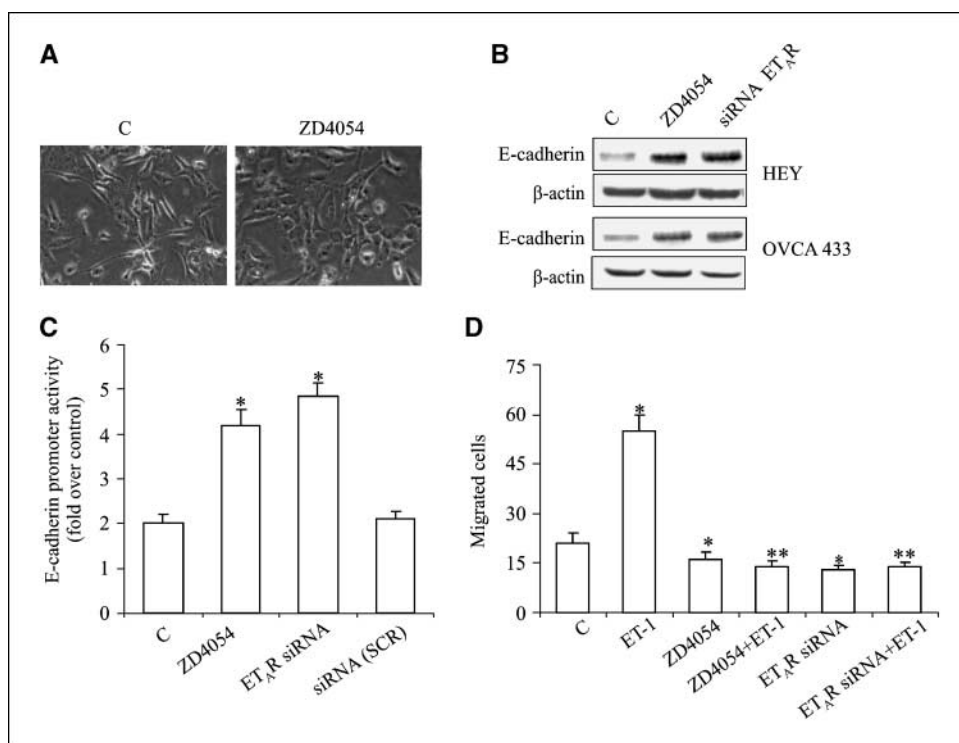


Figure 2. ZD4054 reverts EMT, and restores E-cadherin expression and promoter activity and invasiveness in ovarian cancer cells. **A**, effect of ZD4054 (1 μ mol/L) on serum-starved HEY cell morphology evaluated by phase-contrast microscopy after 48 h of incubation. **B**, effect of ZD4054 or ET_AR siRNA on the expression of E-cadherin evaluated by immunoblotting of HEY and OVCA 433 cells. **C**, effect of ZD4054 or ET_AR siRNA and/or ET-1 (100 nmol/L) on E-cadherin promoter activity as a ratio to β -galactosidase activity in HEY cells. *Columns*, mean of three independent experiments, each done in sextuplicate; *bars*, SD *, $P < 0.001$ compared with control. **D**, effect of ZD4054 or siRNA on basal and ET-1-induced HEY cell invasion. *Columns*, mean of three independent experiments, each done in triplicate; *bars*, SD *, $P < 0.001$ compared with control; **, $P < 0.001$ compared with ET-1.

Statistical analysis. Statistical analyses used the χ^2 test, Student's test, and Fisher's exact test as appropriate. The time course of tumor growth was compared across the groups with the use of two-way ANOVA, with group and time as variables. All statistical tests were two sided. A probability value of <0.05 was considered statistically significant. All statistical analyses were done using commercial software (SPSS).

Results

Combination of ZD4054 and gefitinib inhibits the phosphorylation of EGFR, p42/44 MAPK, and AKT and the proliferation in ovarian carcinoma cells. To evaluate the effect of the ET_AR-specific antagonist ZD4054 on the proliferation of ovarian carcinoma cells, we used two established cell lines, HEY and OVCA 433. They express in fact functional ET-1 and EGF high-affinity receptors (in an average of 45,500 receptors per cell with a K_d 0.10 nmol/L for ET-1 and 52,700 receptors per cell with a K_d 0.25 nmol/L for EGF) and secrete high levels of their corresponding ligands, ET-1, and transforming growth factor α for both of which an autocrine stimulation has been implicated in ovarian tumor progression (5–7, 34). The same autocrine loop occurs in both cell lines as indicated by a significant constitutive EGFR, MAPK, and AKT phosphorylation observed upon serum starvation. In HEY cells, exogenous ET-1 and EGF were able to induce a rapid EGFR transactivation and a further increases in the activation of phospho-p42/44 MAPK and p-AKT (Fig. 1A). Although ZD4054 was able to reduce the ET-1-induced EGFR transactivation, gefitinib significantly inhibited EGF- and ET-1-induced p-EGFR, but incompletely reduced the ET-1-induced phosphorylation of MAPK and AKT (Fig. 1A). This indicates that dual alternative pathways converge on MAPK and AKT activation, one EGFR-dependent, and the other EGFR-independent. Combination of ZD4054 plus gefitinib resulted in a greater inhibition of EGFR, MAPK, and AKT phosphorylation, indicating the critical role of

these interconnected signaling. Similar results were obtained in OVCA 433 cells (Supplementary Fig. S1).

The above results prompted us to analyze the contribution of the gefitinib-sensitive pathway to the mitogenic effect of ET-1 in both ovarian cancer cells. Using the thymidine incorporation assay, we observed that the stimulatory activity of ET-1 was completely blocked in the presence of ZD4054 (Fig. 1B). In both cell lines, combination of ZD4054 with gefitinib, which prevents the interaction of endogenous ligands, caused a significant inhibition of the basal proliferation rate. The activity of 100 nmol/L ET-1 on thymidine incorporation was significantly decreased by combination treatment in both cell lines (60%; $P \leq 0.005$).

The finding that EGFR transactivation in ovarian carcinoma cells is in part responsible for the mitogenic signaling of agonist-induced ET_AR activation, and our previous demonstration that ET-1 exerts additive proliferative effects in the presence of maximally effective EGF concentration (12, 34), suggest that the coexistence of ET-1 and EGF autocrine circuits in tumor cells could provide maximal growth advantage that may be blocked by the combined regimen of ZD4054 and gefitinib.

ZD4054 reverts EMT, restores E-cadherin expression, and inhibits ovarian cancer cell invasion. Based on the report that increased E-cadherin expression enhances the magnitude and duration of EGFR inhibition response (27–29) and that ET-1 promotes EMT selectively through ET_AR (7), we tested whether ZD4054 could revert EMT, increase E-cadherin expression, and thus inhibit ovarian cancer invasiveness. Interestingly, in the presence of ZD4054, a large percentage of HEY (Fig. 2A) and OVCA 433 cells (Supplementary Fig. S2) reverted to an epithelial phenotype. To determine whether EMT-associated molecular alterations have occurred in these cells, we examined the effect of ET_AR blockade, achieved either pharmacologically or by RNA interference (siRNA), on the expression of E-cadherin. ZD4054 and transfection with

ET_AR siRNA, which markedly reduced the expression of ET_AR at mRNA and protein levels (Supplementary Fig. S3), resulted in a significant increase in E-cadherin expression in both cell lines (Fig. 2B). This effect was complemented by an enhancement of E-cadherin promoter activity (Fig. 2C) and by a reduction of the basal and ET-1-induced cell invasion (Fig. 2D). Taken together, these data indicate that ZD4054 could revert EMT-associated morphologic and molecular changes, restoring E-cadherin expression and activity, and inhibiting ovarian cancer cell invasion. This suggested that the addition of ZD4054 to gefitinib treatment may increase the therapeutic activity of the latter.

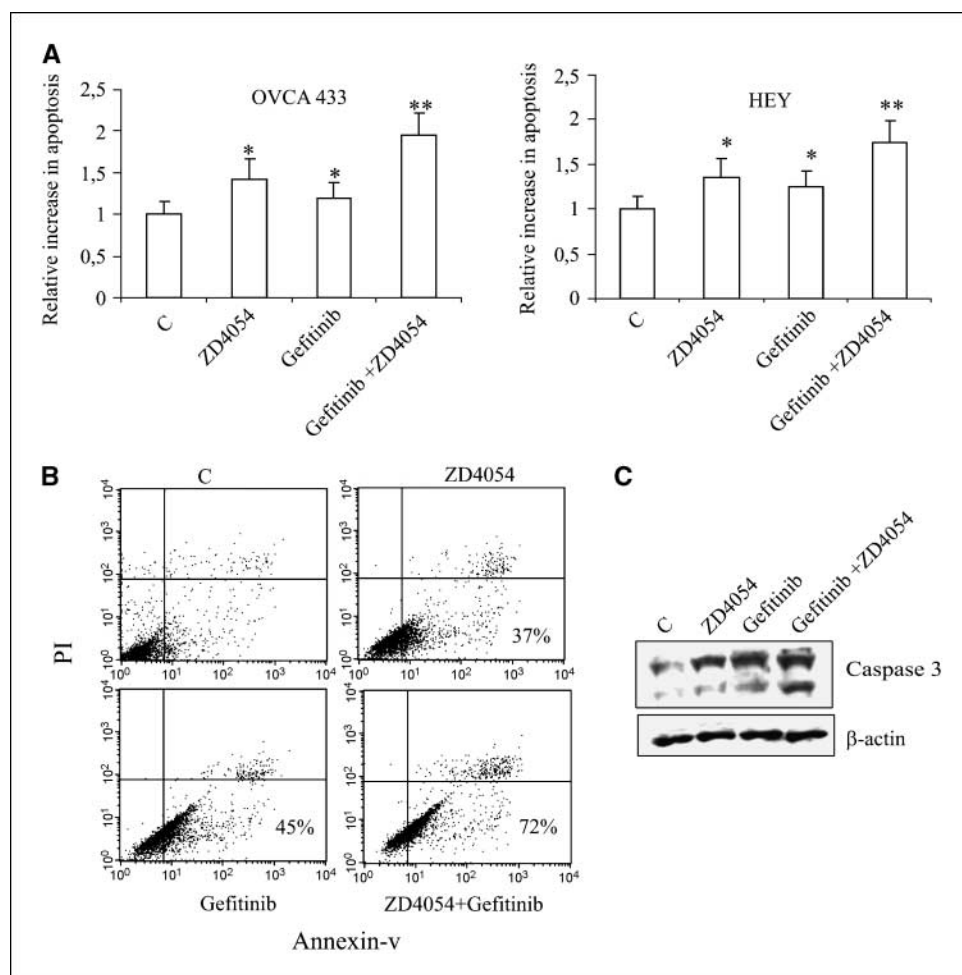
Combination of ZD4054 and gefitinib induces apoptosis. Because ET-1 is a survival factor, we determined whether the antiproliferative effect of ZD4054 resulted in the induction of programmed cell death. As shown in Fig. 3A, ZD4054 treatment increased the percentage of apoptotic HEY and OVCA 433 cells after 48 h of treatment ($P \leq 0.05$ compared with control), by measuring the histone-associated DNA fragments. Next, we evaluated the potential combined proapoptotic effect of treatment with ZD4054 and gefitinib. The addition of ZD4054 significantly increased gefitinib-induced apoptosis ($P \leq 0.005$) in both cell lines (Fig. 3A). Early apoptotic cells were also determined by Annexin V staining. Treatment of HEY cells with ZD4054 and gefitinib increased the number of early apoptotic cells to 37% and 45%, respectively. As expected, the induction of

apoptosis was higher when cells were treated with both drugs (72%; Fig. 3B). In HEY cells, combination treatment also resulted in increased cleaved caspase-3, a marker of apoptosis, compared with single agents (Fig. 3C), suggesting that combination treatment of gefitinib plus ZD4054 in a cooperative fashion activates apoptosis through caspase-dependent pathway. These results established that ET_AR and EGF-activated redundant survival pathways were effectively affected by combined treatment with ZD4054 and gefitinib.

Combination of ZD4054 and gefitinib affects VEGF production and cell invasion. ET_AR and EGFR are capable of regulating angiogenesis by promoting the tumor production of VEGF (8). Untreated HEY and OVCA 433 cells secreted $\sim 400 \text{ pg} \times 10^6$ cells of VEGF. Treatment with either ZD4054 or gefitinib alone caused an $\sim 32\%$ inhibition of VEGF secretion ($P < 0.001$) compared with control. The combination of ZD4054 with gefitinib produced a further inhibitory effect, reaching almost 50% reduction of VEGF secretion ($P < 0.002$; Fig. 4A). Combined treatment significantly inhibited also the basal and ET-1-induced invasion of HEY cells ($P < 0.001$; Fig. 4B). These results show that combined ET_AR and EGF blockade results in reduced expression of VEGF and inhibition of cell invasion.

Combination of ZD4054 and gefitinib inhibits tumor growth in nude mice. We studied whether the combination of ZD4054 and gefitinib could maximally impair tumor xenograft growth

Figure 3. Induction of apoptosis by treatment with ZD4054 alone and in combination with gefitinib in ovarian carcinoma cells. **A**, serum-starved OVCA 433 and HEY cells were treated for 48 h with $1 \mu\text{mol/L}$ ZD4054 or with $1 \mu\text{mol/L}$ gefitinib or in combination (gefitinib+ZD4054). Data are expressed in arbitrary units as relative increase compared with untreated cells considered as 1. Columns, averages of quadruplicate determinations of two separate experiments; bars, SD. *, $P < 0.05$ compared with control; **, $P < 0.001$ compared with ZD4054 or gefitinib. **B**, HEY cells were treated as in (A) and detection of apoptotic cells was evaluated by Annexin V and propidium iodide double staining and analyzed by flow cytometry. **C**, cell lysates of serum-starved HEY cells were processed for Western blot using antibodies capable of recognizing the proforms and activated forms of caspase-3. The membranes were probed with anti- β -actin to ensure the equal amount of proteins.



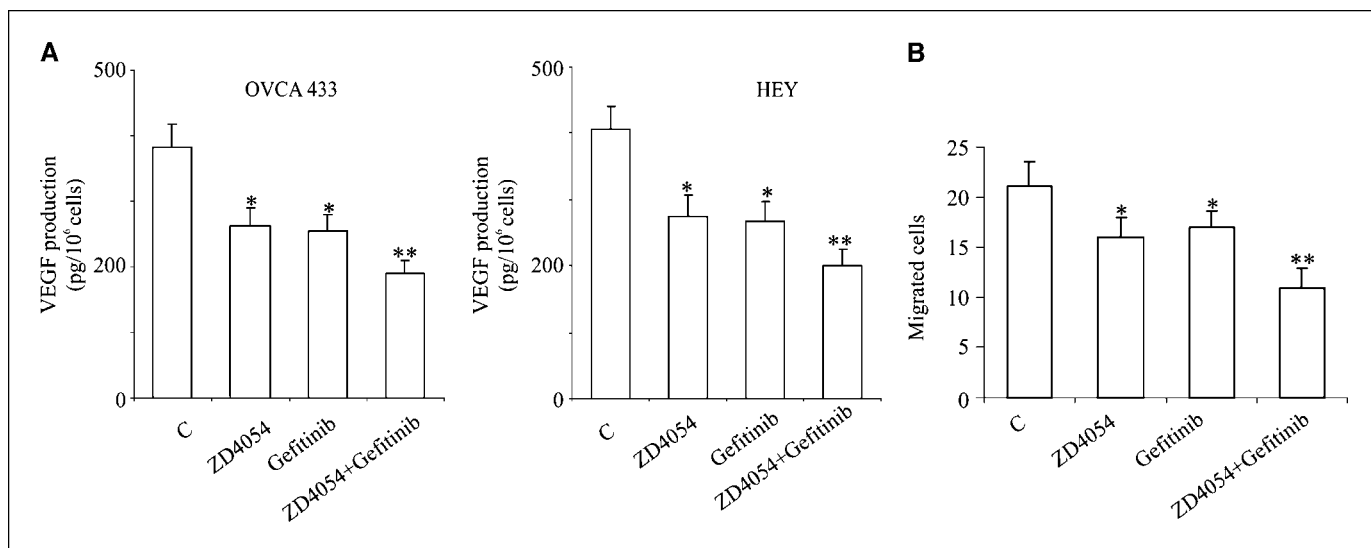


Figure 4. Effects of ZD4054 and/or gefitinib on VEGF production and invasiveness in ovarian cancer cells. **A**, serum-starved OVCA 433 and HEY cells were treated for 48 h with 1 $\mu\text{mol/L}$ ZD4054 or with 1 $\mu\text{mol/L}$ gefitinib or in combination (ZD4054+gefitinib). Columns, VEGF production, means of results from three ELISA determinations, each done in duplicate; bars, SD. *, $P < 0.001$ compared with control; **, $P < 0.002$ compared with ZD4054 or gefitinib. **B**, serum-starved HEY cells were treated as in (A) and cell invasion was measured using an invasion assay. Columns, mean of three independent experiments, each done in triplicate; bars, SD. *, $P \leq 0.001$ compared with control; **, $P < 0.001$ compared ZD4054 or gefitinib.

compared with either treatment alone. To this end, we used HEY cells that display high expression of both ET_AR and EGFR and, as shown above, are highly responsive to both drugs *in vitro*. For combined treatment, we selected a 10 mg/kg/d dose of ZD4054, because capable of inducing a 69% tumor inhibition with no associated toxicity, given i.p. for 21 days in combination with oral administration of gefitinib (125/mg/kg/d for 21 days). Tumor growth inhibition achieved with ZD4054 was comparable with that observed with gefitinib. Further tumor growth inhibition (82% of controls) was elicited by the combined regimen of ZD4054 and gefitinib. The comparison of the time course of tumor-growth curves by two-sided ANOVA was statistically significant ($P < 0.001$; Fig. 5). As shown in Table 1, the combined treatment was highly effective with no histologic evidence of HEY tumors in 4 of 10 mice. The dual treatment at the dose and schedule tested was well tolerated, as evaluated by the absence of weight loss or other signs of acute or delayed toxicity. To establish the duration of the combined ZD4054 and gefitinib treatment bioactivity, we extended the follow-up of the treated animal up to 60 days. As compared with control tumor xenografts, the growth delay in established tumors persisted for up to 4 weeks after the termination of the combined treatment.

Effect of combined treatment of ZD4054 and gefitinib on proliferation, angiogenesis, invasion, and EMT-related effectors. Biochemical and immunohistochemical analyses were done on HEY tumor xenografts, freshly excised on day 40 after tumor cell injection. Analysis of the cell proliferation rate, evaluated by Ki-67 immunostaining, revealed a marked decrease in the tumors treated with gefitinib and ZD4054 (Fig. 5B; Table 1). Ki-67 protein expression was decreased by 37% and 30% in tumors treated with ZD4054 or gefitinib alone, respectively, compared with untreated tumors, whereas Ki-67 was decreased by 46% in tumors treated with ZD4054 and gefitinib. The inhibition of Ki-67 by dual receptor blockade was statistically different from that observed with either ZD4054 or gefitinib treatment alone ($P < 0.02$ and $P < 0.001$, respectively).

Tumor-induced vascularization, which was quantified as microvessel density (MVD) using antibody against CD31, was significantly reduced after treatment with ZD4054. A further significant inhibition of tumor neovascularization (78%; $P < 0.0001$) was observed after combined treatment compared with either ZD4054 (62%; $P < 0.0001$) or gefitinib (45%; $P < 0.001$) treatment alone (Fig. 5B; Table 1). HEY tumor xenografts were also analyzed for VEGF expression by Western blots (Fig. 5C). We observed a marked reduction of VEGF expression in animals treated with combination regimens that was proportional to the reduction of vascularization, quantified as MVD. Activation of ET_AR in HEY cells leads to up-regulation of MMP secretion and activation (10). In this context, we evaluated whether ET_AR blockade inhibits MMP-2 expression. ZD4054 treatment and, above all, the combination of the two agents significantly reduced the percentage of HEY cells positive for MMP-2 (Fig. 5B). Analysis of EMT determinant in ovarian xenografts revealed a marked increase of E-cadherin expression in ZD4054-treated mice and especially in ZD4054 plus gefitinib-treated mice compared with control (Fig. 5C).

We next examined the expression of phospho-EGFR and phospho-p42/44 MAPK in tumors removed from the animals. Both phospho-EGFR and phospho-p42/44 MAPK expression were almost completely abrogated compared with either control or single-agent groups (Fig. 5C).

Discussion

Ovarian cancer remains the most common cause of death among gynecologic malignancies (1). Following cytoreductive surgery, chemotherapy has been the mainstay for ovarian cancer treatment (35). The majority of women with advanced ovarian cancer will develop drug-resistant disease with an overall 5-year survival $\leq 50\%$. The identification of new effective biology-based therapy is therefore mandatory. The $\text{ET}_1/\text{ET}_A\text{R}$ autocrine pathway has a crucial role in stromal-cancer cell interactions that promote tumor growth, neovascularization, EMT, and

metastatic spread (14). Therefore, interfering with ET_AR and their downstream targets might provide an opportunity to develop new mechanism-based strategies for cancer management. Among the small molecules capable of inhibiting the binding of the endogenous ligand to the ET_AR, ZD4054 is a potent and high selective ET_AR antagonist (31). ZD4054 inhibits ET-1-induced mitogenic activity in ovarian carcinoma cell lines (32). Moreover, ZD4054, through the inactivation of bcl-2 and activation of caspase-3 increases apoptosis and inhibits VEGF production in ovarian cancer cells. Preclinical activity was shown *in vivo* by the inhibition of tumor growth in mice bearing HEY ovarian carcinoma xenografts. Of interest, ZD4054 *in vivo* enhances the effects of chemotherapeutic agent such as taxanes, with no side effect (36). Along this line, to offer broader therapeutic prospects, we evaluated the therapeutic potential of ZD4054 in combined

therapy by targeting signaling networks that are selectively exploited in ovarian cancer and oversee multiple aspects of tumor cell maintenance and progression. We have previously shown that in ovarian cancer cells, EGFR transactivation by ET_AR is in part responsible for MAPK activation, prostaglandin E₂ and VEGF secretion, MMP activity, and ovarian cancer cell invasion (37, 38). Cross-talk between cell surface receptors, a main modality to expand the cellular communication signaling network, is also implicated in the acquired resistance to targeted therapies aiming at a single oncogenomic pathway. Therefore, disabling EGFR and ET_AR using gefitinib in combination with ZD4054 could be of benefit by increasing tumor cell death and intersecting escaping signaling networks that may override the effect of EGFR inhibitors. In this study, we showed that not only cell proliferation and survival, but also invasiveness, VEGF

Figure 5. Antitumor activity of ZD4054 treatment in combination with gefitinib on established HEY human ovarian carcinoma xenografts. **A**, mice were given injections of 1.5×10^6 HEY cells s.c. in the dorsal flank. After 7 d, the mice were treated i.p. for 21 d with vehicle (control), or with ZD4054 (10 mg/kg/d) alone, or with gefitinib (125 mg/kg/d) alone, or with combination of ZD4054 and gefitinib. Three different experiments with a total of 40 mice for each experiment were done. In each experiment, each group consisted of 10 mice. The comparison of the time course of tumor growth curves by two-way ANOVA with group-by-time interaction for tumor growth was statistically significant ($P < 0.001$). Columns, averages; bars, SD. **B**, immunohistochemical analysis. Tumors were removed from controls and from treated mice as described in (A), and were snap-frozen in liquid nitrogen on day 40 after tumor injection. Cryostat sections were immunostained for expression of CD31, Ki-67, and MMP-2, as described in Materials and Methods. Original magnification, $\times 200$. **C**, immunoblotting for phospho- and total-EGFR, phospho- and total-p42/44 MAPK, E-cadherin, and VEGF expression in HEY tumor xenografts treated as reported in (A).

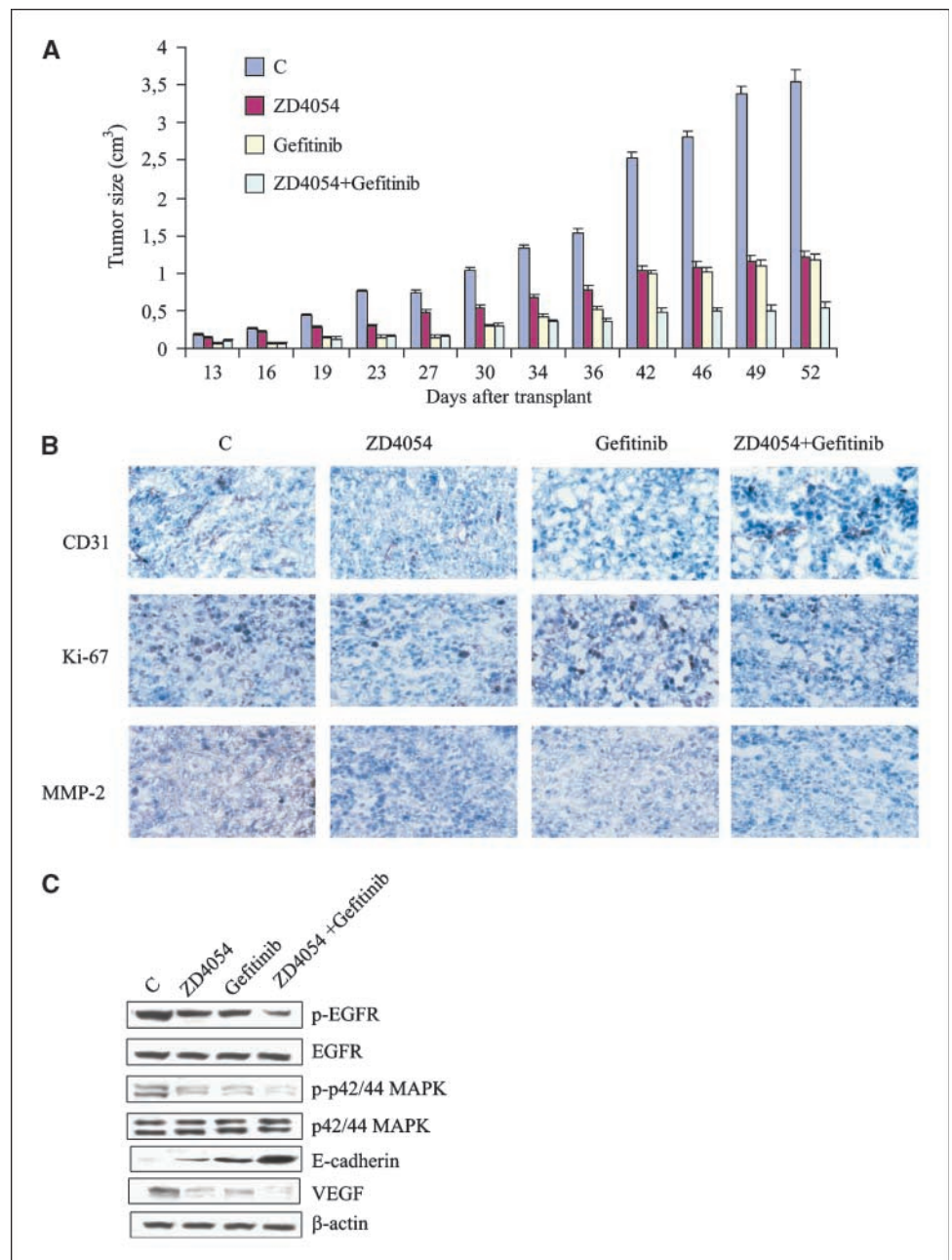


Table 1. Quantitative analysis of HEY ovarian carcinoma xenografts after treatment with ZD4054 and gefitinib

Treatment	Tumor volume (cm ³ ± SD)	Tumor-free mice (n)	Median vessel density*	Proliferation index Ki-67 [†]
Control	2.5 ± 1.29	0/10	67 ± 6	37.3 ± 1.5
ZD4054	1.03 ± 0.39 [‡]	0/10	25 ± 2 [‡]	23.4 ± 3.3 [‡]
Gefitinib	0.99 ± 0.28	0/10	37 ± 2 [‡]	26.6 ± 2.2 [‡]
ZD4054 + gefitinib	0.45 ± 0.1 [§]	4/10	15 ± 2 [§]	20.2 ± 3.3 [§]

NOTE: Mice treated for 21 d with ZD4054 or with gefitinib or with ZD4054 and gefitinib, were sacrificed on day 40 after tumor injection, and immunohistochemical analysis was done. Each group consisted of 10 mice. No histologic evidence of HEY tumor was detected in 4 of 10 mice treated with the combination of ZD4054 and gefitinib.

* Vessel counts were assessed by light microscopy after staining for CD31. Areas containing the highest numbers of capillaries and small venules were identified by scanning at low power, and individual vessel counts were done at ×200.

[†] The relative number of proliferating cells was quantitatively assessed in 10 randomly selected fields (×200).

[‡] *P* < 0.02 compared with control.

[§] *P* < 0.001 compared with ZD4054 or gefitinib.

production, and downstream signaling molecules, including phospho-p42/44 MAPK and p-AKT, can be maximally inhibited *in vitro* and *in vivo* by the combination of ZD4054 and gefitinib, suggesting that a contributing mechanism to tumor growth escape from EGFR inhibition could be the aberrant activation of downstream molecular effectors, and the increased angiogenic and invasive potential. These results are in agreement and extend those of Bianco et al. (39, 40), which have shown, using human GEO colon cancer cells, that EGFR inhibitor-resistant cells exhibit a 5- to 10-fold increase in VEGF production as well as in certain intracellular signal pathways that are downstream to the activated EGFR, compared with sensitive cells.

The recent demonstration that reversion of EMT and restoration of E-cadherin expression increases sensitivity to EGFR inhibitors in lung cancer cell lines may help to develop biomarkers and strategies to overcome resistance to EGFR inhibitors (27–29). EMT is a key step in the progression of tumors toward metastasis and invasion and may well play a role in determining sensitivity to EGFR inhibitors (28). E-cadherin expression is regulated by ET-1/ET_AR autocrine pathway and its expression is increased after ET_AR antagonist treatment *in vitro* and *in vivo* in ovarian cancer (7). In this study, we show that ZD4054 treatment reverts EMT, enhances E-cadherin expression and promoter activity, and inhibits invasiveness in ovarian cancer cells. Thus, blockade of ET_AR by ZD4054, restoring E-cadherin expression, could increase sensitivity to EGFR inhibitors, suggesting that E-cadherin expression not only is a potential marker of response to EGFR inhibitors but also plays a role in the mechanism underlying response to these drugs. Therefore, concomitant blockade of ET_AR and EGFR could represent an attractive therapeutic strategy for overcoming resistance to EGFR inhibitor.

Because response rate to gefitinib seems to be limited to the tumors harboring mutations in the tyrosine kinase domain of EGFR, new approaches are needed to increase the overall efficacy of gefitinib. In this context, combination therapy may be especially beneficial in the majority of tumors lacking mutations of functional significance because it potentially provides a second mechanism for inhibiting the EGFR through reduction of its transactivation by ET_AR and by restoring E-cadherin, resulting into increased response to gefitinib in ovarian cancer cells.

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 (41). In this context, experimental models have been used to show that significant and sustained antitumor activity can be obtained through the combination of selective anti-EGFR agents with other antesignal transduction agents. The tumor growth inhibition, the effect on downstream signaling molecules, and the decreased tumor cellularity and vascularity in response to gefitinib and ZD4054 treatment suggest that this drug combination by simultaneously disabling multiple signaling circuitries activated by EGFR and ET_AR may provide novel perspective in cancer therapy.

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