ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma \textit{in vitro} and \textit{in vivo}

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Abstract

The autocrine endothelin (ET)-1/endothelin A receptor (ET₄R) pathway is an important regulator of several processes involved in ovarian cancer progression, and its overexpression is associated with aggressive disease. These features have led to the proposal of the ET₄R receptor as a potential target for improving ovarian cancer treatment. In this study, we evaluated \textit{in vitro} and \textit{in vivo} the effects of ZD4054, an orally active antagonist that specifically binds ET₄R, as monotherapy, and in combination with paclitaxel. In the human ovarian cancer ET₄R-positive cell lines HEY, OVCA 433, SKOV-3, and A-2780, ZD4054 effectively inhibited the basal and ET-1-induced cell proliferation, associated with the inhibition of AKT and p42/44MAPK phosphorylation, and with increased apoptosis, through the inhibition of bcl-2 and activation of caspase-3 and poly(ADP-ribose) polymerase proteins. ZD4054 treatment also resulted in a reduction of ET₄R-driven angiogenesis and invasive mediators, such as vascular endothelial growth factor, cyclooxygenase-1/2, matrix metalloproteinase (MMP). The combination of ZD4054 and paclitaxel led to the potentiation of all these effects, indicating that ZD4054 and paclitaxel led to the potentiation of all antitumor effects, with 40% of mice remaining tumor-free, supporting a rationale for the clinical use of ZD4054 as monotherapy or in combination with cytotoxic drugs.

Introduction

Among gynecologic malignancies, epithelial ovarian carcinoma is associated with the highest mortality rate in the Western world. Most cases are diagnosed at an advanced stage of the disease resulting into a poor survival rate (1). Although chemotherapy regimens are associated with high remission rate, the disease frequently relapses with drug resistance being a significant barrier to cure (2). To overcome the limitations of cytotoxic chemotherapy, current approaches to drug design in oncology are aimed at modulating specific cell signaling pathways relevant to tumor growth and survival. In this context, clinical trials evaluating targeted therapy have indicated that improved survival can be achieved through combination schedules with chemotherapy. Although cancer cells integrate multiple signaling pathways sustaining tumor progression, therapeutic interest in the endothelin-1 (ET-1) axis is supported by its central role in several human cancers, including ovarian carcinoma (3–6). ET-1 is a member of the endothelin family of three closely related isopeptides, ET-1, ET-2, and ET-3. Two major receptor subtypes that mediate ET’s biological effects belong to the G-protein coupled receptor family: the ET₄ receptor (ET₄R) is considered as the major effector of the ET-1 axis and binds ET-1 and ET-2 with high affinity and ET-3 with low affinity; whereas the ET₃R binds all ET isopeptides with the equal affinity (7). Interestingly, in gene expression profiles of late-stage ovarian cancer, ET₄R was identified as a metastasis-associated gene that activates cell signaling involved in the control of cell migration, spread, and invasion (8, 9). Levels of ET-1 are markedly elevated in the ascites of patients with epithelial ovarian cancer (10) and, together with the ET₄R, are overexpressed and activated in 85% of ovarian tumors, correlating with advanced stages (7, 11, 12). In ovarian cancer cells, the autocrine loop mediated by the ET-1/ET₄R receptor-ligand interaction has been implicated in the parallel activation of several signal transduction pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)–dependent integrin-linked kinase and AKT activation, Src-mediated epidermal growth factor receptor transactivation, and p125 focal adhesion kinase and paxillin modulation (8, 13–15). This cascade of events ultimately induces nuclear transcription of several proto-oncogenes.
promoting cell proliferation and escape from apoptosis, neovascularization, protease production, epithelial-to-mesenchymal transition, and invasiveness, suggesting that ET-1 may also play a role in metastatic competence (6, 12, 16–20). Interestingly, ET-1 protects ovarian cancer cells from paclitaxel-induced apoptosis via a bcl-2–dependent mechanism and involves the activation of Akt. This survival pathway is inhibited by ETAR antagonists, indicating that ETAR blockade could increase the commitment toward apoptosis induced by paclitaxel (16). Importantly, pharmacologic blockade of the ETAR inhibits tumor xenograft growth and molecular determinants involved in tumor progression (11–13, 21). The pleiotropic effects exerted by ET-1 in the development of ovarian cancer and other epithelial tumors, such as prostate and cervical cancers, has led to the development of small molecules that antagonize the binding of ET-1 to the ETAR (22–24). Among various ETAR antagonists, ZD4054 is an orally active ETAR antagonist in early clinical development for the treatment of cancer. The synthesis and molecular characterization of ZD4054 has been described previously (25). ZD4054 binds to the ETAR with high affinity. In preclinical studies, ZD4054 specifically antagonizes ETAR-mediated proliferative and antiapoptotic effects, but not ET _β_ -mediated proapoptotic effects in different cell types (26, 27). We have demonstrated previously that in ovarian cancer cells, ZD4054 antagonizes ET-1–induced cell proliferation (27). Given the encouraging preliminary results, in this study, we investigated the antitumor activity of ZD4054 in ovarian carcinoma by a multiparametric analysis _in vitro_ and _in vivo_. The effects of ETAR antagonism described above, together with evidence of protection against paclitaxel-induced apoptosis, provided the rationale for evaluating the therapeutic activity of ZD4054 in combination with conventional chemotherapeutic agents (16). Preclinical studies using a combination treatment of ETAR antagonist with paclitaxel revealed additive therapeutic efficacy in ovarian, cervical, and prostate carcinomas (21, 22, 28–30). Therefore, we determined _in vitro_ and _in vivo_ the relative contributions of ZD4054 administered alone or in combination with paclitaxel to the inhibition of basal cell proliferation and apoptosis protection. Our findings provided mechanism-based evidence demonstrating that _in vitro_ and _in vivo_ , ZD4054, by blocking the ET-1/ETAR axis and its related signaling cascade, displayed several antitumor activities that were of the same magnitude of effect as paclitaxel. Furthermore, combined ZD4054 and paclitaxel treatment resulted in a greater degree of inhibition of tumor growth and tumor-promoting effects compared with single agents.

**Cells and Cell Culture Conditions**

Human ovarian carcinoma cell lines, HEY, OVCA 433, and A-2780, were a generous gift from Dr. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). The SKOV-3 cell line was obtained from the American Type Culture Collection. All culture reagents were from Invitrogen. The cells were serum starved by incubation for 24 h in serum-free DMEM. ET-1 (Peninsula Laboratories) was used at 100 nmol/L and incubated with the cells for the indicated times. Pretreatment of cells with ZD4054 was performed for 30 min prior to the addition of ET-1.

**Thymidine Incorporation Assay**

Serum-starved HEY, OVCA 433, SKOV-3, and A-2780 cells were treated with ZD4054 (1 μmol/L) and/or paclitaxel (60 nmol/L), and after 24 h, 1 μCi of [methy] [3H]-thymidine (5 Ci/μmol/L) was added to each well. The thymidine incorporation assay was performed as described previously (21). Responses to all treatments were assayed in sextuplicate, and results were expressed as the means of three separate experiments.

**ELISA**

Serum-starved HEY cells were treated for 24 h with 1 μmol/L ZD4054 or 60 nmol/L paclitaxel or in combination. The vascular endothelial growth factor (VEGF) protein levels in the cell-conditioned media were determined in triplicate by ELISA using the Quantikine human VEGF immunoassay kit (R&D Systems). The sensitivity of the assay is less than 5.0 pg/mL. Intra-assay variation is 5.4%, and interassay variation is 7.3%. Gelatinase activities in conditioned medium were determined by a matrix metalloproteinase (MMP) gelatinase activity assay kit (Chemicon) according to the manufacturer’s instructions. The sensitivity of the assay is <5 ng/mL MMP in the range 10–200 ng/mL.

**Apoptosis Assay**

The induction of programmed cell death was determined as described previously (16) by the cell death detection ELISA Plus kit (Boehringer Mannheim). Briefly, 5 × 10⁵ cells per well were seeded into 12-well plates. After a 48-h treatment, cells were lysed, and the supernatants were 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 performed in quadruplicate and repeated twice. For detection of early apoptotic events, nonadherent 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 analyzed by cyttofluorometric analysis.

**Western Blot Analysis**

For protein detection, whole cell lysates were subjected to SDS-PAGE and revealed by Western blotting using antibodies to caspase-3, phospho-AKT and AKT, phospho-p42/44 MAPK, and p42/44 MAPK (Cell Signaling), bcl-2 and poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Inc.), cyclooxygenase-1 (COX-1) and COX-2.
sections in which the incubation with the primary antibody was either omitted or substituted by isotype-matched immunoglobulins. The evaluation of microvessel density was performed by two independent observers on an ×200 field according to the criteria of Weidner et al. (31). Cells positive for Ki-67 were expressed as the percentage of tumor cells with nuclear staining counted in three separate ×40 microscopic fields (at least 200 cells per field were counted).

### Statistical Analysis

Statistical analysis was performed using Student’s *t* test and Fisher’s exact test as appropriate. The time course of tumor growth was compared across the groups using two-way ANOVA, with group and time as variables. All statistical tests were carried out using SPSS software (SPSS). A two-sided probability value of <0.05 was considered statistically significant.

### Results

#### Cell Proliferation Inhibition by ZD4054 and Paclitaxel

The potential antiproliferative effect of ZD4054 alone or in combination with paclitaxel was evaluated on HEY, OVCA 433, SKOV-3, and A2780 cells that release ET-1 and express functional ET<sub>A</sub>R (4, 5). The ET-1 levels released from these cells were within the physiological range needed for the activation of the ET<sub>A</sub>R in an autocrine fashion (12). Serum-starved cell lines were exposed to ZD4054 (1 μmol/L) and/or paclitaxel (60 nmol/L). As measured by the [³H]-thymidine incorporation assay, significant inhibition of cell proliferation was observed in ZD4054-treated cells (Fig. 1A). Furthermore, combination of ZD4054 and paclitaxel led to a greater inhibition of cell growth induced by endogenous ET-1 in comparison to the partial inhibition produced by single agents. This observation suggests that the specific blockade of the ET<sub>A</sub>R with ZD4054 cooperates with conventional cytotoxic drugs, such as paclitaxel, to inhibit ovarian tumor growth (Fig. 1A). Similar results were obtained in the presence of exogenous ET-1 (Supplementary Fig. S1). Because inhibition of cell growth could also be due to the induction of apoptosis induced by ZD4054 or taxol, we therefore investigated whether ZD4054 could potentiate the effect of paclitaxel by inducing apoptosis compared with either single agent. ET<sub>A</sub>R activation by ET-1 triggers multiple signal transduction pathways that include MAPK and AKT pathways (8, 15). In particular, we previously demonstrated that PI3K-dependent Akt pathway has a central role in ET<sub>A</sub>R-mediated protection against paclitaxel-dependent apoptosis in ovarian cancer cells (16). Therefore, we evaluated whether ZD4054 treatment was associated with reduced ET<sub>A</sub>R-driven downstream signaling pathway in HEY cells. As expected, serum-starved HEY cells displayed constitutive levels of activated AKT and p42/44 MAPK; the addition of exogenous ET-1 induced a marked increase in the phosphorylation of p42/44 MAPKs and

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4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
AKT, as assessed by the use of specific antibodies (Fig. 1B). ZD4054 decreased the endogenous ET-1–induced phosphorylation/activation of both kinases, and combination of ZD4054 with paclitaxel exerted a stronger interruption of the two signaling pathways (Fig. 1B). Together, these data suggest that ZD4054, as monotherapy and in combination with paclitaxel, affected ET AR-dependent signal transduction pathways related to cell proliferation and survival.

Effect on Apoptosis

To determine whether the growth-inhibitory effect of ZD4054 could involve the induction of apoptosis, HEY and OVCA 433 cells were treated with ZD4054 or paclitaxel for 48 h and evaluated by ELISA assay, which detected histone-associated DNA fragments. As shown in Fig. 2A, ZD4054 was able to induce an increase in apoptotic cells similar to that induced by paclitaxel (*P < 0.01 compared with control). The combined treatment of ZD4054 plus paclitaxel further increased the percentage of apoptotic cells.

Figure 1. Effect of ZD4054 in monotherapy and combination therapy with paclitaxel on ovarian carcinoma cell proliferation and on MAPK and AKT signaling pathways. A, serum-starved HEY, OVCA 433, SKOV-3, and A-2780 cells were treated with ZD4054 (1 μmol/L) or paclitaxel (PAC, 60 nmol/L) or ZD4054 + PAC before measuring [3H]-thymidine incorporation. Columns, averages of sextuplicate determinations of three separate experiments; bars, SD; *, P < 0.001 compared with control (C); **, P < 0.0001 compared with ZD4054 or paclitaxel. B, whole-cell lysates of HEY cells treated with ET-1 (100 nmol/L) or with ZD4054 (1 μmol/L) or paclitaxel (60 nmol/L) or ZD4054 + paclitaxel were blotted for detection of total and activated p42/44 MAPK and AKT by using specific antibodies.

Figure 2. Induction of apoptosis by treatment with ZD4054 and paclitaxel in ovarian carcinoma cells. A, HEY and OVCA 433 cells were treated for 48 h with ZD4054 (1 μmol/L) or with paclitaxel (PAC, 60 nmol/L) or in combination (PAC + ZD4054). Columns, averages of quadruplicate determinations of two separate experiments; bars, SD; *, P < 0.01 compared with control (C); **, P < 0.001 compared with ZD4054 or paclitaxel. B, apoptotic changes induced by exposure of HEY cells treated as in A, were determined by flow-cytometric analysis of annexin V–propidium iodide staining. C, cell lysates of serum-starved HEY cells treated as in A were processed for Western blot using antibodies capable of recognizing the proforms and activated forms of caspase-3, PARP, and bcl-2. The membranes were probed with anti–β-actin antibody to ensure equal amount of proteins.
paclitaxel markedly increased apoptosis ($P < 0.001$ compared with ZD4054 or paclitaxel alone). HEY cells were also analyzed for the presence of early apoptotic events using annexin V staining (Fig. 2B). Treatment of serum-starved HEY cells with ZD4054 increased the number of early apoptotic cells from $\sim 13\%$ to $\sim 39\%$, confirming our previous findings that, in ovarian cancer cells, endogenous ET-1 induces survival signaling through the ET_{A}R (15). The coadministration of ZD4054 significantly potentiated ($\sim 70\%, P < 0.005$ compared with single agent treatment) the cytotoxic drug-induced apoptotic cell death in an additive manner, suggesting a specific therapeutic window in which combination therapy may be more effective at inducing apoptotic death in ovarian cancer cells. In view of the described effects on apoptosis induction, we examined the effects of ZD4054 on the levels of apoptosis-related protein expression. Treatment of HEY cells with either ZD4054 or paclitaxel alone resulted in little or no detectable cleaved caspase-3. In contrast, combination treatment resulted in the accumulation of processed caspase-3. Moreover, combination treatment also resulted in increased cleaved PARP, a substrate of activated caspase-3, compared with single agents (Fig. 2C), suggesting that paclitaxel and ZD4054 act in a cooperative fashion to activate apoptosis through a caspase-dependent pathway. As commitment to apoptosis is largely a mitochondrial event, controlled by antiapoptotic proteins in the bcl-2 family, we also investigated whether ZD4054 could modulate the expression of bcl-2 induced by paclitaxel. As shown in Fig. 2C, although paclitaxel treatment induced a significant increase in phosphorylation and, thus, inactivation of bcl-2, the addition of ZD4054 potentiated the paclitaxel-induced effect, confirming the potential cooperation of ZD4054 to enhance the therapeutic efficacy of paclitaxel.

**Inhibition of Invasion and Angiogenesis**

We have demonstrated previously that the tumor-promoting effects of ET-1 through the ET_{A}R in ovarian carcinoma cells also includes the secretion and activation of MMPs, such as MMP-2 and MMP-9, which are highly relevant in angiogenesis as well as in tumor cell invasion and metastasis (18, 19). Thus, we investigated whether ZD4054 may inhibit ET-1--induced MMP secretion and activity in ovarian carcinoma cells. Both gelatin zymography and activity measurements demonstrated an increase in MMP-2 and MMP-9 activity after 48 h in ET-1--stimulated HEY cells. Pretreatment with ZD4054 reduced the basal MMP activity (Fig. 3A and B), and this effect was more pronounced when ZD4054 was added in combination with paclitaxel. In ovarian carcinoma cells, ET-1 increases VEGF, COX-1, and COX-2 expression through the ET_{A}R, and these modulate invasive behavior (17, 18). Therefore, we also tested the therapeutic efficacy of ZD4054 alone or in combination with paclitaxel on the endogenous production of VEGF and on COX-1/-2 expression. By ELISA assay, we found that ZD4054 inhibited basal production of VEGF by $\sim 30\%$ (comparable to the effect seen with paclitaxel), confirming the involvement of an autocrine ET-1/ET_{A}R in this effect (Fig. 3C). This inhibitory effect was more evident in the presence of both drugs (reaching $\sim 60\%$), indicating that combination therapy was also effective in preventing endogenous ET-1--induced production

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**Figure 3.** Effects of ZD4054 in mono-therapy and combination therapy with paclitaxel on ovarian carcinoma cell angiogenesis and invasion. A, serum-starved HEY cells were treated with ET-1 (100 nmol/L) or ZD4054 (1 μmol/L) or paclitaxel (PAC, 60 nmol/L) or in combination (PAC + ZD4054) for 48 h. Gelatin zymography was used to determine the activities of MMP-2 and MMP-9. B, MMP activity was also measured by a MMP gelatinase activity assay kit. Bars, SD; *, $P < 0.05$ compared with the control (C); **, $P < 0.001$, compared with ZD4054 or paclitaxel. C, VEGF production measured in HEY and OVCA 433 cells treated as in A is reported as means of results from three ELISA determinations, each performed in duplicate. Bars, SD; *, $P < 0.001$ compared with control; ***, $P < 0.001$ compared with ZD4054 or paclitaxel. D, cell lysates of serum-starved HEY cells, treated as in A, were analyzed for COX-1 and COX-2 protein expression by Western blotting and reprobed with anti-β-actin antibody to ensure equal amount of proteins.
of VEGF leading to tumor neovascularization. Moreover, ET-1 significantly induced COX-1 and COX-2 protein up-regulation. Basal COX-1/2 was inhibited by both ZD4054 and paclitaxel, and this effect was more significantly inhibited when ZD4054 was added in combination with paclitaxel compared with either agent (Fig. 3D). Taken together, these results indicate that ZD4054 alone, and to a greater extent in combination with paclitaxel, reduced the expression of angiogenic and invasive mediators, such as MMPs, VEGF, and COX-1/2 induced by endogenous ET-1, in ovarian carcinoma cells leading to an inhibition of tumor progression.

Inhibition of Growth of Human HEY Ovarian Carcinoma in Nude Mice

The potential antitumor effect of ZD4054 in vivo was assessed in murine tumor xenografts. Human HEY cells were grown as s.c. tumors in nude mice. Following 7 days of tumor growth, when established xenografts were palpable, mice were treated with three different doses of ZD4054 i.p. for 21 days: 10, 25, and 50 mg/kg/day. Treatment with ZD4054 produced a 69% inhibition of tumor growth on day 40 after tumor injection, with either medium (25 mg/kg/day) or high (50 mg/kg/day) doses (both \( P < 0.001 \) compared with control; Fig. 4A). ZD4054 treatment was generally well tolerated, with no detectable signs of acute or delayed toxicity, even at the highest dose. As shown in Fig. 4B, the tumor growth inhibition obtained with ZD4054 (25 mg/kg/day) treatment was comparable with that achieved with paclitaxel (three 20 mg/kg i.v. doses every 4 days). We next evaluated whether the cooperative effects of ZD4054 and paclitaxel in vitro could also be present in vivo. A marked and sustained growth inhibitory effect was observed when ZD4054 (10 mg/kg/day) was used in combination with paclitaxel (three 20 mg/kg i.v. doses every 4 days), causing significant inhibition in tumor growth at the end of the 4-week treatment period in all mice (87%, \( P < 0.002 \)) when compared to mice treated with a single agent. The comparison of the time course of tumor-growth curves by two-sided ANOVA, using group and time as variables, showed that the group-by-time interaction for tumor growth was statistically significant (\( P < 0.0001 \); Fig. 5A). As shown in Table 1, the combined treatment was highly effective, with no histological evidence of HEY tumors in 4 out of 10 mice. The combination treatment at the dose and schedule tested was well tolerated, as indicated by the absence of weight loss or other signs of acute or delayed toxicity. Moreover, as shown by Fig. 5A, the tumor growth inhibition obtained with combination treatment of ZD4054 and paclitaxel persisted for up to 4 weeks after termination of treatment. To demonstrate that the inhibition of tumor growth observed in treated mice was accompanied by a significant reduction in tumor cell proliferation and vascularization, tissue sections of HEY tumors on day 40 after tumor cell injection were analyzed by immunohistochemistry (Fig. 5B and Table 1). Tumor-induced vascularization, quantified by staining of tissue sections with an anti-CD31 antibody, was significantly reduced versus controls, following treatment with either ZD4054 or paclitaxel (~60% and ~54%, respectively; \( P < 0.05 \) compared with control). Moreover, in mice treated with ZD4054 plus paclitaxel, this inhibition reached ~75% \( (P < 0.002 \) compared with single agents). The inhibition of tumor vascularization was associated with the inhibition of the expression of MMP-2 in all treatment schedules (data not shown). We also found that the number of proliferating tumor cells, measured by the number of Ki-67–positive cells, was reduced from 37.3 ± 1.5 in tumors of control mice to 22.4 ± 3.3 and 27.7 ± 2.2 in tumors of mice treated with either ZD4054 or paclitaxel, respectively \( (P < 0.05 \) compared with control), whereas in the combination therapy group, the number of Ki-67–positive cells was reduced to 15.7 ± 3.3 \( (P < 0.002 \) compared with single agents).

Discussion

In recent years, many experimental studies have been performed to develop novel antitumor agents that are capable of selectively interfering with key oncogenic signaling pathways. The ET\(_4\)R has been reported as a potential target for the development of selective antagonists for ET\(_4\)R. In our recent studies, we showed that ZD4054, a selective ET\(_4\)R antagonist, can inhibit tumor growth in vivo in a human ovarian carcinoma xenograft model.

**Figure 4.** Antitumor activity of ZD4054 and paclitaxel on established HEY human ovarian carcinoma xenografts. A, mice were given injections of \( 1.5 \times 10^6 \) HEY cells s.c. into the dorsal flank. After 7 d, mice were treated i.p. for 21 d with vehicle (Control), and three doses of ZD4054 (10, 25, or 50 mg/kg/day). Three different experiments using a total of 40 mice for each experiment were performed. In each experiment, each group consisted of 10 mice. Treatment with ZD4054 produced a 69% inhibition of tumor growth on day 40 after tumor injection, with either medium (25 mg/kg/day) or high (50 mg/kg/day) doses \( (P < 0.001 \) compared with control). Data represent mean tumor volume. B, mice bearing HEY tumor xenografts were treated as follows: one group with 25 mg/kg/day of ZD4054 for 21 d, one group with three i.v. administrations of paclitaxel (PAC, 20 mg/kg) every 4 d, and one group with vehicle (Control). Data represent mean tumor volume.
new therapeutic target in several human cancers, including cervical (32), prostate (33), ovarian (21), breast (34), colon (35), lung (36), renal (37), and thyroid gland (38) malignancies. ETAR signaling has been shown convincingly to regulate stromal-tumor cell interactions promoting tumor growth, neovascularization, and metastatic spread (3, 11, 12, 39). In particular, we have previously demonstrated overexpression of the ET-1/ET AR autocrine loop in primary and metastatic ovarian carcinomas and its contribution to tumor growth and progression (21). In these malignancies, overexpression of the ET-1 axis is associated with advanced-stage disease and has pleiotropic activities including enhanced migration, invasion, and epithelial-to-mesenchymal transition, which could be reversed by specific blockade of the ETAR (10–21). In this respect, interfering with ETAR and its downstream targets may provide an opportunity for the development of new, mechanism-based strategies for cancer treatment, as demonstrated by the preclinical results with small molecules targeting ETAR (21–24). Thus, we demonstrated previously that in vivo inhibition of the ET AR autocrine pathway using ETAR antagonism results in growth reduction of tumor xenografts (11–13, 21). In the present study, we demonstrated the therapeutic relevance of the specific ET AR

Table 1. Immunohistochemical analysis of HEY ovarian carcinoma xenograft after treatment with ZD4054 and paclitaxel

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor volume (cm&lt;sup&gt;3&lt;/sup&gt;), mean ± SD</th>
<th>Mice tumor free (n)</th>
<th>Median vessel density*</th>
<th>Proliferation index Ki-67&lt;sup&gt;†&lt;/sup&gt;</th>
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<tr>
<td>Control</td>
<td>3.7 ± 1.29</td>
<td>0/10</td>
<td>67 ± 6</td>
<td>37.3 ± 1.5</td>
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<tr>
<td>ZD4054</td>
<td>1.78 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/10</td>
<td>25 ± 2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>22.4 ± 3.3&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.06 ± 0.82</td>
<td>0/10</td>
<td>31 ± 2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>27.7 ± 2.2&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZD4054 + Paclitaxel</td>
<td>0.46 ± 0.11</td>
<td>4/10</td>
<td>17 ± 2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>15.7 ± 3.3&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
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NOTE: Nude mice were given a s.c. injection of 1.5 × 10<sup>6</sup> viable HEY cells on day 0. Treatment was started on day 7 after tumor injection. Mice were treated for 21 d i.p. with ZD4054 (10 mg/kg/day) or with paclitaxel (20 mg/kg i.v. every 4 d for three doses) or with a combination of ZD4054 + paclitaxel. Mice were sacrificed on day 40 after tumor injection, and immunohistochemical analysis was performed. Each group consisted of 10 mice. No histological evidence of HEY tumor was detected in 4 of 10 mice treated with the combination of paclitaxel and ZD4054.

<sup>*</sup>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 performed at ×200.

<sup>†</sup>The relative number of proliferating cells was quantitatively assessed in 10 randomly selected fields (×200).

<sup>b</sup>P < 0.05 compared with control.

<sup>x</sup>P < 0.002 compared with single treatments.
antagonist, ZD4054, to block ovarian cancer growth and progression. In ovarian carcinoma cells, pharmacological antagonism of ET\textsubscript{A}R-driven autocrine pathways by ZD4054 resulted in a reduction of cell proliferation and angiogenic and invasive mediators, associated with elevated apoptotic signals. Furthermore, ZD4054 significantly antagonized ET\textsubscript{A}R-associated proliferative and antiapoptotic signaling pathways, demonstrating the potential for inhibiting multiple tumor-promoting signals driven by ET\textsubscript{A}R activation. Consistent with the in vitro results, ZD4054 affected the growth of HEY xenografts in nude mice leading to a significant reduction in tumor growth and neovascularization, with no associated toxicity. Current efforts to improve anticancer treatment are increasing, employing the concept of additive targets, such as the use of targeted agents in combination with cytotoxic drugs. This is critical in ovarian cancer, which, although highly responsive to platinum-based therapy after initial cytoreductive surgery, often recurs because of the emergence of drug resistance to conventional chemotherapy regimens (2). Novel therapeutic agents, in addition to combination regimens, may delay the emergence of resistance, thus prolonging remissions and improving long-term outcomes. In an attempt to understand the biological mechanisms underlying the drug response, we also evaluated the therapeutic efficacy of ZD4054 in combination with paclitaxel in vitro and in vivo. Consistent with previous results demonstrating the role of ET-1 as an antiapoptotic factor implicated in paclitaxel resistance (16), in the present study, we demonstrated that when the two agents were combined, a significant additive antitumor effect occurred compared to a single regimen, which was associated with a dramatic inhibition of the pleiotropic events triggered by ET\textsubscript{A}R activation. The increased commitment toward apoptosis is dependent on the activation of caspase-3 and PARP and inactivation of the antiapoptotic bcl-2 protein, suggesting that ZD4054 enhances the antitumor effect of paclitaxel by blocking survival pathways. The benefit of combining ZD4054 with paclitaxel becomes more pronounced following in vivo treatment, with complete regression of tumors in 40% of treated mice. The therapeutic efficacy of the combination of ZD4054 and paclitaxel is associated with a marked inhibition of tumor neovascularization and reduction in the proliferation index. In summary, our experiments demonstrated that ZD4054 enhances the antitumor effect of paclitaxel in vivo, highlighting the potential benefit of combining two therapeutic agents that have entirely different mechanisms of action. These results complement and extend previous studies published by our and other groups and support the concept that pharmacological blockade of the ET\textsubscript{A}R using small molecule receptor antagonists in combination with cytotoxic drugs is therapeutically advantageous in different tumor types (21, 28–30, 40). Moreover, a recent gene expression profile analysis of intrinsic and/or acquired chemoresistance in ovarian cancers has identified the ET\textsubscript{A}R as one of the genes highly expressed in postchemotherapy samples compared with untreated primary tumors, implicating this receptor as a target for prospective investigations of acquired chemoresistance in ovarian cancers (41). In conclusion, these findings have demonstrated the therapeutic efficacy of ZD4054, which has been shown to induce concomitant antitumor activity and inhibition of neovascularization, providing a rationale for the clinical evaluation of this molecule alone and in combination with cytotoxic drugs in patients with ovarian tumors.

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References


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