Cancer Therapy: Preclinical

Clinical Cancer Research

Acquisition of Chemoresistance and EMT Phenotype Is Linked with Activation of the Endothelin A Receptor Pathway in Ovarian Carcinoma Cells

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Abstract

Purpose: Emerging evidence suggests molecular and phenotypic association between chemoresistance and epithelial–mesenchymal transition (EMT) in cancer. Endothelin-1 (ET-1)/endothelin A receptor (ET_AR) axis is implicated in the pathobiology of epithelial ovarian cancer (EOC) by driving tumor-promoting effects, including EMT. Here, we analyzed how ET_AR regulates chemoresistance and EMT in EOC.

Experimental Design: The effects of ET-1 axis on cell proliferation, drug-induced apoptosis, invasiveness, and EMT were analyzed in cultured EOC cells sensitive and resistant to cisplatinum and taxol. Tumor growth in response to ET_AR antagonist was examined in EOC xenografts. ET_AR expression was examined in 60 human EOC tumors by immunohistochemistry and correlated with chemoresistance and EMT.

Results: In resistant EOC cells ET-1 and ET_AR are upregulated, paralleled by enhanced mitogen activated protein kinase (MAPK) and Akt phosphorylation and cell proliferation. Moreover, in these cells the expression of E-cadherin transcriptional repressors, including Snail, Slug, and Twist, as well as of mesenchymal markers, such as vimentin and N-cadherin, were upregulated and linked with enhanced invasive behavior. Interestingly, ET_AR blockade with zibotentan, a specific ET_AR antagonist, or its silencing, downregulated Snail activity, restored drug sensitivity to cytotoxic-induced apoptosis, and inhibited the invasiveness of resistant cells. *In vivo*, zibotentan inhibited tumor growth of sensitive and resistant EOC xenografts, and sensitized to chemotherapy. Analysis of EOC human tissues revealed that ET_AR is overexpressed in resistant tumors and is associated with EMT phenotype.

Conclusions: Our data provide the first evidence that blockade of ET_AR-driven EMT can overcome chemoresistance and inhibit tumor progression, improving the outcome of EOC patients' treatment. *Clin Cancer Res; 17(8); 2350–60. ©2011 AACR.*

Introduction

Ovarian cancer accounts for the highest tumor-related mortality in women with gynaecologic malignancy (1). The identification of the molecular mechanisms underlying chemoresistance is mandatory to achieve advancement in ovarian cancer therapy (2). Accumulating evidences demonstrated that epithelial–mesenchymal transition (EMT), which modulates cancer progression and metastasis, has also been implicated in the onset of drug resistance and tumor relapses, representing an escape mechanism from apoptosis (3). Therefore, the acquisition of mesenchymal phenotypes engenders tumor cells with a multifaceted capacity to proliferate, migrate, and avoid cell death and permanent arrest, as well as protection from extracellular signals and drug effect activities (4). The hallmark of EMT is loss of the epithelial molecule E-cadherin and gain of mesenchymal markers, such as N-cadherin and vimentin. The E-cadherin repressors, Snail and Slug, which interact with E-box elements located within the proximal region of the E-cadherin promoter, and the basic helix-loop-helix transcription factor Twist, are significant inducers of EMT in cancer cells by repressing E-cadherin expression (5). Several clinical studies have shown that increased expression of E-cadherin is associated with improved survival in several tumor types (6, 7), and silencing of E-cadherin transcriptional suppressors can increase cellular sensitivity to genotoxic stress (8). Since EMT development is driven by key modulators that are directly controlled by numerous extracellular signals and pathways (5), it is becoming clear that the blockade of these signaling pathways is critical for

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Translational Relevance

Overcoming chemoresistance is the major challenging in treating epithelial ovarian cancer (EOC). Our study well explains the effect of endothelin-1 (ET-1) axis on the acquisition of chemoresistance and epithelial–mesenchymal transition (EMT) phenotype and provides possible strategies in clinical oncotherapy. In a combined *in vitro*/*in vivo* model of EOC cells resistant to taxol and cisplatinum, we revealed that ET-1 triggers major tumorigenic signals, including the activation of the EMT-driver Snail. Endothelin A receptor (ET_AR) blockade by a specific ET_AR antagonist results into reverted EMT, restored drug sensitivity, and inhibited cell invasion. Analysis of human EOC tissues validated the preclinical results revealing that ET_AR is overexpressed in the chemoresistant tumors and is associated with EMT marker expression. In summary, this study unraveling opportunities to interfere with major signals involved in the chemoresistance onset and EMT by manipulating ET_AR-mediated pathways, pinpoints that blockade of ET_AR may provide appropriate choice in clinical EOC treatment.

reverting EMT and related biological effects including drug sensitivity. Although cancer cells integrate multiple signaling pathways sustaining tumor progression, therapeutic interest in the endothelin-1 (ET-1)/endothelin A receptor (ET_AR) axis is supported by its central role in several human cancers (9). This axis is highly expressed in a number of human tumors, including epithelial ovarian carcinoma (EOC), where its overexpression correlates with advanced stages (10). In EOC cells, the autocrine loop mediated by the ET-1/ET_AR interaction has been implicated in the sustained activation of several signal transduction pathways, including mitogen activated protein kinase (MAPK) and PI3-K-dependent Akt, thus representing a key driver in promoting cell proliferation, escape from apoptosis, angiogenesis, EMT, invasion, and metastasis (11). Interestingly, in these cells ET-1 acts as a survival factor protecting tumor cells from drug-induced apoptosis via a bcl-2-dependent mechanism that involves the activation of the PI3-K/Akt pathway, suggesting that activated ET_AR can substantially contribute to chemoresistance in ET_AR-positive tumors (12). According with *in vitro* studies, an increase in antitumor activity was observed when ET_AR antagonists, such as zibotentan, were combined with cisplatinum and paclitaxel (13, 14). These data were confirmed by a high-throughput screening analysis of EOC displaying different response to chemotherapy that identified ET_AR as one of the genes associated with chemoresistance (15). Furthermore, a pathway analysis identified the ET-1 signaling among the canonical pathways associated with platinum resistance (16). All these results suggest that ET_AR expression may be regarded as potential marker of resistance in EOC. In view of the above, in this study we evaluated whether response to chemotherapeutics in sensitive, and cisplatin- and taxol-resistant EOC cells and in human tissues is associated with the activation of ET-1/ET_AR signaling, EMT and invasive phenotype and activation of survival signaling pathways. Finally we investigated whether ET_AR blockade by reverting EMT results into restoration of drug sensitivity, thus offering a potential improvement in ovarian cancer management.

Materials and Methods

Cells and cell culture conditions

The human ovarian carcinoma cell line A2780 WT was obtained from European Collection of Cell Cultures. To retain cisplatin (CIS) and paclitaxel (TAX) resistance, 1 μmol/L cisplatin and 60 nmol/L paclitaxel were added to the culture medium A2780 CIS and A2780 TAX, respectively, every 2 passages (17). The 2008 cell line and its cisplatinum resistant subclone 2008C13 (CIS) were kindly provided by Dr. S.B. Howell, University of San Diego, La Jolla, CA). Further details are described in the Supplementary Materials and Methods.

Thymidine incorporation assay

Serum-starved A2780 WT and its resistant sublines, or ET_AR- or scrambled-siRNA transfected cells, were treated with ET-1 and/or zibotentan and after 24 hours, ^{3}[H]-thymidine was used as previously described (13). Responses to all treatments were assayed in sextuplicate, and results were expressed as the means of 3 separate experiments.

Apoptosis assay

For detection of early apoptotic events, cells were double stained with fluorescein isothiocyanate–conjugated annexin-V and propidium iodide using the Vybrant apoptosis kit according to the manufacturer's instructions (Invitrogen). Further details are described in the Supplementary Materials and Methods.

Western blot analysis

Cell lysates or conditioned media obtained from cell cultures were subjected to SDS-PAGE and revealed by Western blotting (WB). Further details are described in the Supplementary Materials and Methods.

Quantitative real-time PCR

Total RNA was isolated using the Trizol (Invitrogen) according to the manufacturer's protocol. RNA (5 μg) was reversed transcribed using SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed by using LightCycler rapid thermal cycler system (Roche Diagnostics) according to the manufacturer's instructions. The primers sets used and further details were shown in the Supplementary Materials and Methods.

Reverse Transcriptase (RT)-PCR

RT-PCR was performed using a Superscript One-Step RT-PCR System (Invitrogen) according to the manufacturer's

instructions. The primers sets used and further details were shown in the Supplementary Materials and Methods.

Luciferase reporter gene assay

To measure the transcriptional activity of Snail and E-cadherin promoter, 3×10^5 cells/well were transiently transfected with 0.5 μg of pGL3-SNA (−869/+59), or with 0.5 μg pGL2 Ecad3/luc construct, or with empty control vectors (Promega). Where indicated, cells were transfected with 100 nmol/L scrambled or ET_AR siRNA duplexes against mRNA (SMART pool) or mock siRNA obtained commercially (Dharmacon). Further details were shown in the Supplementary Materials and Methods.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (18). Details were shown in the Supplementary Materials and Methods.

Invasion assay

Cell invasion assays were carried out using modified Boyden chamber. Details were shown in the Supplementary Materials and Methods.

Xenografts in nude mice

Female athymic (nu+/nu+) mice, 4 to 6 week of age (Charles River Laboratories, Milan, Italy) were treated following the guidelines for animal experimentation of the Italian Ministry of Health. Mice were injected s.c. into 1 flank with 4×10^6 viable A2780 WT, cisplatinum- and taxol-resistant cells. After 7 days, when tumors reached approximately 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 zibotentan (diluted in PBS) at the daily dose of (10 mg/kg/day), 1 group was treated with paclitaxel (20 mg/kg per dose given i.v. 3 times a day every 4 days), 1 group with cisplatinum (5 mg/kg i.p. 1 time on day 1), 1 group with combination of zibotentan and cisplatinum or zibotentan and paclitaxel. Control mice were injected i.p. with vehicle. Three different experiments with a total of 30 mice for each group were performed. On day 45 after tumor injection, tumors were removed from control and treated mice and analyzed for WB. Tumor size was measured with caliper and was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

Patient population

The study included 60 primary untreated ovarian cancer patients admitted to the Gynecologic Oncology Unit, Catholic University of Campobasso and Rome treated with platinum-based chemotherapy, plus paclitaxel. Further details were shown in the Supplementary Materials and Methods.

Immunohistochemistry

Immunohistochemical analysis of ovarian cancers was performed on archival from 60 frozen tumors collected from patient population above described with informed consent, as indicated by Institutional Review Board, and classified according with WHO criteria. Further details were shown in the Supplementary Materials and Methods.

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 2-way ANOVA, with group and time as variables. All statistical tests were carried out using SPSS software (SPSS 11, SPSS Inc.). A 2-sided probability value of less than 0.05 was considered statistically significant.

Results

Expression of ET-1/ET_AR axis and activation of signaling pathways are upregulated in chemoresistant ovarian cancer cells

In order to assess the potential involvement of ET-1 axis in chemoresistance, we used the A2780 (WT) human EOC cell line, the derivative cisplatinum-resistant (A2780 CIS) and taxol-resistant (A2780 TAX) sublines, and the 2008 cell line and its cisplatinum-resistant variant 2008 CIS. The different sensitivity to these drugs was evident after treatment of serum-starved cells with the cytotoxic drugs, cisplatinum and taxol (Supplementary Fig. S1A). In both A2780 and 2008 sensitive cells, cisplatinum or taxol treatment increased the number of early apoptotic cells, while in the resistant sublines the apoptotic pathway was compromised, confirming the acquisition of drug resistance in these cells.

The ET-1 axis is expressed in ovarian cancer cells acting as survival factor against chemotherapeutic drug treatment (12), suggesting that ET-1/ET_AR axis would be upregulated in cisplatinum and taxol-resistant cells. As shown in Figure 1(A–C) and Supplementary Figure S2A, the cisplatinum- and taxol-resistant cells significantly expressed ET_AR at higher levels compared to sensitive cells, and the concentrations of ET-1 released by resistant cells increased of about 8-fold compared to parental cells. All these data show that ET-1 axis is upregulated in EOC resistant cells correlating with the reduced sensitivity of these sublines to cytotoxic drugs.

We next determined the signaling pathways activated by ET-1/ET_AR axis to modulate cell survival. ET-1 activated both MAPK and Akt in sensitive A2780 cells, but a significant higher activation was observed in the resistant sublines that was inhibited when the cells were pretreated with the specific ET_AR antagonist zibotentan (Fig. 1D). Accordingly, ET-1 promoted A2780 cell proliferation, which was significantly inhibited in zibotentan-treated cells. To further verify the role of ET_AR in regulating cell proliferation, we knockdown ET_AR with siRNA and we found that also in ET_AR-silenced cells the ET-1-induced cell proliferation was inhibited (Fig. 1E). Specificity of siRNA oligos was confirmed by WB analysis, which showed a 90% knockdown of ET_AR (Supplementary Fig. S2B).

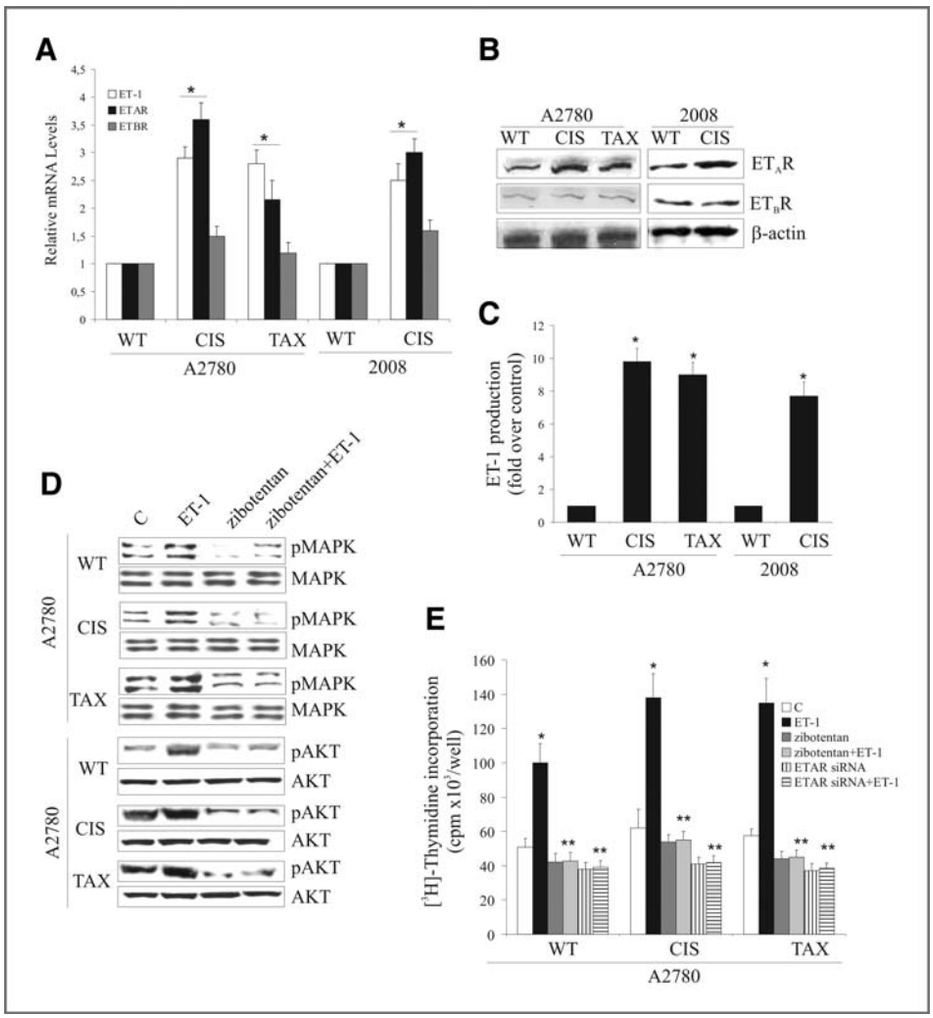

Figure 1. ET-1 and ET_AR expression is upregulated in chemoresistant ovarian cancer cells, associated with enhanced MAPK and Akt activation and cell proliferation. A, expression of ET-1, ET_AR, and ET_BR mRNA in sensitive and resistant A2780 and 2008 cells measured by real-time PCR. Values are presented as mean of 3 measurements ± SD. *, $P < 0.005$ compared to WT cells. B, A2780 and 2008 cell lysates analyzed by WB using anti-ET_AR and anti-ET_BR. β-Actin was used as loading control. C, ET-1 secretion evaluated by ELISA in conditioned media of sensitive and resistant A2780 and 2008 cells. *, $P < 0.05$ compared to WT cells. D, lysates of A2780 cells treated with ET-1 (100 nmol/L) and/or zibotentan (1 μmol/L) was analyzed by WB using anti-pMAPK, anti-MAPK, anti-pAKT, and anti-AKT. E, cell proliferation analyzed by [^{3}H]-Thymidine incorporation in A2780 cells treated for 24 hours with ET-1 (100 nmol/L) and/or zibotentan (1 μmol/L), or transfected with ET_AR siRNA. Columns, averages of sextuplicate determination of 3 separate experiments; bars, ±SD; *, $P < 0.0001$ compared to control; **, $P < 0.005$ compared to ET-1.

These results suggest that in chemoresistant cells the higher activation of ET-1 axis lead to enhanced MAPK and Akt signaling pathways, which promote cell survival and proliferation.

Chemoresistant cells display molecular changes consistent with ET-1-driven EMT and invasiveness

Given the ability of ET-1 to promote EMT machinery in ovarian tumor cells and the association between chemoresistance and acquisition of EMT phenotype in different tumor cells (11, 19–21), we analyzed whether the chemoresistance in A2780 and 2008 cells was associated with molecular changes consistent with EMT and whether the ET_AR pathway is involved in this process. To this end we examined the expression of E-cadherin and its transcriptional repressors, Snail, Slug, and Twist, and other mesenchymal markers, such as vimentin and N-cadherin. Enhanced mRNA expression levels for Snail, Slug, Twist, vimentin, and N-cadherin were observed in resistant sublines compared with parental cells, associated with a concomitant decrease in E-cadherin expression at level of mRNA and protein (Fig. 2A and C). Moreover, ET-1 enhanced the expression of Snail and Twist in both sensitive and resistant cells and the treatment with zibotentan inhibited the ET-1-induced effects, restoring E-cadherin expression (Fig. 2B and C). Remarkably, ET_AR blockade, by both zibotentan or its silencing, reverted the ET-1-induced suppression of E-cadherin promoter activity and in resistant cells prevented that induced at higher level by endogenous ET-1/ET_AR axis (Fig. 2D), suggesting that the transcriptional regulation of E-cadherin may be important to ET_AR-driven EMT and acquisition of chemoresistance. In agreement with above results, ET-1 induced significant induction of *Snail* promoter activity in parental cells, reaching about 4-fold increase in resistant cells, which was significantly inhibited by zibotentan (Fig. 2E), indicating that ET-1/ET_AR controls the transcriptional repression of E-cadherin through Snail in chemoresistant cells. Furthermore, we examined how Snail binds to the E-cadherin gene promoter. As shown in Fig 2F, the E-cadherin promoter sequences were detected bound to Snail upon treatment with ET-1 in a time-dependent manner. The association of Snail with E-cadherin promoter began 5 minutes after ET-1 exposure and declined rapidly in sensitive A2780 cells.

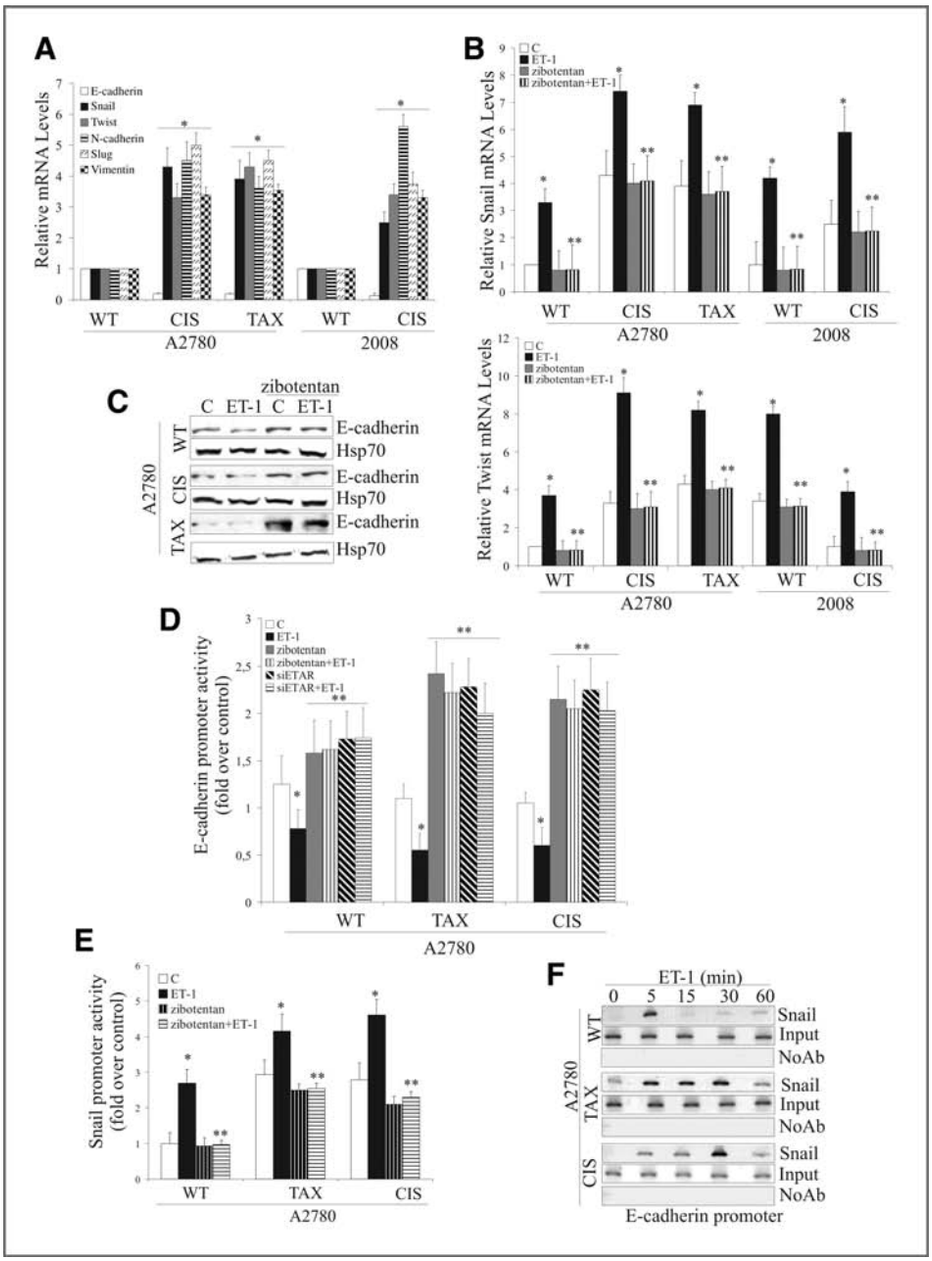

Figure 2. ET_AR-driven EMT process is upregulated in chemoresistant cells A, E-cadherin, Snail, Slug, Twist, vimentin, and N-cadherin expression in sensitive and resistant A2780 and 2008 cells evaluated by real time PCR. Values are presented as mean of 3 measurements ± SD. *, $P < 0.002$ compared to WT cells. B, Snail and Twist expression in sensitive and resistant A2780 and 2008 cells treated with ET-1 and/or zibotentan evaluated by real time PCR. Values are presented as mean of 3 measurements ± SD. *, $P < 0.02$ compared to control cells; **, $P < 0.005$ compared to ET-1-treated cells. C, lysates of A2780 cells treated with ET-1 and/or zibotentan was analyzed by WB using anti-E-cadherin and anti-Hsp70 as loading control. D, E-cadherin transcriptional activity in A2780 cells stimulated with ET-1 and/or zibotentan for 24 hours or cotransfected with ET_AR siRNA, bars ±S.D. *, $P < 0.001$ compared to control; **, $P < 0.05$ compared to ET-1. E, Snail promoter activity in A2780 cells treated with ET-1 and/or zibotentan for 24 hours. *, $P < 0.02$ compared to control cells; **, $P < 0.005$ compared to ET-1-treated cells. F, ChIP analysis performed in A2780 cells treated with ET-1 for different times. Chromatin was incubated either without antibody (NoAb) or with an anti-Snail and analyzed by PCR analysis by using specific primers for E-cadherin promoter.

Interestingly, in resistant cells the recruitment, starting after 5 minutes, was long-lasting with a maximum after 30 minutes, and persisting for 60 minutes, suggesting that the ET-1-dependent and sustained binding of Snail in the E-cadherin promoter might account for the EMT and chemoresistant phenotype of these cells.

To assess whether the acquisition of EMT phenotype correlates with functional changes in resistant cells, we evaluated the expression of several matrix-metalloproteases (MMP) and the invasive potential of EOC cells. As shown in Figure 3A and B, a significant upregulation and activity of MMP-2 and -9 was observed in resistant cells, confirming the association between invasive phenotype and chemoresistant properties of these cells. As demonstrated by invasion assay, the A2780 WT cells showed a significant increase in the number of invading cells after treatment with ET-1 that was inhibited by zibotentan (Supplementary Fig. S3). The invasive capability of chemoresistant sublines was greater, with a ~2-fold increase in the number of invading cells compared with sensitive cells that was blocked by pretreatment with zibotentan (Fig. 3C), demonstrating that ET_AR blockade is associated with a reverted EMT phenotype and reduced invasiveness in chemoresistant cells.

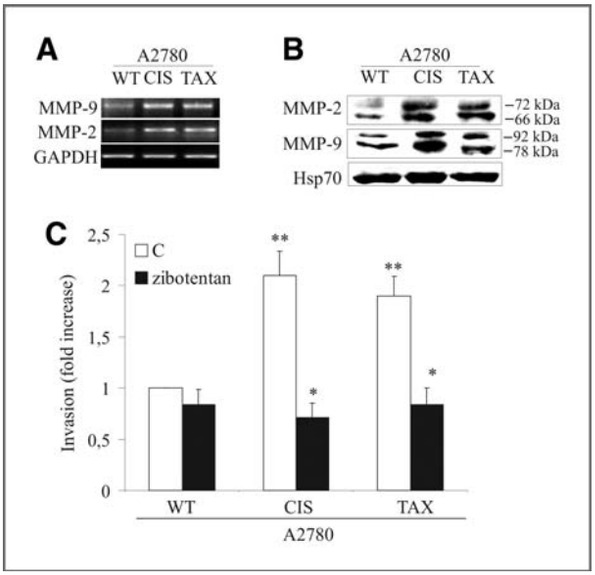

Figure 3. ET_AR-mediated MMP activity and cell invasion is upregulated in chemoresistant cells. A, RT-PCR analysis of MMP-2, -9, and GAPDH expression in sensitive and resistant A2780 cells. B, conditioned media from A2780 cells analyzed for MMP-2 (latent form, 72 kDa; active form, 66 kDa) and for MMP-9 (latent form, 92 kDa; active form, 86 kDa) by WB. Hsp70 was used as loading control. C, invasion assay of A2780 WT, CIS- and TAX-resistant cells exposed to zibotentan. *, $P < 0.02$ compared to untreated cells; **, $P < 0.002$ compared to control of A2780 WT cells.

ET_AR antagonist sensitizes chemoresistant ovarian cancer cells to drug-induced apoptosis

To determine whether the ET_AR blockade might sensitize A2780 and 2008 cells to cisplatinum-induced apoptosis, we evaluated the drug-induced apoptosis in resistant cells pretreated with zibotentan. As shown in Figure 4, treatment of serum-starved A2780 WT cells with zibotentan increased the number of early apoptotic cells, to an extent similar to that obtained by cisplatinum, confirming that endogenous ET-1 induces survival signaling through the ET_AR (12). Interestingly, the coadministration of zibotentan significantly potentiated ($P < 0.005$ and $P < 0.002$ compared with single agent treatment in A2780 and 2008 cells, respectively) the cytotoxic drug-induced apoptotic cell death in sensitive cells. Most importantly, the addition of zibotentan plus cisplatinum to both cisplatinum-resistant cells enhanced their susceptibility to chemotherapy ($P < 0.001$ in A2780 and 2008 cells). Similar results were obtained in A2780 taxol-resistant cells (data not shown). This suggests that a combination therapy may be effective at inducing apoptotic death, overcoming resistance in EOC cells.

Antitumor effects of zibotentan in chemoresistant EOC xenografts are associated with reduction of EMT marker expression

We next determined whether ET_AR blockade resulted in the inhibition of ET-1-induced EMT effectors and tumor growth by treating mice bearing A2780 WT, cisplatinum- and taxol-resistant tumors with zibotentan. This treatment was generally well tolerated with no detectable signs of acute or delayed toxicity, and produced a 55% inhibition of tumor growth on day 41 after tumor injection in A2780 WT bearing mice ($P \leq 0.005$; Fig. 5A). A similar growth inhibitory effect was observed when zibotentan was used in both taxol (58%) and cisplatinum (54%)-resistant ovarian carcinoma xenografts, causing significant inhibition in tumor growth at the end of the 4-week treatment period in all mice ($P < 0.002$ for A2780 CIS and $P < 0.005$ for A2780 TAX; Fig. 5B). Most importantly, a further significant growth inhibitory effect was observed when zibotentan was used in combination with cisplatinum (71%) or in combination with paclitaxel (81%), in resistant EOC xenografts. Remarkably, a marked increase of E-cadherin expression in zibotentan-treated mice, especially in xenografts from resistant cells, which was paralleled to the reduction of N-cadherin expression (Fig. 5C). These results indicate a specific therapeutic window in which blockade of ET_AR with zibotentan, in combination with chemotherapeutic drugs, controlling the EMT and aggressive phenotype of chemoresistant cells, is therapeutic effective in resistant EOC cells, increasing sensitivity to the chemotherapy.

ET_AR is overexpressed in resistant human ovarian tumors

Finally we analyzed the importance of ET_AR as potential marker of resistance in human EOC tissues from patients treated with platinum-based chemotherapy, plus paclitaxel. Clinicopathologic characteristics of the overall series are summarized in Table 1. Forty-seven cases (78.3%) were stage III to IV disease, and serous histotype was documented in the vast majority ($n = 44$, 73.3%) of cases. Optimal cytoreduction (residual tumor ≤ 1 cm) was achieved in 32 (53.3%) patients. Thirty-one (51.7%) cases were defined as platinum resistant, while 29 patients (48.3%) were platinum sensitive. Patients optimally cytoreduced at first surgery were more likely to be platinum sensitive compared to patients judged as unresectable (62.5% vs. 32.1%, $P = 0.017$). Patients with no ascites were more frequently platinum sensitive than patients with ascites at primary laparotomy (66.7% vs. 33.3%, $P = 0.018$). We analyzed the ET_AR levels by immunohistochemistry (IHC) in these human ovarian cancer specimens encompassing platinum sensitivity and platinum resistance. Overall, ET_AR was significantly upregulated in 66% ($P < 0.017$) of resistant ovarian cancers compared to sensitive tumors (Table 1 and Fig 5D). Consistent with our observations in EOC cell cultures and xenografts, overexpression of ET_AR in platinum-resistant human ovarian cancer tissues was associated with the downregulation of E-cadherin ($P < 0.002$) and with enhanced expression of mesenchymal N-cadherin ($P < 0.07$; Fig. 5D). These results are consistent with our previous findings in primary ovarian tumor tissues (10), indicating the biological relevance of ET_AR in the regulation of EMT in the tumor context.

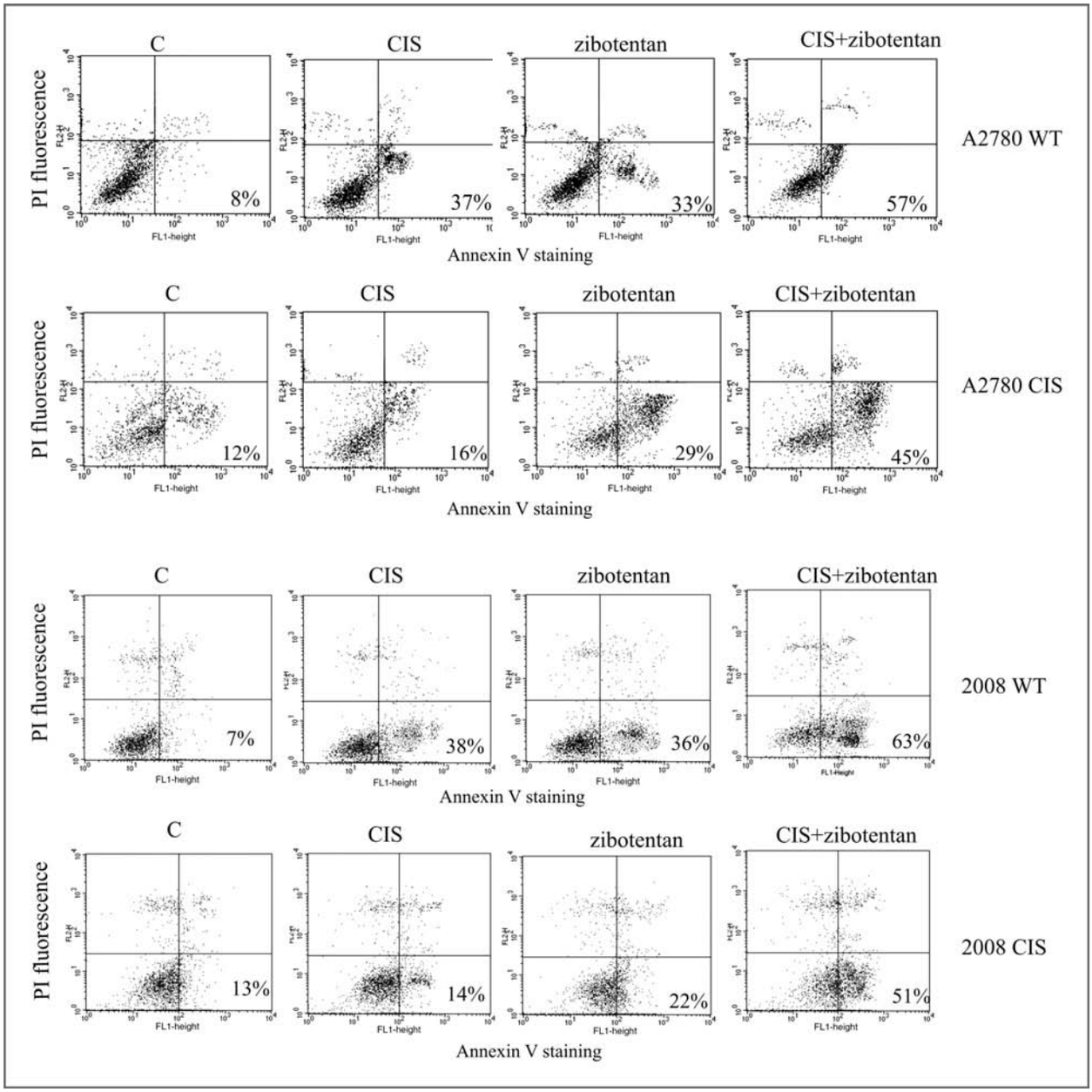

Figure 4. ET_AR blockade sensitizes chemoresistant cells to drug-induced apoptosis. Sensitive and cisplatinum-resistant A2780 and 2008 cells were maintained for 48 hours in absence or presence of cisplatinum and/or zibotentan. Apoptosis was measured by Annexin V and PI staining and expressed as percentage of apoptotic cells.

Discussion

Development of drug resistance remains the major therapeutic barrier in EOC. Consequently an in depth understanding of the mechanisms underlying the development of chemoresistance is of utmost importance for improving the therapeutic regimens (2, 21). Since ET-1/ET_AR axis is critically involved in EOC growth and progression, in the present study we investigated the pathobiological role of ET-1 and ET_AR in developing drug resistance. Our results provide evidence that ET-1/ET_AR overexpression, by regulating EMT and invasive behavior, endows EOC cells with an increased survival capacity and resistance to 2 presently employed chemotherapic agents, such as cisplatinum and taxol. The blockade of ET_AR by zibotentan reverted EMT, restored drug sensitivity, and inhibited invasiveness and growth of ovarian tumor xenografts. Finally, through the analysis of primary advanced EOC patients, we reported that the overexpression of ET_AR correlates with clinical chemoresistance and EMT phenotype.

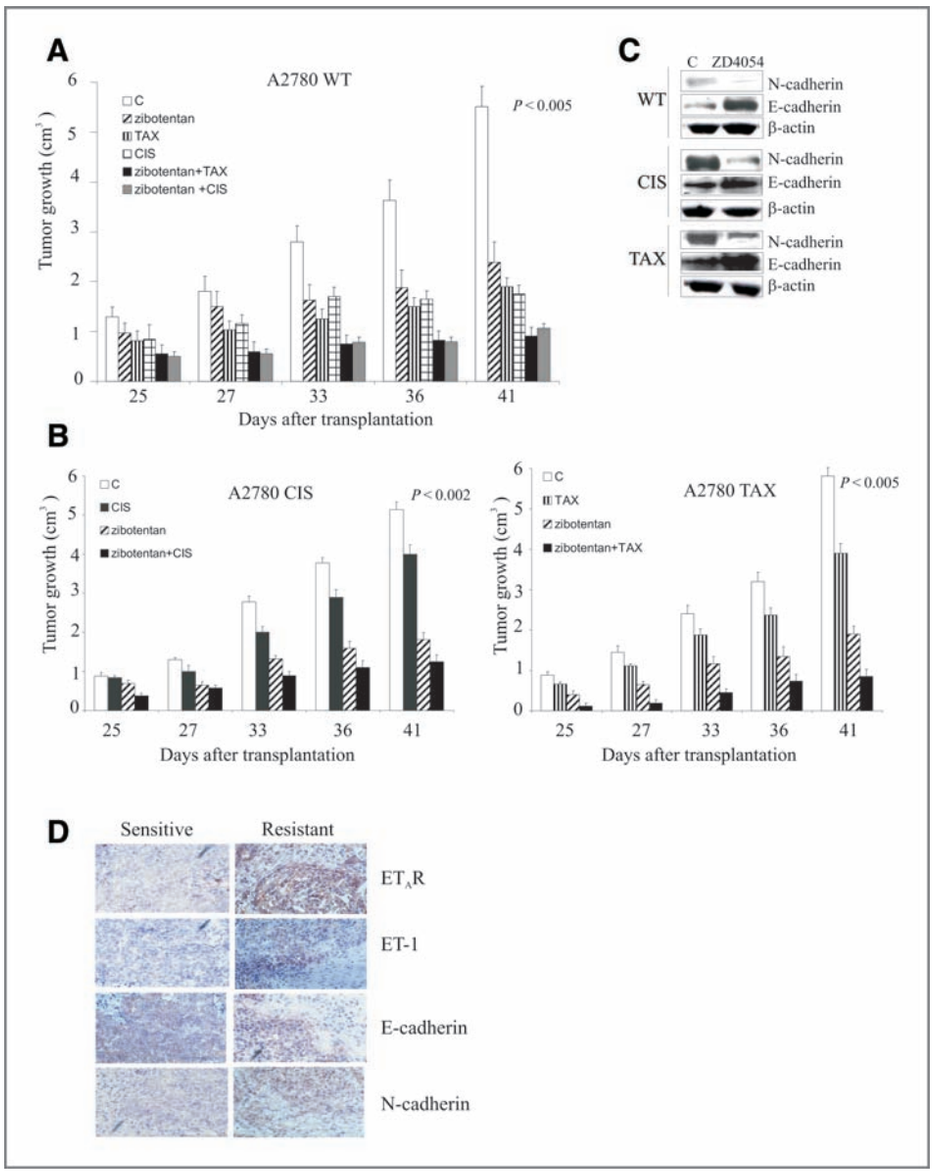

Figure 5. Antitumor effects of zibotentan in A2780 sensitive and resistant xenografts is associated with reverted EMT phenotype. A2780 WT (A), CIS and TAX (B) cells were injected into nude mice. After 7 days, the mice were treated as indicated for 21 days. Each experiment was performed 3 times with a total of 30 mice per group. The comparison of the time course of tumor growth curves by 2-way ANOVA with group-by-time interaction for tumor growth was statistically significant in both sensitive and resistant A2780 xenografts. Data points, averages; bars, ± SD. C, E-cadherin and N-cadherin expression in tumors from A2780 xenografts treated as described in A and B. β-actin was used as loading control. D, the expression patterns of ET_AR and EMT markers in the 60 platinum sensitive and resistant human ovarian cancer samples were determined by IHC. Representative immunohistochemical staining in sensitive and resistant human primary ovarian carcinoma samples for ET-1, ET_AR, E-Cadherin, and N-cadherin expression (original magnification, ×200).

Drug resistance is a multifactorial phenomenon involving interrelated and/or independent pathways (22). The overall apoptotic balance within a given cell population relies on a number of signaling pathways differentially regulated between chemosensitive and chemoresistant cancer cells. In this context, it has been demonstrated that ET-1 acts as an antiapoptotic factor, modulating cell survival pathways through a PI3-K-mediated Akt activation, which is considered to be a molecular "crutch" to escape cell death (12). The onset of chemoresistance of EOC cells can also be related to the overexpression of the ET-1/ET_AR signaling pathway that, in turn, may control the apoptotic response in resistant cells through the activation of survival pathways, such as MAPK or the PI3K/AKT signaling. It is becoming clear that EMT may reflect an ultimate adaptation of cancer cells to survive to cytotoxic drug activity, thus being responsible for chemosensitivity and that EMT inducing transcription factors such as Snail, Slug, and Twist which confer resistance to cell death (3, 23–29). In human EOC, changes in the expression of Snail, Slug, and Twist play an important role in ovarian tumorigenesis and progression, and are significantly higher in advanced stages and metastatic lesions (30–33). Moreover, it was reported that cellular morphology, motility, and molecular changes consistent with EMT, including enhanced Snail and Twist expression, were related to paclitaxel-resistance in EOC cells (34, 35). Recent work has suggested that may be a link between the cancer stem cell (CSC) phenotype and that induced by the process of EMT (36). The CSC isolated from EOC samples express markers associated with stem cells and EMT, including Snail and Slug, suggesting that EOC cells, by going through an EMT, acquire stemness

Table 1. Clinicopathologic characteristics and platinum sensitivity in the current series of patient population

Characteristics	No. of patients	Platinum resistant no. (%)	Platinum sensitive no. (%)	P^a
All cases	60	31 (51.7)	29 (48.3)	
Age (y)				
≤65	45	24 (53.3)	21 (46.7)	
>65	15	7 (46.7)	8 (53.3)	n.s
FIGO stage				
I–II	13	3 (23.1)	10 (76.9)	
III–IV	47	28 (59.6)	19 (40.4)	0.028
Grade				
G1–2	17	9 (52.9)	8 (47.1)	
G3	35	18 (51.4)	17 (48.6)	n.s
NA	8	–	–	
Histotype				
Serous	44	24 (54.5)	20 (45.5)	
Other	16	7 (51.4)	9 (56.2)	n.s
Residual tumor at first surgery				
<1 cm	32	12 (37.5)	20 (62.5)	
Exploratory laparotomy	28	19 (67.8)	9 (32.1)	0.017
Ascites				
No	27	9 (33.3)	18 (66.7)	
Yes	33	22 (66.7)	11 (33.3)	0.018
ET_AR expression				
Low	25	8 (32.0)	17 (68.0)	
High	35	23 (65.7)	12 (34.3)	0.017

Abbreviation: NA, not available.

[a]Fisher's exact test was used to analyze the distribution of cases between groups.

characteristics qualifying them to acquire chemoresistance by overcoming p53-mediated apoptosis (16, 37–41). In this context, recent data suggest that Twist1 may also be an important regulator of "stemness" in EOC cells (42), indicating that the initiation of the EMT programme may be critical for the acquisition of stem cell-like characteristics resulting in chemoresistance (43). In this regard, a recent pathway analysis revealed ET-1 signaling among the canonical pathways significantly ($P < 0.02$) associated with resistance to platinum-based chemotherapy, whose several are linked to EMT and stemness reinforcing the relationship of both processes with therapy resistance (16).

The present study reveals the molecular mechanisms of resistance activated by ET-1/ET_AR axis through EMT transcriptional programs. Thus, the transcription activity of Snail, upon its recruitment on E-cadherin promoter, is essential in ET_AR-mediated E-cadherin repression in resistant EOC cells. The upregulation of EMT transcription factors in chemoresistant cells is associated with enhanced MMP activity and invasiveness, suggesting that these factors might control acquisition of EMT in cancer cells to induce motility in response to adverse environmental changes facilitating cancer progression and therapeutic resistance (44, 45). Under this scenario EMT initiating signals would be an ideal target as they are the seeds for metastasis and recurrence. Of clinical relevance, knockdown of ET_AR levels by siRNA, or blockade by zibotentan, reverted EMT phenotype, inhibited invasive behavior, and increased susceptibility to chemotherapeutic agents, suggesting that ET_AR-mediated EMT signaling can represent a "salvage pathway" occurring during chemoresistance development. The significant association between ET_AR overexpression and the resistant phenotype, identified for the first time ET_AR as predictor of chemoresistance in human EOC tissues and its relationship with EMT marker expression in the resistant tumor context.

In summary, our study provides evidence that targeting ET_AR with zibotentan, in combination with chemotherapy can sensitize tumor to chemotherapeutics by preventing EMT-associated escape signaling thus offering a rationale for the clinical evaluation of this target-based drug as modulator of both chemoresistance and tumor progression.

Disclosure of Conflicts of Interest

No potential conflicts of interest were disclosed.

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