Therapeutics, Targets, and Chemical Biology

Cancer Research

Endothelin A Receptor/β-Arrestin Signaling to the Wnt Pathway Renders Ovarian Cancer Cells Resistant to Chemotherapy

Laura Rosanò[1], Roberta Cianfrocca[1], Piera Tocci[1], Francesca Spinella[1], Valeriana Di Castro[1], Valentina Caprara[1], Elisa Semprucci[1], Gabriella Ferrandina[2], Pier Giorgio Natali[1,3], and Anna Bagnato[1]

Abstract

The high mortality of epithelial ovarian cancer (EOC) is mainly caused by resistance to the available therapies. In EOC, the endothelin-1 (ET-1, EDN1)–endothelin A receptor (ET_AR, EDNRA) signaling axis regulates the epithelial–mesenchymal transition (EMT) and a chemoresistant phenotype. However, there is a paucity of knowledge about how ET-1 mediates drug resistance. Here, we define a novel bypass mechanism through which ET_AR/β-arrestin-1 (β-arr1, ARRB1) links Wnt signaling to acquire chemoresistant and EMT phenotype. We found that ET_AR/β-arr1 activity promoted nuclear complex with β-catenin and p300, resulting in histone acetylation, chromatin reorganization, and enhanced transcription of genes, such as *ET-1*, enhancing the network that sustains chemoresistance. Silencing of β-arr1 or pharmacologic treatment with the dual ET_AR/ET_BR antagonist macitentan prevented core complex formation and restored drug sensitivity, impairing the signaling pathways involved in cell survival, EMT, and invasion. *In vivo* macitentan treatment reduced tumor growth, vascularization, intravasation, and metastatic progression. The combination of macitentan and cisplatinum resulted in the potentiation of the cytotoxic effect, indicating that macitentan can enhance sensitivity to chemotherapy. Investigations in clinical specimens of chemoresistant EOC tissues confirmed increased recruitment of β-arr1 and β-catenin to *ET-1* gene promoter. In these tissues, high expression of ET_AR significantly associated with poor clinical outcome and chemoresistance. Collectively, our findings reveal the existence of a novel mechanism by which ET_AR/β-arr1 signaling is integrated with the Wnt/β-catenin pathway to sustain chemoresistance in EOC, and they offer a solid rationale for clinical evaluation of macitentan in combination with chemotherapy to overcome chemoresistance in this setting. *Cancer Res; 74(24); 7453–64. ©2014 AACR.*

Introduction

Chemoresistance heralding tumor recurrence is the major cause of poor survival rates of patients with ovarian cancer (1). Epithelial ovarian cancer (EOC) cells in fact activate autocrine programs that may opt as survival mechanisms in response to chemotherapy (1, 2). Understanding the distinct mechanisms that facilitate survival and propagation is therefore central for improving the clinical outcome for patients with EOC. Emerging evidences suggest that epithelial–mesenchymal transition (EMT) plays a crucial role in the aggressiveness of EOC, because it increases migration and invasion ability, contributing to chemoresistance and cancer stem cell (CSC) populations (3, 4). Among the proteins driving tumor progression and EMT, numerous studies have identified G protein–coupled receptors (GPCR) as the most prominent validated pharmacologic targets in biomedicine (5). Of particular interest, the endothelin-1 (ET-1, EDN1)–endothelin A receptor (ET_AR, EDNRA) axis is aberrantly activated in EOC to stimulate cell proliferation, survival, angiogenesis, and invasion, and increased ET_AR expression has been correlated with platinum resistance and EMT marker expression (2, 6–9). In EOC, also ET_BR (EDNRB) appears to have protumorigenic activity by promoting tumor survival through the evasion of immune response. Indeed, ET_BR signaling is capable to impair antitumor immunity by preventing T-cell recruitment to tumors (10, 11). In addition, ET_BR plays a role in inducing tumor angiogenesis and lymphangiogenesis by inducing in blood and lymphatic endothelial cell proliferation, survival, and migration (12, 13). Hence, ET_AR and ET_BR, which are heterogeneously expressed in EOC cells (14, 15), have emerged as key targets for cancer therapy. A complex cross-talk between ET-1 signaling and other growth factor pathways drives tumor progression via the scaffold protein β-arrestin

[1]Molecular Pathology Laboratory, Regina Elena National Cancer Institute, Rome, Italy. [2]Gynecologic Oncology Unit, Catholic University of Rome, Italy. [3]Consorzio Interuniversitario Nazionale per la Bio-Oncologia (CINBO) Laboratories, University Gabriele d'Annunzio, Chieti, Italy.

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

Corresponding Authors: Anna Bagnato and Laura Rosanò, Molecular Pathology Laboratory, Regina Elena National Cancer Institute, Via Elio Chianesi, 53, 00144 Rome, Italy. Phone: 39-06-52662565; Fax: 39-06-52662600; E-mail: bagnato@ifo.it and rosano@ifo.it

doi: 10.1158/0008-5472.CAN-13-3133

©2014 American Association for Cancer Research.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

(β-arr, ARRB1) that serves as molecular hub to organize complex signaling network (16–19). Some noteworthy cross-talk is the ET-1–mediated transactivation of receptor tyrosine kinases and β-catenin signaling (17, 19). The canonical Wnt pathway results in β-catenin nuclear accumulation and transcriptional activation of target genes. Aberrant accumulation of β-catenin correlates with EOC tumor grade and poor survival (20, 21) and deregulation of Wnt/β-catenin signaling is a key factor in inducing and maintaining chemoresistance in EOC (22, 23). Although recent evidence suggests that downstream of ET_AR, β-arr1 serves as component of functional complexes for β-catenin stabilization, and invasive behavior (18, 19), a complete picture of the underlying molecular mechanisms leading to chemoresistance remains to be elucidated. In this study, we reveal a signaling framework relevant for chemoresistance, providing evidence for a novel integration between ET_AR/β-arr1 signaling and the β-catenin pathway, leading to chemoresistance onset. Of translational interest is the finding that the dual ET_AR/ET_BR antagonist macitentan, a potent inhibitor of ET_AR with significant affinity for the ET_BR (24), results into inhibition of tumor growth, neovascularization, intravasation, and metastatic progression and chemoresistance.

Materials and Methods

Cells and cell culture conditions

The human ovarian carcinoma cell line, A2780, and its cisplatinum and taxol-resistant subclones, A2780 cisplatinum and A2780 paclitaxel, and 2008 cell line and its resistant cisplatinum subclone, 2008 cisplatinum, were cultured as previously described (6). ET-1 (100 nmol/L), BQ123, cyclo (-D-Trp–D-Asp–Pro–D-Val–Leu; 1 μmol/L), and BQ788, N-cis-2,6-dimethylpiperidinocarbonyl-4-methyl-Leu-D-Trp(1-methoxycarbonyl)-D-Nle-OH (1 μmol/L), were purchased from Bachem. Zibotentan, ZD4054, and N-(3-methoxy-5-methylpyrazin-2-yl)-2-(4-[1,3,4-oxadiazol-2-yl]phenyl) pyridine-3-sulfonamide (1 μmol/L) were kindly provided by AstraZeneca. Macitentan, also called ACT-064992 or N-(5-[4-bromophenyl]-6-{2-[5-bromopyrimidin-2-yloxy]ethoxy}pyrimidin-4-yl)-N′-propylsulfamide (1 μmol/L), was kindly provided by Actelion Pharmaceuticals, Ltd. (25).

Immunoblotting and immunoprecipitation

Cells were lysed in lysis buffer [250 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, and protease inhibitors]. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) were used to separate cytoplasmic and nuclear fractions.

Luciferase reporter gene assay

Reporter activity was measured using the Luciferase assay system (Promega) in cells cotransfected with 1 μg pTOP/Flash or pFOP/Flash (Upstate Biotech) or 1 μg ET-1 promoter reporter (ETP) sequence and 100 ng pCMV-β-galactosidase (Promega) vectors using LipofectAMINE 2000 reagent (Life Technologies). Luciferase assays were carried out according to standard procedures.

Chromatin immunoprecipitation assays

Chromatin was extracted from cells and chromatin immunoprecipitation (ChIP) assays were performed as previously described (18).

Chemoinvasion assay

Chemoinvasion assays were carried out using Transwell membrane filter inserts with 8-μm size polycarbonate membrane precoated with polymerized collagen (placed in a 24-well plate; BD Biosciences), according to the manufacturer's instructions.

Xenografts in nude mice

Female athymic (nu^+/nu^+) mice, 4 to 6 week of age (Charles River Laboratories), were injected i.p. with 2×10^6 viable 2008- and A2780-sensitive and cisplatinum- and taxol-resistant cells, following the guidelines for animal experimentation of the Italian Ministry of Health. Ten days later, animals were randomized into different groups of 10 mice undergoing the following treatments for 4 weeks: (i) vehicle, (ii) macitentan (30 mg/kg, oral daily), (iii) paclitaxel (5 mg/kg, i.p. weekly), (iv) cisplatinum (8 mg/kg, i.p. weekly), (v) macitentan plus cisplatinum, (vi) zibotentan (10 mg/kg). Two weeks after termination of treatment, all mice were euthanized and intraperitoneal organs were analyzed. Tumor volume was calculated using the formula: $\pi/6$ larger diameter $\times$ (smaller diameter)^2. The number and sizes of visible metastases, and tumor location were noted and the removed tumors were measured, frozen, and analyzed for immunoblotting, ChIP and immunohistochemical (IHC) analysis. In a different set of experiments, mice injected with 2008 and 2008 cisplatinum cells were randomized into four different groups of 5 mice undergoing the treatments with macitentan (30 mg/kg, oral daily) in monotherapy or combination with cisplatinum (8 mg/kg, i.p. weekly) for 4 weeks, sacrificed after the end of treatment, and peritoneal tumors were harvested and measured. Values represent the mean $\pm$ SD of 10 mice for group from three independent experiments.

Patient population

The study included 68 patients with ovarian cancer admitted to the Gynecologic Oncology Units, Catholic University of Rome/Campobasso (Rome, Italy).

Immunohistochemistry

IHC analysis on tumors from xenografts was done as previously described (7). IHC analysis of human EOC was performed on archival frozen tumors collected from the described patient population. The tissues were obtained and handled as indicated by Institutional Review Board, and classified according with WHO criteria.

Statistical analysis

Statistical analysis was performed using the Student *t* test and Fisher exact test to compare *in vitro* experiments. 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 the SPSS software (SPSS 11,

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

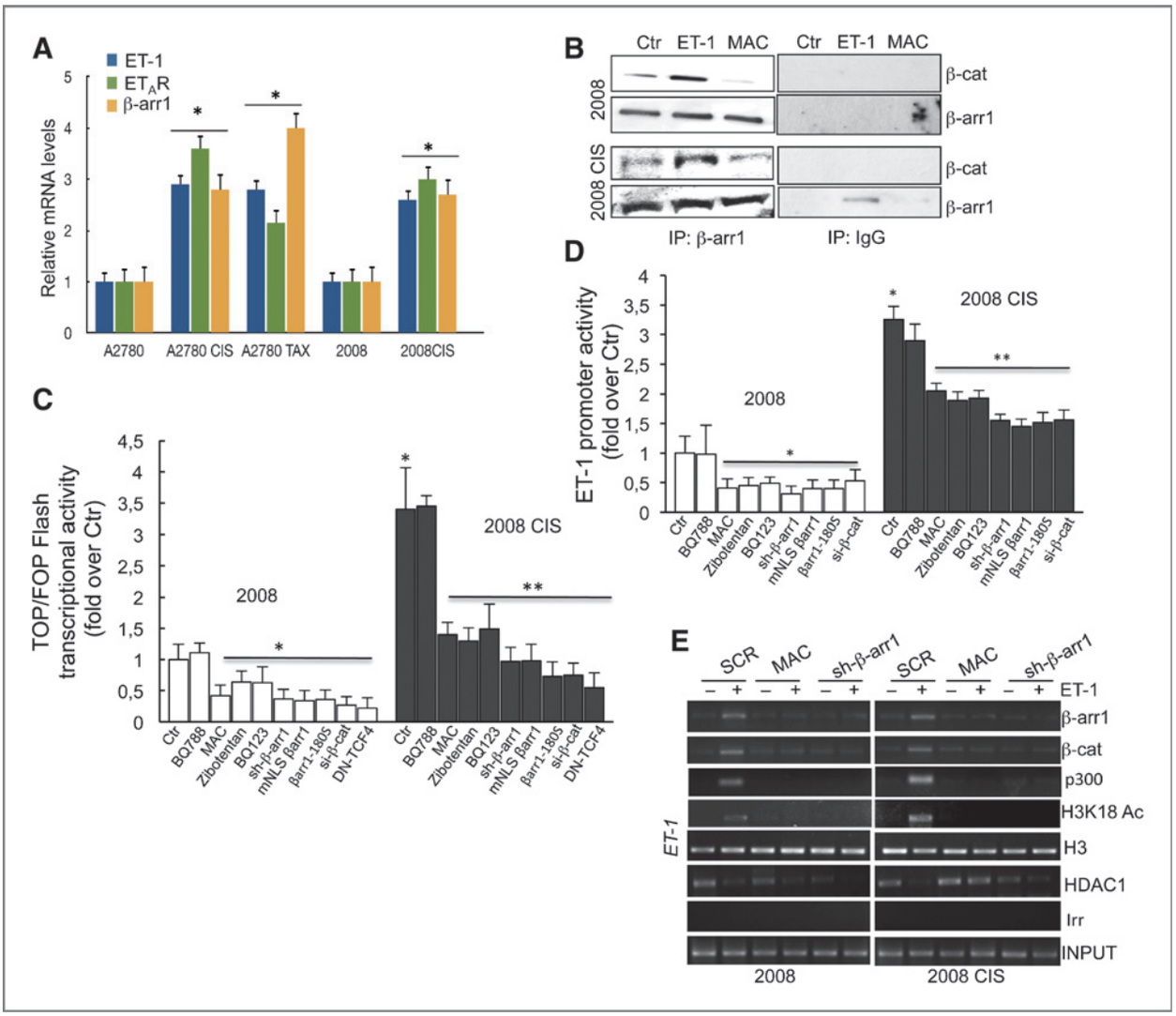

Figure 1. ETAR-driven nuclear β-arr1/β-catenin association controls ET-1 expression in chemoresistant EOC cells. A, qPCR analysis in A2780 and 2008 cells. Values are the mean ± SD ($n = 3$; *, $P < 0.05$). B, immunoprecipitation (IP) and immunoblot analysis of nuclear extracts from 2008 cells treated with 100 nmol/L ET-1 and/or with 1 μmol/L macitentan (MAC) for 15 minutes. C, β-catenin transcriptional activity evaluated in 2008 cells treated as indicated. Values are the mean ± SD ($n = 6$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). D, ET-1 promoter activity evaluated in 2008 cells treated as indicated. Values are the mean ± SD ($n = 6$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). E, ChIP assay in 2008 cells transfected with scramble (SCR) or sh-β-arr1 and treated with ET-1 and/or macitentan for 15 minutes. Ctr, control.

SPSS Inc.). A two-sided probability value of <0.05 was considered statistically significant.

For more detailed methods, see Supplementary Materials and Methods.

Results

ET_AR activation promotes β-arr1–β-catenin association in chemoresistant EOC cells to regulate ET-1 expression

To establish the role of β-arr1 in ET-1–induced chemoresistance, we used the A2780 and 2008 human EOC cell lines, and their cisplatinum- or taxol-resistant variants (A2780 cisplatinum, A2780 paclitaxel, and 2008 cisplatinum). Immunocytochemical analysis of these EOC cells showed expression of ET_AR and ET_BR (Supplementary Fig. S1A). Resistant cells expressed higher levels of β-arr1 and of ET_AR (1- to 8- and 2- to 4-fold in A2780 cisplatinum and paclitaxel, respectively, vs. sensitive cells and 2- to 3-fold in 2008 cisplatinum vs. sensitive cells; Fig. 1A; Supplementary Fig. S1B), and released high concentrations of ET-1 (6). Moreover, β-arr1 accumulated in the nuclear compartment after 100 nmol/L ET-1 challenge in A2780 (1.1- and 2.9-fold increase at 15 minutes vs. time zero in sensitive and resistant cells, respectively; Supplementary Fig. S1C). Following ET-1 stimulation, there was an increased nuclear association between β-catenin and β-arr-1 in both sensitive and resistant cells (Fig. 1B and Supplementary Fig. S1D). This

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

association was inhibited by macitentan as well as by the expression of mNLS-β-arr1, a mutant unable of nuclear import (26), or with βarr1-180S mutant (27) lacking of the structural determinant for β-catenin binding (Fig. 1B and Supplementary Fig. S1E). Nuclear β-catenin mediates effects on target genes via its interaction with TCF/LEF transcription factors (28). β-Catenin transcriptional activity was significantly increased in resistant cells compared with sensitive cells (Fig. 1C and Supplementary Fig. S1F and S1G). This activity was significantly inhibited by silencing of β-arr1, or expression of mNLS-β-arr1, or βarr1-180S or by macitentan, or by both ET_AR antagonists BQ123 and zibotentan. Similar effects were observed upon silencing of β-catenin or expression of dominant-negative TCF4 (DN-TCF4). On the contrary, the addition of the ET_BR antagonist BQ788 did not decrease β-catenin transcriptional activity (Fig. 1C and Supplementary Figs. S1F, S1G, and S2A–S2C). Altogether, these findings demonstrate that ET-1 acts through ET_AR to control the nuclear trafficking of β-arr1 and to modulate β-catenin transcriptional activity in chemoresistant EOC cells.

Given the role of β-arr1 to regulate β-catenin target gene expression in EOC cells (18), we investigated whether the enhanced nuclear recruitment of β-arr-1 in chemoresistant EOC cells could result in the upregulation of ET-1 that has previously been identified as downstream β-catenin target gene (18, 29). By using a reporter plasmid with *ET-1* promoter sequence containing a functional TCF-binding element (TBE; ref. 29), we found that the *ET-1* promoter activity in chemoresistant cells was significantly upregulated compared with sensitive cells and it was completely inhibited when the cells were treated with macitentan, BQ123, zibotentan, or silenced for β-arr1 or β-catenin, or upon rescue with either mNLS-β-arr1 or βarr1-180S, but not when treated with BQ788 (Fig. 1D and Supplementary Fig. S2D). Concordantly, ChIP experiments demonstrated that both β-arr1 and β-catenin were recruited on TBE of *ET-1* promoter loci (Fig. 1E and Supplementary Fig. S2F). Moreover, the silencing of β-arr1 as well as macitentan or BQ123 treatment, but not BQ788, negatively controlled β-arr1 and β-catenin recruitment (Fig. 1E and Supplementary Fig. S2E and S2F). A similar effect was also found for *MMP-2* and *Cyclin D1* promoters (Supplementary Fig. S2G). To evaluate the involvement of β-arr1 in controlling dynamic regulation of histone acetylation and deacetylation, we observed a striking decrease in histone deacetylase (HDAC)1 association to *ET-1* promoter upon ET-1 challenge, rendering the chromatin less compact and transcriptionally active (Fig. 1E and Supplementary Fig. S2F). In parallel, ET-1 induced acetylation at Lysine residue 18 in histone 3 (H3K18) as well as p300 recruitment at this promoter, indicating that the presence of p300 is required with β-arr1 and β-catenin for the epigenetic regulation of *ET-1* in chemoresistant EOC cells. All these effects were reverted in cells silenced for β-arr1 or treated with macitentan (Fig. 1E and Supplementary Fig. S2F). Collectively, these results indicate that activation of ET_AR promotes nuclear association of β-arr1 with β-catenin that directly upregulates ET-1 expression in chemoresistant EOC cells by inducing H3 acetylation, leading to chromatin reorganization and enhanced transcription of *ET-1*.

ET_AR/β-arr1–driven signaling sustains EMT, stemness features, and promotes cell invasion

EOC cells with CSC-like properties and EMT features become resistant to chemotherapy (30–32). Consistent with previous reports demonstrating that ET_AR is specifically expressed in $CD133^+$ ovarian CSC cell lines and patient samples (33), we observed that chemoresistant EOC cells expressed stemness genes, including *Nanog*, *CD44*, *Notch*, *Oct4*, *Bmi1*, and *HES1* (Fig. 2A) and EMT markers, including E-cadherin, N-cadherin, and Snail (Fig. 2B). Then, because macitentan reduced the formation of tumor spheres from $CD133^+$ ascites cells of a patient with platinum refractory EOC (33), we analyzed the changes in the expression of EMT effectors upon silencing of β-arr-1 or treatment with macitentan. Both treatments restored E-cadherin expression, and inhibited that of N-cadherin, and Snail (Fig. 2B). Because ET-1 triggers an intricate network of cross-talk through the transactivation of receptor tyrosine kinases (2, 19), we investigated whether ET-1 is able to activate vascular endothelial growth factor receptor-2 (VEGFR-2), which has been reported to be necessary for EOC growth and drug sensitivity (34, 35). As shown in Fig. 2C, ET-1 enhanced tyrosine phosphorylated form of VEGFR-2, becoming evident 2 minutes after ET-1 stimulation, and decreasing after 5 minutes (Supplementary Fig. S3A). In the presence of macitentan, or in cells silenced for β-arr-1, the ET-1–induced VEGFR-2 phosphorylation was strongly inhibited (Fig. 2D and E), demonstrating for the first time that ET-1 induces a rapid transactivation of VEGFR-2 through β-arr1.

In line with these results, chemoresistant EOC cells showed an increased capability to invade compared with sensitive cells (Fig. 2F). Treatment with macitentan, or BQ123, or silencing of β-arr1 or β-catenin, resulted in a strong inhibition of cell invasiveness that was unaffected by BQ788 (Fig. 2F and Supplementary Fig. S3B). Altogether these results highlight the critical role of ET_AR/β-arr1 in promoting EMT and cell invasion, which can be downregulated by macitentan.

Macitentan chemosensitizes EOC cells to drug-induced apoptosis

ET-1–ET_AR axis modulates cell survival pathways in sensitive and chemoresistant EOC cells (6). A2780- and 2008-resistant cells showed weak sensitivity to cytotoxic drugs (Fig. 3A and Supplementary Fig. S4A). In contrast, the resistant A2780 paclitaxel and A2780 cisplatinum showed similar sensitivity as the parental A2780 cells to cisplatinum and taxol, respectively (Supplementary Fig. S4A), suggesting that the altered phenotype was restricted to the specific drug of the induced resistance. In line with these results, we observed increased basal growth rate in resistant EOC cells compared with sensitive cells that was inhibited by treatment with macitentan, indicating that ET-1 autocrine loop present on these cells is functional and delivers signals that modulate cell growth (Fig. 3A and Supplementary S4A). In the presence of macitentan, cell proliferation, as evaluated by Ki67 staining (Supplementary Fig. S4B) and by ^{3}H-thymidine incorporation assay (Supplementary Fig. S4C), was significantly reduced. Similarly, the addition of BQ123 or zibotentan, but not the treatment with BQ788, inhibited cell proliferation (Fig. 3A and Supplementary S4D).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

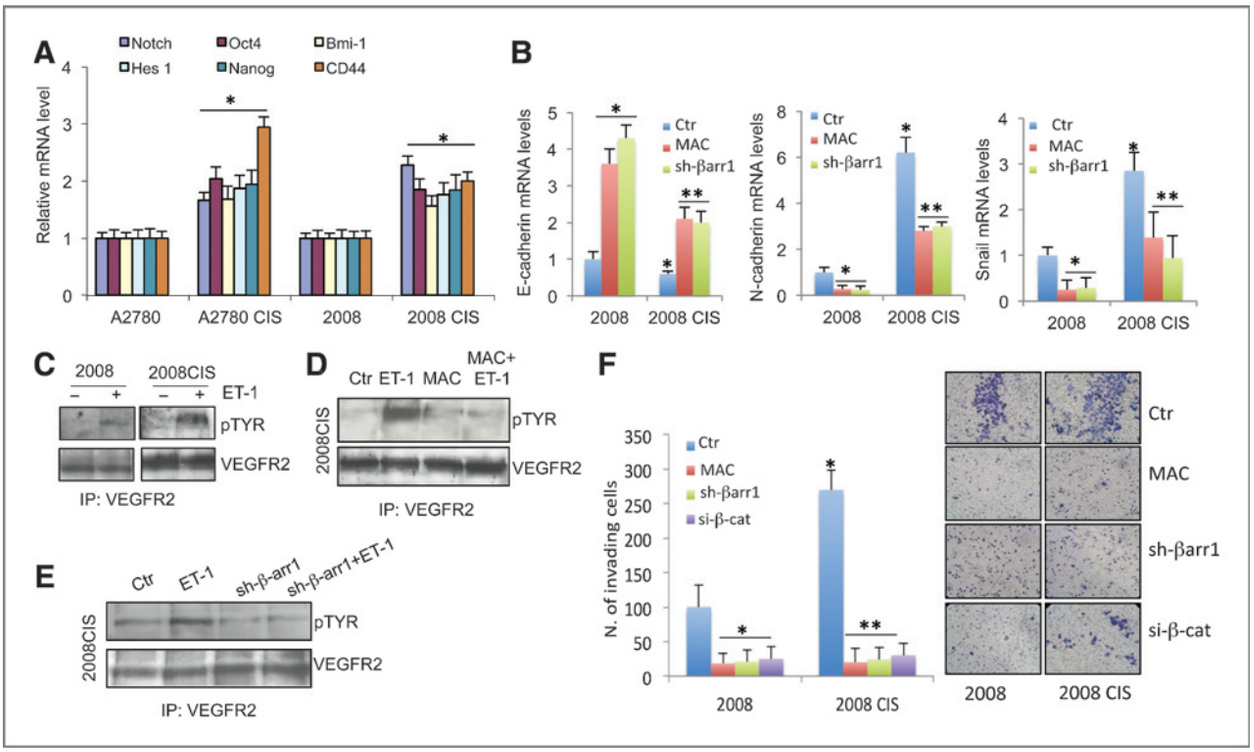

Figure 2. ET_AR/β-arr1–driven signaling sustains EMT and invasion in chemoresistant EOC cells. qRT-PCR analysis of CSC (A) or EMT (B) markers. Values are the mean ± SD ($n = 3$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). C, immunoprecipitation (IP) and immunoblot analysis of total protein extracts from 2008 cells treated for 2 minutes with ET-1 and/or with macitentan (MAC; D) or transfected with SCR (Ctr) or sh-β-arr1 (E). F, chemoinvasion assay of 2008 cells treated as indicated. The crystal violet–stained invasive cells were photographed (right) and counted (left). Magnification, ×40. Columns show the mean ± SD ($n = 3$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). Ctr, control.

Interestingly, cells silenced for β-arr1 were significantly less viable than untrasfected cells (Supplementary Fig. S4E). Furthermore, sensitive 2008 cells that overexpressed β-arr1 showed poor sensitivity to cisplatinum (Fig. 3C), with IC_{50} value of 3.5 μmol/L, compared with parental 2008 cells with IC_{50} value of 1 μmol/L for effect of cisplatinum (Supplementary Fig. S4F). Cytotoxic activity of both macitentan or silencing of β-arr1 was explained by apoptosis induction (Fig. 3B). The cotherapy of macitentan with cisplatinum, as well as the addition of cisplatinum in cells silenced for β-arr1, led to enhanced sensitivity to chemotherapeutics not only in sensitive but also in chemoresistant cells, inducing synergistic cytotoxic effect (Fig. 3D). This confirms that ET-1–ET_AR axis activates, through β-arr1, survival signals against chemotherapeutic drug treatment (36). To better characterize pathways downstream of ET-1 receptors that may be involved in resistance to apoptosis, we observed that the expression of cleaved PARP or caspase-3 increased in cells treated with macitentan or cisplatinum, and even more in combination treatment (Fig. 3E). We also found that ET-1–induced Bcl-xL expression was reduced by treatment with macitentan and was fully inhibited upon cotreatment of macitentan and cisplatinum (Fig. 3E). This implies that activation of ET_AR/β-arr1 signaling might render these cells more resistant to chemotherapeutic agents, and that treatment with macitentan sensitizes cells to apoptosis by modulating survival pathways.

Macitentan sensitizes EOC xenografts to chemotherapeutic drugs

Next, we evaluated the *in vivo* ability of macitentan alone or in combination with chemotherapeutic drugs to control tumorigenic and metastatic behavior of sensitive and resistant 2008 and A2780 cells orthotopically implanted in nude mice. All mice developed solid peritoneal tumors, which heterogeneously expressed both ET_AR and ET_BR. As revealed by IHC analysis, ET_BR were also detected in vessels and stromal components (Fig. 4A). Metastatic intraperitoneal spread was detected on the peritoneal surface, omentum, small bowel, mesentery, and ovaries (Supplementary Fig. S5A). Tumor weight in mice treated with macitentan significantly decreased not only in sensitive but also in resistant 2008 and A2780 xenografts (Fig. 4B; Supplementary Fig. S5B; Table 1).

Most importantly, a superior growth-inhibitory effect was observed when macitentan was used in combination with cisplatinum in sensitive and resistant 2008 xenografts (80% and 77%, respectively), at the end of the 4-week treatment period (Table 1). Moreover, the tumor weight inhibition obtained with macitentan, both in monotherapy or combination with cisplatinum, persisted for up to 2 weeks after termination of therapy (Table 1). Of interest, in tumors from macitentan-treated mice, a significant inhibition of p42/44 MAPK, AKT, and VEGFR-2 activation was observed (Fig. 4C), indicating that macitentan may control the apoptotic response

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

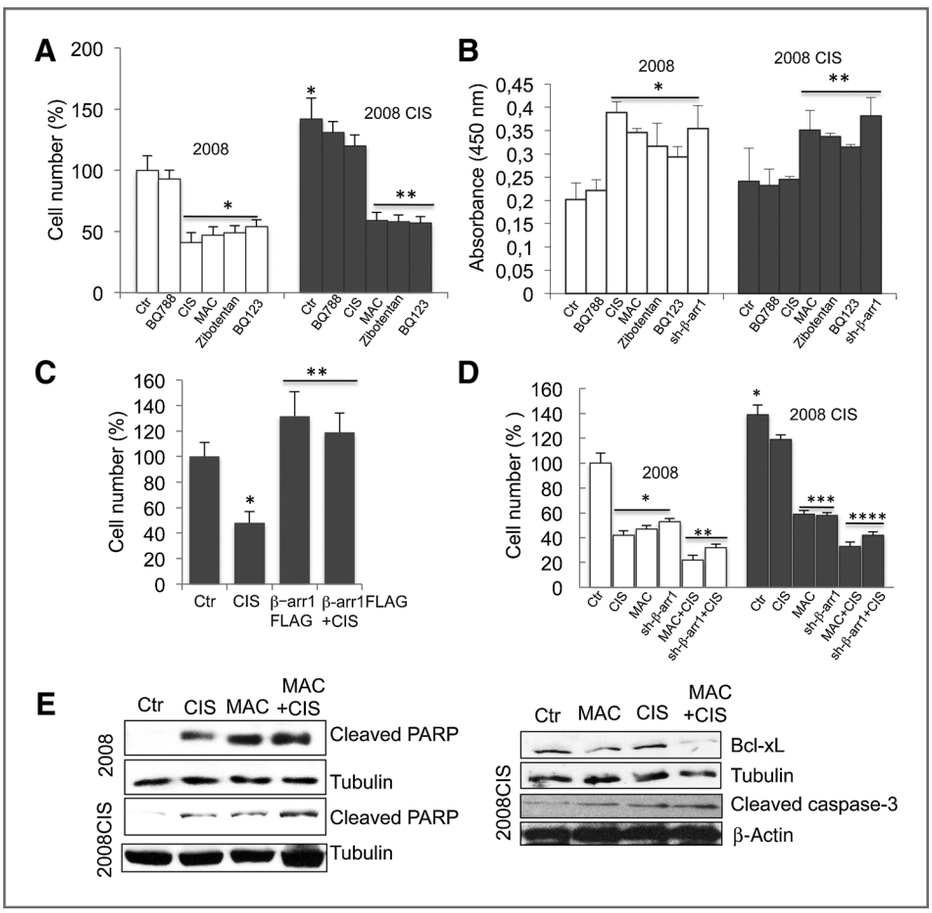

Figure 3. Macitentan sensitizes chemoresistant EOC cells to drug-induced apoptosis. A, cell viability of 2008 cells cultured for 72 hours in serum-free medium (Ctr) or treated as indicated. Values are the mean ± SD ($n = 6$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). B, apoptosis in 2008 cells cultured for 72 hours as indicated. Values are the mean ± SD ($n = 3$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. Ctr of resistant cells). C, cell viability in 2008 cells, transfected with SCR (Ctr) or β-arr1-FLAG and treated with cisplatinum. Values are the mean ± SD ($n = 6$; *, $P < 0.05$ vs. Ctr cells. **, $P < 0.05$ vs. cisplatinum-treated cells). D, cell viability in 2008 cells treated as indicated. ($n = 6$; *, $P < 0.05$ vs. Ctr of sensitive cells; **, $P < 0.05$ vs. cisplatinum-treated sensitive cells; ***, $P < 0.05$ vs. Ctr of resistant cells; ****, $P < 0.05$ vs. cisplatinum-treated resistant cells). E, IB analysis of total extracts from 2008 cells treated as indicated for 48 hours. Ctr, control.

in resistant cells through the inhibition of survival pathways, as well as the transactivation of VEGFR-2. Macitentan treatment induced a significant decrease of microvascular density (MVD) and cell proliferation, as evaluated by CD31 and Ki67 staining, in sensitive and resistant xenografts (Fig. 4D), which paralleled its ability to reduce tumor growth. In view of these results, we further explored the antiangiogenic activity of macitentan using an *in vivo* Matrigel plug assay. As shown in Fig. 4E, plugs containing conditioned medium (CM) of 2008 and 2008 cisplatinum cells exhibited an enhanced angiogenic response compared with PBS-infused (control) plugs, indicating that angiogenic factors released by sensitive and resistant EOC cells formed functional vasculatures inside the Matrigel. The addition of macitentan to the plugs inhibited vascular formation (Fig. 4E). Quantification analysis of the angiogenesis, by determination of the hemoglobin content of the plugs, indicated that macitentan significantly reduced vascular formation *in vivo*. In agreement with these results, treatment with macitentan induced apoptosis in tumor-associated endothelial cells and in surrounding tumor cells, as detected by colocalization of terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) and CD31 staining (Supplementary Fig. S5C). These data extend those in earlier reports (14, 15, 33), providing evidence that macitentan might target EOC and endothelial cells.

Besides intraperitoneal seeding, recent results demonstrated that EOC cells are able to metastatize hematogenously (37). Therefore to metastasize, EOC cells acquire the ability to invade surrounding tissues and intravasate to enter the systemic circulation. In view of this, we examined whether macitentan also impaired tumor cell intravasation from the primary site. Notably, macitentan treatment diminished the presence of circulating tumor cells (54% in sensitive and 63% in resistant xenografts; Fig. 4F). In line with these findings, the treatment with macitentan significantly decreased the number of visible metastatic lesions in EOC xenografts. Interestingly, the specific ET_AR antagonist zibotentan was less efficacious to decrease the numbers of nodules in 2008 xenografts, compared with macitentan (Supplementary Fig. S5D). Furthermore, the cotherapy of macitentan and cisplatinum demonstrated a significant improvement in the inhibition of metastasis formation compared with macitentan or cisplatinum monotherapy (Supplementary Fig. S5D). Moreover, to demonstrate the role of β-arr1 during metastatization, mouse i.p. xenograft model was established by implanting A2780 cells transfected with SCR or shRNA-β-arr1 and followed their colonization pattern. β-arr1 silencing significantly inhibited metastasis formation, in a manner that mimicked the effect of macitentan (Supplementary Fig. S5E). Collectively, these results suggest that macitentan in combination with platinum-based therapy

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

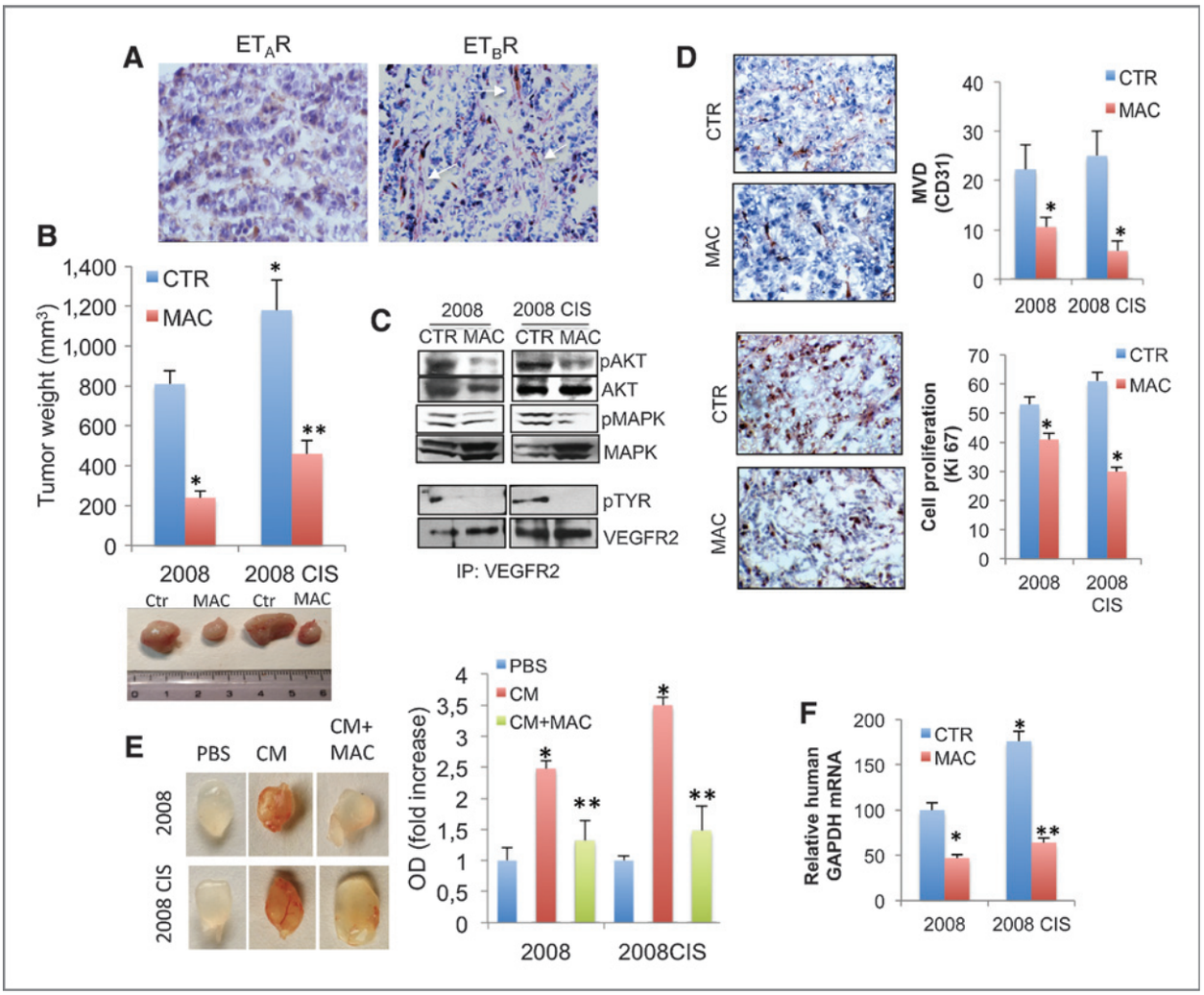

Figure 4. Macitentan treatment inhibits growth, neovascularization, and metastatic progression in EOC xenografts. A, IHC staining of representative 2008 peritoneal tumors for expression of ET_AR and ET_BR. Arrows, tumor vessels (original magnification, ×250). B, size of intraperitoneal nodules grown in 2008 xenografts was reported as the mean ± SD of 10 mice for group ($n = 3$); *, $P < 0.05$ vs. Ctr of sensitive xenografts; **, $P < 0.05$ vs. Ctr of resistant xenografts. Bottom, representative intraperitoneal nodules located on the peritoneal wall of 2008 xenografts. C, immunoblot analysis of total extracts from tumors of 2008 and 2008 cisplatinum xenografts. IP, immunoprecipitation. D, IHC and quantification analysis of tumors from 2008 cisplatinum xenografts for expression of CD31 (top) and Ki67 (bottom). Magnification, ×200. Bars ± SD; *, $P < 0.05$ vs. Ctr. E, *in vivo* Matrigel plug assay. Right, representative photographs of Matrigel plugs containing PBS or CM of 2008 and 2008 cisplatinum cells. Macitentan (MAC; 1 μmol/L) was added to the plugs. Left, quantification of blood vessel formation through measurement of hemoglobin content. Results are expressed as fold increase relative to plugs containing PBS ± SD of 6 mice for group ($n = 3$); *, $P < 0.001$ vs. PBS or CM of sensitive cells; **, $P < 0.05$ vs. CM. F, qRT-PCR analysis of human-specific *GAPDH* expression relative to murine β-actin from circulating tumor cells. Values are the mean ± SD ($n = 3$); *, $P < 0.05$ vs. Ctr of sensitive 2008 xenografts; **, $P < 0.05$ vs. Ctr of resistant xenografts). Ctr, control.

could be effective in enhancing sensitivity to the chemotherapy, by targeting aggressive resistant EOC cells and tumor-associated endothelial cells.

β-arr1/β-catenin nuclear complexes in human ovarian carcinomas

To more directly probe the *in vivo* association between nuclear β-arr1/β-catenin to *ET-1*-responsive promoters in human EOC, ChIP assays were performed on 26 sensitive and resistant tumors. This analysis showed increased levels of β-arr1 and β-catenin (92% and 100%, respectively) recruited on *ET-1* promoter in resistant tumors compared with sensitive samples (76% and 84%, respectively; Fig. 5A and Supplementary Fig. S6B), further supporting a direct and functional association between β-arr1 and β-catenin in human EOC on this promoter. To explore the pathophysiologic function of ET_AR and β-arr1 in chemoresistant EOC, a cohort of 24 primary tumors were assayed by IHC for ET_AR and β-arr1 expression. Notably, the expression of ET_AR, as well as of β-arr1, and their coexpression increased in chemoresistant compared with sensitive EOC. Moreover, 43% of β-arr1–positive chemoresistant tumors were positive for ET_AR (6 of 14), whereas 20% of sensitive tumors coexpressed β-arr1 and ET_AR (2 of 10; Supplementary Fig. S6A and S6C).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

Table 1. Treatment of 2008 and 2008 cisplatinum xenografts with macitentan in monotherapy and combination with cisplatinum

	2008		2008 cisplatinum	
Treatment group	Tumor weight at the end of treatment (mm^3), mean ± SD	Tumor weight 2 weeks after the end of treatment (mm^3), mean ± SD	Tumor weight at the end of treatment (mm^3), mean ± SD	Tumor weight 2 weeks after the end of treatment (mm^3), mean ± SD
CTR	560 ± 97	800 ± 178	928 ± 169	1,215 ± 253
MAC	298 ± 56[a]	305 ± 66[a]	500 ± 99[a]	498 ± 91[a]
CIS	312 ± 57[a]	335 ± 37[a]	891 ± 174	1017 ± 204
MAC+CIS	115 ± 21[b]	120 ± 16[b]	211 ± 19[b]	189 ± 11[b]

NOTE: Data were reported as the mean ± SD of 5 mice for group.
[a]$P < 0.05$ vs. CTR.
[b]$P < 0.001$ vs. cisplatinum (CIS) or macitentan (MAC) treatment.

ET_AR expression is associated with chemoresistance and survival in EOC patients

To strengthen the rationale for targeted therapy of ET_AR, we determined the prognostic value of ET_AR expression in patients with EOC, whose clinicopathologic characteristics are summarized in Supplementary Table S1. The expression of ET_AR and ET_BR in human EOC samples was investigated using IHC. This analysis clearly demonstrated the heterogeneous

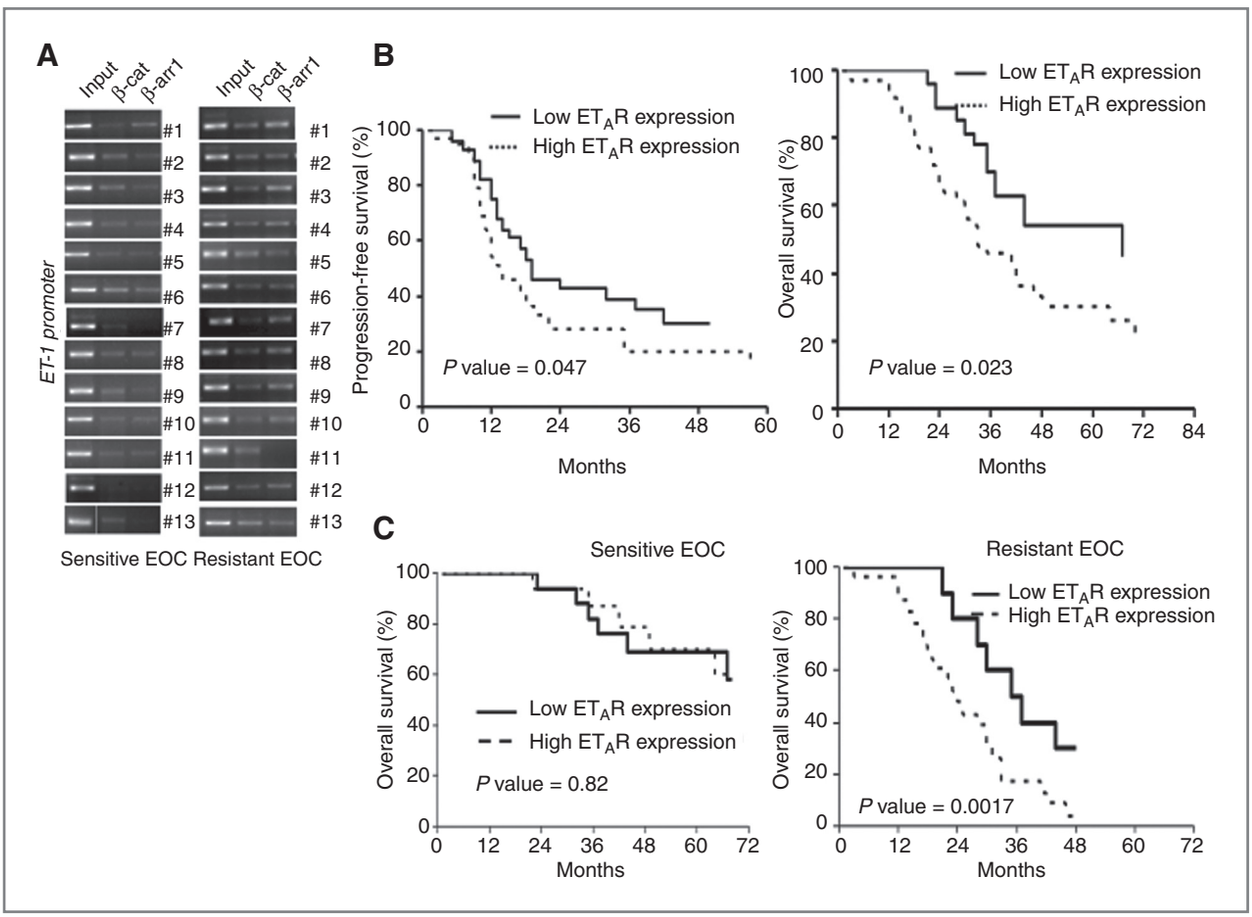

Figure 5. ET_AR expression is associated with chemoresistance and survival in patients with EOC. A, the occupancy of β-arr1 and β-catenin to *ET-1* promoter was measured by ChIP assays in 26 human sensitive and resistant EOC tissues. B, PFS and OS curves according to ET_AR expression. C, OS according to status of ET_AR expression in platinum sensitive and in platinum-resistant patients with EOC.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

Table 2. Patient characteristics and distribution of ET_AR expression according to clinicopathologic features

Characteristics	All cases (*N*)	Low ET_AR expression, *n* (%)	High ET_AR expression, *n* (%)	*P*[a]
All	68	28 (41.2)	40 (58.8)	
Age, y				
≤65	49	19 (38.8)	30 (61.2)	0.58
>65	19	9 (47.4)	10 (52.6)	
Histotype				
Serous	50	21 (42.0)	29 (58.0)	
Other	18	7 (38.9)	11 (61.1)	0.96[b]
Grade				
G1–2	14	6 (42.9)	8 (57.1)	
G3	46	17 (37.0)	29 (63.0)	0.75
n.a.	8			
Stage				
I–II	13	5 (38.5)	8 (61.5)	
III–IV	55	23 (41.8)	32 (58.2)	0.92
Residual tumor				
<1 cm	37	13 (35.1)	24 (64.9)	
Exploratory laparotomy	31	15 (48.4)	16 (51.6)	0.19
Ascites				
No	29	16 (55.2)	13 (44.8)	
Yes	38	11 (28.9)	27 (71.0)	0.044
Platinum sensitivity				
Resistant	35	10 (28.6)	25 (71.4)	
Sensitive	33	18 (54.5)	15 (45.4)	0.047

[a]Calculated by the Fisher exact test for proportion.
[b]Calculated after grouping serous versus other histologic subtypes.

expression of these two receptors in tumor cells. ET_BR expression was weak in tumor cells and highly detectable in tumor vessels and stromal components (Supplementary Fig. S6C). Table 2 shows the distribution of cases with low versus high ET_AR expression; in the whole series, high ET_AR expression was documented in 40 cases (58.8%). There was no statistically significant difference in the percentage of cases with high ET_AR expression according to age, histotype, grade, stage of disease, as well as residual tumor at first surgery; on the other hand, we documented a significantly higher proportion of cases with high ET_AR expression in patients with ascites versus patients without ascites (71.0% vs. 44.8%; $P = 0.044$). Moreover, high ET_AR expression characterized the platinum-resistant disease compared with the platinum-sensitive tumors (71.4% vs. 45.4%, respectively; $P = 0.047$).

ET_BR expression was analyzed in 39 cases; high ET_BR expression was documented in 13 cases (33.3%), while negative or low ET_BR expression was found in 15 (38.5%), and in 11 (28.2%) of cases, respectively. For purpose of analysis, cases were subgrouped in low versus high ET_BR expression (Supplementary Table S2); there was no difference in the distribution of cases with high versus low ET_BR expression according to different clinicopathologic factors including platinum resistance.

As far as survival analysis is concerned, recurrence/progression of disease was observed in 51 (75.0%) cases, while death of disease was documented in 41 patients (60.3%); Fig. 5B shows the progression-free survival (PFS) and overall survival (OS) curves according to ET_AR expression: the 3-year PFS was 39% in patients exhibiting low ET_AR expression versus 20% in cases with high ET_AR expression ($P = 0.047$). A significantly worse OS was found in patients with high versus low ET_AR expression (3-year OS 70% vs. 46%, respectively; $P = 0.023$). We also run a separate analysis of OS according to status of ET_AR expression in platinum-sensitive and -resistant disease (Fig. 5C). Although in platinum-sensitive cases, there was no difference in the 3-year OS between cases with low versus high ET_AR expression (82% vs. 87%, respectively; $P = 0.82$), in platinum-resistant tumors, high ET_AR expression was significantly associated with worse prognosis (3-year OS 50.0% vs. 17%, respectively; $P = 0.017$). On the other hand, there was no difference in terms of PFS or OS according to ET_BR expression (Supplementary Fig. S7). Finally, Supplementary Table S3 shows the univariate and multivariate analysis of clinicopathologic parameters as prognostic factors for OS in the whole series: advanced stage of disease, not optimal primary cytoreduction, serous tumor histotype, presence of ascites as well as short progression-free interval (PFI) and high ET_AR expression were significantly

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

associated with poor clinical outcome. In multivariate analysis, high ET_AR expression still maintained its unfavorable prognostic role together with advanced stage of disease, not optimal primary cytoreduction, and the shorter PFI.

Discussion

Considering the poor prognosis for patients with EOC, mainly because of late diagnosis and low response to chemotherapy (1, 30–32), the identification of key hub players and downstream signaling pathways that could modulate the response to chemotherapy might help in the development of more efficacious combinatorial regimens. Here, we report that ET_AR/β-arr1 is a critical mediator of the chemoresistant phenotype linking β-catenin signaling. Our findings provide a molecular explanation into how β-arr1/β-catenin–mediated epigenetic modification endows EOC cells with increased ET_AR-driven bypass signaling pathways. The small-molecule macitentan controlled nuclear function of β-arr1, inhibited β-catenin-transcriptional activity, restored drug sensitivity, and inhibited growth, vascularization, and progression to metastatization in EOC xenografts. Interestingly, we reported that the overexpression of ET_AR in human EOC correlates with chemoresistance and poor prognosis, indicating ET_AR as a potential predictive marker of chemoresistance.

The findings presented here reveal integration between ET_AR and the Wnt/β-catenin pathway mediated by β-arr1. We show that activation of ET_AR by ET-1 promotes a direct interaction between β-arr1 and β-catenin to regulate epigenetic modifications driving EOC chemoresistance onset through forming a multiprotein complexes. In particular, our data indicate an important role for β-arr1 downstream of ET_AR in promoting the compartmentalization of β-catenin signaling in chemoresistant cells. This includes the nuclear association between β-arr1 and β-catenin, and the recruitment of β-catenin on the TCF4-binding sites. Indeed, β-arr1 is involved in the recruitment of β-catenin on the *ET-1* proximal promoter, and histone modification patterns associated with *ET-1* gene transcription. Of interest, our findings support a positive feedback mechanism in which ET-1 stabilizes β-catenin, resulting in the autoregulatory β-catenin–mediated transcription of *ET-1* itself (7, 17, 18, 29, 38). The enhanced expression of ET-1 results in the amplification of its autocrine loop that, in turn, sustains cell viability, survival pathways, and EMT phenotype of chemoresistant cells.

Consistent with previous report demonstrating that ET_AR modulates chemoresistance in EOC stem cells (33), we demonstrated that resistant EOC cells, expressing high levels of ET_AR/β-arr1, together with stemness and EMT-associated markers, are capable to invade through activation of ET_AR/β-arr1–mediated pathway. Moreover, our findings unveil that β-arr1 might act as a signaling platform regulating also the cross-talk between ET_AR and VEGFR-2, indicating that β-arr1 could interact with different factors orchestrating the network that regulates chemoresistant onset.

The approved drug macitentan, by impairing the ET_AR pleiotropic signaling capable of regulating epigenetic changes in β-catenin–driven chemoresistant behavior, contributes to sensitize EOC cells to apoptosis. Of clinical relevance, treatment with macitentan results into inhibition of tumor growth, vascularization, intravasation, and metastatic dissemination. Most importantly, this study reveals the opportunity of macitentan to interfere with two tissues involved in the chemoresistance onset, promoting apoptosis in tumor-associated endothelial cells and surrounding tumor cells. Furthermore, these data complement and add greater relevance to previous studies (14, 15, 33), demonstrating that the addition of macitentan with cytotoxic drugs to resistant EOC cells sensitizes them to chemotherapy, thus providing a solid rationale for using macitentan in combination with chemotherapy. Differently from previous studies (14, 15, 33), which failed to demonstrate antitumor activity of macitentan when used as a single agent, here we report that this small molecule impairs tumor growth by an extent comparable with that achieved with chemotherapy. The differences could be attributed to the experimental conditions (i.e., route of cell injection, number of cells inoculated, dosage of macitentan, and duration of treatment) and to the EOC cell types used.

The difficulty in monitoring intraperitoneal disease formation and progression *in vivo* is one major limitation of xenograft models (39, 40). Therefore, in attempt to prioritize and guide future macitentan clinical studies, further studies by using new EOC patient–derived xenograft models, together with novel *in vivo* imaging techniques, are warranted.

The strengths of our preclinical data are highly supported by the analyses performed on EOC tissues from sensitive and resistant patients, demonstrating that the association between high ET_AR expression and poor survival is to be ascribed to the unfavorable prognostic role played by high ET_AR expression in the subset of platinum-resistant cases. Moreover, coexpression of ET_AR and β-arr1 and the co-occupancy of β-arr1 and β-catenin on *ET-1* gene promoter appear to be indicative of the chemoresistant phenotype of primary human EOC, further supporting the pathobiologic relevance of ET_AR/β-arr1/β-catenin in the regulation of chemoresistance. Overall, these findings provide important insights in the development of new prognostic tools and will likely lead to an improved treatment for patients with EOC. Our findings also provide a potential explanation as to why the use of selective ET_AR antagonists in clinical trials did not achieve satisfactory results (2, 41–43). Selective ET_AR blockade could tilt the balance toward ET_BR signaling in the tumor microenvironment, including the recruitment of antitumor T cells (10, 11). Besides the intrinsic mechanisms activated in EOC cells, acquisition of chemoresistance could be dependent also by the tumor microenvironment (44). In this regard, recent studies demonstrated that endothelial cells chemoprotect tumor cells through activation of ET-1 axis (45). Therefore, we can hypothesize that other mechanisms activated by ET_BR, heterogeneously expressed on tumor cells as well as on endothelial cells, might also contribute to sensitize tumor cells to chemotherapy. Therefore, macitentan, interfering with ET_AR and with ET_BR, might offer a more efficacious "two-hit" therapeutic strategy because it might target aggressive EOC cells, disabling multiple signaling circuits activated by ET_AR in a β-arr1–dependent manner, and microenvironment-associated elements expressing ET_BR

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

(10–15, 33). The activity of macitentan in EOC preclinical models associated with a well-tolerated toxicity profile, suggest that this approved small molecule can be used in a clinical setting for future development of combination regimens aimed at sensitizing tumor to chemotherapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Rosanò, A. Bagnato
Development of methodology: L. Rosanò, R. Cianfrocca, V. Di Castro, V. Caprara, E. Semprucci, P.G. Natali
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Rosanò, P. Tocci, F. Spinella, V. Di Castro, G. Ferrandina
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Cianfrocca, P. Tocci, F. Spinella, G. Ferrandina, P.G. Natali, A. Bagnato
Writing, review, and/or revision of the manuscript: L. Rosanò, P.G. Natali, A. Bagnato
Study supervision: P.G. Natali, A. Bagnato

Acknowledgments

The authors thank Dr. Rosanna Sestito for critically reviewing the article, Maria Rita Nicotra for her skillful contribution for IHC studies, Aldo Lupo for technical assistance, and Maria Vincenza Sarcone for secretarial support.

Grant Support

This work was supported by Associazione Italiana Ricerca sul Cancro (AIRC) to A. Bagnato (AIRC 14199) and L. Rosanò (AIRC 12852). R. Cianfrocca is recipient of a Fondazione Umberto Veronesi fellowship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 4, 2013; revised September 29, 2014; accepted October 1, 2014; published OnlineFirst November 6, 2014.

References

1. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003;3:502–16.
2. Rosanò L, Spinella F, Bagnato A. Endothelin 1 in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 2013; 13:637–51.
3. Huang RY, Chung VY, Thiery JP. Targeting pathways contributing to epithelial–mesenchymal transition (EMT) in epithelial ovarian cancer. Curr Drug Targets 2012;13:1649–53.
4. Marchini S, Fruscio R, Clivio L, Beltrame L, Porcu L, Nerini IF, et al. Resistance to platinum-based chemotherapy is associated with epithelial to mesenchymal transition in epithelial ovarian cancer. Eur J Cancer 2013;49:520–30.
5. Lappano R, Maggiolini M. G protein–coupled receptors: novel targets for drug discovery in cancer. Nat Rev Drug Discov 2011;10:47–60.
6. Rosanò L, Cianfrocca R, Spinella F, Di Castro V, Nicotra MR, Lucidi A, et al. Acquisition of chemoresistance and EMT phenotype is linked with activation of the endothelin A receptor pathway in ovarian carcinoma cells. Clin Cancer Res 2011;17:2350–60.
7. Rosanò L, Spinella F, Di Castro V, Nicotra MR, Dedhar S, de Herreros AG, et al. Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells. Cancer Res 2005;65:11649–57.
8. Jazaeri AA, Awtrey CS, Chandramouli GV, Awtrey CS, Chandramouli GV, Chuang YE, et al. Gene expression profiles associated with response to chemotherapy in epithelial ovarian cancers. Clin Cancer Res 2005;11:6300–10.
9. Helleman J, Smid M, Jansen MP, van der Burg ME, Berns EM. Pathway analysis of gene lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture. Gynecol Oncol 2010;117:170–6.
10. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med 2008;14: 28–36.
11. Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G. Endothelin B receptor, a new target in cancer immune therapy. Clin Cancer Res 2009;15:4521–8.
12. Salani D, Taraboletti G, Rosanò L, Di Castro V, Borsotti P, Giavazzi R, et al. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. Am J Pathol 2000;157:1703–11.
13. Spinella F, Garrafa E, Di Castro V, Rosanò L, Nicotra MR, Caruso A, et al. Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. Cancer Res 2009;69:2669–76.
14. Kim SJ, Kim JS, Kim SW, Brantley E, Yun SJ, He J, et al. Macitentan (ACT-064992), a tissue-targeting endothelin receptor antagonist, enhances therapeutic efficacy of Paclitaxel by modulating survival pathways in orthotopic models of metastatic human ovarian cancer. Neoplasia 2011;13:167–79.
15. Kim SJ, Kim JS, Kim SW, Yun SJ, He J, Brantley E, et al. Antivascular therapy for multidrug-resistant ovarian tumors by macitentan, a dual endothelin receptor antagonist. Transl Oncol 2012;5:39–47.
16. Shenoy SK, Lefkowitz RJ. β-Arrestin–mediated receptor trafficking and signal transduction. Trends Pharmacol Sci 2011;32:521–33.
17. Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, et al. Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. Proc Natl Acad Sci U S A 2009;106:2806–11.
18. Rosanò L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Spadaro F, et al. β-Arrestin-1 is a nuclear transcriptional regulator of endothelin-1-induced β-catenin signaling. Oncogene 2013;32:5066–77.
19. Spinella F, Caprara V, Di Castro V, Rosanò L, Cianfrocca R, Natali PG, et al. Endothelin-1 induces the transactivation of vascular endothelial growth factor receptor-3 and modulates cell migration and vasculogenic mimicry in melanoma cells. J Mol Med 2013;91:395–405.
20. Gamallo C, Palacios J, Moreno G, Calvo de Mora J, Suárez A, Armas A. Beta-catenin expression pattern in stage I and II ovarian carcinomas: relationship with beta-catenin gene mutations, clinicopathological features, and clinical outcome. Am J Pathol 1999;155:527–36.
21. Lee CM, Shvartsman H, Deavers MT, Wang SC, Xia W, Schmandt R, et al. Beta-catenin nuclear localization is associated with grade in ovarian serous carcinoma. Gynecol Oncol 2003;88:363–8.
22. Chau WK, Ip CK, Mak AS, Lai HC, Wong AS. c-Kit mediates chemoresistance and tumor-initiating capacity of ovarian cancer cells through activation of Wnt/β-catenin-ATP-binding cassette G2 signaling. Oncogene 2013;32:2767–81.
23. Arend RC, Londoño-Joshi AI, Straughn JM Jr, Buchsbaum DJ. The Wnt/β-catenin pathway in ovarian cancer: a review. Gynecol Oncol 2013;131:772–9.
24. Bolli MH, Boss C, Binkert C, Buchmann S, Bur D, Hess P, et al. The discovery of N-[5-bromophenyl)-6-[2-[(5-bromo-2-pyrimidinyl)oxy-]ethoxy]-4-pyrimidinyl]-N′-propylsulfamide (Macitentan), an orally active, potent dual endothelin receptorantagonist. J Med Chem 2012; 55:7849–61.
25. Iglarz M, Binkert C, Morrison K, Fischli W, Gatfield J, Treiber A, et al. Pharmacology of macitentan, an orally active tissue targeting dual endothelin receptor antagonist. J Pharmacol Exp Ther 2008;327: 736–45.
26. Hoeppner CZ, Cheng N, Ye RD. Identification of a nuclear localization sequence in b-arrestin-1 and its functional implications. J Biol Chem 2012;287:8932–43.
27. Yang M, He RL, Benovic JL, Ye RD. Beta-arrestin1 interacts with the G-protein subunits beta1gamma2 and promotes beta1gamma2-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025

dependent Akt signaling for NF-kappaB activation. Biochem J 2009;417:287–96.
28. Schuijers J, Mokry M, Hatzis P, Cuppen E, Clevers H. Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF. EMBO J 2014;33:146–56.
29. Kim TH, Xiong H, Zhang Z, Ren B. Beta-catenin activates the growth factor endothelin-1 in colon cancer cells. Oncogene 2005;24: 597–604.
30. Latifi A, Abubaker K, Castrechini N, Ward AC, Liongue C, Dobill F, et al. Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell–like profile. J Cell Biochem 2011;11:22850–64.
31. Ahmed N, Abubaker K, Findlay J, Quinn M. Epithelial mesenchymal transition and cancer stem cell–like phenotypes facilitate chemoresistance in recurrent ovarian cancer. Curr Cancer Drug Targets 2010;10:268–78.
32. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009;9:265–73.
33. Coffman L, Mooney C, Lim J, Bai S, Silva I, Gong Y, et al. Endothelin receptor-A is required for the recruitment of antitumor T cells and modulates chemotherapy induction of cancer stem cells. Cancer Biol Ther 2013;14:184–92.
34. Vecchione A, Belletti B, Lovat F, Volinia S, Chiappetta G, Giglio S, et al. A microRNA signature defines chemoresistance in ovarian cancer through modulation of angiogenesis. Proc Natl Acad Sci U S A 2013;110:9845–50.
35. Adham SA, Sher I, Coomber BL. Molecular blockade of VEGFR2 in human epithelial ovarian carcinoma cells. Lab Invest 2010;90:709–23.
36. Del Bufalo D, Di Castro V, Biroccio A, Varmi M, Salani D, Rosanò L, et al. Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for Akt activation. Mol Pharmacol 2002;61:524–32.
37. Pradeep S, Kim SW, Wu SY, Nishimura M, Chaluvally-Raghavan P, Miyake T, et al. Hematogenous metastasis of ovarian cancer: rethinking mode of spread. Cancer Cell 2014;26:77–91.
38. Sun P, Xiong H, Kim T H, Ren B, Zhang Z. Positive inter-regulation between β-catenin/T cell factor—4 signaling and endothelin—1 signaling potentiates proliferation and survival of prostate cancer cells. Mol Pharmacol 2006;69:520–31.
39. Shaw TJ, Senterman MK, Dawson K, Crane CA, Vanderhyden BC. Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. Mol Ther 2004;10: 1032–42.
40. Lengyel E, Burdette JE, Kenny HA, Matei D, Pilrose J, Haluska P, et al. Epithelial ovarian cancer experimental models. Oncogene 2014;33: 3619–33.
41. Cognetti F, Bagnato A, Colombo N, Savarese A, Scambia G, Sehouli J, et al. A phase II, randomized, double-blind study of zibotentan (ZD4054) in combination with carboplatin/paclitaxel versus placebo in combination with carboplatin/paclitaxel in patients with advanced ovarian cancer sensitive to platinum-based chemotherapy (AGO-OVAR 2.14). Gynecol Oncol 2013;130:31–7.
42. Said N, Smith S, Sanchez-Carbayo M, Theodorescu D. Tumor endothelin—1 enhances metastatic colonization of the lung in mouse xenograft models of bladder cancer. J Clin Invest 2011;121: 132–47.
43. Kohan DE, Cleland JG, Rubin LJ, Theodorescu D, Barton M. Clinical trials with endothelin receptor antagonists: what went wrong and where can we improve? Life Sci 2012;91:528–39.
44. Thibault B, Castells M, Delord JP, Couderc B. Ovarian cancer microenvironment: implications for cancer dissemination and chemoresistance acquisition. Cancer Metastasis Rev 2014;33:17–39.
45. Kim SW, Chai HJ, Lee H-J, He J, Wu Q, Langley RR, et al. Role of endotheln axis in astrocyte- and endothelial cell-mediated chemoprotection of cancer cells. Neuro-Oncology 2014;0:1–14.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/74/24/7453/2711898/7453.pdf by guest on 24 March 2025