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

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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 (ETAR, 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 ETAR/β-arrestin1–ARRB1 links Wnt signaling to acquire chemoresistant and EMT phenotype. We found that ETAR/β-arrestin1 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-arrestin1 or pharmacologic treatment with the dual ETAR/ETBR 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-arrestin1 and β-catenin to ET-1 gene promoter. In these tissues, high expression of ETAR significantly associated with poor clinical outcome and chemoresistance. Collectively, our findings reveal the existence of a novel mechanism by which ETAR/β-arrestin1 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 (ETAR, EDNRA) axis is aberrantly activated in EOC to stimulate cell proliferation, survival, angiogenesis, and invasion, and increased ETAR expression has been correlated with platinum resistance and EMT marker expression (2, 6–9). In EOC, also ETBR (EDNRB) appears to have protumorigenic activity by promoting tumor survival through the evasion of immune response. Indeed, ETBR signaling is capable to impair antitumor immunity by preventing T-cell recruitment to tumors (10, 11). In addition, ETAR plays a role in inducing tumor angiogenesis and lymphangiogenesis by inducing in blood and lymphatic endothelial cell proliferation, survival, and migration (12, 13). Hence, ETAR and ETBR, 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.
(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. Ablation 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 ETAR, 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-arrestin signaling and the β-catenin pathway, leading to chemoresistance onset. Of translational interest is the finding that the dual ET AR/ETBR antagonist macitentan, a potent inhibitor of ET AR with significant affinity for the ETBR (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-Drp(1-methoxyacarbonyl)-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 Actelion. Macitentan, also called ACT-064992 or N-(5-[4-bromophenyl]-6-{2-azin-2-yl})-2-(4-[1,3,4-oxadiazol-2-yl]phenyl) pyridine-3-sulfonamide (1 μmol/L), was kindly provided by AstraZeneca. Macitentan, also called ACT-064992 or N-(5-[4-bromophenyl]-6-[2-[5-bromopyrimidin-2-yl]oxy]ethyl)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 × 10⁶ 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 intra-peritoneal organs were analyzed. Tumor volume was calculated using the formula: π/6 larger diameter × (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 ± 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,
SPSS Inc.). A two-sided probability value of <0.05 was considered statistically significant.

For more detailed methods, see Supplementary Materials and Methods.

Results

\( \beta \)-arr1 promotes \( \beta \)-catenin association in chemoresistant EOC cells

To establish the role of \( \beta \)-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\(_{A}\)R and ET\(_{B}\)R (Supplementary Fig. S1A). Resistant cells expressed higher levels of \( \beta \)-arr1 and of ET\(_{B}\)R (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, \( \beta \)-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 \( \beta \)-catenin and \( \beta \)-arr1 in both sensitive and resistant cells (Fig. 1B and Supplementary Fig. S1D). This
association was inhibited by macitentan as well as by the expression of mNLS-β-arr1, a mutant unable of nuclear import (26), or with βarr1-1805s 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 Figs. S1F and S1G). This activity was significantly inhibited by silencing of β-arr1, or expression of mNLS-β-arr1, or βarr1-1805s or by macitentan, or by both ETAR 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 ETAR 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 ETAR 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-arr1 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 βarr1-1805s, 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 Figs. 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 ETAR 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.

**ETAR/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 ETAR 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-arr1 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-arr1, 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 ETAR/β-arr1 in promoting EMT and cell invasion, which can be downregulated by macitentan.

**Macitentan chemosensitizes EOC cells to drug-induced apoptosis**

ET-1–ETAR 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 3H-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).
Interestingly, cells silenced for β-ar1 were significantly less viable than untransfected cells (Supplementary Fig. S4E). Furthermore, sensitive 2008 cells that overexpressed β-ar1 showed poor sensitivity to cisplatinum (Fig. 3C), with IC₅₀ value of 3.5 μmol/L, compared with parental 2008 cells with IC₅₀ value of 1 μmol/L for effect of cisplatinum (Supplementary Fig. S4F). Cytotoxic activity of both macitentan or silencing of β-ar1 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-ar1, 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–ET₄R axis activates, through β-ar1, 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 ET₄R/β-ar1 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 heterogeneous-ly expressed both ETAR and ETBR. As revealed by IHC analysis, ETBR 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...
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 by 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.

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 = 6); *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 of sensitive 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 resistant cells). E, IB analysis of total extracts from 2008 cells treated as indicated for 48 hours. Ctr, control.

Besides intraperitoneal seeding, recent results demonstrated that EOC cells are able to metastasize 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_A 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...
could be effective in enhancing sensitivity to the chemotherapy, by targeting aggressive resistant EOC cells and tumor-associated endothelial cells.

**β-ar1/β-catenin nuclear complexes in human ovarian carcinomas**

To more directly probe the *in vivo* association between nuclear β-ar1/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-ar1 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-ar1 and β-catenin in human EOC on this promoter. To explore the pathophysiologic function of ET_3_R and β-ar1 in chemoresistant EOC, a cohort of 24 primary tumors were assayed by IHC for ET_3_R and β-ar1 expression. Notably, the expression of ET_3_R, as well as that of β-ar1, and their coexpression increased in chemoresistant compared with sensitive EOC. Moreover, 43% of β-ar1–positive chemoresistant tumors were positive for ET_3_R (6 of 14), whereas 20% of sensitive tumors coexpressed β-ar1 and ET_3_R (2 of 10; Supplementary Fig. S6A and S6C).

**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_3_R and ET_3_B. 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 K67 (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.
ETAR expression is associated with chemoresistance and survival in EOC patients

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

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor weight at the end of treatment (mm³), mean ± SD</th>
<th>Tumor weight 2 weeks after the end of treatment (mm³), mean ± SD</th>
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<tr>
<td>CTR</td>
<td>560 ± 97</td>
<td>800 ± 178</td>
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<td>MAC</td>
<td>298 ± 56ₜ</td>
<td>305 ± 66ₜ</td>
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<tr>
<td>CIS</td>
<td>312 ± 57ₜ</td>
<td>335 ± 37ₜ</td>
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<tr>
<td>MAC+CIS</td>
<td>115 ± 21ᵇ</td>
<td>120 ± 16ᵇ</td>
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NOTE: Data were reported as the mean ± SD of 5 mice for group.

ₜP < 0.05 vs. CTR.

ᵇP < 0.001 vs. cisplatinum (CIS) or macitentan (MAC) treatment.
expression of these two receptors in tumor cells. ETBR 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 ETAR expression; in the whole series, high ETAR expression was documented in 40 cases (58.8%). There was no statistically significant difference in the percentage of cases with high ETAR 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 ETAR expression in patients with ascites versus patients without ascites (71.0% vs. 44.8%; \(P = 0.044\)). We also run a separate analysis of OS according to status of ETAR 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 ETAR expression (82% vs. 87%, respectively; \(P = 0.82\)), in platinum-resistant tumors, high ETAR 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 ETBR 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 ETAR expression were significantly

<table>
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<th>Characteristics</th>
<th>All cases (N)</th>
<th>Low ETAR expression, n (%)</th>
<th>High ETAR expression, n (%)</th>
<th>(P^a)</th>
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<tr>
<td>G3</td>
<td>46</td>
<td>17 (37.0)</td>
<td>29 (63.0)</td>
<td></td>
</tr>
<tr>
<td>n.a.</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>13</td>
<td>5 (38.5)</td>
<td>8 (61.5)</td>
<td>0.92</td>
</tr>
<tr>
<td>III–IV</td>
<td>55</td>
<td>23 (41.8)</td>
<td>32 (58.2)</td>
<td></td>
</tr>
<tr>
<td>Residual tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 cm</td>
<td>37</td>
<td>13 (35.1)</td>
<td>24 (64.9)</td>
<td>0.19</td>
</tr>
<tr>
<td>Exploratory laparotomy</td>
<td>31</td>
<td>15 (48.4)</td>
<td>16 (51.6)</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>16 (55.2)</td>
<td>13 (44.8)</td>
<td>0.044</td>
</tr>
<tr>
<td>Yes</td>
<td>38</td>
<td>11 (28.9)</td>
<td>27 (71.0)</td>
<td></td>
</tr>
<tr>
<td>Platinum sensitivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>35</td>
<td>10 (28.6)</td>
<td>25 (71.4)</td>
<td>0.047</td>
</tr>
<tr>
<td>Sensitive</td>
<td>33</td>
<td>18 (54.5)</td>
<td>15 (45.4)</td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Calculated by the Fisher exact test for proportion.
\(b\)Calculated after grouping serous versus other histologic subtypes.

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 ETAR expression: the 3-year PFS was 39% in patients exhibiting low ETAR expression versus 20% in cases with high ETAR expression (\(P = 0.047\)). A significantly worse OS was found in patients with high versus low ETAR expression (3-year OS 70% vs. 46%, respectively; \(P = 0.023\)). We also run a separate analysis of OS according to status of ETAR 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 ETAR expression (82% vs. 87%, respectively; \(P = 0.82\)), in platinum-resistant tumors, high ETAR 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 ETBR 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 ETAR expression were significantly
associated with poor clinical outcome. In multivariate analysis, high ET$_A$R 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$_A$R/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$_A$R-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$_A$R in human EOC correlates with chemoresistance and poor prognosis, indicating ET$_A$R as a potential predictive marker of chemoresistance.

The findings presented here reveal integration between ET$_A$R and the Wnt/β-catenin pathway mediated by β-arr1. We show that activation of ET$_A$R 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$_A$R 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$_A$R modulates chemoresistance in EOC stem cells (33), we demonstrated that resistant EOC cells, expressing high levels of ET$_A$R/β-arr1, together with stemness and EMT-associated markers, are capable to invade through activation of ET$_A$R/β-arr1–mediated pathway. Moreover, our findings unveil that β-arr1 might act as a signaling platform regulating also the cross-talk between ET$_A$R 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$_A$R pleiotropic signaling capable of regulating epigenetic changes in β-catenin–driven chemoresistant behavior, contributes to sensitise 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$_A$R expression and poor survival is to be ascribed to the unfavorable prognostic role played by high ET$_A$R expression in the subset of platinum-resistant cases. Moreover, coexpression of ET$_A$R 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$_A$R/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$_A$R antagonists in clinical trials did not achieve satisfactory results (2, 41–43). Selective ET$_A$R blockade could tilt the balance toward ET$_A$R signaling in the tumor microenvironment, including the recruitment of antitumor 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$_A$R, 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$_A$R and with ET$_A$R, might offer a more efficacious “two-hit” therapeutic strategy because it might target aggressive EOC cells, disabling multiple signaling circuits activated by ET$_A$R in a β-arr1–dependent manner, and microenvironment-associated elements expressing ET$_A$R.
(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.

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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, bios-statistics, computational analysis): R. Cianfrocca, 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

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

Laura Rosanò, Roberta Cianfrocca, Piera Tocci, et al.


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