Therapeutics, Targets, and Chemical Biology

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

Laura Rosanò¹, Roberta Cianfrocca¹, Piera Tocci¹, Francesca Spinella¹, Valeriana Di Castro¹, Valentina Caprara¹, Elisa Sempucci¹, Gabriella Ferrandina², Pier Giorgio Natali¹,³, and Anna Bagnato¹

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-arrestin-1 (β-ar1, ARRB1) links Wnt signaling to acquire chemoresistant and EMT phenotype. We found that ETAR/β-ar1 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-ar1 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, invasatation, 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-ar1 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-ar1 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.

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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.

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

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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: bagnato0@ffo.it and rosano@ffo.it
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(β-ar, 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. Ablent 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<sub>R</sub>, β-ar1 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<sub>R</sub>B/β-ar1 signaling and the β-catenin pathway, leading to chemoresistance onset. Of translational interest is the finding that the dual ET<sub>R</sub>B/ET<sub>R</sub>A antagonist macitentan, a potent inhibitor of ET<sub>R</sub>B with significant affinity for the ET<sub>R</sub>A (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 cisplatin- 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-methylpyrrolizin-2-yl)-2-(4-[1,3,4-oxadiazol-2-yl]-phenyl) pyridine-3-sulfonamide (1 μmol/L) were 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 (EFP) 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<sup>−</sup>/nu<sup>−</sup>) mice, 4 to 6 weeks of age (Charles River Laboratories), were injected i.p. with 2 × 10<sup>6</sup> 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: \( \pi/6 \times (\text{larger diameter} \times \text{smaller diameter})^2 \). 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,
A two-sided probability value of \(< 0.05\) was considered statistically significant.

For more detailed methods, see Supplementary Materials and Methods.

**Results**

**\(\beta\)-Arr-1 Mediates Signaling of ET\(\alpha\)R in Chemoresistance**

**ET\(\alpha\)R activation promotes \(\beta\)-arr1–\(\beta\)-catenin association in chemoresistant EOC cells to regulate ET-1 expression**

To establish the role of \(\beta\)-arr1 in ET\(\alpha\)R-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\(\alpha\)R and ET\(\alpha\)R (Supplementary Fig. S1A). Resistant cells expressed higher levels of \(\beta\)-arr1 and of ET\(\alpha\)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. IA; 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 \(\beta\)-arr-1 in both sensitive and resistant cells (Fig. 1B and Supplementary Fig. S1D).
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 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 βarr1-180S, 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 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.

**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 cisplatin showed similar sensitivity as the parental A2780 cells to cisplatin 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 Fig. 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 untrasfected cells (Supplementary Fig. S4E). Furthermore, sensitive 2008 cells that overexpressed β-ar1 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-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–ETAR 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). This implies that activation of ETAR/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 heterogeneously 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
Macitentan 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 in vivo that macitentan significantly reduced vascular formation 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 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 ETAR antagonist zibotentan was less efﬁcacious to decrease metastatic lesions in EOC xenografts. These data extend those in earlier reports (14, 15, 33), providing evidence that macitentan might target EOC and endothelial cells.

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 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 ETAR 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-ar1 during metastatization, mouse i.p. xenograft model was established by implanting A2780 cells transfected with SCR or shRNA-β-ar1 and followed their colonization pattern. β-ar1 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 pathobiologic function of ET-aR and β-ar1 in chemoresistant EOC, a cohort of 24 primary tumors were assayed by IHC for ET-aR and β-ar1 expression. Notably, the expression of ET-aR, 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-aR (6 of 14), whereas 20% of sensitive tumors coexpressed β-ar1 and ET-aR (2 of 10; Supplementary Fig. S6A and S6C).

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**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. D, immunoprecipitation 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.
ETαR expression is associated with chemoresistance and survival in EOC patients
To strengthen the rationale for targeted therapy of ETαR, we determined the prognostic value of ETαR expression in patients with EOC, whose clinicopathologic characteristics are summarized in Supplementary Table S1. The expression of ETαR and ETβR in human EOC samples was investigated using IHC. This analysis clearly demonstrated the heterogeneous

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

<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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<tbody>
<tr>
<td>CTR</td>
<td>560 ± 97</td>
<td>800 ± 178</td>
</tr>
<tr>
<td>MAC</td>
<td>298 ± 56a</td>
<td>305 ± 66a</td>
</tr>
<tr>
<td>CIS</td>
<td>312 ± 57a</td>
<td>335 ± 37a</td>
</tr>
<tr>
<td>MAC+CIS</td>
<td>115 ± 21b</td>
<td>120 ± 16b</td>
</tr>
</tbody>
</table>

NOTE: Data were reported as the mean ± SD of 5 mice for group.

*P < 0.05 vs. CTR.

**P < 0.001 vs. cisplatinum (CIS) or macitentan (MAC) treatment.

Figure 5. ETαR 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 ETαR expression. C, OS according to status of ETαR expression in platinum sensitive and in platinum-resistant patients with EOC.

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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

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 analyzed 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>
<thead>
<tr>
<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>
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</tr>
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<td>&lt;65</td>
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<td>30 (61.2)</td>
<td>0.58</td>
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<td>19</td>
<td>9 (47.4)</td>
<td>10 (52.6)</td>
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<tr>
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<td>21 (42.0)</td>
<td>29 (58.0)</td>
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<td>G3</td>
<td>46</td>
<td>17 (37.0)</td>
<td>29 (63.0)</td>
<td>0.75</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>

\( ^a \text{Calculated by the Fisher exact test for proportion.} \)

\( ^b \text{Calculated after grouping serous versus other histologic subtypes.} \)
associated with poor clinical outcome. In multivariate analysis, high ETAR 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 combinational regimens. Here, we report that ENαR/β-ar1 is a critical mediator of the chemoresistant phenotype linking β-catenin signaling. Our findings provide a molecular explanation into how β-ar1/β-catenin–mediated epigenetic modification endows EOC cells with increased ENαR-driven bypass signaling pathways. The small-molecule macitentan controlled nuclear function of β-ar1, 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 ENαR in human EOC correlates with chemoresistance and poor prognosis, indicating ENαR as a potential predictive marker of chemoresistance.

The findings presented here reveal integration between ENαR and the Wnt/β-catenin pathway mediated by β-ar1. We show that activation of ENαR by ET-1 promotes a direct interaction between β-ar1 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-ar1 downstream of ENαR in promoting the compartmentalization of β-catenin signaling in chemoresistant cells. This includes the nuclear association between β-ar1 and β-catenin, and the recruitment of β-catenin on the TCF4-binding sites. Indeed, β-ar1 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 ENαR 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 ENαR modulates chemoresistance in EOC stem cells (33), we demonstrated that resistant EOC cells, expressing high levels of ENαR/β-ar1, together with stemness and EMT-associated markers, are capable to invade through activation of ENαR/β-ar1–mediated pathway. Moreover, our findings unveil that β-ar1 might act as a signaling platform regulating also the cross-talk between ENαR and VEGFR-2, indicating that β-ar1 could interact with different factors orchestrating the network that regulates chemoresistant onset.

The approved drug macitentan, by impairing the ENαR 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 ENαR expression and poor survival is to be ascribed to the unfavorable prognostic role played by high ENαR expression in the subset of platinum-resistant cases. Moreover, coexpression of ENαR and β-ar1 and the co-occupancy of β-ar1 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 ENαR/β-ar1/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 ENαR antagonists in clinical trials did not achieve satisfactory results (2, 41–43). Selective ENαR blockade could tilt the balance toward ENαR 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 ENαR, heterogeneously expressed on tumor cells as well as on endothelial cells, might also contribute to sensitize tumor cells to chemotherapy. Therefore, macitentan, interfering with ENαR and with ENαR, might offer a more efficacious “two-hit” therapeutic strategy because it might target aggressive EOC cells, disabling multiple signaling circuits activated by ENαR in a β-ar1–dependent manner, and microenvironment–associated elements expressing ENαR.
(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, F. Spinella, G. Ferrandina, P.G. Natali, A. Bagnato

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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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