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# Macitentan blocks endothelin-1 receptor activation required for chemoresistant ovarian cancer cell plasticity and metastasis

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*Aims:* In epithelial ovarian cancer (EOC), activation of endothelin-1 (ET-1)/endothelin A receptor ($ET_AR$) and ET-1/$ET_BR$ signaling is linked to many tumor promoting effects, such as proliferation, angiogenesis, invasion, metastasis and chemoresistance. Understanding how to hamper the distinct mechanisms that facilitate epithelial plasticity and propagation is therefore central for improving the clinical outcome for EOC patients.
*Main methods:* The phosphorylation status of Akt and MAPK was evaluated by immunoblotting in A2780 and 2008 EOC cell lines and their cisplatinum-resistant variants. Vasculogenic mimicry was analyzed by vascular tubules formation assay. Tumor growth and metastases inhibition was performed in chemoresistant EOC xenografts.
*Key findings:* We found that the dual $ET_AR$/$ET_BR$ antagonist macitentan was able to inhibit the ET-1-induced activation of Akt and MAPK signaling pathways in chemoresistant EOC cells. Moreover, chemoresistant EOC cells displayed higher capability to engage vasculogenic mimicry compared to sensitive cells that was inhibited after treatment with macitentan. Finally, the specific $ET_AR$ antagonist zibotentan was less efficacious compared to macitentan to suppress tumor growth in chemoresistant EOC xenografts and the co-treatment of macitentan and cisplatinum reduced the metastatic progression.
*Significance:* Our findings better clarify the ET-1-induced molecular mechanisms underlying the aggressive behavior of chemoresistant EOC cells. These results also support the use of macitentan in combination with chemotherapy as a rational therapeutic strategy for circumventing drug resistance in EOC.

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## 1. Introduction

Epithelial ovarian cancer (EOC) is the leading cause of gynecologic cancer mortality worldwide [1]. The drug-resistant nature of EOC cells means that effective chemotherapies are lacking, which contributes to the high mortality in patients diagnosed with EOC [2]. Understanding the distinct mechanisms that sustain tumor metastasis and therapeutic resistance is therefore central for improving the clinical outcome for EOC patients. An aberrant activation of endothelin-1 (ET-1) axis, which consists of the ligand ET-1 and its G-protein coupled receptors (GPCR) endothelin A receptor ($ET_AR$) and $ET_BR$, is now recognized as a common mechanism underlying the tumor progression and the acquisition of chemoresistance and epithelial-mesenchymal transition (EMT) phenotype of EOC [3]. ET-1 axis activates several mechanisms, including mitogenesis, cell survival, invasiveness and angiogenesis [3]. In particular, the ET-1/$ET_AR$ interaction triggers the activation of different pathways through the scaffold protein β-arrestin-1 that serves as molecular hub to organize complex signaling network [4–6]. In this regard, $ET_AR$/β-arrestin-1 cooperates with Wnt signaling to acquire a chemoresistant phenotype through the amplification of ET-1 autocrine loop, thus sustaining EMT, stemness features, cell invasion and metastasis [7]. In addition to $ET_AR$, $ET_BR$, which is highly expressed in blood and lymphatic endothelial cells, is implicated in angiogenesis and lymphangiogenesis [8,9], and controls immune response by preventing T cell recruitment to tumors [10,11]. Therefore, the use of dual $ET_AR$ and $ET_BR$ antagonists in EOC therapy could offer many advantages due to their capability to target not only cancer cells (which typically express $ET_AR$), but also tumor-associated stromal elements, such as vascular, lymphatic, inflammatory cells and fibroblasts, which all express $ET_BR$. The approved drug macitentan, by impairing the $ET_AR$ pleiotropic signaling capable of regulating chemoresistant behavior, contributes to sensitize EOC cells to apoptosis [7]. Of clinical relevance, treatment with macitentan results into inhibition of tumor growth, vascularization, intravasation, and metastatic dissemination [12–15].

Considerable body of research has demonstrated that tumor vasculature is not necessarily derived from endothelial cell sprouting; instead, cancer tissue can generate its vasculature from diverse origins by alternative mechanisms, namely, vasculogenesis [16] and vascular mimicry

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(VM) [17]. Sood et al. demonstrated that aggressive ovarian cancer cells are able to display *in vitro* VM [18]. The existence of tumor cell line vasculature has been demonstrated in approximately 30% of invasive ovarian cancers and its association with aggressive tumor features [19].

In this study, we elucidated the role of ET-1/$ET_AR$ axis in epithelial cell plasticity and in tumor growth and metastatization. Our findings demonstrate for the first time that macitentan blocks ET-1-driven EOC cell plasticity, related to chemoresistance onset, and metastasis in monotherapy and even more in combination with cisplatinum, thus supporting the use of macitentan as an important therapeutic approach for EOC in combination with chemotherapy.

## 2. Materials and methods

### *2.1. Cells and cell culture conditions*

The human ovarian carcinoma cell line A2780 was obtained from European Collection of Cell Cultures (Salisbury, UK). The 2008 cell line and its cisplatinum-resistant subclone 2008C13 (CIS) were kindly provided by Dr. S.B. Howell (University of San Diego, La Jolla, CA, USA). To retain cisplatinum (CIS) resistance, 1 μM cisplatinum (Pfizer, Italy) was added to the culture medium every two passages [20]. All cells were passed in our laboratory for fewer than 3 months after resuscitation and were tested routinely for cell proliferation as well as mycoplasma contamination, and they showed similar growth rate and negative mycoplasma during the experiments. Cells were serum starved by incubation in serum-free medium for 24 h. ET-1 was used at 100 nM and was purchased from Bachem (Switzerland). Macitentan, also called ACT-064992 or N-(5-[4-bromophenyl]-6-{2-[5-bromopyrimidin-2-yloxy]ethoxy}pyrimidin-4-yl)-N′-propylsulfamide, was added 30 min before ET-1 at a dose of 1 μM and was kindly provided by Actelion Pharmaceuticals, Ltd. (Switzerland). Zibotentan, ZD4054, N-(3-methoxy-5-methylpyrazin-2-yl)-2-(4-[1,3,4-oxadiazol-2-yl]phenyl) pyridine-3-sulfonamide, was kindly provided by AstraZeneca (UK).

### *2.2. Western blot analysis*

Whole cell lysates were prepared using a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a mixture of protease and phosphatase inhibitors. Total proteins were subjected to SDS-PAGE, and the antibodies (Abs) used for the study were as follows: anti-p-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK, anti-pAkt (S473) and anti-Akt were from Cell Signaling Technology (MA, USA). Western blotting filters were developed using the ECL-plus detection system or, otherwise, the SuperSignal West Femto kit (Thermo Scientific, IL, USA). Western blots for pAkt and pMAPK were quantified by densitometric analysis using ImageJ v.1.34s software (http://rsb.info.nih.gov/ij/).

### *2.3. Vasculogenic mimicry and Immunofluorescence*

The ability of tumor cells to form capillary-like structure called vasculogenic mimicry (VM) formation has been assessed on cells cultured on Cultrex (basal membrane extract; Trevigen, MD, USA), as previously described [18]. Images were analyzed with ImageJ software for determining the length of the tubes and the number of intersections. Representative images were captured with Olympus Ix70 microscope at 20× magnification. For immunofluorescence staining, tubes were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 followed by incubation with Abs to CD31 (Dako, Carpinteria, CA, USA), VE-cadherin (BD Transduction Laboratories, CA, USA), $ET_AR$ (Santa Cruz Biotechnology, CA, USA), and VEGFR-2 (Santa Cruz Biotechnology), and secondary Alexa Fluor 488 and 594 antibodies (Life Technologies, Italy). 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Life Technologies), was added to stain nucleus. Images of representative cells were captured at 10× magnitude with a Leica DMIRE2 deconvolution microscope equipped with a Leica DFC 350FX camera and elaborated by a Leica FW4000 deconvolution software (Leica, Germany).

### *2.4. Xenografts in nude mice*

$2 \times 10^6$ viable A2780 cisplatinum-resistant cells were injected subcutaneously into the flank of female athymic (nu+/nu+) mice, 4–6 week of age (Charles River Laboratories, Italy), following the guidelines for animal experimentation of the Italian Ministry of Health. After two weeks, when the tumors were detectable, mice were randomized in 10 for group to receive the following treatments for 4 weeks: vehicle (0.05% (wt/wt) methylcellulose solution containing 0.05% (v/v) Tween 80; Ctr), zibotentan (10 mg/kg, oral daily), and macitentan (MAC, 30 mg/kg, oral daily). For oral administration, macitentan was reconstituted in 200 μl of vehicle before use. Tumor volume was measured with caliper and was monitored every 4 days and the tumor growth curves were plotted. Tumor volume was calculated using the formula: $\pi/6$ larger diameter × (smaller diameter)$^2$. At the end of experiments all mice were euthanized, the site of tumors was noted, and the removed tumors were frozen and analyzed for Western blotting. In a different set of experiments, female nude mice were injected intraperitoneally (i.p.) with $2 \times 10^6$ viable A2780 cisplatinum-resistant cells. Ten days after, animals were randomized into different groups of 10 mice undergoing the following treatments for 4 weeks, as previously described [7]: 1) vehicle (Ctr), 2) macitentan (MAC, 30 mg/Kg, oral daily), 3) cisplatinum (CIS, 8 mg/kg, i.p. weekly), 4) macitentan plus cisplatinum. After the end of treatment, all mice were euthanized and intraperitoneal organs were analyzed for visible metastases.

### *2.5. Statistical analysis*

Student's t-test (unpaired, two-tailed) was used for comparing statistical differences. Differences were considered statistically significant when $p < 0.05$. Statistical analysis was carried out using SPSS software (SPSS II, SPSS Inc., IL, USA) and SOLO (BMDP Statistical Software, CA, USA).

## 3. Results

### *3.1. ET-1-receptor blockade by macitentan impairs activation of Akt/MAPK signaling pathways in chemoresistant EOC cells*

To better clarify the signaling pathways activated by ET-1 and involved in chemoresistance, we used the A2780 human EOC cell line and the derivative cisplatinum-resistant (A2780 CIS) subline. ET-1 activated both mitogen activated protein kinase (MAPK) and Akt in sensitive A2780 cells, and significant higher activation was observed in the resistant subline (Fig. 1). Importantly, the small molecule macitentan, a potent $ET_AR$ antagonist with significant affinity for $ET_BR$, reduced ET-1-induced phosphorylation of both p42/p44 MAPK and Akt in A2780 sensitive and cisplatinum-resistant cells (Fig. 1). These *in vitro* data demonstrated that macitentan inhibits the activation of these survival pathways in resistant EOC cells.

### *3.2. Endothelin-1-induced vasculogenic mimicry of chemoresistant EOC cells is blocked by macitentan*

Because EOC cells with cancer stem cell (CSC)-like properties, such as vasculogenic mimicry and EMT features, become resistant to chemotherapy [21–27], we examined whether EOC cells might form vascular tubules *in vitro* in ET-1-dependent manner. Both sensitive and cisplatinum-resistant A2780 and 2008 cells plated on matrigel form a network of vascular tubules (Fig. 2A). Notably, quantification analysis demonstrated that the length of tubes and the number of their intersection in chemoresistant EOC cells increased significantly

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