Macitentan (ACT-064992), a Tissue-Targeting Endothelin Receptor Antagonist, Enhances Therapeutic Efficacy of Paclitaxel by Modulating Survival Pathways in Orthotopic Models of Metastatic Human Ovarian Cancer\textsuperscript{1,2}

Abstract
Potential treatments for ovarian cancers that have become resistant to standard chemotherapies include modulators of tumor cell survival, such as endothelin receptor (ETR) antagonist. We investigated the therapeutic efficacy of the dual ETR antagonist, macitentan, on human ovarian cancer cells, SKOV3ip1 and IGROV1, growing orthotopically in nude mice. Mice with established disease were treated with vehicle (control), paclitaxel (weekly, intraperitoneal injections), macitentan (daily oral administrations), or a combination of paclitaxel and macitentan. Treatment with paclitaxel decreased tumor weight and volume of ascites. Combination therapy with macitentan and paclitaxel reduced tumor incidence and further reduced tumor weight and volume of ascites when compared with paclitaxel alone. Macitentan alone occasionally reduced tumor weight but alone had no effect on tumor incidence or ascites. Immunohistochemical analyses revealed that treatment with macitentan and macitentan plus paclitaxel inhibited the phosphorylation of ETRs and suppressed the survival pathways of tumor cells by decreasing the levels of pVEGFR2, pAkt, and pMAPK. The dose of macitentan necessary for inhibition of phosphorylation correlated with the dose required to increase antitumor efficacy of paclitaxel. Treatment with macitentan enhanced the cytotoxicity mediated by paclitaxel as measured by the degree of apoptosis in tumor cells and tumor-associated endothelial cells. Collectively, these results show that administration of macitentan in combination with paclitaxel prevents the progression of ovarian cancer in the peritoneal cavity of nude mice in part by inhibiting survival pathways of both tumor cells and tumor-associated endothelial cells.

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Introduction
In 2009, ovarian cancer was the leading cause of death from gynecologic cancer in the United States [1]. Despite initial response rates that can exceed 80\% [2,3], most patients with advanced ovarian cancer ultimately relapse with drug-resistant disease [4,5]. Because the response rate to second-line agents is approximately 15\% to 20\% [6], new therapeutic regimens are urgently needed for this devastating cancer. Recent approaches to overcoming tumor resistance to chemotherapy include modulation of cell signaling pathways involved in tumor cell growth and survival and of the interaction of tumor cells with the organ microenvironment [7,8].
One intriguing therapeutic possibility is based on antagonists of the endothelin (ET) family of small peptides consisting of ET-1, -2, and -3 [9,10]. ETs share structural homology and initiate signaling by binding to G protein–coupled receptors ETAR and ETBR [11–14]. ETs, initially defined as potent vasoconstrictors and non-peptidic small molecule ET receptor (ETR) antagonists [14,15], were developed to treat cardiovascular diseases. For example, the dual ETAR and ETBR inhibitor, bosentan, is now used to treat pulmonary arterial hypertension [15–17].

It is important to note that ETs, beyond inducing vasoconstriction, act as paracrine or autocrine tissue factors to regulate biologic processes such as tissue remodeling and repair [18], smooth muscle cell proliferation [19], and inflammation [20]. ETs and their receptors are expressed in many tumor types [21] and are involved in tumor cell proliferation, migration, invasiveness, and vascular differentiation [21–25]. In addition, activated ETRs have also been reported to be involved in inhibition of apoptosis, matrix remodeling, and bone deposition [10,11,13,14,16,21,26], in prostate cancer [27,28], lung cancer [29,30], colon cancer [31,32], renal cancer [33], cervical cancer [34,35], brain tumors [36–38], ovarian cancer [25,39–48], and other tumors [49,50]. ET production has been demonstrated in many human tumor cell lines and human tumors. In the tumor vasculature, the endothelium is the main source of ET [49–51]. In most carcinoma cells, the dominating receptor is the ETAR receptor [50], whereas in melanoma cells and glioblastoma cells, the ETBR receptor is highly expressed [50,52]. Vascular endothelial cells express high levels of ETAR receptors [53], and ETBR receptor signaling has been associated with endothelial cell proliferation, migration, differentiation, and vascular endothelial growth factor (VEGF) induction [43,54–57].

The ET axis in ovarian cancer has been broadly studied [25,44–46,58]. Examination of clinical specimens revealed increased expression of ET-1 and ETBR in ovarian cancer cells, indicating their involvement in an autocrine loop [44]. In another study using primary and metastatic ovarian carcinomas, ETAR receptors were localized to carcinoma cells and intratumoral vessels, whereas ETBR were mainly found in the endothelial cells. VEGF production from cancer cells was found to be stimulated by ET in vitro through induction of hypoxia-inducible factor-1α [59], leading to VEGF-mediated neovascularization in vivo [44,54,56]. The level of ET-1 is also increased in peritoneal ascitic fluid of patients with ovarian carcinoma, suggesting a correlation with the level of VEGF, whereas effusions from patients without cytologic disease had low or undetectable levels of ET-1 [43]. Collectively, these data indicate that the expression of ET-1 and ETAR correlates with advanced stages of the disease [24,58,60].

In the present study, we determined whether the daily oral administration of the dual ETR receptor antagonist macitentan [61], combined with once-weekly intraperitoneal injections of paclitaxel, produced significant therapeutic effects in intraperitoneally transplanted human ovarian tumor xenografts in nude mice. Therapeutic effects were correlated with induction of apoptosis in both tumor cells and tumor-associated endothelial cells and the inhibition of phosphorylation of proteins involved in signal transduction and cell survival.

Materials and Methods

Human Ovarian Cancer Cell Lines

Highly metastatic human ovarian cancer cell lines, SKOV3ip1 [61] and IGROV1 [62,63], were maintained as monolayer cultures in Eagle minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc, Grand Island, NY), l-glutamine, pyruvate, non-essential amino acids, two-fold vitamins, and penicillin-streptomycin (Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO2 and 95% air. Immortal mouse lung endothelial cells were incubated at 33°C [64]. All reagents used for tissue culture were free of endotoxin, Mycoplasma, and viral pathogens, reovirus type 3; pneumonia virus; K virus; Theliers’ encephalitis virus; Sendai virus; min virus; mouse adenovirus; mouse hepatitis virus; lymphocytic choriomeningitis virus; ectromelia virus; and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

Reagents

Macitentan, also called ACT-064992 or (N-[5-(4-bromophenyl)ethoxy]pyrimidin-4-yl)-N’-propylaminosulfonamide), was provided by Actelion Pharmaceuticals, Ltd (Allschwil, Switzerland) as powder. For oral administration, macitentan was reconstituted in 0.05% (wt/wt) methylcellulose solution containing 0.05% (vol/vol) Tween 80 and diluted to different concentrations in 200 μl of vehicle before use. Paclitaxel (Taxol), purchased from Bristol-Myers Squibb (Princeton, NJ), was diluted in distilled water for intraperitoneal injection.

Animals

Female athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute – Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with all current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health. The mice were used in these experiments in accordance with institutional guidelines when they were 8 to 12 weeks old.

Orthotopic Implantation of Ovarian Cancer in Animal Models

To produce tumors, SKOV3ip1 and IGROV1 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped by replacing the trypsin-EDTA with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Ca2+/Mg2+-free Hank’s balanced salt solution. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of more than 95% viability were used for injection. In a preliminary experiment, 1 × 106 cells in 200 μl of Ca2+/Mg2+-free Hank’s balanced salt solution were injected into the peritoneal cavity of female nude mice. Ten days after the injection, three mice were randomly selected and examined by necropsy to confirm development of tumors (Figure W1).

Therapy Experiments

To induce peritoneal tumors, SKOV3ip1 (1 × 106) or IGROV1 (1 × 106) cells were injected into the peritoneal cavity of female nude mice. Ten days later, the mice were randomized into treatment groups (n = 10). In the first set of experiments, we investigated the therapeutic effect of macitentan. Mice were implanted with SKOV3ip1 and randomized into the following treatment groups: 1) daily oral administration and weekly intraperitoneal injection of vehicle (control), 2) weekly intraperitoneal injections of paclitaxel (5 mg/kg) and daily oral
administration of vehicle, 3) daily oral administration of macitentan (100 mg/kg) and weekly intraperitoneal injection of paclitaxel (5 mg/kg), and 4) daily oral administration of macitentan (100 mg/kg) and weekly intraperitoneal injection of paclitaxel (5 mg/kg). These treatments were continued for 4 weeks.

In this first set of experiments, the therapeutic effect of macitentan was confirmed, but serious toxicities, such as weight loss, poor skin turgor, dehydration, diarrhea, and sluggishness, were observed. Therefore, in the second set of experiments, we determined the biologic optimal dose of macitentan. Ten days after the intraperitoneal implantation of SKOV3ip1 (1 × 10³), mice were randomized into five groups (n = 10): 1) daily oral administration and weekly intraperitoneal injection of vehicle (control), 2) weekly intraperitoneal injection of paclitaxel (5 mg/kg) and daily oral administration of vehicle, 3) weekly intraperitoneal injection of paclitaxel (5 mg/kg) and daily oral administration of macitentan (30 mg/kg), 4) weekly intraperitoneal injection of paclitaxel (5 mg/kg) and daily oral administration of macitentan (10 mg/kg), and 5) weekly intraperitoneal injection of paclitaxel (5 mg/kg) and daily oral administration of macitentan (5 mg/kg).

In the third set of experiments, we determined whether blockade of the ET axis with the ET antagonist macitentan could increase the sensitivity of cancer cells to lower doses of paclitaxel. Ten days after the intraperitoneal implantation of SKOV3ip1 (1 × 10³), mice were randomized into six groups (n = 10): 1) daily oral administration and weekly intraperitoneal injections of vehicle (control), 2) weekly intraperitoneal injections of nontherapeutic dose of paclitaxel (2 mg/kg) and daily oral administration of vehicle, 3) weekly intraperitoneal injections of therapeutic dose of paclitaxel (5 mg/kg) and daily oral administration of vehicle, 4) daily oral administration of macitentan (50 mg/kg) and weekly intraperitoneal injections of vehicle, 5) daily oral administration of macitentan (50 mg/kg) and weekly intraperitoneal injections of nontherapeutic dose of paclitaxel (2 mg/kg), and 6) daily oral administration of macitentan (50 mg/kg) and weekly intraperitoneal injections of therapeutic dose of paclitaxel (5 mg/kg). These treatments were continued for 4 weeks.

From these three sets of experiments, we concluded that 50 mg/kg of macitentan is a biologically optimal dose, and so further therapy experiments were repeated with the 50-mg/kg dose. To determine whether therapeutic effects were not limited to SKOV3ip1, we did experiments using an additional human ovarian cancer cell line, IGROV1.

Necropsy Procedures and Preparation of Tissues

After 4 weeks of treatment, mice were injected in the tail vein with 100 μl of BrdU (Sigma; 25 mg/ml). Two hours later, the mice were killed by intramuscular injection of Nembutal (1 g/kg) and examined by necropsy. Tumor incidence, tumor weight, and volume of ascites were recorded (Figure W2). Tumor tissues were embedded in OCT compound (Miles, Inc, Elkhart, IN) and rapidly frozen in liquid nitrogen or fixed in 10% buffered formalin for 24 hours and processed for paraffin block.

Immunohistochemical Analyses and TUNEL Assay

Primary antibodies used in this study were: mouse anti-BrdU monoclonal antibody (1:400; Becton Dickinson, San Jose, CA), rat antimouse CD31 monoclonal antibody (1:600; BD Pharmingen, San Diego, CA), goat antihuman ET-1 polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), goat antihuman ET-2 polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc), goat antihuman ET₄R polyclonal antibody (1:200, immunohistochemistry experiments; Santa Cruz Biotechnology, Inc), rabbit anti-ET₆R (1:200, immunohistochemistry experiments; Acris, Herford, Germany), goat antihuman ET(B)R polyclonal antibody (1:200, immunofluorescence experiments; Santa Cruz Biotechnology, Inc), rabbit anti-ET(B)R (1:200, immunohistochemistry experiments; Acris), rabbit antimonouse phosphorylated Akt monoclonal antibody (1:200; Cell Signaling Technology, Boston, MA), rabbit antihuman phosphorylated-p44/42 MAP kinase (Thr202/Tyr204) polyclonal antibody (1:200; Cell Signaling Technology), and rabbit antihuman phosphorylated VEGF receptor 2 (VEGFR2) monoclonal antibody (1:200; Cell Signaling Technology). HRP-conjugated donkey antigoat IgG (1:400; Santa Cruz Biotechnology), goat antirat Alexa 594 IgG (1:400; Invitrogen), rabbit antigoat Alexa 488 IgG (1:400; Invitrogen), rabbit antigoat FITC IgG (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA), and goat antirabbit Alexa 488 IgG (1:400; Invitrogen) were purchased for use as secondary antibodies. TUNEL assay was performed using a commercial apoptosis detection kit (Promega Corp, Madison, WI) with modification [9]. For color reaction of HRP-conjugated secondary antibodies, stable 3′,3-diaminobenzidine (Research Genetics, Huntsville, AL) was used.

Immunofluorescence Staining

To determine the expression of ET-1, ET-2, ET₄R, ET(B)R, phosphorylated VEGFR2 (pVEGFR2), phosphorylated Akt (pAkt), and phosphorylated MAP kinase (pMAPK) on tumor cells and/or tumor-associated endothelial cells, tissues were stained with anti-CD31 antibody and anti-ET-1, anti-ET-2, anti-ET₄R, anti-ET(B)R, anti-pVEGFR2, or anti-pAkt antibodies. In brief, frozen slides were air-dried and fixed in acetone. After washing with phosphate-buffered saline (PBS), blocking for nonspecific protein was done with 4% fish gel. The slides were incubated with primary antibodies for 18 hours, washed with PBS three times, and incubated with compatible secondary antibodies for 2 hours at room temperature. During incubation, the slides were shielded from ambient light. For examination of the endothelium, colocalization staining with CD31 was performed followed by staining with the second primary antibodies as described previously [8].

Proliferative and apoptotic indices of tumor-associated endothelial cells as well as tumor cells were determined by colocalization of CD31 and anti-BrdU or TUNEL staining. Images were captured by an Olympus BX-51 microscope (Olympus America, Inc, Center Valley, PA).

Double Immunofluorescence Staining for ETRs and Phospho-Serine in Tumor Tissues

Because specific antibodies to detect activated, serine/threonine–phosphorylated ETRs were not available, we used double immunofluorescence staining with anti-ET₄R or ET(B)R and anti-phospho-serine antibodies. Tissue sections (6-8 μm) of frozen samples were mounted on positively charged slides and air-dried for 30 minutes. The sections were fixed in cold acetone for 10 minutes, washed three times with PBS for 3 minutes, and incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes at room temperature. The slides were incubated with a primary antibody against ET₄R (1:100, 12977; Abcam, Cambridge, MA) or ET(B)R (1:100, 65972; Abcam) at 4°C overnight, washed three times with PBS, and then incubated with Alexa Fluor 594 goat anti–rabbit secondary antibody (1:600 dilution, A11037; Invitrogen). The slides were then incubated with the blocking solution at room temperature
for 30 minutes and then incubated at 4°C overnight with primary antibody against phospho-serine (1:100, sc-81514; Santa Cruz Biotechnology). The slides were then washed three times with PBS for 3 minutes and incubated at room temperature for 1 hour with Alexa Fluor 488 goat antimouse secondary antibody (1:600 dilutions, A11029; Invitrogen). All slides were rinsed, incubated with Hoechst 33342 (H3570; Invitrogen) to visualize nuclei, and then mounted with a glycerol/PBS solution containing 0.1 M propyl gallate to minimize fluorescent bleaching. All images were captured with an Olympus microscope (BX-51) with an attached DP71 digital camera and processed with DP Controller and DP Manager software (Olympus).

**Quantification of Proliferating Cells and Apoptotic Cells**

To quantify proliferating cells and apoptotic cells, BrdU-positive cells or TUNEL-positive cells in 10 random 0.159-mm² fields of the tumors were counted at 100x magnification as described previously [8].

**Radioligand Binding Assay**

Radioligand binding assays on cell lines were performed by diluting cells in Dulbecco modified Eagle medium, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin (Sigma). Cells were then incubated with 125I-labeled ET-1 in the presence or absence of competing ligands on ice for 4 hours in a total volume of 250 μl in 96-well microwells. BQ-123 (10 μM final concentration) (Sigma) and Sarafotoxin 6c (1 μM final concentration; Sigma) were used as competitive ligands for ETAR- and ETBr-specific binding, respectively. The bound ligand was separated from free ligand by filtration through GF/C filters pre-soaked with binding buffer by cell harvester (Filtermate; Packard Bioscience, Meriden, CT). After the addition of ReadySafe (Beckman, Fullerton, CA) scintillation fluid, bound radioactivity was quantitated using the TopCountNXT (Packard).

Radioligand binding assays were performed on tumor sections by equilibrating slides to room temperature before blocking twice for 10 minutes each in assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 5% skimmed milk). Slides were then briefly dipped in distilled water and dried under a stream of cold air for 2 minutes. Sections were incubated for 4 hours at room temperature with 100 pM 125I-ET-1 in assay in the presence or absence of competitive ligands. BQ-123 (50 μM final; Sigma) and Sarafotoxin 6c (10 μM final concentration; Sigma) were used as competitive ligands for ETAR- and ETBr-specific binding, respectively. After incubation, sections were washed four times for 2 minutes in ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂), briefly dipped in distilled water at 4°C, and dried under a stream of cold air. Sections were exposed to BAS2500 imaging plate (Fuji Photo Film, Kanazawa, Japan) overnight and then Kodak Biomax MS film (Rochester, NY) for 2 days.

**ET Secretion**

ET levels were quantified using the QuantiGlo ET-1 Immunoassay (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. For quantification of ET produced by cell lines, 50,000 cells in Dulbecco modified Eagle medium, 25 mM HEPES, pH 7.4, and endothelial cells stained positive for ETAR. In contrast, expression of ETAR was weak and heterogeneous in tumor cells and more pronounced in tumor-associated endothelial cells and possibly in infiltrating leukocytes [65]. ETAR and ETBr were also found to be expressed in tumors derived from IGROV1 cells growing in the peritoneal cavity of nude mice as determined by immunofluorescence (data not shown). Collectively, these results indicate that the expression of ETARs was stronger in ovarian cancer cells growing in vivo than in vitro.

We next determined the level of ET secretion by the cell lines growing in vitro. As shown in Figure 1A, SKOV3ip1 and IGROV1 cells expressed levels of ET (35 and 25 pg/ml, respectively) that were similar to those measured in immortal mouse lung endothelial cells (26 pg/ml). The level of ET secretion in SKOV3ip1 tumors was determined by measuring the level of ETs in ascitic fluids (Figure 1D). ETs could be detected in ascites of the three mice tested with levels ranging from 6.9 to 16 pg/ml.

**Therapy for SKOV3ip1 and IGROV1 with Macitentan and Paclitaxel**

In the first set of therapy experiments, treatment of SKOV3ip1 with paclitaxel significantly decreased tumor weight as compared with the control group (Table 1A; median [range]: 0.4 g [0.1-0.5 g] vs 1.1 g [0-1.8 g], P < .05). Tumor weight in mice treated with only macitentan was not reduced. The combination of paclitaxel and macitentan further decreased tumor weight (0.1 g [0-0.3 g], P < .01). Treatment with a combination of paclitaxel and macitentan also significantly decreased tumor incidence as compared with the control group (5/9 vs 8/10, P < .05). The incidence and volume of ascites were significantly decreased by the treatment with paclitaxel (4/9 vs 8/10, P < .05; and median [range] of 0.4 ml [0-0.9 ml] vs 0.1 ml [0-0.2 ml], P < .01, respectively) and the combination therapy completely inhibited development of ascites (0/9 vs 8/10, P < .01). The combination of macitentan with paclitaxel significantly enhanced the therapeutic efficacy of paclitaxel as measured by tumor weight (0.4 g [0.1-0.5 g] vs 0.1 g [0-0.3 g], P < .01), tumor incidence (9/9 vs 5/9, P < .05), and incidence of ascites (4/9 vs 0.9, P < .05).

**Results**

**ETR and ET Expression in Ovarian Cancer Cell Lines**

We first assessed the expression level of ETAR and ETBr and the level of secreted ETs in two ovarian cancer cell lines, SKOV3ip1 and IGROV1. In both an ETAR- and an ETBr-specific radioligand assay, ETAR was not expressed at the cell surface of either cell line (Figure 1A). In contrast, ETBr was weakly expressed in SKOV3ip1 cells (ratio of specific to nonspecific binding, 1.8) but absent in IGROV1. CHO cells overexpressing ETAR or ETBr were used as a positive control (ratio of specific binding to nonspecific binding, 17 to 22), and CHO mock-transfected cells were used as a negative control. Next, we determined the level of ETR expression in SKOV3ip1 tumors growing in the peritoneal cavity of nude mice using ETAR- and ETBr-specific radioligand assays (Figure 1B). ETAR was highly expressed in the three tumors (ratio of specific to nonspecific binding, 5.9 to 15.2). ETBr was also expressed in tumors although at a lower level (ratio of specific to nonspecific binding, 2 to 2.8). The expression of the ETRs in SKOV3ip1 tumors was also investigated using immunohistochemistry (Figure 1C). Both receptors were detected in the cytoplasm and/or plasma membranes of tumor cells but not in the nucleus. Consistent with the radioligand assay, both tumor cells and endothelial cells stained positive for ETAR. In contrast, expression of ETBr was weak and heterogeneous in tumor cells and more pronounced in tumor-associated endothelial cells and possibly in infiltrating leukocytes [65]. ETAR and ETBr were also found to be expressed in tumors derived from IGROV1 cells growing in the peritoneal cavity of nude mice as determined by immunofluorescence (data not shown). Collectively, these results indicate that the expression of ETRs was stronger in ovarian cancer cells growing in vivo than in vitro.
In a repeated therapy experiment using 50 mg/kg macitentan, similar therapeutic effects were found in mice treated with the combination of paclitaxel and macitentan (data not shown).

In mice bearing IGROV1 tumors in the peritoneal cavity, treatment with paclitaxel significantly decreased tumor weight as compared with control mice (0.5 g [0.0-0.9 g] vs 1.1 g [0.4-2.1 g], \( P < .05 \); Table 1B). Treatment with macitentan alone did not produce a significant therapeutic effect. The combination of macitentan (50 mg/kg) and paclitaxel (5 mg/kg) further decreased the weight of the tumors (0.3 g [0.0-0.6 g] vs 1.1 g [0.4-2.1 g], \( P < .01 \); Table 1B). Macitentan combined with paclitaxel significantly enhanced the therapeutic efficacy of paclitaxel as measured by tumor weight (0.5 g [0.0-0.9 g] vs 0.3 g [0.0-0.6 g], \( P < .05 \)). Mice treated with macitentan at 100 mg/kg exhibited poor skin turgor, dehydration, diarrhea, and sluggishness. For this reason, in the next set of experiments, we determined the biologically optimal dose of macitentan. Treatment with paclitaxel (5 mg/kg) and 3 or 10 mg/kg macitentan did not produce enhanced therapeutic effects (data not shown). In contrast, treatment with 5 mg/kg paclitaxel combined with 30 mg of macitentan significantly reduced tumor weight but not tumor incidence (data not shown). For these reasons, all future experiments used a macitentan dose of 50 mg/kg administered daily to treat SKOV3ip1 and IGROV.

**Table 1.** Treatment of Human Ovarian Cancers SKOV3ip1 (A) and IGROV1 (B) Growing in the Peritoneal Cavity of Female Nude Mice with Macitentan and Paclitaxel.

<table>
<thead>
<tr>
<th>A</th>
<th>Cell Lines</th>
<th>ET secretion (B/NSB)</th>
<th>ET(_A)R expression (B/NSB)</th>
<th>ET(_B)R expression (B/NSB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV3ip1</td>
<td>35 pg/ml</td>
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<tr>
<td>IGROV1</td>
<td>25 pg/ml</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>ND</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>CHO-EDNRA</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>CHO-EDNRB</td>
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<td>17</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>26 pg/ml</td>
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<table>
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<tr>
<th>B</th>
<th>Tumors</th>
<th>ET(_A)R expression (B/NSB)</th>
<th>ET(_B)R expression (B/NSB)</th>
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<td>SKOV3 T1</td>
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<tr>
<td>SKOV3 T3</td>
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**Figure 1.** Expression level of ET\(_A\)R, ET\(_B\)R, and secreted ETs by SKOV3ip1 and IGROV1 cells growing in vitro and in vivo. (A) ET secretion was determined by ELISA in culture supernatant of SKOV3ip1, IGROV1, and mouse lung endothelial cells in vitro 48 hours after seeding. ET\(_A\)R and ET\(_B\)R expression was determined using a radioligand binding method. Nonspecific binding (NSB) was determined by incubating cells with BQ-123 or Sarafotoxin 6c. The ratio binding-B/NSB indicates the specific binding. ET\(_A\)R and ET\(_B\)R expression was measured by radioligand binding (B) and immunohistochemistry (C) in SKOV3ip1 tumors growing in the peritoneal cavity of nude mice (arrows indicating endothelial cells). (D) ET secretion was measured by ELISA in ascites from three control mice and three mice injected intraperitoneally with SKOV3ip1 cells and treated with macitentan (50 mg/kg).

In mice bearing IGROV1 tumors in the peritoneal cavity, treatment with paclitaxel significantly decreased tumor weight as compared with control mice (0.5 g [0.0-0.9 g] vs 1.1 g [0.4-2.1 g], \( P < .05 \); Table 1B). Treatment with macitentan alone did not produce a significant therapeutic effect. The combination of macitentan (50 mg/kg) and paclitaxel (5 mg/kg) further decreased the weight of the tumors (0.3 g [0.0-0.6 g] vs 1.1 g [0.4-2.1 g], \( P < .01 \); Table 1B). Macitentan combined with paclitaxel significantly enhanced the therapeutic efficacy of paclitaxel as measured by tumor weight (0.5 g [0.0-0.9 g] vs 0.3 g [0.0-0.6 g], \( P < .05 \)). Mice treated with macitentan at 100 mg/kg exhibited poor skin turgor, dehydration, diarrhea, and sluggishness. For this reason, in the next set of experiments, we determined the biologically optimal dose of macitentan. Treatment with paclitaxel (5 mg/kg) and 3 or 10 mg/kg macitentan did not produce enhanced therapeutic effects (data not shown). In contrast, treatment with 5 mg/kg paclitaxel combined with 30 mg of macitentan significantly reduced tumor weight but not tumor incidence (data not shown). For these reasons, all future experiments used a macitentan dose of 50 mg/kg administered daily to treat SKOV3ip1 and IGROV.

**Table 1.** Treatment of Human Ovarian Cancers SKOV3ip1 (A) and IGROV1 (B) Growing in the Peritoneal Cavity of Female Nude Mice with Macitentan and Paclitaxel.

(A) Treatment Group | Tumor Incidence | Tumor Weight, Median (Range), g | Ascite Incidence | Ascites, Median (Range), ml
---|---|---|---|---|
Control | 8/10 | 1.1 (0-1.8) | 8/10 | 0.4 (0-0.9) |
Paclitaxel, 5 mg/kg | 9/9 | 0.4 (0-0.5) | 4/9 | 0.1 (0-0.2) |
Macitentan, 100 mg/kg | 7/10 | 2.3 (0-4.6) | 7/10 | 0.4 (0-4.7) |
Paclitaxel + macitentan | 5/9+2 | 0.1 (0-0.3) | 0/9+2 | 0+2 |

(B) Treatment Group | Tumor Incidence | Tumor Weight, Median (Range), g
---|---|---|
Control | 10/10 | 1.1 (0-4.2) |
Paclitaxel, 5 mg/kg | 9/10 | 0.5 (0-0.9) |
Macitentan, 50 mg/kg | 10/10 | 0.7 (0-4.3) |
Paclitaxel + macitentan | 9/10 | 0.3 (0-0.6) |

Mice were injected intraperitoneally with 1 × 10⁶ SKOV3ip1 or IGROV1 cells. Ten days later, treatment began with vehicle, paclitaxel, macitentan, or a combination of macitentan and paclitaxel. Treatment continued for 4 weeks when mice were examined by necropsy. Tumor incidence, weight, ascites incidence, and volume were recorded.

*Statistically significant compared with the control group, \( P < .05 \).
†Statistically significant compared with the control group, \( P < .01 \).
‡Statistically significant compared with the paclitaxel group, \( P < .05 \).
Table 2. Treatment of Human Ovarian Cancer, SKOV3ip1, with Macitentan and Paclitaxel Growing in the Peritoneal Cavity of Female Nude Mice.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Tumor Incidence</th>
<th>Tumor Weight, Median (Range), g</th>
<th>Ascites Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10/10</td>
<td>1.9 (0.5-4.8)</td>
<td>10/10</td>
</tr>
<tr>
<td>Paclitaxel, 2 mg/kg</td>
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<td>2.5 (1.2-3.3)</td>
<td>10/10</td>
</tr>
<tr>
<td>Paclitaxel, 5 mg/kg</td>
<td>10/10</td>
<td>0.4 (0.2-1.3)*</td>
<td>5/10*</td>
</tr>
<tr>
<td>Macitentan, 50 mg/kg</td>
<td>9/10</td>
<td>0.6 (0.1-1.3)*</td>
<td>7/10</td>
</tr>
<tr>
<td>Paclitaxel, 2 mg/kg + macitentan</td>
<td>5/10(^{1,3})</td>
<td>0.3 (0.0-0.7)*(^{1,3})</td>
<td>4/10(^{1,3})</td>
</tr>
<tr>
<td>Paclitaxel, 5 mg/kg + macitentan</td>
<td>5/10(^{1,3})</td>
<td>0.05 (0.0-0.5)(^{1,3})</td>
<td>0/10(^{1,3})</td>
</tr>
</tbody>
</table>

Mice were injected intraperitoneally with 1 × 10⁶ SKOV3ip1 cells. Ten days later, treatment with vehicle, paclitaxel, macitentan, or macitentan and paclitaxel began and continued for 4 weeks. At the end of the experiments, the mice were examined by necropsy. Tumor incidence, weight, and incidence and volume of ascites were recorded.

*Statistically significant compared with the control group, \(P < .01\).
†Statistically significant compared with the control group, \(P < .05\).
‡Statistically significant compared with the 2-mg/kg paclitaxel-treated group and 5-mg/kg paclitaxel-treated group, respectively, \(P < .05\).
§Statistically significant compared with the control group, \(P < .01\).

Sensitization or Ovarian Cancer Cells to Paclitaxel with Macitentan

Mice were implanted with 1 × 10⁶ SKOV3ip1 cells, and 10 days later when tumors were established, treatment began either with non-therapeutic (2 mg/kg) or therapeutic (5 mg/kg) doses of paclitaxel (Table 2). Nontherapeutic doses of paclitaxel (2 mg/kg) did not decrease the weight of the tumors (2.5 g [1.2-3.3 g]) compared with the control group (1.9 g [0.5-4.8 g], \(P > .05\)). However, the combination of macitentan (50 mg/kg) with a nontherapeutic dose of paclitaxel (2 mg/kg) significantly decreased the tumor weight (0.3 g [0.0-0.7 g], \(P < .001\)) compared with control mice or mice treated with only a nontherapeutic dose of paclitaxel (2.5 g [1.2-3.3 g], \(P < .001\); Table 2). Tumor incidence (5/10 vs 10/10) and incidence of ascites (4/10 vs 10/10) were also significantly decreased \((P < .05)\) implying that administration of macitentan at 50 mg/kg increases the sensitivity of tumor cells to paclitaxel. Interestingly, whereas treatment with 100 mg/kg of macitentan as a single agent did not show therapeutic benefits (Table 1), mice treated with a nontoxic dose of 50 mg/kg macitentan as a single agent had a decrease in tumor weight (0.6 g [0-1.1 g] vs 1.9 g [0.5-4.8 g], \(P < .05\)). The full therapeutic efficacy in this study, however, was produced by combining 5 mg/kg paclitaxel with 50 mg/kg macitentan (0.1 g [0-0.5 g], \(P < .001\)).

From these three sets of experiments, we conclude that 50 mg/kg macitentan administered once a day is the biologic optimal dose, and less than 10 mg/kg is an ineffective dose in the SKOV3ip1 tumor. The 50-mg/kg dose of macitentan did not produce toxicity.

Immunohistochemical Analyses of the ET Axis and Signaling Molecules in SKOV3ip1 and IGROV1

Tumor cells and tumor associated endothelial cells of SKOV3ip1 (Figure 2) and IGROV1 (not shown) peritoneal tumors were analyzed for expression of ET-1, ET-2, ETₐR, and ET₉R. Treatment with paclitaxel (5 mg/kg), macitentan (100 mg/kg), or a combination of paclitaxel and macitentan did not alter the expression of the ligands or the receptors in both tumor cells and tumor-associated endothelial cells (Figure 2, A and B). ET-1, ET-2, ETₐR, and ET₉R produced green fluorescence, and endothelial cells (CD31) produced red fluorescence. Thus, colocalization of ET-1, ET-2, ETₐR, or ET₉R with CD31 produced a yellow stain (Figure 2, A and B).

Figure 2. Immunohistochemical analyses of human ovarian carcinoma SKOV3ip1 growing in the peritoneal cavity of nude mice treated with macitentan and paclitaxel. SKOV3ip1 tumor tissues were harvested and processed for the frozen sections. Tissues were stained with anti-CD31 antibody (red) and anti–ET-1, ET-2, ETₐR, or ET₉R antibody (green). Expression of ET-1, ET-2, ETₐR, or ET₉R on tumor-associated endothelial cells yielded yellow signal by colocalization of red and green. Experimental conditions; see Table 14. (A) ET-1 and ET-2 were expressed on tumor cells (green) as well as tumor-associated endothelial cells (yellow) and treatment with ETR antagonist, macitentan, with or without paclitaxel, did not affect the expression of ET-1 or ET-2. (B) ETₐR and ET₉R were expressed on tumor cells (green) as well as tumor-associated endothelial cells (yellow), and treatment with ETR antagonist, macitentan, with or without paclitaxel, did not affect the expression of ETₐR or ET₉R.
The ET receptor antagonist, macitentan, did not affect the expression of ET-1, ET-2, ET₄R, or ET₆R on the tumor cells or tumor-associated endothelial cells.

Treatment with macitentan alone (50 mg/kg) or treatment with a combination of macitentan and paclitaxel (5 mg/kg) significantly decreased the levels of pVEGFR2, pAkt, and pMAPK in tumor cells and in tumor-associated ET cells (note decreased yellow signal as compared with the control group) (IGROV1; Figure 3).

When pVEGFR and pAkt were analyzed in SKOV3ip1 tumors from mice treated with different doses of macitentan, the 10 mg/kg administered daily did not inhibit Akt and VEGFR phosphorylation, whereas the 30 mg/kg administered daily strongly inhibited Akt phosphorylation and, to a lesser extent, VEGFR phosphorylation. When macitentan was administered at 100 mg/kg, both pAkt and pVEGFR were completely inhibited, and this correlated with the stronger efficacy in terms of tumor incidence (data not shown).

Because β-arrestin is recruited to the ETRs [48] via binding to serine/threonine phosphorylated sites at the cytoplasmic C-terminus of the receptor proteins, we applied double immunofluorescence staining for phosphorylated serine and ETRs to tumor tissues to...
Table 3A and Figure 5). Treatment with macitentan (50 or 100 mg/kg) alone also increased the number of apoptotic tumor cells and endothelial cells but did not decrease the number of proliferating tumor cells (Figure 5). Combination therapy with macitentan and paclitaxel significantly increased the number of apoptotic tumor cells compared with paclitaxel or macitentan alone. Furthermore, the combination therapy also increased apoptosis in tumor-associated endothelial cells (Figure 5). Proliferating and apoptotic cells were quantified by counting BrdU-positive cells and TUNEL-positive cells, respectively, in 10 random 0.159-mm² fields at 100× magnification (Table 3). In SKOV3ip1 tumors, paclitaxel (5 mg/kg) significantly increased the number of TUNEL-positive cells compared with control treatment. Treatment with macitentan (50 mg/kg) also significantly increased the number of apoptotic cells. The combination of paclitaxel and macitentan yielded the highest level of apoptosis (with statistical significance compared with the paclitaxel group [195.3 ± 42.6 vs 150.0 ± 38.3], P < .01). The number of proliferating cells was significantly decreased by treatment with paclitaxel; however, treatment with macitentan alone did not significantly decrease the number of proliferating cells. The combination of paclitaxel and macitentan further decreased the number of proliferating cells, but it was not statistically significant compared with tumors treated with only paclitaxel (18.5 ± 11.1 vs 30.8 ± 14.9, P < .05; Table 3A).

In IGROV1 tumors (Table 3B), treatment with paclitaxel (5 mg/kg) significantly increased the number of apoptotic cells, and treatment with macitentan (50 mg/kg) alone also significantly increased the apoptotic cells (69 ± 13.1 vs 5.7 ± 3.3, P < .05) compared with the control group. The combination of paclitaxel and macitentan yielded the best outcome compared with paclitaxel-treated mice (181.4 ± 32.9 vs 139.0 ± 29.7, P < .05). The number of proliferating cells was significantly decreased by the treatment with paclitaxel compared with the control group. Treatment with macitentan alone did not induce a significant decrease in the number of proliferating cells. Treatment with both paclitaxel and macitentan further decreased cell proliferation as compared with the paclitaxel group, but the decrease was not statistically significant (19.3 ± 8.8 vs 28.2 ± 10.0, P = .057; Table 3B).

In the third set of experiments, treatment with the nontherapeutic dose (2 mg/kg) of paclitaxel did not induce significant apoptosis of tumor cells (green) or tumor-associated endothelial cells (yellow), but the combination of macitentan (50 mg/kg) with 2 mg/kg paclitaxel induced significant apoptosis in both the tumor cells and tumor-associated endothelial cells (Figure 6). The number of apoptotic cells

**Proliferation and Apoptosis of Cells in Tumors**

Treatment of SKOV3 tumors with paclitaxel alone at 5 mg/kg significantly decreased the number of BrdU-positive proliferating cells (green tumor cells and yellow endothelial cells) and increased the number of apoptotic tumor cells (TUNEL-positive, green) and TUNEL-positive endothelial cells (TUNEL-positive, yellow) (SKOV3ip1;
induced by the therapeutic dose of paclitaxel (5 mg/kg) was increased by combining the paclitaxel with macitentan (Figure 6).

**Discussion**

The purpose of the present study was to determine whether blockade of ETRs by macitentan, coupled with administration of paclitaxel, decreases the progressive growth of human ovarian cancer cells implanted into the peritoneal cavity of nude mice and to identify potential pharmacodynamic markers, which could be useful in dose finding and therapy. Multiple studies indicate that ET and ETRs contribute to the progressive growth of ovarian cancer [25,39–48] and expression of the ETAR has been shown to correlate with advanced stages of ovarian cancer in patients [39,66,67]. Gene expression profiles of advanced ovarian cancer further indicate that ETAR expression is associated with cell migration and invasion [66,67]. ET-1 has also been shown to promote survival of cells through Bcl-2-dependent mechanisms and inhibit paclitaxel-mediated apoptosis [39]. In the current study, we demonstrated, using two ovarian cancer cell lines grown in vitro and in vivo, that ETRs were preferentially expressed when tumors were grown in vivo. Both tumors produced ET in vitro and in vivo in ascites (SKOV3ip1). Immunohistochemistry confirmed the expression of ETRs on tumor cells (ETAR) and on tumor-associated vascular endothelial cells (ETBR). As demonstrated in therapy experiments, inhibition of ET signaling sensitized not only tumor cells but also tumor-associated endothelial cells to killing by paclitaxel.

Results obtained in preclinical cancer models [49,68] and in analyzing clinical tumor specimens led to clinical studies of ETR antagonists, mainly using ETAR-selective antagonists in hormone-refractory and metastatic prostate cancer [49,69,70]. Clinical data obtained with ETαR selective antagonists in prostate cancer patients, in particular with zibotentan, justify additional studies of these antagonists, both in prostate cancer and in other tumors [70,71]. The dual receptor antagonist, bosentan, has been studied in melanoma patients in a phase 2 trial [72] based on data that indicate a fundamental role of the ETβR in melanocyte physiology [73] and a correlation of ETβR overexpression in melanoma with tumor progression [74]. The single agent phase 2 study suggested that additional studies of the agent in combination with other anticancer drugs were warranted.

A double-blind, placebo-controlled study of high-dose bosentan, in combination with dacarbazine, was performed in patients with stage IV metastatic melanoma. In this study, the addition of bosentan had no effect on time to tumor progression or other measures of efficacy [75]. No pharmacodynamic markers were applied to determine the drug action in tumors. The inability of this drug to affect response to dacarbazine may be due to poor tissue penetration by bosentan because ET and ETRs are expressed in cancer cells and tumor stroma. Macitentan was selected for our study because of its high tissue targeting properties and for targeting both the ETαRs and ETβRs [61].

In pathologic situations, both ETA and ETB receptors have been shown to mediate vasoconstriction [76], smooth muscle proliferation [77], and inflammation [78]. These findings, together with those we describe regarding the highly expressed ET axis in tumors, make further evaluation of dual ET receptor antagonists in oncology warranted.

The efficacy of ETαR-selective inhibitors have been tested in ovarian cancer xenografts. ABT-627 (atrasentan) has shown antitumor efficacy in subcutaneously transplanted HEY ovarian tumors alone...
and in combination with Taxol. The antitumor effect was accompanied with reduced production of VEGF, reduced expression of matrix metalloproteinase 2, and an increase in the number of apoptotic tumor cells [79]. In other studies using the HEY tumor model, ABT-627 efficacy was confirmed, and in one case, the antitumor effect was associated with the reversal of markers typical for the epithelial-to-mesenchymal transition [80] and in another case with the reduction of integrin-linked kinase expression and reduced AKT and GSK-3β phosphorylation [81]. The ETAR selective antagonist ZD4054 (zibotentan) showed antitumor activity in subcutaneous HEY ovarian tumor xenografts alone and in combination with paclitaxel [65] and the epidermal growth factor receptor kinase inhibitor gefitinib [40]. Administration of ZD4054 reduced VEGF expression and phosphorylation of p42/44 MAP kinase and inhibited growth of metastatic nodules after intraperitoneal transplantation of HEY ovarian cancer cells [48]. In this case, the metastatic phenotype was linked to ETAR-mediated β-arrestin signaling and activation of the wnt pathway.

In the present study, we show that blockade of the ET axis by the dual ETR antagonist macitentan inhibits the growth of human ovarian cancer cells, SKOV3ip1 and IGROV1, in the peritoneal cavity of nude mice (orthotopic site). SKOV3ip1 and IGROV1 cells were both sensitive to paclitaxel and highly express ETRs in vivo. The SKOV3ip1 tumor also produces ET in ascites fluid. Treatment with macitentan did not change the expression levels of ET-1, ET-2, ETAR, and ETβR in tumor cells, it significantly decreased the phosphorylation of ETRs and such cell survival pathway markers as pVEGFR2, pAkt, and pMAPK
These data extend those in earlier reports [39,40,79,81]. Treatment with macitentan alone increased the number of apoptotic cells, and its combination with paclitaxel significantly enhanced apoptosis in tumor cells and the tumor-associated endothelial cells compared to each agent alone, indicating loss of blood vessels. Similarly, paclitaxel and the drug combination significantly decreased the number of proliferating cells, whereas macitentan alone did not. Collectively, these results suggest that blockade of the ET axis may suppress antiapoptotic mechanisms in tumor cells and associated endothelial cells by affecting survival pathways through the inhibition of phosphorylation of pAkt, pMAPK, and pVEGFR2. Thus, treatment with macitentan rendered tumor cells and tumor-associated endothelial cells more sensitive to paclitaxel, even when paclitaxel was administered at the dose of 2 mg/kg, which is less than half of its therapeutic dose (5 mg/kg). This suggests that the combination of the ET antagonist macitentan with a suboptimal dose of the chemotherapeutic agent may be just as effective as paclitaxel alone at the higher therapeutic dose.

The efficacy of macitentan was correlated with inhibition of protein phosphorylation in a dose–response study. Doses of macitentan that did not increase the efficacy of paclitaxel did not downregulate expression of pAkt, pMAPK, or pVEGFR. These markers can therefore be used as pharmacodynamic biomarkers to determine the minimal effective dose in other animal models or patients. Importantly, a direct measurement of the activation state of the ETRs would be a most desirable biomarker. It has recently been shown that ETRs also signal through the β-arrestin pathway [48]. β-Arrestin binding to an activated G protein–coupled receptor requires phosphorylation of the C-terminal intracellular part of the receptor at the amino acids serine or threonine by a G protein–coupled receptor kinases [84]. Because antibodies that are able to detect phospho-serine or phospho-threonine in an ETR–specific peptide sequence are not available, we determined the phosphorylation of ETRs by colocalization of ETRs and phosphorylated serine using anti-ETR and nonspecific anti-phospho-serine antibodies. Colocalized signals of ETRs and phosphorylated serine were decreased in a dose-dependent manner in tumors of animals treated with efficacious doses of macitentan, indicating blockage of ETR activation by the receptor antagonist. This colocalization assay is highly dependent on the specificity of the antibodies. To increase the link of this assay with the activation status of ETR, in vitro cell culture experiments with SKOV3ip1 cells were performed. In these experiments, concentrations as low as 100 nM macitentan, which has been shown to functionally inhibit ETR signaling in cellular assays [61], were able to block ET-1–induced colocalization of the antibodies (data not shown). Preliminary determination of macitentan level in the plasma correlated with the localization of ETR-pSer in the tumors. These measurements may therefore serve as biomarkers most closely linked to ETR activation.

Our present study clearly demonstrates that treatment of mice with macitentan and paclitaxel inhibited phosphorylation of ETRs in tumor cells and tumor-associated vascular endothelial cells and produced apoptosis in both cell types. Previous data from our laboratory demonstrated that therapy for ovarian cancer [62], colon cancer [85], and prostate cancer [86,87] can be mediated by targeting the tumor vasculature.

In summary, this study confirms the efficacy of inhibiting cell signaling pathways and tumor growth by ETR antagonists in combination with paclitaxel in orthotopic models of ovarian cancer. Of importance, macitentan decreased the effective dose of paclitaxel. Efficacy is associated not only with tumor cell killing but also with destruction of tumor-associated vascular endothelial cells. The contribution to paclitaxel efficacy of the tissue-targeting dual ETR antagonist macitentan was correlated with inhibition of phosphorylation of signaling and survival proteins. Therefore, the degree of protein phosphorylation might be used as a pharmacodynamic biomarker in future studies and supports the finding of the biologically active therapeutic dose.

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References
tions 102, 1434–1440.


Figure W1. SKOV3ip1 growing in the peritoneal cavity of nude mice. Ten days after the intraperitoneal injection of $1 \times 10^6$ SKOV3ip1 cells, three mice were randomly selected and necropsied. Tumor burden was documented before the start of treatment.

Figure W2. Necropsy procedure. At the end of the experiment, mice were necropsied. Peritoneal tumors were collected and weighed. (A) Control group of SKOV3ip1. (B) Control group of IGROV1. Range of the tumor size was within the normal distribution curve. *One mouse in the IGROV1 control group became moribund and was killed 1 day before the completion of the experiment.
Figure W3. Colocalization of pSer with ET₄R (A) and ET₉R (B). Immunofluorescence analyses were performed in SKOV3ip1 tumors growing in the peritoneal cavity of nude mice. Phosphorylated serine signal was coded green, and receptors were coded red. Colocalizations yield yellow signals. Phosphorylation of endothelin receptors was inhibited by ACT-064992 in a dose-dependent manner. Endothelin receptors A and B were phosphorylated in tumors treated with vehicle, paclitaxel, and paclitaxel combined with 3 or 10 mg/kg ACT-064992. In contrast, phosphorylation of endothelin receptors A and B was significantly inhibited in tumors treated with paclitaxel combined with 30 or 50 mg/kg ACT-064992.