Antivascular Therapy for Multidrug-Resistant Ovarian Tumors by Macitentan, a Dual Endothelin Receptor Antagonist[1]

Sun-Jin Kim[*,2], Jang Seong Kim[*,2], Seung Wook Kim[*,2], Seok Joong Yun[*,2], Junqin He[*], Emily Brantley[*], Dominic Fan[*], Panja Strickner[†], François Lehembre[†], Urs Regenass[†] and Isaiah J. Fidler[*]

*Department of Cancer Biology, Metastasis Research Laboratory, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; †Actelion Pharmaceuticals, Ltd, Allschwil, Switzerland

Abstract

Endothelin receptors (ETRs) are often overexpressed in ovarian tumors, which can be resistant to conventional therapies. Thus, we investigated whether blockage of the ETR pathways using the dual ETR antagonist macitentan combined with taxol or cisplatinum can produce therapy for orthotopically growing multidrug-resistant (MDR) human ovarian carcinoma. In several studies, nude mice were injected in the peritoneal cavity with HeyA8-MDR human ovarian cancer cells. Ten days later, mice were randomized to receive vehicle (saline), macitentan (oral, daily), taxol (intraperitoneal, weekly), cisplatinum (intraperitoneal, weekly), macitentan plus taxol, or macitentan plus cisplatinum. Moribund mice were killed, and tumors were collected, weighed, and prepared for immunohistochemical analysis. The HeyA8-MDR tumors did not respond to taxol, cisplatinum, or macitentan administered as single agents. In contrast, combination therapy with macitentan and taxol or macitentan and cisplatinum significantly decreased the tumor incidence and weight and significantly increased the survival of mice and their general condition. Multiple immunohistochemical analyses revealed that treatment with macitentan and macitentan plus taxol or cisplatinum inhibited the phosphorylation of ETRs, decreased the levels of pVEGFR2, pAkt, and pMAPK in tumor cells after 2 weeks of treatment and induced a first wave of apoptosis in tumor-associated endothelial cells followed by apoptosis in surrounding tumor cells. Our study shows that ovarian cancer cells, which express the endothelin axis and are multidrug resistant, are exquisitely sensitive to treatment with a dual ET antagonist and can be resensitized to both taxol and cisplatinum. This combined therapy led to a significant reduction in tumor weight.

Translational Oncology (2012) 5, 39–47

Introduction

Ovarian cancer is the leading cause of mortality among women with gynecologic cancers [1]. Whereas taxane- and cisplatinum-based chemotherapies induce an initial favorable response in patients with advanced ovarian cancer [2], the prognosis is grave owing to disease recurrence and the emergence of drug-resistant disease [3]. Tumor cells are genetically unstable, leading to biologic heterogeneity [4], which is largely responsible for the emergence of multidrug-resistant (MDR) cells that often express the *MDR1* gene and its product, P-glycoprotein (P-gp) [5]. The growth and spread of tumor cells in the body are dependent on the continuous interaction of tumor cells with the organ

microenvironment, which the tumor cells can exploit [4]. Targeting the organ microenvironment in general and the tumor-associated vascular system, in particular, is one approach to treating tumor cells, regardless of their sensitivity or resistance to chemotherapeutic drugs [6,7].

An exciting approach for targeting tumor cells and the tumor vasculature is through the endothelin (ET) pathway [7]. ETs are a family of small peptides consisting (ET-1, -2, and -3) [8,9], which share structural homology and initiate signaling by binding to the G protein–coupled receptors ET_AR and ET_BR [10]. ETs were classically defined as potent vasoconstrictors, a role that led to considerable efforts to develop nonpeptide ET receptor (ETR) antagonists to treat cardiovascular diseases [11,12]. Further research, however, revealed that the ETs regulate diverse biologic processes such as tissue remodeling, tissue repair, cellular differentiation [13], smooth muscle cell proliferation [14], and inflammation [15]. Many tumors have been found to express ETs and ETRs, and ET pathways have been shown to play important roles in tumor cell proliferation, migration, and invasion and vascular differentiation [16–19]. Activation of ETRs has been shown to play a role in the inhibition of apoptosis, matrix remodeling, and bone deposition in prostate cancer [20]. The ET axis has also been reported to be of relevance in lung cancer [21], colon cancer [22], renal cancer [23], cervical cancer [24], brain tumors [25], and ovarian cancer [26–28].

The role of the ETs and ETRs in the biology and therapy for ovarian carcinoma has been described [29], and increased expression of ET-1 and ET_AR on ovarian carcinoma cells and ET_BR on intratumoral vessels has been reported, as well as a relationship between the expression of ET-1 and vascular endothelial growth factor (VEGF) in the development of ascites and resistance of cells to therapy [29].

We recently reported that the tissue-targeting ETR antagonist macitentan (ACT-064992) [30] enhances the therapeutic efficacy of paclitaxel against orthotopically implanted human ovarian carcinoma by modulating the survival pathways of the tumor cells [7]. Because the major cause of death from ovarian cancer is due to drug-resistant tumor cells, we wished to determine whether macitentan is also effective in treating MDR ovarian cancer cells. Because all cells in the body depend on an adequate supply of nutrients and oxygen for their survival, therapeutic regimens that target tumor-associated endothelial cells can kill tumor cells regardless of their response to chemotherapeutic drugs [31], and the induction of apoptosis in tumor-associated endothelial cells has been shown to induce apoptosis in MDR prostate cancer cells growing in the prostate of nude mice [6]. In the present study, we determined whether the daily oral administration of the dual ETR antagonist macitentan combined with once-weekly intraperitoneal injection of taxol or cisplatinum can produce therapy for MDR human ovarian tumors growing in the peritoneal cavity of nude mice.

Materials and Methods

Human Ovarian Cancer Cell Lines
The human ovarian cancer cell line HeyA8 and the MDR variant HeyA8-MDR cell line were maintained as a monolayer culture in Eagle minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc, Grand Island, NY) as described previously [7,32].

Reagents
Macitentan, also called ACT-064992 or N-(5-(4-bromophenyl)-6-{2-(5-bromopyrimidin-2-yloxy)ethoxy}pyrimidin-4-yl)-N'-propylsulfamide, was provided by Actelion Pharmaceuticals, Ltd (Allschwill, Switzerland) as powder. For oral administration, macitentan was reconstituted in a 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. Taxol (taxol-injectable; Bristol-Myers Squibb, Seattle, WA) and cisplatinum (APP Pharmaceuticals, LLC, Schaumburg, IL) were diluted in distilled water for intraperitoneal injections.

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. 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. Mice were used in these experiments in accordance with institutional guidelines when they were 8 to 12 weeks old.

Rhodamine Efflux Assay in a Drug-Resistant Cell Line
HeyA8 and HeyA8-MDR cells were dissociated using cell dissociation buffer (Gibco, Rockville, MD) and incubated in Eagle minimal essential medium with 10% fetal calf serum, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, and penicillin/streptomycin (Gibco) with the desired compounds (2 μM elacridar, 10 μM macitentan, 10 μM ACT-132577 or dimethyl sulfoxide). After 30 minutes, 10 μM rhodamine 123 (R123; Invitrogen, Carlsbad, CA) was added for 60 minutes at 37°C in 5% CO_2. Subsequently, cells were washed once with wash buffer (phosphate-buffered saline, 1% bovine serum albumin, and 2 mM ethylenediaminetetraacetic acid) and resuspended in wash buffer, and intracellular rhodamine fluorescence was determined.

Rhodamine fluorescence was measured on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488-nm argon laser and 530-nm band-pass filter. At least 10,000 events were collected for all samples, and debris was eliminated by gating on forward *versus* side scatter.

Orthotopic Implantation of Ovarian Cancer Cells in Animal Models
To produce tumors, HeyA8-MDR cells were harvested from subconfluent cultures as described previously [7]. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of more than 95% viability were used for injection. The minimal tumorigenic dose was determined to be 1×10^6 cells, and this number of cells was used in all further experiments.

Therapy Experiments
In the first set of experiments, 1×10^6 HeyA8-MDR cells were injected into the peritoneal cavity of female nude mice. Ten days later, mice were randomized into treatment groups ($n = 10$/group) as follows: 1) daily oral administration and weekly intraperitoneal injection of vehicle (control), 2) weekly intraperitoneal injection of taxol (5 mg/kg) and daily oral administration of vehicle, 3) daily oral administration of macitentan (50 mg/kg) and once-weekly intraperitoneal injection of vehicle, 4) weekly intraperitoneal injection of taxol (5 mg/kg) and daily

oral administration of macitentan (50 mg/kg), 5) weekly intraperitoneal injection of taxol (5 mg/kg) and daily oral administration of macitentan (10 mg/kg), and 6) weekly intraperitoneal injection of taxol (5 mg/kg) and daily oral administration of macitentan (1 mg/kg).

In the second set of experiments, we determined the therapeutic effect of macitentan combined with either taxol or cisplatinum against the HeyA8-MDR peritoneal tumors. Ten days after intraperitoneal injection of 1×10^6 cells, mice were randomized into 10 treatment groups (n = 10/group) as follows: 1) daily oral administration and weekly intraperitoneal injections of vehicle (control), 2) weekly intraperitoneal injections of taxol (5 mg/kg) and daily oral administration of vehicle, 3) daily oral administration of macitentan (50 mg/kg) and weekly intraperitoneal injections of vehicle, 4) daily oral administration of macitentan (10 mg/kg) and weekly intraperitoneal injections of vehicle, 5) daily oral administration of macitentan (5 mg/kg) and weekly intraperitoneal injections of vehicle, 6) daily oral administration of macitentan (50 mg/kg) and weekly intraperitoneal injections of taxol (5 mg/kg), 7) daily oral administration of macitentan (10 mg/kg) and weekly intraperitoneal injections of taxol (5 mg/kg), 8) daily oral administration of macitentan (5 mg/kg) and weekly intraperitoneal injections of taxol (5 mg/kg), 9) weekly intraperitoneal injections of cisplatinum (8 mg/kg) and daily oral administration of vehicle, and 10) daily oral administration of macitentan (10 mg/kg) and weekly intraperitoneal injections of cisplatinum (8 mg/kg). The treatments continued until all control mice became moribund (8 weeks after injection of tumor cells; 6 weeks of treatment). This allowed an evaluation of 10 mice per group.

To determine the sequence of microscopic changes in tumor cells and tumor-associated endothelial cells, three additional mice from each of the treatment groups were necropsied after 1, 2, or 3 weeks of treatment. Tumors were prepared for histopathologic and immunohistochemical analyses.

Necropsy Procedures and Preparation of Tissues

Moribund mice or mice receiving 4 or 6 weeks of treatment were killed by intramuscular injection of nembutal (1 mg/kg) and autopsied. The presence of tumors (tumor incidence) in the peritoneal cavity and tumor weights were recorded. The 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 to be embedded in a paraffin block.

Immunohistochemical Analyses and TUNEL Assay

The primary antibodies used in this study were goat anti-human ET-1 polyclonal antibody and goat anti-human ET-2 polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Inc) was used as the secondary antibody. A TUNEL assay was performed using a commercial apoptosis detection kit (Promega Corp, Madison, WI) with modifications as described previously [6,7]. For the color reaction of the HRP-conjugated secondary antibodies, stable 3′,3-diaminobenzidine (Research Genetics, Huntsville, AL) was used.

Immunofluorescence Staining

To measure the expression of ET_AR, ET_BR, phosphorylated VEGFR2 (pVEGFR2), phosphorylated Akt (pAkt), and phosphorylated MAP kinase (pMAPK) in tumor cells and/or tumor-associated endothelial cells, tissues were costained with the following pri-

mary antibodies: rat anti-mouse CD31 monoclonal antibody (BD Pharmingen, San Diego, CA), goat anti-human ET_AR polyclonal antibody (Santa Cruz Biotechnology, Inc), rabbit anti-ET_AR antibody (Acris Antibodies, Herford, Cambridge, United Kingdom), goat anti-human ET_BR polyclonal antibody (Santa Cruz Biotechnology, Inc), rabbit anti-ET_BR antibody (Acris), rabbit anti-mouse pAkt monoclonal antibody (Cell Signaling Technology, Beverly, MA), rabbit anti-human phosphorylated-p44/42 MAP kinase (Thr202/Thr204) polyclonal antibody (Cell Signaling Technology), rabbit anti-human pVEGFR2 monoclonal antibody (Cell Signaling Technology), and Ki-67 (sc-81514; Santa Cruz Biotechnology, Inc). Goat anti-rat Alexa 594 IgG (Invitrogen), rabbit anti-goat Alexa 488 IgG (Invitrogen), rabbit anti-goat fluorescein-isothiocyanate IgG (Jackson Immuno Research Laboratories, West Grove, PA), and goat anti-rabbit Alexa 488 IgG (Invitrogen) were purchased for use as secondary antibodies [6,7].

Double Immunofluorescence Staining for ETRs and Phosphoserine in Tumor Tissues

Because specific antibodies to detect activated, serine/threonine–phosphorylated ETRs were not available, we used double immunofluorescence staining with anti-ET_AR or anti-ET_BR antibodies and anti-phosphoserine antibodies as previously described [7]. Anti-ET_AR (1:100; 12977; Abcam, Cambridge, MA), ET_BR (1:100; 65972; Abcam), and anti-phosphoserine (1:100; sc-81514; Santa Cruz Biotechnology) were used as the primary antibodies, and Alexa Fluor 488 goat anti-mouse antibody (1:600; A11029; Invitrogen) was used as the secondary antibody. Nuclei were stained with Hoechst 33342 dye (H3570; Invitrogen) [7].

Statistical Analyses

Tumor incidence, the incidence of ascites (χ^2 test), tumor weight (Mann-Whitney t test), and the number of Ki-67–positive and TUNEL-positive cells (unpaired Student's t test) were compared across the treatment groups (SPSS program; SPSS Inc, Chicago, IL).

Results

Effects of Macitentan and Its Metabolite (ACT-132577) on Rhodamine 123 Accumulation in HeyA8 and HeyA8-MDR Cells

P-gp, an efflux pump for many drugs, is highly expressed in HeyA8-MDR cells but not in the parental HeyA8 cells [33]. We used P-gp–mediated efflux of the fluorescent dye R123 as an indicator assay for P-gp activity. We detected strong intracellular accumulation of R123 in the parental HeyA8 cells (data not shown), indicating low drug efflux activity. In contrast, in the absence of elacridar, a P-gp inhibitor, R123 did not accumulate within the HeyA8-MDR cells, indicating active drug efflux. When elacridar was present, the intracellular R123 accumulation was restored. Treatment with macitentan or with ACT-132577, the major active metabolite of macitentan, did not lead to intracellular accumulation of R123 in HeyA8-MDR, showing that these compounds are not P-gp inhibitors. Macitentan and ACT-132577 were tested at concentrations ranging from 10 nM to 50 μM, and none of the drug concentrations were able to inhibit drug efflux.

Therapy for HeyA8-MDR Tumors with Macitentan and Taxol

In the first set of *in vivo* experiments, we determined whether administration of taxol, macitentan, as well as macitentan and taxol

Table 1. Treatment of HeyA8-MDR Human Ovarian Tumors Growing in the Peritoneal Cavity of Female Nude Mice with Macitentan and Taxol.

Treatment Group	Body Weight (g), Mean ± SD	Tumor Incidence	Tumor Weight (g), Median (Range)
Control	25.7 ± 1.9	10/10	1.4 (1.0-2.0)
Macitentan, 50 mg/kg	25.3 ± 1.9	7/7	1.6 (0.9-2.9)
Taxol, 5 mg/kg	25.6 ± 2.6	10/10	1.5 (1.0-2.2)
Taxol + macitentan, 50 mg/kg	26.2 ± 2.0	5/10*	0.5 (0-0.7)[†]
Taxol + macitentan, 10 mg/kg	25.5 ± 2.8	7/10	0.1 (0-0.6)[†]
Taxol + macitentan, 1 mg/kg	25.6 ± 2.8	9/10	1.1 (0-1.8)

Mice were injected intraperitoneally with 1×10^6 HeyA8-MDR cells. Ten days later, treatment with vehicle, taxol, macitentan, or a combination of macitentan and taxol was initiated. Treatment continued for 4 weeks, and the mice were necropsied. Tumor incidence and weight were recorded.
*Statistically significant compared with the control group, $P < .05$.
[†]Statistically significant compared with the control group, $P < .01$.

reduced the incidence and weight of HeyA8-MDR peritoneal tumors (Table 1). As expected, taxol alone did not reduce the tumor incidence or median weight of the tumors compared with the untreated control group (median = 1.5 g, range = 1.0-2.2 g vs median = 1.4 g, range = 1.0-2.0 g; $P > .05$). Likewise, mice treated with macitentan alone at 50 mg/kg had no decrease in tumor incidence or median tumor weight (median = 1.6 g, range = 0.9-2.9 g vs median = 1.4 g, range = 1.0-2.0 g; $P > .05$). By contrast, mice treated with the combination of taxol and 10 or 50 mg/kg macitentan, but not 1 mg/kg macitentan, had significantly reduced tumor weights (median = 0.1 g, range = 0-0.6 g and median = 0.5 g, range = 0-0.7 g, respectively, vs median = 1.4 g, range = 1.0-2.0 g; $P < .01$). We did not observe any toxic effects (weight loss) of the drugs in any of the above groups. The administration of 1 mg/kg macitentan plus taxol had no therapeutic effects. These data clearly show that the daily oral administration of 10 mg/kg macitentan, coupled with once-per-week 5 mg/kg taxol, significantly reduces the weight (size) of HeyA8-MDR tumors.

In the next set of experiments, we repeated the macitentan and taxol treatment and also studied whether the therapeutic effects could be extended to another chemotherapeutic drug, cisplatinum (Table 2). All control mice developed large peritoneal tumors and were necropsied after 6 weeks of treatment (median = 4.5 g, range = 3.2-5.1 g). Treatment with taxol alone (5 mg/kg), macitentan alone (5, 10, or 50 mg/kg), or cisplatinum alone (8 mg/kg) produced no therapeutic benefits. Once again, treatment of mice with a combination of taxol and either 10 or 50 mg/kg (but not 5 mg/kg) macitentan significantly reduced the median tumor weight (median = 1.4 g, range = 0-2.5 g and median = 1.5 g, range = 0-2.6 g, respectively, vs median = 4.5 g, range = 3.2-5.1 g; $P < .01$). Furthermore, the combination of cisplatinum and 10 mg/kg

macitentan also significantly reduced the tumor weights (median = 1.4 g, range = 0-2.1 g vs median = 4.5 g, range = 3.2-5.1 g; $P < .01$).

Immunohistochemical Analyses of HeyA8-MDR Tumors

HeyA8-MDR cells growing in the peritoneal cavity of nude mice were analyzed for expression of ET-1, ET-2, ET_AR, and ET_BR. Similar to other human ovarian cancer cells, such as SKOV3ip1 and IGROV1 [7], the HeyA8-MDR tumor cells express ET-1, ET-2, ET_AR, and ET_BR (data not shown). Costaining for ET_AR and ET_BR within the HeyA8-MDR tumors clearly demonstrates the heterogeneity of the expression of these two receptors. Some cells express ET_AR, some cells express ET_BR, and many cells express both receptors (data not shown).

Treatment with taxol alone had no effect on the phosphorylation of VEGFR2, Akt, or MAPK (Figure 1). In contrast, treatment with macitentan at 10 or 50 mg/kg (but not 1 mg/kg) alone or in combination with taxol significantly decreased the expression of pVEGFR2, pAkt, and pMAPK in HeyA8-MDR cells. In addition, the combination of macitentan (at 10 or 50 mg/kg) and taxol produced a significant increase in the number of apoptotic cells, as measured by TUNEL staining (Figure 1).

In the last set of experiments, we used a colocalization assay to study the macitentan-mediated inhibition of phosphorylation of ET_AR (Figure 2A) and ET_BR (Figure 2B) in HeyA8-MDR tumors. Treatment of mice with 10 or 50 mg/kg macitentan, with or without taxol or cisplatinum, significantly inhibited the colocalization of serine phosphorylation and both ETR receptors, whereas treatment of mice with only taxol or 5 mg/kg macitentan did not. Proteins phosphorylated on serine but unrelated to the ETRs were still phosphorylated. Complete

Table 2. Treatment of HeyA8-MDR Human Ovarian Tumors Growing in the Peritoneal Cavity of Female Nude Mice with Macitentan and Either Taxol or Cisplatinum.

Treatment Group	Body Weight (g), Mean ± SD	Tumor Incidence	Tumor Weight (g), Median (Range)
Control	24.9 ± 2.6	10/10	4.5 (3.2-5.1)
Taxol, 5 mg/kg	24.1 ± 2.2	10/10	4.0 (2.9-5.2)
Macitentan, 50 mg/kg	25.6 ± 1.8	10/10	3.6 (2.9-4.8)
Macitentan, 10 mg/kg	23.6 ± 1.6	10/10	3.1 (2.2-4.6)
Macitentan, 5 mg/kg	25.4 ± 2.0	10/10	3.5 (2.5-5.3)
Macitentan, 50 mg/kg + taxol	26.0 ± 1.7	9/10	1.5 (0-2.6)*
Macitentan, 10 mg/kg + taxol	24.5 ± 2.3	8/10	1.4 (0-2.5)*
Macitentan, 5 mg/kg + taxol	23.1 ± 1.6	10/10	3.0 (2.0-5.0)
Cisplatinum, 8 mg/kg	19.6 ± 3.1	10/10	3.5 (2.5-4.2)
Macitentan, 10 mg/kg + cisplatinum	21.3 ± 3.2	8/10	1.4 (0-2.1)*

Mice were injected intraperitoneally with 1×10^6 HeyA8-MDR cells. Ten days later, treatment with vehicle, taxol, macitentan, and cisplatinum began and was continued until mice in the control group became moribund (6 weeks). Tumor incidence and weight were recorded.
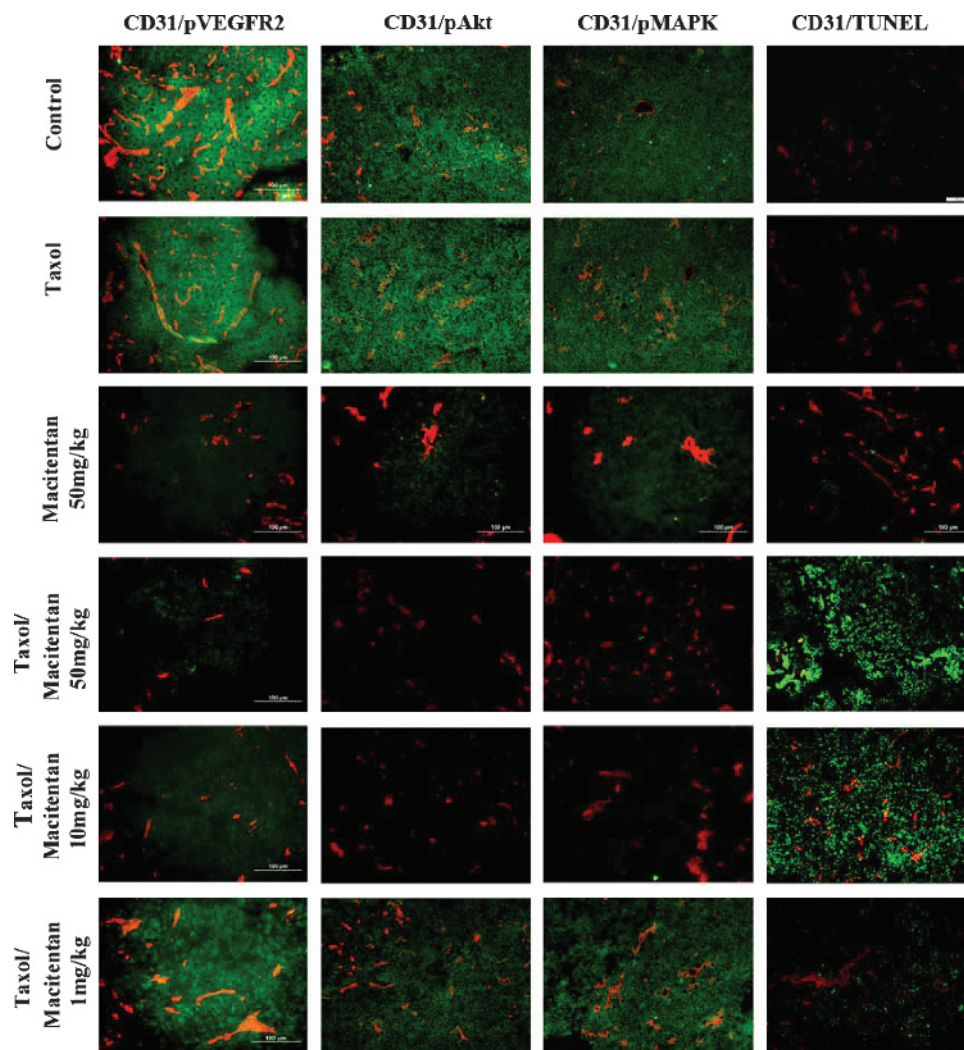*Statistically significant compared with the control group, $P < .01$.

Figure 1. Immunohistochemical analyses of orthotopic HeyA8-MDR ovarian tumors. pVEGFR2, pAkt, pMAPK, and TUNEL-positive cells are stained green. CD31-positive endothelial cells are stained red and endothelial cells positive for pAkt, pMAPK, and TUNEL are stained yellow. Treatment with macitentan at 10 or 50 mg/kg (but not 1 mg/kg) alone or in combination with taxol inhibited phosphorylation of VEGFR2, Akt, and MAPK. The combination of macitentan at 10 or 50 mg/kg and taxol induced apoptosis in tumor-associated endothelial cells (yellow) and tumor cells (green).

inhibition of ETR phosphorylation was achieved 2 weeks after the initiation of the treatment with 10 or 50 mg/kg macitentan (Figure 2, A and B).

Proliferation and Apoptosis of HeyA8-MDR Cells and Tumor-Associated Endothelial Cells

Treatment of mice with taxol, cisplatinum, or macitentan (5, 10, or 50 mg/kg) did not reduce the number of Ki-67–positive cells compared with the controls (Figure 3). Combination therapy with macitentan (10 or 50 mg/kg, but not 5 mg/kg) and taxol or macitentan (10 mg/kg) and cisplatinum significantly reduced the number of proliferating Ki-67–positive cells (mean ± SD = 32.6 ± 12.3, 31.6 ± 9.9, and 35.8 ± 8.9, respectively, vs 66.7 ± 19.5; $P < .05$).

Treatment of mice with taxol, cisplatinum, or macitentan (5, 10, or 50 mg/kg) alone did not increase the number of apoptotic tumor cells or apoptotic tumor-associated endothelial cells (by TUNEL staining) compared with control tumors. In contrast, treatment of peritoneal HeyA8-MDR tumors with the combination of macitentan (10 or 50 mg/kg) and taxol significantly increased the number of apoptotic

cells compared with the control group (mean ± SD = 119.6 ± 32.7 and 125.0 ± 29.0, respectively, vs 7.4 ± 2.6; $P < .01$). The combination of macitentan (10 mg/kg) and cisplatinum also significantly increased the number of TUNEL-positive cells compared with the control group (mean ± SD = 120.8 ± 35.2 vs 7.4 ± 2.6; $P < .01$).

Time Course of Apoptosis Induction in HeyA8-MDR Tumor Cells and Tumor-Associated Endothelial Cells

Tumor tissues were harvested after 1, 2, and 3 weeks of treatment and analyzed by TUNEL and for colocalization of TUNEL with CD31. One week after treatment, no apoptosis could be detected in tumor cells and endothelial cells from mice treated with macitentan either alone or in combination with taxol or cisplatin (Figure 4). By the second week of treatment with macitentan (10 and 50 mg/kg but not 5 mg/kg) and taxol, apoptosis was induced in the tumor-associated endothelial cells and surrounding tumor cells. Three weeks after beginning the combination treatments, apoptotic cells could be detected all over the tumor and in endothelial cells (taxol plus macitentan, 10 and 50 mg/kg, but not 5 mg/kg). Likewise, treatment with 10 mg/kg
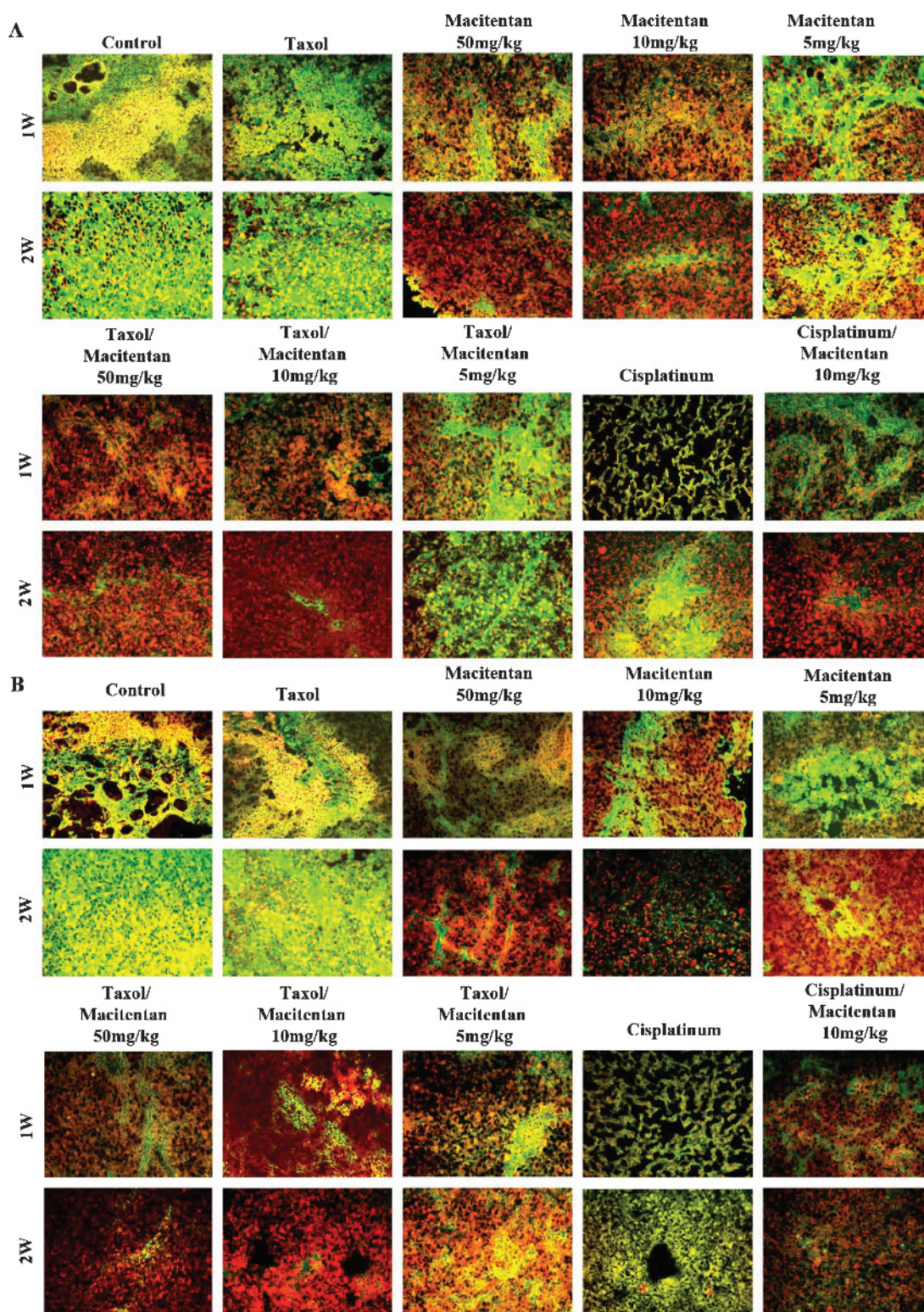
Figure 2. Double immunofluorescence staining for phosphorylated ETRs. HeyA8-MDR tumor sections were stained with an anti-phosphoserine antibody (red), (A) anti-ET_AR antibody (green), or (B) ET_BR antibody; the phosphorylated receptors are stained yellow. Treatment with macitentan at 10 or 50 mg/kg (but not 5 mg/kg), administered alone or with taxol, inhibited phosphorylation of ET_AR and ET_BR. Macitentan (10 mg/kg) plus cisplatinum also inhibited the phosphorylation of the receptors. Therapeutic effective inhibition of receptor phosphorylation was achieved after 2 weeks of treatment.

macitentan and cisplatinum induced apoptosis of tumor-associated endothelial cells and surrounding tumor cells 3 weeks after the initiation of treatment (Figure 4).

Discussion

Development of resistance to platinum compounds and taxanes, the most effective treatments used in ovarian cancer today, is based on many but different molecular mechanisms [34]. In the present study, we used the MDR ovarian cancer cell line HeyA8-MDR as a representative model for MDR ovarian cancer. The resistant cell line was generated by *in vitro* exposure to increasing levels of taxol [32]. HeyA8-MDR cells overexpress P-gp and show a higher level of taxol efflux than the parental cell line [33], making them highly resistant to taxol. Furthermore, HeyA8-MDR human ovarian cancer cells growing in the peritoneal cavity of female nude mice were resistant to systemic administration of taxol or cisplatinum. Our results show that the observed resensitization of the tumors to chemotherapy by macitentan is unlikely to be due to efflux pump inhibition.

The low responsiveness to cisplatinum must be associated with orthotopic growth *in vivo* because the cells are platinum-sensitive *in vitro*. The cisplatinum dose used led to weight loss in animals (Table 2) and was therefore a biologically active, maximally tolerated dose. *In vivo*, many mechanisms leading to resistance to platinum drugs have been described, ranging from impaired drug delivery to the up-regulation of survival pathways [35]. Of relevance to ETRs and GPCRs, it has been recently shown that chemoresistance to both taxanes and platinum compounds in ovarian cancer cells, including HeyA8-MDR, can be associated with the amplification of survival pathways by the down-regulation of regulators of G protein signaling (RGS) proteins, which activate GPCR signaling [36]. Therefore, antagonizing G protein receptor signaling, such as ET/ETR signaling,

may resensitize cells to chemotherapeutics *in vivo*. In this context, it is interesting to note that the minimal effective macitentan dosage in models of chemosensitive ovarian tumors was determined to be 30 mg/kg daily, whereas HeyA8-MDR tumors could be resensitized to chemotherapy, including expression of biomarkers with a dosage of 10 mg/kg daily.

ETs also play important roles in cell survival and apoptosis. A recent work suggests that ET-1 is necessary for the survival of lung fibroblasts and rapidly activates numerous critical survival pathways *in vitro*, including the phosphatidylinositol 3-kinase/Akt and p38 MAPK pathways [37]. These downstream pathways are also thought to be important for regulating tumor cell survival as well. ET-1 acts as a survival factor for both colon and ovarian cancer cells by inhibiting chemotherapy-induced apoptosis through activation of the phosphatidylinositol 3-kinase/Akt pathway [38,39]. Indeed, the ET signaling axis can provide protective effects against chemotherapy *in vivo*. For example, mice treated with a pharmacologic ET_AR inhibitor in combination with docetaxel had significantly smaller pancreatic tumor burdens and growth rates, compared with those treated with vehicle alone [40].

Systemic administration of macitentan (as a single agent or combined with chemotherapy) did not affect the expression ETs and ETRs but inhibited the phosphorylation of the ETRs as well as the Akt, MAPK, and VEGFR2 pathways [7]. Thus, inhibition of cell survival pathways may be another mechanism by which macitentan sensitizes tumor cells to chemotherapy.

Our data with macitentan confirm the antitumor effect of ETR antagonists using macitentan in combination with chemotherapy. In contrast to other ETAs, zibotentan, and ABT-627, which showed significant antitumor effects as single agents [19,33], macitentan alone decreased the expression of survival pathways but was ineffective or was only marginally effective alone as an antitumor agent, therefore leading to strong synergism with chemotherapy.
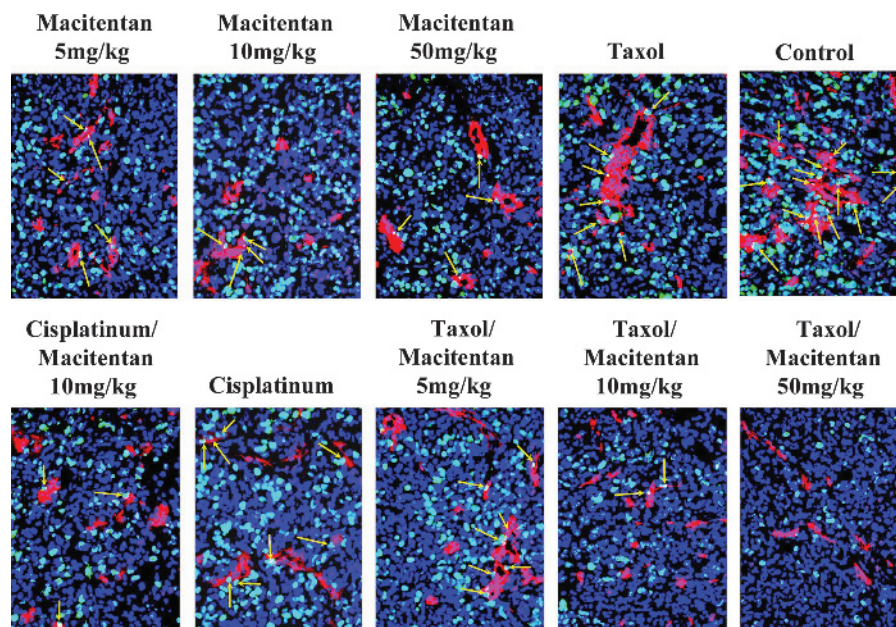

Figure 3. Inhibition of cell proliferation in orthotopic HeyA8-MDR tumors. Nuclei 4′,6-diamidino-2-phenylindole, dihydrochloride are stained dark blue and Ki-67–positive tumor cells are green, dark blue, and green, yielding a sky blue color for dividing cells. CD31-positive vessels are stained red, and dividing endothelial cells are white (red and green results in white color, arrows). Note the significant decrease in the number of Ki-67–positive cells in tumor and endothelial cells (arrows) in tumors of mice treated with taxol and macitentan (10 or 50 mg/kg), as well as those treated with macitentan (10 mg/kg) and cisplatinum.
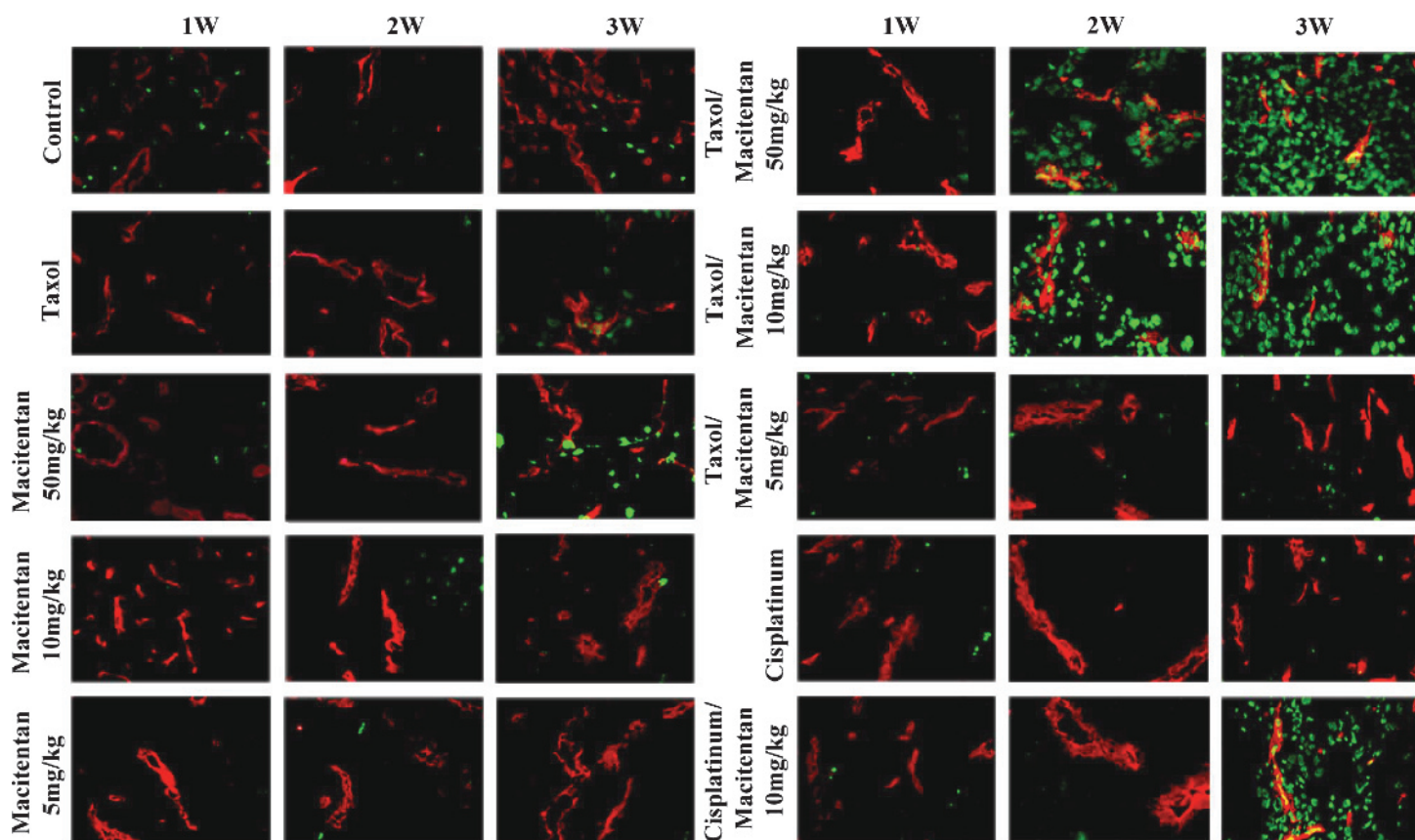
Figure 4. Induction of apoptosis in orthotopic HeyA8-MDR tumors. To detect apoptotic cells, tissues were stained with CD31 antibodies (red) and TUNEL (green). Treatment with macitentan (10 or 50 mg/kg, but not 5 mg/kg) and taxol induced apoptosis in tumor (green) and endothelial cells (yellow). Similar findings were found for macitentan (10 mg/kg) and cisplatinum. Two weeks after the start of treatment with macitentan and taxol and 3 weeks after the start of macitentan and cisplatinum treatment, the first wave of apoptosis was induced in tumor-associated endothelial cells (yellow) and surrounding tumor cells.

Regardless of the sensitivity to chemotherapeutic drugs, the survival of all cells depends on a viable vasculature [41]. Specifically, 2 weeks after the initiation of treatment with macitentan and taxol, and 3 weeks after the initiation of treatment with macitentan and cisplatinum, the first wave of apoptosis in the HeyA8-MDR tumors was detected in tumor-associated endothelial cells, which led to subsequent apoptosis in the surrounding tumor cells that depend on the tumor-associated blood vessels for oxygen and nutrients [6]. The reason for the slower induction of apoptosis in tumor-associated endothelial cells by the macitentan-cisplatinum combination needs further investigation, but induction of apoptosis in tumor-associated endothelial cells was a common denominator. Because inhibition of phosphorylation of ETR, Akt, MAPK, and VEGFR2 was correlated with therapeutic efficacy, they may be used as biologic markers in the follow-up biopsies in possible clinical studies to determine an effective dose and schedule. HeyA8-MDR cells do not produce peritoneal ascites, but VEGFR2 in tumor cells and tumor-associated endothelial cells was phosphorylated, and treatment with macitentan inhibited its phosphorylation. Thus, the function of VEGFR in HeyA8-MDR cells may be related to the survival and migration of endothelial cells rather than vascular permeability.

In summary, several mechanisms can lead to drug resistance *in vivo*, and macitentan is able to resensitize tumors expressing the ET axis to chemotherapy. Modulation of survival and proliferation pathways by macitentan, as demonstrated by a reduction in phosphorylated ETRs and cell signaling phosphoproteins in tumor-associated endothelial

cells and MDR cancer cells render them highly sensitive to taxol or cisplatinum. Macitentan combined with chemotherapeutic drugs induces apoptosis in tumor-associated endothelial cells followed by apoptosis of cancer cells after 2 weeks of therapy, regardless of the mechanism of therapy resistance. Thus, targeting ETR with macitentan, a dual tissue-targeting ETR antagonist, in combination with chemotherapy, offers an attractive new modality to treat MDR ovarian tumors. Macitentan targets not only cancer cells but also tumor-associated endothelial cells that might sensitize tumors to chemotherapy irrespective of the drug resistance mechanism.

Acknowledgments

References
[1] Jemal A, Siegal R, Ward E, Hao Y, Xu J, and Thun MJ (2009). Cancer statistics, 2009. *CA Cancer J Clin* **59**, 225–249.
[2] Cannistra SA (1993). Cancer of the ovary. *N Engl J Med* **329**, 1550–1559.
[3] Gore ME (2001). Treatment of relapsed epithelial ovarian cancer. In *American Society of Clinical Oncology 2001 Education Book*. American Society of Clinical Oncology, Alexandria, VA. pp. 468–476.
[4] Talmadge JE and Fidler IJ (2010). AACR Centennial Series: the biology of cancer metastasis: historical perspective. *Cancer Res* **70**, 5649–5669.

[5] Gottesman MM and Pastan I (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**, 385–427.

[6] Kim SJ, Uehara H, Yazici S, Busby JE, Nakamura T, He J, Maya M, Logothetis CJ, Mathew P, Wang X, et al. (2006). Targeting platelet-derived growth factor receptor on endothelial cells of multidrug-resistant prostate cancer. *J Natl Cancer Inst* **98**, 783–793.

[7] Kim SJ, Kim JS, Kim SW, Brantley E, Yun SJ, He J, Maya M, Zhang F, Wu Q, Lehembre F, et al. (2011). Macitentan (ACT-064992), a tissue-targeting endothelin receptor antagonist, enhances therapeutic efficacy of paclitaxel by modulating survival pathways in orthotopic models of metastatic human ovarian cancer. *Neoplasia* **13**, 167–179.

[8] Levin ER (1995). Endothelins. *N Engl J Med* **333**, 356–363.

[9] Bloch KD, Friedrich SP, Lee ME, Eddy RL, Shows TB, and Quertermous T (1989). Structural organization and chromosomal assignment of the gene encoding endothelin. *J Biol Chem* **264**, 10851–10857.

[10] Goldie RG (1999). Endothelins in health and disease: an overview. *Clin Exp Pharmacol Physiol* **26**, 145–148.

[11] Opgenorth TJ (1995). Endothelin receptor antagonism. *Adv Pharmacol* **33**, 1–65.

[12] Clozel M (2000). Endothelin receptor antagonists: current status and perspectives. *J Cardiovasc Pharmacol* **35**, S65–S68.

[13] Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, duBois RM, Black CM, et al. (2004). Endothelin-1 promotes myofibroblasts induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt–dependent pathway and is essential for the enhanced contractile phenotype of fibro tic fibroblasts. *Mol Biol Cell* **15**, 2707–2719.

[14] Yang Z, Krasnici N, and Lüscher TF (1999). Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation* **100**, 5–8.

[15] Hocher B, Schwarz A, Fagan KA, Thöne-Reineke C, El-Hag K, Kusserow H, Elitok S, Bauer C, Neumayer HH, Rodman DM, et al. (2000). Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Am J Respir Cell Mol Biol* **23**, 19–26.

[16] Nelson J, Bagnato A, Battistini B, and Nisen P (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer* **3**, 110–116.

[17] Spinella F, Garrafa E, Di Castro V, Rosano L, Nicotra MR, Caruso A, Natali PG, and Bagnato A (2009). Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. *Cancer Res* **69**, 2669–2676.

[18] Sonveaux P, Dessy C, Martinive P, Havaux X, Jordan BF, Gallez B, Grégoire V, Balligand JL, and Feron O (2004). Endothelin-1 is a critical mediator of mitogenic tone in tumor arterioles: implications for cancer treatment. *Cancer Res* **64**, 3209–3214.

[19] Rosanò L, Spinella F, Di Castro V, Nicotra MR, Dedhar S, de Herreros AG, Natali PG, and Bagnato A (2005). Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells. *Cancer Res* **65**, 11649–11657.

[20] Pristivishalil G and Nelson JB (2000). Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate* **44**, 44–77.

[21] Ahmed SI, Thompson J, Coulson JM, and Woll PJ (2000). Studies on the expression of endothelin, its receptor subtypes, and converting enzymes in lung cancer and in human bronchial epithelium. *Am J Respir Cell Mol Biol* **22**, 422–431.

[22] Asham E, Shankar A, Loizidou M, Fredericks S, Miller K, Boulos PB, Burnstock G, and Taylor I (2001). Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. *Br J Cancer* **85**, 1759–1763.

[23] Pflug BR, Zheng H, Udan MS, D'Antonio JM, Marshall FF, Brooks JD, and Nelson JB (2007). Endothelin-1 promotes cell survival in renal cell carcinoma through the ET(A) receptor. *Cancer Lett* **246**, 139–148.

[24] Bagnato A, Cirilli A, Salani D, Simeone P, Muller A, Nicotra MR, Natali PG, and Venuti A (2002). Growth inhibition of cervix carcinoma cells *in vivo* by endothelin A receptor blockade. *Cancer Res* **62**, 6381–6384.

[25] Egidy G, Eberl LP, Valdenaire OV, Irmler M, Majdi R, Diserens A-C, Fontana A, Janzer R-C, Pinet F, and Juillerat-Jeanneret L (2000). The endothelin system in human glioblastoma. *Lab Invest* **80**, 1681–1689.

[26] Rosanò L, Di Castro V, Spinella F, Nicotra MR, Natali PG, and Bagnato A (2007). ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma *in vitro* and *in vivo*. *Mol Cancer Ther* **6**, 2003–2011.

[27] Rayhman O, Klipper E, Muller L, Davidson B, Reich R, and Meidan R (2008). Small interfering RNA molecules targeting endothelin-converting enzyme-1 inhibit endothelin-1 synthesis and the invasive phenotype of ovarian carcinoma cells. *Cancer Res* **68**, 9265–9273.

[28] Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, Salvati E, Nicotra MR, Natali PG, and Bagnato A (2009). β-Arrestin links endothelin A receptor to β-catenin signaling to induce ovarian cancer cell invasion and metastasis. *Proc Natl Acad Sci USA* **106**, 2806–2811.

[29] Bagnato A, Loizidou M, Pflug BR, Curwen J, and Growcott J (2011). Role of the endothelin axis and its antagonists in the treatment of cancer. *Br J Pharmacol* **163**, 220–233.

[30] Iglarz M, Binkert C, Morrison K, Fischli W, Gatfield J, Treiber A, Weller T, Bolli MH, Boss C, Buchmann S, et al. (2008). Pharmacology of macitentan, an orally active tissue targeting dual endothelin receptor antagonist. *J Pharmacol Exp Ther* **327**, 736–745.

[31] Fidler IJ (2003). The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited (*Timeline*). *Nat Rev Cancer* **3**, 453–458.

[32] Thaker PH, Yazici S, Nilsson MB, Yokoi K, Tsan RZ, He J, Kim SJ, Sood AK, and Fidler IJ (2005). Antivascular therapy for orthotopic human ovarian carcinoma through blockade of the vascular endothelial growth factor and epidermal growth factor receptors. *Clin Cancer Res* **11**, 4923–4933.

[33] Zahedi P, De Souza R, Huynk L, Piquette-Miller M, and Allen C (2011). Combination drug delivery strategy for the treatment of multidrug resistant ovarian cancer. *Mol Pharm* **8**, 260–269.

[34] Agarwal R and Kaye SB (2003). Ovarian cancer: strategies for overcoming resistance to chemotherapy. *Nat Rev Cancer* **3**, 502–516.

[35] Stewart DJ (2007). Mechanisms of resistance to cisplatin and carboplatin. *Crit Rev Oncol Hematol* **63**, 12–31.

[36] Hooks SB, Callihan P, Altman MK, Hurst JH, Ali MW, and Murph MM (2010). Regulators of G-protein signaling RGS10 and RGS17 regulate chemoresistance in ovarian cancer cells. *Mol Cancer* **9**, 289–304.

[37] Kulasekaran P, Scavone CA, Rogers DS, Arenburg DA, Thannickal VJ, and Jorowitz JC (2009). Endothelin-1 and transforming growth factor-β1 independently induce fibroblast resistance to apoptosis via AKT activation. *Am J Respir Cell Mol Biol* **41**, 484–493.

[38] Eberl LP, Egidy G, Pinet F, and Juillerat-Jeanneret L (2000). Endothelin receptor blockade potentiates FasL-induced apoptosis in colon carcinoma cells via the protein kinase C pathway. *J Cardiovasc Pharmacol* **36**, S354–S356.

[39] Vacca F, Bagnato A, Catt KJ, and Tecce R (2000). Transactivation of the epidermal growth factor receptor in endothelin-1–induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res* **60**, 5310–5317.

[40] Akhavan A, McHugh KH, Guruli G, Bies RR, Zamboni WC, Strychor S, Nelson JB, and Pflug B (2006). Endothelin receptor A blockade enhances taxane effects in prostate cancer. *Neoplasia* **8**, 725–732.

[41] Fidler IJ (1995). Modulation of the organ microenvironment for the treatment of cancer metastasis. *J Natl Cancer Inst* **87**, 1588–1592.