Treatment of experimental human breast cancer and lung cancer brain metastases in mice by macitentan, a dual antagonist of endothelin receptors, combined with paclitaxel

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See the editorial by Kerbel, on pages 459–461.

Background. We recently demonstrated that brain endothelial cells and astrocytes protect cancer cells from chemotherapy through an endothelin-dependent signaling mechanism. Here, we evaluated the efficacy of macitentan, a dual endothelin receptor (ETAR and ETBR) antagonist, in the treatment of experimental breast and lung cancer brain metastases.

Methods. The effect of macitentan on astrocyte- and brain endothelial cell-mediated chemoprotective properties was measured in cytotoxic assays. We compared survival of mice bearing established MDA-MB-231 breast cancer or PC-14 non–small cell lung cancer (NSCLC) brain metastases that were treated with vehicle, macitentan, paclitaxel, or macitentan plus paclitaxel. Cell division, apoptosis, tumor vasculature, and expression of survival-related proteins were assessed by immunofluorescent microscopy.

Results. Cancer cells and tumor-associated endothelial cells expressed activated forms of AKT and MAPK in vehicle- and paclitaxel-treated groups in both metastasis models, but these proteins were downregulated in metastases of mice that received macitentan. The survival-related proteins Bcl2L1, Gsta5, and Twist1 that localized to cancer cells and tumor-associated endothelial cells in vehicle- and paclitaxel-treated tumors were suppressed by macitentan. Macitentan or paclitaxel alone had no effect on survival. However, when macitentan was combined with paclitaxel, we noted a significant reduction in cancer cell division and marked apoptosis of both cancer cells and tumor-associated endothelial cells. Moreover, macitentan plus paclitaxel therapy significantly increased overall survival by producing complete responses in 35 of 35 mice harboring brain metastases.

Conclusions. Dual antagonism of ETAR and ETBR signaling sensitizes experimental brain metastases to paclitaxel and may represent a new therapeutic option for patients with brain metastases.

Keywords: brain metastases, endothelin, macitentan, endothelial cells, astrocytes.

An estimated 200,000 cases of brain metastases occur each year in the United States.1 The majority of brain metastases are derived from tumors that originate in the lung (40%–50%) and breast (15%–20%).2 Brain metastasis is associated with poor prognosis and diminished quality of life, and usually signifies a fatal outcome for patients with solid cancers.3,4 There are no targeted therapies specific for brain metastases, and the blood-brain barrier can pose a physiologic impediment to many cytotoxic drugs and antibody-based therapies.5,6 Whole brain radiotherapy, the mainstay treatment for patients with brain metastases, has an overall survival rate of ~4–6 months.7,8 Given that the incidence of brain metastasis is expected to continue to increase,9,10 much effort is currently directed toward the development of novel therapeutic approaches to improve clinical outcomes.

We recently discovered that brain endothelial cells and astrocytes protect cultured breast cancer cells and lung cancer cells from the microtubule-stabilizing drug, paclitaxel, through an endothelin-dependent signaling mechanism that leads to upregulation of antiapoptotic proteins in cancer cells.11 The chemoprotective effect requires physical interaction between cancer cells and endothelial cells or astrocytes and can be...
abolished by combined antagonism of ET_{A}R and ET_{B}R signaling.\textsuperscript{11} Endothelins (ET-1, ET-2, ET-3) are small peptides that bind to 2 G-protein-coupled receptors, ET_{A}R and ET_{B}R, and elicit a number of biological responses.\textsuperscript{12} ET-1 plays a critical role in maintaining cardiovascular homeostasis by virtue of its ability to elicit vascular smooth muscle contraction.\textsuperscript{13} More recent studies have determined that aberrant endothelin signaling contributes to a variety of pathophysiologic processes,\textsuperscript{17} including cancer.\textsuperscript{14} ET-1 is expressed in \textasciitilde 40\%–60\% of human breast cancers,\textsuperscript{15,16} and elevated expression of ET-1, ET_{A}R, and ET_{B}R is associated with a reduction in both disease-free survival and overall survival in breast cancer.\textsuperscript{15} Expression of ET-1 and endothelin receptors correlate with vascular endothelial growth factor (VEGF) expression and angiogenesis in breast cancer,\textsuperscript{16} and expression of ET_{A}R predicts an unfavorable response to neoadjuvant chemotherapy in locally advanced tumors.\textsuperscript{17} ET-1 expression is also associated with poor prognosis in non–small cell lung cancer (NSCLC).\textsuperscript{18} Evidence suggests that an intact endothelin-signaling pathway may be particularly important for cancer cell survival in the brain. Expression of ET_{B}R has been shown to be a key determinant in the progression of melanoma cells to the brain metastatic phenotype,\textsuperscript{19} and inhibition of ET_{B}R on glioblastoma stem cells was found to lead to loss of tumor-initiating properties and cell death.\textsuperscript{20} Peritumoral astrocytes overexpress endothelin in 85\% of human brain metastasis cases.\textsuperscript{21} Herein, we sought to determine the extent that endothelin signaling is critical for the growth of experimental breast and lung cancer brain metastases by treating mice with macitentan, a dual endothelin receptor antagonist, alone and in combination with paclitaxel.

Materials and Methods

Cell Lines

Human PC-14 lung adenocarcinoma cells,\textsuperscript{22} human MDA-MB-231 breast cancer\textsuperscript{11} cells, and murine NIH 3T3 fibroblasts\textsuperscript{11} were purchased from the American Type Culture Collection and maintained in complete Eagle’s minimal essential medium (MEM) supplemented with 10\% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies), and a penicillin/streptomycin mixture containing 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies), and a penicillin/streptomycin mixture.

Reagents

The following reagents were used in this study: anti-ET_{A}R, anti-ET_{B}R, anti-Ki67 (Abcam); anti-CD31 (BD Biosciences); anti-ET_{A}R, anti-ET_{B}R, anti-phospho-serine (Santa Cruz Biotechnology); anti-TWIST1, anti-BCL2L1, anti-phospho-AKT (pAKT), anti-phospho-MAPK (pMAPK) (Thr-202 and Tyr-204) (Cell Signaling); anti-glutathione S-transferase A5 (GSTAS) (Novus Biologicals); anti-glial fibrillary acidic protein (GFAP) (BioCare Medical); anti-a smooth muscle actin (a-SMA) (Abcam); anti-rabbit Alexa 594, anti-rabbit Alexa 488 (Life Technologies); Hoechst 33342 (Invitrogen) was used as a nuclear counterstain, and 3,3′-diaminobenzidine was used for colorimetric immunohistochemistry of GFAP. Macitentan was provided by Actelion Pharmaceuticals Ltd., and paclitaxel was purchased from Bristol-Myers Squibb. A terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining kit was purchased from Promega.

Luciferase Transfection

A lentiviral vector containing both the enhanced green fluorescent protein (eGFP) gene and the luciferase gene was used to generate firefly luciferase-expressing MDA-MB-231 breast cancer cells and PC-14 NSCLC cells. Experimental details are provided in the Supplementary material.

Cytotoxicity Studies

To determine if astrocytes and brain endothelial cells protect PC-14 NSCLC cells from paclitaxel, we conducted chemoprotection assays as previously described.\textsuperscript{11} We also evaluated the ability of endothelial cells derived from the mouse lung and lymphatic vasculature to protect cancer cells from paclitaxel. In brief, murine astrocytes, murine brain endothelial cells, murine lung endothelial cells, murine lymphatic endothelial cells, and murine NIH 3T3 fibroblasts were transfected with GFP genes and coincubated with MDA-MB-231 cells (positive reference) or PC-14 NSCLC cells (cancer cell: test cell plating ratio of 1:2 for astrocytes and 3T3 fibroblasts and 1:3 for endothelial cells) for 24 hours. The media were then replaced with fresh MEM, MEM containing 15 ng/mL of paclitaxel, or MEM containing 100 nM of macitentan. To determine whether any observed chemoprotection was the result of endothelin-mediated signaling, several cultures containing the coincubated cells were treated with 100 nM of macitentan for 2 hours prior to incubation in the paclitaxel-containing media. After 48 hours, the GFP-labeled cells were separated from the cancer cells by fluorescence-activated cell sorting (FACS) and the apoptotic index of the cancer cells was determined by FACS analysis of propidium iodide-stained DNA.

Mice

Female athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute- Frederick Cancer Research and Development Center. The mice were maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the National Institutes of Health. Mice were used in accordance with institutional guidelines when they were 8–12 weeks old.

Experimental Brain Metastasis Models

Experimental brain metastases were generated as described previously\textsuperscript{17} and as detailed in the Supplementary material. Metastases were established by injecting 100 \mu L of HBSS containing 2 \times 10^6 MDA-MB-231 cancer cells or 1 \times 10^6 PC-14 cancer cells into the carotid artery.

Therapy Studies

Based on the results obtained from pilot studies, we selected a dose of 8 mg/kg of paclitaxel for treating brain metastases originating from MDA-MB-231 cells and 5 mg/kg paclitaxel for brain
metastases derived from PC-14 cells. Mice were randomly assigned into 4 treatment groups: (i) control: daily oral administration of vehicle (0.1% Tween 80-based PBS) and once-weekly i.p. injection of PBS; (ii) paclitaxel: daily oral administration of vehicle and once-weekly i.p. injection of paclitaxel; (iii) macitentan: daily oral administration of 10 mg/kg macitentan and once-weekly i.p. injection of PBS; and (iv) combination group: daily oral administration of 10 mg/kg macitentan and once-weekly i.p. injection of paclitaxel. Macitentan was reconstituted in 0.05% (wt) methylcellulose solution containing 0.05% (vol) Tween 80. Mice were monitored for weight loss, dehydration, and abnormal neurological signs including lethargy, hyperkinesis, or circling behavior. Animals that exhibited any of these signs were euthanized by i.p. injection of Nembutal (1 g/kg). Survival durations were recorded, and all brains were harvested for further evaluation.

In some studies, we labeled MDA-MB-231 cancer cells and PC-14 cancer cells with luciferase to monitor tumor responsiveness to therapy and to ensure that metastases were established prior to initiating therapy. We collected bioluminescence images each week on anesthetized (2% inhaled isoflurane) mice. Mice were injected (i.p.) with 15 mg/mL of D-luciferin and imaged with an IVIS 100 imaging system. We used Living Image software (Xenogen Corp.) to grid the imaging data and integrate the total bioluminescence signals in each boxed region.

**Immunohistochemical Methods**

Immunofluorescence staining of tissues for ETAR, ETBR, GFAP, phosphorylated AKT (pAKT), pMAPK, pETAR, pETBR, CD31, Bcl2L1, Gsta5, Twist1, and Ki67 was performed as described previously and is detailed in the Supplementary material. TUNEL was performed using an apoptosis detection kit according to the manufacturer’s instruction (Promega). Methods for quantifying ETAR, ETBR, Ki67, and TUNEL-positive cells are detailed in the Supplementary material.

**Pericyte Coverage and Microvascular Density**

Pericyte coverage of tumor-associated blood vessels was determined by staining brain sections from mice that had been treated with the different therapies for 21 days (n = 3 mice/group). Sections were stained with antibodies directed against CD31 (red) and α-smooth muscle actin (α-SMA; green) and visualized with an Olympus BX-51 microscope. Four fields were randomly selected at a magnification of X400, and blood vessels that were at least 50% covered by green α-SMA-positive cells were considered to be positive for pericyte coverage. Tumor microvascular density (MVD) was determined using the method described by Weidner and as detailed in the Supplementary material.

**Statistical Analysis**

Statistical analysis of cytotoxicity and cell division/apoptosis studies was performed with the Prism program (GraphPad Software, version 6.01) using the Student t test. P < .05 was considered statistically significant. Kaplan-Meier survival plots were generated, and the log-rank (Mantel-Cox) test was performed to test the difference in survival between groups.

**Results**

**Expression of GFAP and Endothelin Receptors in Clinical and Experimental Breast Cancer and Lung Cancer Brain Metastases**

Both clinical and experimental breast and lung cancer brain metastases were surrounded and infiltrated by GFAP-positive astrocytes (Fig. 1). ETAR and ETBR were heterogeneously expressed in...
Macitentan in Combination with Paclitaxel Significantly Increases Overall Survival of Mice with Experimental Brain Metastases

Next, we performed several survival analyses in which mice harboring either breast or lung cancer brain metastases were treated with vehicle, macitentan, paclitaxel, or macitentan plus paclitaxel. In the first study, we initiated treatment 2 weeks after injection of MDA-MB-231 cancer cells into the carotid artery of mice (n = 10 mice/group). Two mice in the paclitaxel plus macitentan group were euthanized during the course of the study for nonmalignancy-related causes. Single-agent treatment with either paclitaxel or macitentan had no effect on survival, and only mice that were treated with combined macitentan and paclitaxel were alive when the study concluded on day 150 (P < .001, log-rank test) (Fig. 2A). These mice were determined to be metastasis-free based on histological examinations of brain sections.

To verify that metastases were established prior to starting therapy, we performed a second survival analysis using MDA-MB-231 cancer cells that were transfected with luciferase (n = 10 mice/group). Therapy began on day 14, when all mice had measurable brain metastases as determined by bioluminescence imaging. One mouse receiving macitentan plus paclitaxel was euthanized for rectal prolapse during the course of the study. By day 125, all mice from the control, paclitaxel, and macitentan groups were dead, whereas 9 mice in the combined macitentan plus paclitaxel group were alive (P < .0001, log-rank test) (Fig. 2B). We were unable to detect luciferase signals in surviving mice, and no breast cancer cells were identified by histological analysis of brain sections. Representative results from the bioluminescence imaging analysis are shown in Fig. 2C.

To determine whether the antitumor effect of macitentan plus paclitaxel was unique to MDA-MB-231 brain metastases, we studied the survival of mice harboring experimental PC-14 brain metastases. Mice were randomized into the 4 treatment groups, and treatment began 2 weeks after injection of the PC-14 cancer cells into the carotid artery (n = 11 mice/group). Two mice in the macitentan plus paclitaxel group were euthanized for nonmalignancy-related causes. The study was terminated on day 155, when only mice that received macitentan plus paclitaxel were alive (P < .001, log-rank test) (Fig. 3A). Single-agent treatment with either macitentan or paclitaxel had no effect on overall survival. Routine histological analyses of brain sections from mice that received the combination therapy were negative for metastases (data not shown).

To confirm that PC-14 metastases were established when we initiated therapy and to monitor any tumor response to treatment, we performed a second survival analysis using PC-14 cancer cells that were transfected with luciferase. Treatment was started on day 15, when tumors were considered established by bioluminescence imaging (n = 7 or 8 mice/group). One mouse in the macitentan plus paclitaxel group was euthanized during the course of the study due to rectal prolapse. The study concluded on day 125 when only mice in the macitentan plus paclitaxel treatment group were alive (P < .001, log-rank test) (Fig. 3B). These mice were determined to be tumor-free based on bioluminescence imaging and subsequent histological examination of brain sections. Representative bioluminescence images collected from mice belonging to the different treatment groups are shown in Fig. 3C. These images were obtained 75 days after injecting cancer cells into mice.
and ET\textsubscript{B}R (Supplementary material, Figs. S3A and B). ET\textsubscript{A}R and ET\textsubscript{B}R phosphorylation was markedly attenuated by the administration of macitentan. The results from our previous cell-based assays indicated that dual antagonism of ET\textsubscript{A}R and ET\textsubscript{B}R signaling abrogates astrocyte- and brain endothelial cell-induced activation of AKT/MAPK-signaling pathways in cancer cells.\textsuperscript{11} Therefore, we next evaluated the expression of AKT/MAPK pathways in the microenvironment of MDA-

**Fig. 2.** Combination therapy with macitentan and paclitaxel significantly increases overall survival in mice with breast cancer brain metastases. (A) Kaplan-Meier plot of mice bearing experimental MDA-MB-231 brain metastases. Mice were randomly assigned into the following 4 groups: control (n = 11), paclitaxel (n = 10), macitentan (n = 11), and paclitaxel plus macitentan (n = 10). (B) Kaplan-Meier plot of mice bearing experimental MDA-MB-231 brain metastases. In this experiment, luciferase-labeled MDA-MB-231 cancer cells were used to generate metastases in order to permit noninvasive monitoring of therapeutic response (n = 10 mice/group). (C) Representative bioluminescent images from mice bearing experimental MDA-MB-231 brain metastases that were treated with vehicle (control), paclitaxel, macitentan, or macitentan plus paclitaxel. Images were collected 91 days after cancer cells were injected into the carotid artery. *Euthanized due to nonmalignancy-related cause. †Euthanized due to neurologic symptom.
MB-231 and PC-14 brain metastases from mice that were treated for 21 days with vehicle, paclitaxel, macitentan, or macitentan plus paclitaxel. We observed marked expression of the activated forms of AKT and MAPK on cancer cells and tumor-associated endothelial cells in vehicle- and paclitaxel-treated mice in both MDA-MB-231 (Fig. 4A) and PC-14 (Fig. 4B) experimental brain metastasis models. However, expression of phosphorylated AKT (pAKT) and pMAPK were dramatically downregulated in tumors from mice that received macitentan. We noted that a similar pattern of expression emerged when we evaluated the distribution of survival-related proteins Bcl2L1, Gsta5, and Twist1 in the brain metastases. Bcl2L1, Gsta5, and Twist1 were readily detected on cancer cells and tumor-associated endothelial cells of MDA-MB-231

Fig. 3. Combination therapy with macitentan and paclitaxel significantly increases overall survival in mice with lung cancer brain metastases. (A) Kaplan-Meier plot of mice bearing experimental PC-14 non–small cell lung cancer (NSCLC) brain metastases (n = 11 mice/group). (B) Kaplan-Meier plot of mice bearing experimental PC-14 brain metastases. Mice were injected with luciferase-labeled PC-14 cancer cells and then randomly assigned to control (n = 8), paclitaxel (n = 7), macitentan (n = 8), or paclitaxel plus macitentan (n = 8). (C) Representative bioluminescent images from mice bearing experimental PC-14 brain metastases. Images were collected 75 days after cancer cells were injected into the carotid artery. *Euthanized due to nonmalignancy-related cause. †Euthanized due to neurologic symptom.
Fig. 4. Dual antagonism of ET$_{A}$R and ET$_{B}$R inhibits phosphorylation of AKT and MAPK in cancer cells and tumor-associated endothelial cells in experimental breast cancer and lung cancer brain metastases. Mice were treated with the indicated therapies for a period of 21 days. Hematoxylin and eosin (H&E) staining was performed on tumors from individual treatment groups and on the brain sections collected from non-tumor-bearing (normal) mice. Multiple fields were analyzed for each tumor and these are representative immunofluorescent images of phosphorylated AKT (pAKT) and phosphorylated MAPK (pMAPK) (both depicted in green) in MDA-MB-231 breast cancer (A) and PC-14 non–small cell lung cancer (NSCLC) brain metastases (B). Mice were treated with the indicated therapies for a period of 21 days. Endothelial cells were labeled with an antibody directed against CD31 (red). Scale bar = 50 μm. n = 3 mice/group.

Combined Macitentan and Paclitaxel Therapy Reduces Cancer Cell Division and Stimulates Apoptosis of Cancer Cells and Tumor-associated Endothelial Cells

Next, we measured the number of Ki67-positive cells and TUNEL-positive cells in MDA-MB-231 and PC-14 brain metastases from mice that had been treated for 21 days with vehicle, paclitaxel, macitentan, or macitentan plus paclitaxel. Single-agent macitentan had a more profound effect on cell division in MDA-MB-231 brain metastases (Fig. 5A) when compared with PC-14 metastases (Fig. 5B). Macitentan decreased the number of Ki67 positive cells by 46% in MDA-MB-231 metastases ($P < .001$) and by 21% in PC-14 brain metastases ($P < .01$) (Table 1). Paclitaxel therapy alone had no significant effect on cell division in either metastasis model. In both the MDA-MB-231 and PC-14 brain metastasis models, the combined administration of macitentan plus paclitaxel therapy elicited the most profound reduction in cell division (57% and 69%, respectively), when compared with cell division in metastases from vehicle-treated mice.

We observed a low basal level of apoptosis in vehicle-treated MDA-MB-231 and PC-14 brain metastases (Table 1). Paclitaxel alone elicited a modest but significant increase in apoptosis in MDA-MB-231 brain metastases and a 6-fold increase in the number of apoptotic cells in PC-14 brain metastases. PC-14 metastases were more sensitive to single-agent macitentan therapy when compared with MDA-MB-231 metastases, suggesting that the former may be more reliant on endothelin signaling for their survival. The combination of macitentan plus paclitaxel produced a striking increase in the number of apoptotic cells in both MDA-MB-231 (25-fold increase) and PC-14 (11-fold increase) brain metastases. Moreover, in both brain metastasis models, the combination of macitentan plus paclitaxel therapy produced apoptosis of both cancer cells and tumor-associated endothelial cells.

Combined Macitentan and Paclitaxel Therapy Decreases the MVD of Experimental MDA-MB-231 and PC-14 Brain Metastasis

Because macitentan plus paclitaxel therapy resulted in apoptosis of tumor-associated endothelial cells, we further studied the
Fig. 5. Downregulation of survival proteins, inhibition of cell division, and increased apoptosis in cancer cells and endothelial cells mediated by treatment with macitentan and paclitaxel. Representative immunofluorescent images of sections that were stained for expression of Bcl2L1, Gsta5, and Twist1 proteins (each depicted in green) in MDA-MB-231 (A) and PC-14 non–small cell lung cancer (B) experimental brain metastases. The sections were collected from mice that were treated with the indicated therapies for a period of 21 days. Proliferating (Ki67) and apoptotic (TUNEL) cells are also depicted in green. Blood vessels were labeled with an antibody directed against CD31 (red). Scale bar = 50 μm. n = 3 mice/group.
encompassing 0.065-mm², was recorded. Data are represented as number of positive staining cells in 6 randomly selected regions, each covering a 0.065-mm² area.

Two tumors from each group were selected for analysis, and the MVD of MDA-MB-231 and PC-14 brain metastases were measured. The MVD of MDA-MB-231 and PC-14 brain metastases was significantly less than the MVD of normal brain metastases. The MVD of MDA-MB-231 and PC-14 brain metastases was significantly less than the MVD of normal brain metastases.

The observation that macitentan downregulated expression of antiangiogenic proteins in cancer cells and tumor-associated endothelial cells is consistent with previous reports demonstrating that ET-1 functions as a survival factor for various types of cancer cells. The activation of endothelin receptors is followed by phosphorylation of AKT and MAPK leading to upregulation of multiple genes, among which are GSTA5, BCL2L1, and TWIST1. Cancer cells expressing the protein products of these genes have significantly decreased vulnerability to chemotherapeutic drugs. We have also reported that treating cells with the dual endothelin receptor antagonist macitentan prevents the expression of these survival proteins and thus increases the vulnerability of dividing cancer cells to chemotherapy.

Effects of macitentan on the tumor vasculature of MDA-MB-231 and PC-14 brain metastases. First, we evaluated the maturity of the tumor-associated blood vessels from mice bearing MDA-MB-231 or PC-14 brain metastases that were treated with vehicle, macitentan, paclitaxel, or macitentan plus paclitaxel for a period of 3 weeks. The MVD of MDA-MB-231 and PC-14 experimental metastases was considered covered by pericytes (Supplementary material, Fig. S4A). None of the therapies used in our study had a significant effect on pericyte coverage of tumor blood vessels.

Next, we measured the MVD of experimental metastases from mice that had been treated with the different therapies for a period of 3 weeks. The MVD of MDA-MB-231 and PC-14 brain metastases was significantly less than MVD of normal brain tissue (P < .001) (Supplementary material Fig. S4B). Only combined macitentan plus paclitaxel therapy significantly reduced the MVD of MDA-MB-231 and PC-14 brain metastases (P < .05). These results are consistent with the results from our TUNEL analysis, which demonstrated that combined macitentan plus paclitaxel leads to apoptosis of tumor-associated endothelial cells. Collectively, these data indicate that the tumor-associated blood vessels of MDA-MB-231 breast cancer and PC-14 NSCLC brain metastases are immature and, moreover, vulnerable to combined therapy with macitentan and paclitaxel.

Discussion

Previously, we demonstrated that brain endothelial cells and astrocytes release ET-1 that binds to endothelin receptors on cancer cells. The activation of endothelin receptors is followed by phosphorylation of AKT and MAPK leading to upregulation of multiple genes, among which are GSTA5, BCL2L1, and TWIST1. Cancer cells expressing the protein products of these genes have significantly decreased vulnerability to chemotherapeutic drugs. We have also reported that treating cells with the dual endothelin receptor antagonist macitentan prevents the expression of these survival proteins and thus increases the vulnerability of dividing cancer cells to chemotherapy.

Table 1. Quantification of Ki67- and TUNEL-positive cells in experimental models of MDA-MB-231 and PC-14 brain metastases in mice that were treated with vehicle, paclitaxel, macitentan, or macitentan plus paclitaxel

<table>
<thead>
<tr>
<th></th>
<th>Ki67</th>
<th>TUNEL</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>65.6 ± 17.3</td>
<td>2.2 ± 1.4</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>61.3 ± 12.9</td>
<td>7.1 ± 4.3*</td>
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<tr>
<td>Macitentan</td>
<td>35.4 ± 10.2**</td>
<td>2.7 ± 2.4</td>
</tr>
<tr>
<td>Macitentan + paclitaxel</td>
<td>28.3 ± 6.3**</td>
<td>54.5 ± 11.4**</td>
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<tr>
<td>PC-14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>144.3 ± 27.6</td>
<td>10.6 ± 6.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>134.6 ± 32.7</td>
<td>63.0 ± 19.5**</td>
</tr>
<tr>
<td>Macitentan</td>
<td>113.4 ± 28.8*</td>
<td>37.3 ± 13.1**</td>
</tr>
<tr>
<td>Macitentan + paclitaxel</td>
<td>44.5 ± 16.8**</td>
<td>117.0 ± 28.8**</td>
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Two tumors from each group were selected for analysis, and the number of positive staining cells in 6 randomly selected regions, each encompassing 0.065-mm², was recorded. Data are represented as mean ± SD.

*P < .01 compared with vehicle-treated metastases (Student t test).
**P < .001 compared with vehicle-treated metastases (Student t test).

The decision to evaluate a dual endothelin receptor antagonist in the present report was based on our previous experience with type-specific endothelin receptor antagonists. In those analyses, we demonstrated that the administration of a single ETAR or ETBR antagonist had no significant effect on astrocyte- or brain endothelial cell-induced activation of AKT and MAPK effector proteins in cancer cells. That observation, along with the present finding that ETAR and ETBR are heterogeneously expressed within the same tumor in clinical cases of brain metastases, suggests that antagonism of both endothelin receptors in combination with chemotherapy is much more likely to yield a clinical benefit for patients with brain metastases than a therapeutic regimen employing a single type-selective endothelin receptor antagonist.

To date, the use of antiangiogenic agents for the treatment of CNS tumors has proved disappointing. Therapies that target VEGF do not provide a survival benefit for patients with newly diagnosed glioblastoma. Indeed, glioblastomas treated with anti-VEGF therapies have been shown to adopt a resistant, more infiltrative phenotype. One of the adverse effects of anti-VEGF/VEGFR therapies is the development of hypertension, which has been attributed to activation of the ET receptors.
endothelin system.\textsuperscript{40,41} Whether or not anti-VEGF/VEGFR agents increase ET-1 levels in the microenvironment of CNS tumors to the extent it confers resistance to cancer cells and endothelial cells remains unknown.

Studies evaluating efficacy of chemotherapy in experimental breast cancer brain metastases have shown the blood-tumor barrier remains a major obstacle to therapy.\textsuperscript{42} Paclitaxel concentrations varied widely in experimental 4T1 brain metastases and were a fraction of that found in peripheral metastases.\textsuperscript{42} Recently, we examined the combined effects of macitentan and temozolomide on orthotopically implanted glioblastomas and found that macitentan does not increase temozolomide uptake in experimental brain tumors.\textsuperscript{13} Combined macitentan plus temozolomide was very effective in treating temozolomide-resistant experimental glioblastomas, which prompted us to conclude that the pharmacologic effects on tumor-associated stromal cells may be critical to the success of this therapy.\textsuperscript{43} The current finding that both lung and lymphatic endothelial cells can protect cancer cells through an endothelin-dependent process suggests that macitentan may have utility in treating metastases in other tissues.

Astrocytes are among the first cells to respond to brain-homing cancer cells,\textsuperscript{23} and they can even detect a disseminating cancer cell once it becomes arrested in the cerebral microvasculature.\textsuperscript{34} Studies examining the kinetics of cancer cell-stromal cell interactions in brain metastasis have shown that the number of reactive astrocytes associated with a given metastasis is directly proportional to the mass of the tumor.\textsuperscript{23,44} It has been known for 2 decades now that the peritumoral astrocytes that surround almost all human brain metastases express elevated levels of endothelin.\textsuperscript{21} The results generated from our cell-based assays indicate that astrocyte-derived endothelin can have a profound impact on the efficacy of chemotherapy by rendering cancer cells resistant to therapy.

Vascular endothelial cells are the major cellular source of endothelin in the human body\textsuperscript{42} and play a decisive role in determining the fate of cancer cells in the brain. Real-time imaging studies of experimental brain metastasis revealed that failure to co-opt cerebral capillaries is fatal for melanoma cells, whereas the inability to trigger the angiogenic switch signals for the demise of lung cancer cells.\textsuperscript{45} Over recent years, there has been a growing recognition that tumor-associated blood vessels serve as a niche for brain cancer stem cells\textsuperscript{46} and that the physical interactions between cancer cells and endothelial cells protect the former from chemotherapy and radiation through an as-yet unknown mechanism.\textsuperscript{47} Our data suggest that endothelial cell-derived endothelin protein warrants consideration as a candidate chemoprotective mediator for brain cancer stem cells residing within a vascular niche. We have previously shown that the chemoprotective effects of brain endothelial cells for NSCLC cells and breast cancer cells are dependent on heterotypic endothelial cell–cancer cell adhesion,\textsuperscript{11} and other investigators have also reported on the importance of physical interactions between cancer cell and endothelial cells in sustaining the viability of brain metastases. Indeed, a recent examination of human brain metastases from primary tumors of varied origin concluded that 98% of early metastasis growth occurs through physical interactions with the existing neurovasculature.\textsuperscript{48} The combination of macitentan and paclitaxel therapy disrupts cancer cell–vascular endothelial cell interactions by stimulating apoptosis of tumor-associated endothelial cells. The combination therapy is well tolerated in mice and may provide a new therapeutic approach for the treatment of patients with brain metastases.

**Supplementary Material**

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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**References**


