Macitentan, a Dual Endothelin Receptor Antagonist, in Combination with Temozolomide Leads to Glioblastoma Regression and Long-term Survival in Mice

Sun-Jin Kim, Ho Jeong Lee, Mark Seungwook Kim, Hyun Jin Choi, Junqin He, Qiuyu Wu, Kenneth Aldape, Jeffrey S. Weinberg, W.K. Alfred Yung, Charles A. Conrad, Robert R. Langley, François Lehembre, Urs Regenass, and Isaiah J. Fidler

Abstract

Purpose: The objective of the study was to determine whether astrocytes and brain endothelial cells protect glioma cells from temozolomide through an endothelin-dependent signaling mechanism and to examine the therapeutic efficacy of the dual endothelin receptor antagonist, macitentan, in orthotopic models of human glioblastoma.

Experimental Design: We evaluated several endothelin receptor antagonists for their ability to inhibit astrocyte- and brain endothelial cell-induced protection of glioma cells from temozolomide in chemoprotection assays. We compared survival in nude mice bearing orthotopically implanted LN-229 glioblastomas or temozolomide-resistant (LN-229Res and D54Res) glioblastomas that were treated with macitentan, temozolomide, or both. Tumor burden was monitored weekly with bioluminescence imaging. The effect of therapy on cell division, apoptosis, tumor-associated vasculature, and pathways associated with cell survival was assessed by immunofluorescent microscopy.

Results: Only dual endothelin receptor antagonism abolished astrocyte- and brain endothelial cell-mediated protection of glioma cells from temozolomide. In five independent survival studies, including temozolomide-resistant glioblastomas, 46 of 48 (96%) mice treated with macitentan plus temozolomide had no evidence of disease (P < 0.0001), whereas all mice in other groups died. In another analysis, macitentan plus temozolomide therapy was stopped in 16 mice after other groups had died. Only 3 of 16 mice eventually developed recurrent disease, 2 of which responded to additional cycles of macitentan plus temozolomide. Macitentan downregulated proteins associated with cell division and survival in glioma cells and associated endothelial cells, which enhanced their sensitivity to temozolomide.

Conclusions: Macitentan plus temozolomide are well tolerated, produce durable responses, and warrant clinical evaluation in glioblastoma patients.

Introduction

Glioblastoma is a devastating disease characterized by local invasion, microvascular proliferation, and therapeutic resistance (1, 2). The highly infiltrative nature of glioma cells makes complete surgical resection unlikely, and 90% of tumors recur (3). Resistance to alkylating agents via the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) also remains a barrier to the successful treatment of patients with malignant glioma (4). Efforts to control glioblastoma growth by therapeutic targeting of genetic alterations that drive tumor progression have been hampered by the molecular diversity of the disease (5), which is further augmented by chemotherapy (6). No less than four transcriptional subtypes of glioblastoma have been identified (7), and an individual tumor may be maintained by multiple intermixed populations of cells, each with amplification of a different tyrosine kinase receptor (8). Consequently, it is doubtful that targeting a single oncogenic pathway will improve clinical outcomes in a randomly selected patient population. New treatment strategies for glioblastoma are under investigation, including therapies that target tumor vasculature.

High-grade gliomas are among the most angiogenic of all tumors (9). Tumor blood vessels provide a niche for brain cancer stem cells, where signal-releasing endothelial cells promote their renewal (10). Glioma stem cells ensure their blood supply by releasing VEGF into the microenvironment (11) and by differentiating into endothelial cells (12) and pericytes (13). VEGF promotes glioma vascularization, oxygenation, and growth (14), and its hyperpermeabilizing properties contribute to...
Translational Relevance

Glioblastoma is a fatal disease and efforts to control its growth by surgery, chemotherapy, and radiotherapy have not significantly improved clinical outcome. Previously, we reported that astrocyte- and brain endothelial cell–derived ET-1 protects cancer cells from chemotherapy by upregulating expression of antiapoptotic proteins in cancer cells. Here, we demonstrate that dual antagonism of ET₄R and ET₃R signaling abolishes astrocyte- and brain endothelial cell–induced chemoprotection of glioblastoma cells in vitro and, moreover, downregulates the expression of proteins associated with cancer cell growth and survival in vivo in orthotopic models of glioblastoma. Indeed, when macitentan was combined with temozolomide, we observed marked apoptosis of both glioma cells and tumor-associated endothelial cells, which resulted in marked regression of glioblastomas and durable responses in different models, including those that are resistant to temozolomide. This combination therapy represents an important new therapeutic approach for the treatment of glioblastoma.

Materials and Methods

Cell lines

Human glioblastoma LN-229 cells, murine NIH 3T3 fibroblasts, murine astrocytes (34), and murine brain endothelial cells (35) were maintained as monolayer cultures in a complete MEM supplemented with 10% FBS (HyClone), L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin–streptomycin (all from Life Technologies). The human glioblastoma D54 temozolomide-resistant (D54 develop) cells were generated from the D54 glioma cell line (36) and were maintained in identical media. The human cell lines were tested at the MD Anderson Characterized Cell Line Core Facility using short tandem repeats DNA profiling. The cell lines were free of Mycoplasma and pathogenic murine viruses (assayed by Scientific Applications International Co.).

Antibodies and reagents

The following antibodies were titrated and used in this study: anti-CD31, anti-ET₄R (BD Biosciences); anti-ET₃R (Santa Cruz Biotechnology); anti-glial fibrillary acidic protein (GFAP; BioCare Medical); anti-glutathione S-transferase A5 (GSTA5; Novus Biologicals); anti-Ki67, anti-alpha smooth muscle actin (α-SMA; AbCam); anti-AKT, anti–phospho-AKT (Ser473); anti-MAPK, anti–phospho-MAPK (Thr202 and Tyr204), anti-Twist1, anti-Bcl2L1, anti–β-actin (Cell Signaling Technology); goat-anti-rat Alexa 594, goat-anti-rabbit Alexa 488, rabbit-anti-goat Alexa 488 (Invitrogen); rabbit-anti-goat FITC (Jackson ImmunoResearch Laboratories).

The selective ET₄R antagonist BQ123, selective ET₃R antagonist BQ788, temozolomide [4-methyl-5-oxo-2,3,4,6,8-pentaazabicyclo[4.3.0]-nona-2,7,9-triene-9-carboxamide], and 3,3′-diaminobenzidine were purchased from Sigma-Aldrich. Macitentan [N-[5′-4-Bromophenyl]-6-[2-[(5-bromo-2-pyrimidinyl) ethoxy]-2-pyrimidinyl] N′-propylsulfamide] was provided by Actelion Pharmaceuticals, Ltd. Atrasentan was purchased from Diverchim SA. Zibotentan was purchased from APAC Pharmaceutical LLC.

Immunofluorescence microscopy and TUNEL assay

Sections were fixed in ice-cold acetone for 15 minutes, incubated in protein blocking solution for 20 minutes, and then at 4°C overnight with primary antibodies (1:100) in blocking solution. Control samples were incubated with corresponding IgG isotype antibodies (1:100). Samples were rinsed three times in PBS, incubated in blocking solution for 20 minutes, and then incubated with corresponding secondary antibodies (1:1500) in blocking solution for 1 hour and then mounted. TUNEL was performed using a commercial apoptosis detection kit according to the manufacturer’s instructions (Promega). Formalin-fixed paraffin sections of clinical and experimental glioblastoma (LN-229) were processed and stained with anti–GFAP antibody (1:400). GFAP-positive cells were detected with stable 3′,3′-diaminobenzidine (Research Genetics). All images were captured with an Olympus BX-51 microscope equipped with a DP71 digital camera and then processed with DP Controller and DP Manager software (Olympus America Inc.).
Chemoprotection assay

To evaluate the effect of astrocytes and brain endothelial cells on the sensitivity of LN-229 glioma cells to temozolomide, we performed a series of in vitro chemoprotection assays, as previously described (33). In brief, murine astrocytes, endothelial cells, and 3T3 fibroblasts were transfected with GFP genes and then plated along with LN-229 glioma cells (cancer cell: test cell plating ratio of 1:2) onto individual wells of sterile 6-well plates and allowed to stabilize overnight. In some experiments, the cocultured cells were treated with 100 nmol/L of type-specific endothelin antagonists, atrasentan (ETAR), zibotentan (ETAR), BQ123 (ETR), BQ788 (ETBR), BQ123 and BQ788, or with 100 nmol/L of the dual endothelin receptor antagonist, macitentan, for 2 hours before being challenged with 20 μg/mL temozolomide. After 72 hours, the GFP-labeled cells were separated from LN-229 glioma cells by FACs, and the apoptotic fraction of glioma cells was determined by propidium iodide-stained DNA, as previously described (33).

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) and housed and maintained in specific pathogen-free conditions. The facilities are approved by the American Association for Accreditation of Laboratory Animal Care and meet all current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and NIH. The mice were used in accordance with institutional guidelines when they were 8- to 12-week-old.

Orthotopic implantation of human glioblastoma cells in nude mice

Glioma cells (LN-229, LN-229ebb, and D54ebb cells) were harvested in log-phase growth by briefly exposing glioma cell cultures to a solution containing 0.25% trypsin and 0.02% EDTA. The cells were washed and resuspended in Ca2+-/Mg2+-free Hanks’ balanced salt solution (HBSS). Glioblastomas were produced by stereotactically implanting either 1 × 106 cells or 2 × 106 cells in 4 μL of HBSS into the brain parenchyma of female nude mice as previously described (37).

Luciferase transfection and IVIS imaging

LN-229 cells were plated onto 24-well plates at a density of 5 × 104 cells per well in MEM containing 10% FBS and placed in a 37°C incubator overnight. Firefly luciferase lentivirus (Capital Biosciences) was diluted in MEM with polybrene (Millipore) to a final concentration of 8 μg/mL and added to each well. After an overnight incubation period, the media was replaced with polybrene-free MEM. The infected LN-229 cells were selected using puromycin (0.5 μg/mL) and individual clones were screened for luciferase activity by measuring their light emission with the Xenogen IVIS-100 system after adding D-luciferin (150 μg/mL). Bioluminescent imaging of orthotopically implanted luciferase-labeled glioma cells was achieved by i.p. injection of 150 mg/kg D-luciferin to mice. Measurements were collected on a calibrated instrument and photon flux from the tumor was monitored each week. The exposure time, F-stop, and pixel binning were optimized in Living Image software (Xenogen Corp.) and the bioluminescent signal was displayed as an intensity map.

Therapy experiments

Therapy was initiated when orthotopically implanted glioblastomas were considered established (21–24 days after implantation) as determined by bioluminescent imaging analysis using the Xenogen IVIS Imaging System. Temozolomide was administered daily using an oral dose of 7.5 mg/kg at different schedules. Macitentan was administered daily using an oral dose of 10 mg/kg. Zibotentan was administered daily using an oral dose of 20 mg/kg, whereas atrasentan was administered daily by i.p. injection at a dose of 10 mg/kg. Throughout the course of the survival studies, mice were monitored for weight loss, dehydration, and onset of any abnormal neurologic signs, such as lethargy, hyperkthesis, tilted neck, or circling. Animals that exhibited these signs were euthanized by i.p. injection of 1 g/kg of nembutal. All survival durations were recorded and all brains were harvested and examined using routine histologic and immunohistochemical techniques. Reconstitution formulae were 0.5% DMSO for temozolomide, 0.25N NaHCO3 for atrasentan, 0.5 mg/mL PBS for zibotentan, and 0.05% (wt) methylcellulose solution containing 0.05% (vol) Tween 80 for macitentan.

Western blot analysis

Western blot analyses for AKT and MAPK and their phosphorylated forms were carried out as previously described (33). LN-229 glioma cells were orthotopically implanted into brains of nude mice and 21 days later the mice were treated with vehicle (n = 6) or 10 mg/kg macitentan (n = 6) each day for a period of 3 weeks. Mice were euthanized and proteins were extracted from the glioblastomas with lysis buffer consisting of 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 1% DTT, a phosphatase inhibitor (Roche), and a protease inhibitor cocktail (Roche).

MGMT promoter methylation assay

We evaluated the methylation status of the MGMT promoter using a method previously described (38). Thirty-six female nude mice harboring 3-week-old LN-229, LN-229ebb, or D54ebb orthotopically implanted glioblastomas were randomized into the following treatment groups: vehicle, temozolomide, macitentan, macitentan plus temozolomide. Treatment was started 21 days following glioma cell implantation. Macitentan was administered daily, whereas temozolomide was administered daily on a 1-week-on 2-weeks-off schedule. Therapy was administered for 4 weeks and DNA was extracted from glioblastoma tissues 3 hours after the final treatment.

Sodium fluorescein permeability assay

We assessed the permeability of the tumor vasculature to the fluorescent tracer sodium fluorescein (Sigma: NaFl: MW 376 Da), as previously described (39). In brief, we implanted 2 × 106 LN-229 cells into the brain parenchyma of female nude mice and then randomized the mice into 2 treatment groups: control (vehicle; n = 5) and macitentan (n = 5). Tumors were allowed to develop for 21 days after which the mice were treated daily with vehicle or 10 mg/kg of macitentan for a period of 2 weeks. Four hours after the final dose of macitentan, the mice were injected i.p. with 20 mg of NaFl in 200 μL of sterile normal saline. The NaFl was allowed to circulate for 10 minutes and then the mice were euthanized and their brains processed for frozen sections. Sections were stained for CD31 expression as described above.
Small-animal magnetic resonance imaging

MRI monitoring to evaluate the effect of macitentan treatment on the uptake of contrast agent in orthotopically implanted LN-229 glioblastoma tumors in Swiss nude mice (Charles River Laboratories) was performed by Oncodesign. Twenty-four hours before glioma cell implantation, the mice were irradiated with a γ-source (whole-body irradiation, 2.5 Gy, 60Co, INRA). Glioblastomas were generated as described above and 21 days following the implantation of glioma cells, the mice were randomized into two groups and were administered vehicle (n = 4) or 10 mg/kg macitentan (n = 4) once daily for 21 consecutive days. Images were obtained weekly once treatment started using optimized parameters defined with and without contrast agent. Imaging was performed on a 4.7T horizontal magnet (PharmaScan, Bruker Biospin GMBH, Germany) equipped with an actively shielded gradient system. MR images were acquired using ParaVision (PV5.0, Bruker Biospin). Mice were anesthetized using 2% isoflurane and positioned supine in a mouse cradle that was inserted in a volume coil within the PharmScan. Scout images were acquired for calibration purposes and adjustments were performed to optimize shim, RF power, and amplification of the MR signal. T2 and then T1 pre-contrast images were collected and mice were administered a bolus injection of 0.2 mmol/kg gadopentetate dimeglumine (Gd-DTPA, Magnevist, Bayer Healthcare Pharmaceuticals) through a 24-gauge catheter that was inserted in the caudal vein. Three minutes after the contrast agent was administered, T1 post-enhancement images were obtained. MR images were analyzed with ImageJ and the percentage of enhancement following contrast was calculated by voxelwise subtraction of T1 pre-signal from T1 post-signal.

Pericyte coverage and microvascular density

To determine the extent of pericyte coverage of tumor-associated blood vessels, we stained tumor sections from each of the treatment groups with CD31 (red) and α-SMA (green; Abcam) antibodies and then with their corresponding secondary antibodies. Four fields from each section were randomly selected at a magnification of ×400, and those 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 as previously described (40). Brain sections from mice that had been treated with vehicle, macitentan, temozolomide, or combined macitentan plus temozolomide for a period of 21 days were processed for immunofluorescence microscopy and labeled with anti-CD31 antibodies and then with Alexa594 secondary antibodies. A minimum of 3 tumors from each of the treatment groups was used in the analysis and brains harvested from 3 normal (non-tumor bearing) mice were also included in the analysis.

Statistical analysis

Statistical analyses for the chemoprotection assay, pericyte coverage, and tumor MVD were performed with Prism 6.01 (GraphPad Software) using the Student t test. A P value of <0.05 was considered statistically significant. For in vivo studies, Kaplan–Meier survival plots were generated and comparisons between survival curves were made using the log-rank statistic.

Results

Heterogeneous expression of ET₄R and ET₆R in experimental LN-229 glioblastomas patterns their distribution in clinical tumors

We compared expression of GFAP, ET₄R, and ET₆R in LN-229 glioblastomas that were orthotopically implanted in nude mice with expression in clinical glioblastoma specimens. GFAP is the prototypical marker for immunohistochemical identification of reactive astrocytes (41). Experimental and clinical tumors were highly invasive and surrounded and infiltrated by GFAP⁺ astrocytes (Fig. 1). ET₄R and ET₆R were heterogeneously expressed on glioma cells and tumor-associated endothelial cells in both experimental and clinical tumors.

Macitentan abolishes astrocyte- and brain endothelial cell–mediated protection of LN-229 glioma cells from temozolomide

Reports suggest that astrocytes (33) and brain endothelial cells (33, 42) may contribute to treatment failure by protecting cancer cells from chemotherapy. We found that murine astrocytes and murine brain endothelial cells cocultured with LN-229 glioma...
cells protected the cancer cells from temozolomide. The apoptotic index of LN-229 glioma cells coincubated with murine astrocytes and treated with temozolomide was significantly reduced in comparison with LN-229 glioma cells cultured alone in temozolomide (P < 0.05; Fig. 2A). To determine whether astrocyte-mediated protection of glioma cells was mediated through endothelin signaling, we treated cell cultures for 2 hours with type-specific endothelin receptor antagonists BQ123 (ET\textsubscript{AR}), BQ788 (ET\textsubscript{BR}), BQ123, and BQ788, or with the dual antagonist, macitentan, and then further incubated the cells in temozolomide. The chemoprotective effect was still observed in LN-229 glioma cells that were coincubated with astrocytes and treated with either BQ123 or BQ788. However, treatment with both BQ123 and BQ788 or with macitentan nullified the protection.

The apoptotic index of LN-229 cells coincubated with brain endothelial cells in temozolomide was also significantly reduced when compared with LN-229 cells growing alone in temozolomide (P < 0.01; Fig. 2B). Neither BQ123 nor BQ788 alone had any effect on brain endothelial cell–induced chemoprotection, whereas the protective effect was abolished by the combined administration of BQ123 and BQ788 or macitentan. Murine fibroblasts had no effect on the chemosensitivity of glioma cells (Fig. 2C).

Combination therapy with macitentan and temozolomide produces regression of established orthotopic LN-229 glioblastomas

Next, we conducted several survival studies to test the efficacy of macitentan and temozolomide in established glioblastomas in brains of nude mice (i.e., therapy as opposed to prevention studies). To ensure that tumors were established before administering therapy, we labeled LN-229 glioma cells with luciferase and monitored their intracranial growth using noninvasive bioluminescence imaging. In the first study, we implanted 2 \( \times \) 10\textsuperscript{5} luciferase-labeled LN-229 cells into the brain parenchyma of nude mice and then randomized the mice into 4 treatment groups (\( n = 10 \) mice/group): vehicle (control), temozolomide, macitentan, and macitentan plus temozolomide. Temozolomide was administered daily using a 7-days-on/14-days-off regimen and macitentan was administered daily. Treatment was initiated 24 days after implantation of glioma cells and the study was terminated on day 98. Macitentan therapy alone had no effect on survival. Temozolomide prolonged survival (P < 0.05), but only mice that received macitentan plus temozolomide were alive at the culmination of the study (P < 0.0001, log-rank test; Fig. 3A). We were unable to detect luminescence signals in mice that received the combination treatment and no glioblastoma cells were identified by histologic analysis of brain sections. Results from the bioluminescence imaging analysis are shown in Supplementary Fig. S1. Representative gross images of coronal sections from the brains of mice harboring LN-229 glioblastomas and treated with the different therapies are shown in Supplementary Fig. S2A. The top of Supplementary Fig. S2B shows a representative histologic image of a normal mouse brain and the brain of a mouse 3 weeks after implantation of LN-229 glioma cells. The bottom of Supplementary Fig. S2B depicts representative images of brains from LN-229 tumor-bearing mice that received short-term (3 weeks) therapy. Representative images from the histologic analysis of brain sections from mice harboring LN-229 glioma cells, as well images from mice harboring temozolomide-resistant glioblastomas (LN-229\textsubscript{Res} and D54\textsubscript{Res}) from later experiments, are depicted in Supplementary Fig. S2C.

In the second study, we implanted 1 \( \times \) 10\textsuperscript{5} luciferase-labeled LN-229 cells into the brains of nude mice (\( n = 10 \) mice/group) and initiated treatment 28 days later. Temozolomide was administered using a dose-dense 7-days-on/7-days-off regimen. The study was terminated on day 130. Macitentan administered alone did not prolong survival. The dose-dense temozolomide schedule prolonged survival in the temozolomide-treated group (P < 0.01), but by day 120, all mice that had received only temozolomide were dead (Fig. 3B). In contrast, all mice treated with macitentan plus temozolomide therapy were alive on day 130 (P < 0.0001,
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do endothelial cells in vehicle- and temozolomide-treated mice, but AKT and MAPK were expressed by glioma cells and tumor vascular
associated endothelial cells in all mice (Fig. 4A). The activated forms of ETAR and ETBR were expressed on glioma cells and tumor-asso-
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We performed biomarker analyses on LN-229 glioblastomas from mice that were treated for a period of 3 weeks. Temozolo-
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ciated endothelial cells in all mice (Fig. 4A). The activated forms of AKT and MAPK were expressed by glioma cells and tumor vascular
endothelial cells in vehicle- and temozolomide-treated mice, but were deficient in tumors from mice treated with macitentan or macitentan plus temozolomide. We confirmed that macitentan downregulates expression of the phosphorylated forms of AKT and MAPK in LN-229 in orthotopically implanted glioblastomas using Western blot analysis (Fig. 4B). The antiapoptotic proteins Bcl2L1, Gsta5, and Twist1 localized to glioma cells and tumor-associated endothelial cells in mice from control and temozolomide groups, but were downregulated in tumors from mice that received macitentan (Fig. 4C). We observed robust glioma cell division in mice treated with vehicle, temozolomide alone, and macitentan alone. However, only few dividing glioma cells were present in tumors from mice treated with macitentan plus temozolomide. Only mice treated with macitentan plus temozolomide had large numbers of apoptotic glioma cells and apoptotic tumor-associated endothelial cells.
Macitentan plus temozolomide produces durable responses; rare recurrent glioblastomas remain responsive to therapy
Next, we questioned whether mice treated with macitentan plus temozolomide would remain disease free after cessation of treat-
ment and, if not, whether recurrent tumors would respond to additional cycles of macitentan and temozolomide. Treatment
commenced 3 weeks after the orthotopic implantation of luciferase-labeled LN-229 glioma cells into mice. Temozolomide was
administered using the 7-days-on/14-days-off regimen. Treatment was stopped on day 98, when only those mice treated with
macitentan plus temozolomide were alive (P < 0.0001, log-rank test; Supplementary Fig. S3A). These mice did not exhibit signs or
symptoms of a brain mass and no tumors were detected using bioluminescence imaging. We continued weekly imaging on
these mice and on day 146 (48 days after cessation of therapy), 2 of 16 mice developed recurrent glioblastoma (Supplementary
Fig. S3B) and were returned to therapy. One mouse died on day 167, whereas the other mouse responded well to treatment and
had no evidence of detectable disease on day 181. On day 195 (97 days after cessation of therapy), a third mouse had a relapse of
glioblastoma (Supplementary Fig. S3C). Therapy with macitentan plus temozolomide was restarted and the disease was stable on
day 302 when the mouse was euthanized.

Figure 3.
Combination therapy with macitentan and temozolomide (TMZ) significantly increases overall survival in mice with established LN-229 glioblastomas. A, the Kaplan–Meier plot of mice bearing orthotopically implanted luciferase-
labeled LN-229 glioma cells that were treated with vehicle (control), temozolomide, macitentan, or combination therapy with macitentan plus temozolomide (n = 10 mice/group). Treatment was initiated on day 24 when Tumors were established as determined by bioluminescence imaging. Temozolomide was administered daily using a 7-days-on/7-days-off regimen and macitentan was administered daily. B, the Kaplan–Meier plot of mice that was started on treatment 4 weeks after tumor cell implantation (n = 10 mice/group). Temozolomide was administered daily using a 7-days-on/7-days-off regimen and macitentan was administered daily.
log-rank test) and had no evidence of intracranial disease as determined by bioluminescence imaging.
Macitentan downregulates survival pathways in glioma cells and tumor-associated endothelial cells, which enhances their
sensitivity to temozolomide
To determine the efficacy of macitentan plus temozolomide in treating temozolomide-resistant tumors, we established the temo-
zolomide-resistant LN-229 cell line (LN-229Res) by exposing mono-
layers of LN-229 cells to increasing concentrations of temozolomide for the course of 9 months. LN-229Res cells are approximately four times more resistant to temozolomide when compared with LN-
229 parental cells (IC50 15 μg/mL vs. IC50 3.5 μg/mL, respectively).
Therapy was initiated on 2-week-old LN-229Res brain tumors using a 1-week-on/2-weeks-off temozolomide schedule. One mouse in the macitentan plus temozolomide group was euthanized on day 97 due to rectal prolapse. The study was terminated on day 112 when only mice treated with macitentan plus temozolomide were alive (P < 0.0001, log-rank test; Fig. 5A). Histologic examinations of the brains from mice treated with combination therapy revealed no evidence of disease (Supplementary Fig. S2C).
Next, we evaluated macitentan and temozolomide in D54 temozolomide-resistant (D54Res) tumors. D54Res glioma cells
(IC50 200 μg/mL) are almost 60 times more resistant to temozolomide when compared with LN-229Res cells. Treatment was
initiated 2 weeks after orthotopic implantation of D54Res cells. After 3 weeks of therapy, 3 mice were randomly selected from each
group, euthanized, and their brains processed for immunofluo-
rescence studies. By day 60, all mice in the control and temozolomide groups had died (Fig. 5B). Two of the 8 mice that received macitentan plus temozolomide also died during the study (on days 62 and 70). Six of the 8 mice in the combination treatment group were still alive on day 90 when the study concluded

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Macitentan downregulates survival pathways on glioma cells and tumor-associated endothelial cells in LN-229 glioblastomas. A, representative images from the different experimental groups after mice had received 21 days of therapy. ETα and ETβ and phosphorylated AKT (pAKT) and phosphorylated MAPK (pMAPK) are depicted in green. Blood vessels were labeled with an antibody directed against CD31 (red). B, Western blot analysis of phosphorylated AKT and phosphorylated MAPK expression in orthotopically implanted LN-229 glioblastomas. Mice were treated with vehicle or 10 mg/kg macitentan each day for a period of 3 weeks. C, combination therapy with macitentan and temozolomide (TMZ) leads to targeted destruction of glioma cells and tumor-associated endothelial cells. Representative images were collected from the different experimental groups following 21 days of therapy. Macitentan downregulates expression levels of Bcl2L1, Gsta5, and Twist1 proteins, which are labeled green. 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.
therapy was initiated 2 weeks later when tumors were established. Kaplan glioma cells are 57 times more resistant to temozolomide (IC50 in vitro – 15 μg/mL) than parental LN-229 glioma cells (IC50 in vitro – 200 μg/mL). tumor mass on subsequent histologic analysis of brain sections from mice in virtually all tumor sections collected from mice that received atrasentan or zibotentan. Tumors from mice treated with macitentan plus temozolomide appeared to be supported by the fewest number of blood vessels. Indeed, we observed tumor-associated endothelial cell apoptosis in tumor sections from mice in virtually all tumor sections collected from mice in the macitentan plus temozolomide group.

Methylation status of the MGMT promoter correlates with glioblastoma resistance to temozolomide

Expression levels of MGMT in glioblastomas may determine a response of the tumors to alkylating agents. Epigenetic silencing of the MGMT gene via promoter methylation decreases MGMT expression in tumor cells (4). Compromising the MGMT DNA repair mechanism can, therefore, increase chemosensitivity (43). We evaluated the MGMT promoter methylation status in LN-229, LN-229Res, and D54Res glioblastomas that were harvested from the brains of mice that had been treated for 4 weeks with vehicle, temozolomide, macitentan, or temozolomide plus macitentan. Methylation of the MGMT promoter progressively decreased in GBM cells that were more resistant to temozolomide (Supplementary Fig. S5). However, we did not find a correlation of selective ETaR antagonist, atrasentan, and with the type-specific ETbR antagonist, zibotentan. Similar to our initial analysis, the apoptotic index of LN-229 glioma cells that were coincubated with astrocytes and cultured in temozolomide was significantly reduced in comparison with LN-229 glioma cells cultured alone in the presence of temozolomide (Fig. 6A). Treatment with 100 nmol/L of macitentan eliminated the astrocyte-mediated chemoprotective effect, whereas neither 100 nmol/L of atrasentan nor 100 nmol/L of zibotentan decreased astrocyte-induced chemoprotection.

We performed a survival study to compare the therapeutic efficacy of combining temozolomide with atrasentan or zibotentan with temozolomide plus macitentan therapy. Nude mice harboring 21-day-old glioblastomas were randomized into 5 treatment groups (n = 13 mice/group): control, temozolomide, atrasentan plus temozolomide, zibotentan plus temozolomide, and macitentan plus temozolomide. Three mice from the macitentan plus temozolomide treatment group were randomly removed and euthanized after 3 weeks of therapy and their brains harvested for immunohistochemical studies. This time point was selected to coincide with the initial decline in bioluminescent signal observed in this treatment group and also because we were unable to locate tumors for analysis in mice that received extensive combination therapy. For comparison, we collected the brains of 3 randomly selected mice from the other treatment groups when they were euthanized due to their disease. Temozolomide was administered according to the 1-week-on/2-weeks-off schedule. The survival study concluded on day 120 when only mice in the macitentan plus temozolomide group were alive (P < 0.0001, log-rank test; Fig. 6B). The results of this study demonstrate that the addition of atrasentan or zibotentan to temozolomide does not provide a survival benefit for mice with orthotopically implanted experimental glioblastomas when compared with temozolomide alone.

The immunofluorescent labeling results of dividing (Ki67) and apoptotic (TUNEL) cells in LN-229 were remarkably similar to our findings generated on D54Res glioblastomas. Glioma cell division was readily apparent in tumors from all mice, with the exception of mice that received macitentan plus temozolomide therapy (Fig. 6C). We also noted very few apoptotic cells in tumor sections from mice that received atrasentan or zibotentan. Tumors from mice treated with macitentan plus temozolomide appeared to be supported by the fewest number of blood vessels. Indeed, we observed tumor-associated endothelial cell apoptosis in tumor sections from mice in virtually all tumor sections collected from mice in the macitentan plus temozolomide group.
ET$_{A}$R antagonists alone have no effect on astrocyte-mediated chemoprotection of LN-229 glioma cells. A, comparison of the apoptotic index of LN-229 glioma cells that were pretreated with 100 nmol/L of the type-selective ET$_{A}$R antagonist, atrasentan, or the ET$_{A}$R type–specific antagonist zibotentan, or with 100 nmol/L of the dual endothelin receptor antagonist, macitentan, before coincubation with astrocytes. Each individual single-cell test condition was compared with its corresponding coculture condition. Each assay was conducted in triplicate with $n=3$ in each group. Statistical analysis was performed using the Student $t$ test; *, $P<0.05$. Combination therapy with temozolomide (TMZ) plus atrasentan or zibotentan does not provide a survival benefit when compared with temozolomide alone. (Continued on the following page.)
methylated in LN-229Res glioblastomas from temozolomide-treated mice, but these mice did not have a significant survival benefit when compared with vehicle-treated mice with LN-229Res.

**Combined macitentan plus temozolomide reduces the MVD of LN-229 experimental glioblastomas**

Macitentan relaxes pulmonary blood vessels and is currently used for the treatment of pulmonary arterial hypertension. We studied the effects of macitentan on the vasculature of experimental glioblastomas. We first questioned whether macitentan might be affecting the permeability of tumor-associated blood vessels and noted that similar amounts of NaF accumulated in tumors from both vehicle- and macitentan-treated groups of mice, suggesting that macitentan had little effect on the permeability of tumor-associated blood vessels (Supplementary Fig. S6A). We also performed a series of MRI studies in which mice harboring established LN-229 gliomas were treated with vehicle or macitentan each day for a period of three weeks. Mice were imaged in weekly intervals once the treatment started for a period of four weeks using optimized imaging parameters defined with and without contrast agent. No contrast agent was observed in vehicle- or macitentan-treated tumors when the tumor bulk was less than 60 mm$^3$ (Supplementary Fig. S6B, top). However, when the tumors exceeded 60 mm$^3$, both vehicle- and macitentan-treated tumors became permeable to the contrast agent (Supplementary Fig. S6B, bottom). These results suggest that effectiveness of combined macitentan plus temozolomide therapy was not likely due to the ability of macitentan to enhance delivery of temozolomide to LN-229 glioblastomas.

Next, we evaluated the maturation status of the tumor-associated blood vessels from mice harboring LN-229 glioblastomas that had been treated with vehicle, macitentan, temozolomide, or macitentan plus temozolomide for a period of 3 weeks. We used pericycle coverage as an index for vessel maturation and vessels that were at least 50% covered by pericytes were considered to be positive for pericycle coverage. We noted that approximately 80% of the blood vessels in normal brain sections were covered by pericytes, whereas only 5% of tumor-associated blood vessels were considered positive for pericycle coverage (Supplementary Fig. S6C). None of the treatments used in our study altered pericycle coverage.

Finally, we evaluated the MVD of the LN-229 glioblastomas from mice that had been treated with the different therapies for a period of 3 weeks. The MVD of LN-229 glioblastomas was significantly less than MVD of normal brain tissue ($P < 0.0001$; Supplementary Fig. S6D). Only combined macitentan plus temozolomide therapy significantly reduced the MVD of LN-229 glioblastomas. These results are consistent with our Tunel analysis demonstrating that combined macitentan plus temozolomide produced apoptosis of tumor-associated endothelial cells.

**Discussion**

Accumulating evidence suggests that glioma stem cells reside in a vascular niche, where they are the recipients of endothelial cell-derived signals that maintain their self-renewal properties (10), protect them from temozolomide (42), and promote their expansion (10, 44). Efforts to impede tumor growth by eradicating the vascular niche have proved challenging; glioblastoma-associated endothelial cells are refractory to radiation and temozolomide treatment (42), and anti-VEGF therapies increase glioma cell invasion (17). Here, we demonstrate that inactivation of the endothelin receptor signaling pathway with dual endothelin receptor antagonists, including macitentan, abolishes astrocyte- and brain endothelial cell–mediated chemoprotection of glioma cells and downregulates survival pathways in glioma cells and associated endothelial cells. The elimination of these protective barriers renders glioma cells and their vasculature sensitive to temozolomide resulting in durable responses in mice.

Previously, we reported that the collective modulation of a subset of antiapoptotic proteins could provide an index of cancer cells sensitivity to chemotherapy (45). The results of the present study support that notion and indicate that the absence of Bcl2L1, Gsta5, and Twist1 expression in glioblastomas signified a tumor that is susceptible to chemotherapy. Whether Bcl2L1, Gsta5, and Twist1 can serve as predictive biomarkers and identify other types of tumors that will respond to chemotherapy is currently being evaluated.

The observation that macitentan downregulates these antiapoptotic proteins in tumor blood vessels and glioma cells could be expected, given that endothelin is a known survival factor for endothelial cells (46, 47) and cancer cells (21, 44), including those of glial origin (27, 28).

Although macitentan produced a profound effect on survival-related protein expression in the glioblastoma microenvironment, it had no antitumor activity as a single agent. This finding may help to explain the disappointing results from clinical trials of single-agent endothelin receptor antagonists in cancer patients (21). For example, atrasentan produced only partial responses (8%) in a phase I trial in patients with recurrent glioblastoma (48). Combination studies of type-selective endothelin receptor antagonists with chemotherapy have also failed to demonstrate improved clinical benefit (49). Indeed, our results predict that the addition of a type-selective endothelin receptor antagonist to temozolomide would not provide a benefit beyond that of chemotherapy alone. The heterogeneous expression of ET$_4$R and ET$_R$ in glioblastomas reported here, and elsewhere (27), and the finding that both ET$_4$R and ET$_R$ contribute to astrocyte- and brain-endothelial cell–mediated chemoprotection of glioma cells.
advocate the use of a dual endothelin receptor antagonist combined with temozolomide for clinical evaluation in glioblastoma. It should be noted, however, the only clinical trials evaluating a dual receptor endothelin antagonist in combination with chemotherapy have also proved unsatisfactory. In a phase II trial in patients with stage IV melanoma, the combination of the dual endothelin receptor antagonist, bosentan, with dacarbazine therapy had no effect on tumor progression (50). In comparison with bosentan, macitentan has a significantly lower dissociation rate and a 15-fold greater receptor occupancy half-life, which results in increased potency and sustained target blockade (51, 52). When macitentan is administered in vivo, it is metabolized into a major active metabolite, ACT-132577, that has a long half-life (40–65 hours in humans) and functions as a dual endothelin receptor antagonist (51, 53).

Acquired resistance to temozolomide remains a major barrier for effective treatment of glioblastomas; approximately 90% of recurrent glioblastomas are refractory to additional temozolomide therapy (1). One mechanism whereby glioma cells become resistant to temozolomide involves the selection of pre-existing temozolomide-resistant cells in the parental tumor (54). Our data suggest that combination therapy with macitentan and temozolomide does not necessarily select for a drug-tolerant phenotype. In the 3 mice with recurrent disease following cessation of therapy, one tumor responded to additional cycles of macitentan and temozolomide, whereas another tumor did not progress after treatment was resumed. Macitentan plus temozolomide therapy also produced durable responses in almost 90% of mice with temozolomide-resistant tumors. As mentioned above, the response of LN-229 tumor did not appear to be related to hypermethylation of the MGMT promoter. Rather, the significant results of combined macitentan plus temozolomide obtained in temozolomide-resistant glioblastomas suggest that the pharmacologic effects on supporting stromal cells are critical to the success of this therapy. Disruption of the communication between endothelial cells and glioma cells, which is thought to be essential for glioma progression and survival (10, 39, 41, 55), may be responsible for the profound glioma cell death observed in our models. The escape mechanism that allowed two temozolomide-resistant tumors to progress on macitentan plus temozolomide therapy is an area of active investigation in our laboratory.

The results from the control groups of mice in our survival analyses are consistent with those of other investigators using the LN-229 model (56). However, we are unaware of any previous study that has demonstrated durable therapeutic responses in experimental LN-229 glioblastomas. For comparison, targeted inhibition of focal adhesion kinase and insulin-like growth factor-1 receptor with a dual tyrosine kinase inhibitor increased median survival in the LN-229 glioblastoma model from 26 days in the vehicle-treated group to 47 days in the experimental arm (56). Our experience with LN-229 and D54 glioblastoma models indicates that tumor regression occurs gradually and usually becomes manifest after approximately 30 to 40 days of continuous treatment with macitentan plus temozolomide, which may be related to the kinetics of tumor cell and endothelial cell division in glioblastoma (9). Regardless, the durable responses observed in the different glioblastoma models suggest that combination therapy with macitentan and temozolomide might represent an important new therapeutic approach for the treatment of glioblastoma.

Disclosure of Potential Conflicts of Interest

W.K.A. Yung is a consultant/advisory board member for Actelion and Merck. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-J. Kim, H.J. Lee, H.J. Choi, J. He, Q. Wu, K. Aldape, J.S. Weinberg, W.K.A. Yung, U. Regenass, I.J. Fidler


Writing, review, and/or revision of the manuscript: S.-J. Kim, H.J. Lee, H.J. Choi, J. He, Q. Wu, I.J. Fidler

Study supervision: S.-J. Kim, F. Lehembre, I.J. Fidler

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Sun-Jin Kim, Ho Jeong Lee, Mark Seungwook Kim, et al.


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