

# Increased expression of glutamate transporter GLT-1 in peritumoral tissue associated with prolonged survival and decreases in tumor growth in a rat model of experimental malignant glioma

## Laboratory investigation

RITA SATTLER, PH.D.,<sup>1,2</sup> BETTY TYLER, B.A.,<sup>3</sup> BENJAMIN HOOVER, B.A.,<sup>1</sup> LUKE T. CODDINGTON, B.S.,<sup>1</sup> VIOLETTE RECINOS, M.D.,<sup>3</sup> LEE HWANG, M.D.,<sup>3</sup> HENRY BREM, M.D.,<sup>3</sup> AND JEFFREY D. ROTHSTEIN, M.D., PH.D.<sup>1,2</sup>

<sup>1</sup>Department of Neurology, <sup>2</sup>Brain Science Institute, and <sup>3</sup>Department of Neurosurgery, Johns Hopkins University School of Medicine, Baltimore, Maryland

**Object.** Gliomas are known to release excessive amounts of glutamate, inducing glutamate excitotoxic cell death in the peritumoral region and allowing the tumor to grow and to expand. Glutamate transporter upregulation has been shown to be neuroprotective by removing extracellular glutamate in a number of preclinical animal models of neurodegenerative diseases, including amyotrophic lateral sclerosis and Parkinson disease as well as psychiatric disorders such as depression. The authors therefore hypothesized that the protective mechanism of glutamate transporter upregulation would be useful for the treatment of gliomas as well.

**Methods.** In this study 9L gliosarcoma cells were treated with a glutamate transporter upregulating agent, thiamphenicol, an antibiotic approved in Europe, which has been shown previously to increase glutamate transporter expression and has recently been validated in a human Phase I biomarker trial for glutamate transporter upregulation. Cells were monitored in vitro for glutamate transporter levels and cell proliferation. In vivo, rats were injected intracranially with 9L cells and were treated with increasing doses of thiamphenicol. Animals were monitored for survival. In addition, postmortem brain tissue was analyzed for tumor size, glutamate transporter levels, and neuron count.

**Results.** Thiamphenicol showed little effects on proliferation of 9L gliosarcoma cells in vitro and did not change glutamate transporter levels in these cells. However, when delivered locally in an experimental glioma model in rats, thiamphenicol dose dependently (10–5000 μM) significantly increased survival up to 7 days and concomitantly decreased tumor size from 46.2 mm<sup>2</sup> to 10.2 mm<sup>2</sup> when compared with lesions in nontreated controls. Furthermore, immunohistochemical and biochemical analysis of peritumoral tissue confirmed an 84% increase in levels of glutamate transporter protein and a 72% increase in the number of neuronal cells in the tissue adjacent to the tumor.

**Conclusions.** These results show that increasing glutamate transporter expression in peritumoral tissue is neuroprotective. It suggests that glutamate transporter upregulation for the treatment of gliomas should be further investigated and potentially be part of a combination therapy with standard chemotherapeutic agents. (<http://thejns.org/doi/abs/10.3171/2013.6.JNS122319>)

**KEY WORDS** • glioblastoma • EAAT2 • thiamphenicol • glutamate • oncology • rat • GLT-1

MALIGNANT glioma, or glioblastoma multiforme, is the most common and most aggressive brain tumor in adults, affecting approximately 8.2 of every 100,000 people in the US each year. Standard of care treatments, including surgery followed by radiotherapy and chemotherapy, increase survival up to 2 years, and, therefore, there is still a need for improved therapies. In the search for therapeutic targets, extensive research in glioblastoma biology led to the identification of numerous cell-signaling pathways, especially those related to activated tyrosine kinases, growth factors, and tumor sup-

pressor proteins.<sup>8,26,29</sup> Another attractive therapeutic target has been the neurotransmitter glutamate, whose role in tumor growth and expansion has been studied extensively over the last decade. Glioblastomas have been shown to release glutamate via the glutamate-cystine exchanger system X<sub>c</sub><sup>-</sup>.<sup>38,49,50</sup> The subsequent increase in extracellular glutamate promotes tumor growth and tissue invasion due to activation of glutamate receptors on the glioma cell itself and activation of glutamate receptors on neighboring healthy neurons. This leads to peritumoral seizurelike activity<sup>3,51</sup> and eventually excitotoxic cell death,

*Abbreviations used in this paper:* ALS = amyotrophic lateral sclerosis; DIV = days in vitro; FBS = fetal bovine serum; PBS = phosphate-buffered saline; TAP = thiamphenicol.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

## Neuroprotective glutamate transporters in glioblastoma

creating space in the peritumoral vicinity for the tumor to expand.<sup>35,43,44</sup> This process is facilitated by the fact that glioma cells lack glutamate transporters, which, under physiological conditions, buffer the released glutamate and keep extracellular glutamate at homeostatic levels.<sup>6,49</sup> This increase in extracellular glutamate in glioma cells has not only been shown in *in vitro* and *in vivo* animal models but was also reported in human studies that involved the use of microdialysis probes in the brains of ambulatory glioblastoma patients, confirming excess glutamate concentrations at the tumor margins.<sup>27</sup>

Here we describe a novel approach to prevent glutamate-mediated tumor progression by enhancing glutamate transporter-mediated glutamate uptake. Glioma cells have reduced levels of glutamate transporter expression that are inversely correlated with the tumor grade: Grade IV glioblastomas have significantly lower levels of glutamate transporter protein than Grade III and Grade II tumors.<sup>9</sup> This lack of glutamate uptake is thought to facilitate glioma growth and expansion. Interestingly, enhancing glutamate transport by overexpressing glutamate transporter EAAT2/GLT-1 in glioma cells *in vitro* and *in vivo* reduced cell proliferation and tumor growth in 2 independent studies.<sup>9,48</sup> Furthermore, increased glutamate uptake in peritumoral tissue is hypothesized to protect glutamate-mediated neuronal cell death and thereby prevent tumor invasion into healthy tissue.<sup>35</sup> These experiments strongly suggest that restoring glutamate uptake offers a therapeutic intervention for glioblastoma patients. The approach of enhancing glutamate uptake as a neuroprotective measure has recently been applied in our laboratory to studies in ALS, both *in vitro* and *in vivo*.<sup>36</sup> A drug screen for glutamate transporter activators led to the discovery of ceftriaxone, a  $\beta$ -lactam antibiotic, as a transcriptional activator of EAAT2/GLT-1. Cells treated with ceftriaxone showed increased levels of EAAT2/GLT-1 protein and function, and when ceftriaxone was administered to an animal model of ALS disease, the *SOD1* mutant mouse, the animals showed increased survival compared with vehicle-treated controls.<sup>36</sup> Based on these results, ceftriaxone is currently being studied in a Phase I/II clinical trial for ALS (ClinicalTrials.gov ID: NCT00349622). Meanwhile, overexpression of EAAT2/GLT-1 with ceftriaxone has been shown to be neuroprotective in a number of other animal models for neurodegenerative and psychiatric diseases, including Huntington disease, Parkinson disease, stroke, and depression.<sup>18,22,25,28,31,42,45</sup> Based on these observations, we studied the efficacy and toxicity of another glutamate transporter upregulating agent, thiamphenicol, in an experimental rat model of glioma. While ceftriaxone and thiamphenicol both showed moderately significant activity in our previous studies,<sup>36,39</sup> thiamphenicol was among the top hits arising from our library screen for CNS-penetrable transporter modulators (complete data set of library screen: <http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi>; Bioassay AID: 1587) and has been tested *in vitro*, *in vivo*, and in a Phase I biomarker trial for its glutamate transporter upregulating activity.<sup>36,39</sup> Based on its ability to increase EAAT2 in humans and its blood-brain barrier penetration, we considered thiamphenicol the compound of choice for the present “proof of principle” study.<sup>2,12,30,39</sup>

This current study shows that *in vitro*, thiamphenicol does not increase glutamate transporter levels in a glioblastoma cell line, nor does it have a significant impact on glioma cell proliferation. However, local delivery of thiamphenicol increased protein levels of EAAT2/GLT-1 in peritumoral tissue, increased neuron counts in peritumoral tissue, and dose dependently decreased tumor growth *in vivo*, increasing survival of tumor-bearing rats.

## Methods

Approval for the study was obtained from the Johns Hopkins University Institutional Animal Care And Use Committee.

### *In Vitro Analysis of Thiamphenicol Treatment on Glioma Cell Proliferation*

Thiamphenicol was tested for its cytotoxic properties against the 9L gliosarcoma cell line. The 9L cells were obtained from UCSF Brain Tumor Tissue Bank (San Francisco, CA). The cells were maintained in DMEM with 10% FBS and grown in a humidified environment at 37°C and 5% CO<sub>2</sub>. To test for effects on proliferation, 9L cells were grown on 96-well plates at 2.5 × 10<sup>3</sup> cells/well and treated with thiamphenicol at increasing concentrations (range 0.1–100 μM) for 72 hours. Cell viability was determined using the CellTiter 96 AQ<sub>ueous</sub> One Solution Proliferation Assay (Promega).

### *In Vitro Analysis of Thiamphenicol Treatment on Glutamate Transporter Levels*

To measure thiamphenicol’s efficacy in upregulating glutamate transporter gene expression in the 9L gliosarcoma cell line, 9L cells grown to confluence on 6-well plates were treated with increasing concentrations of thiamphenicol (3–100 μM) for 3 days and 5 days. For protein analysis, cultured cells were lysed directly with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X, and 0.1% SDS). Total protein concentrations of cell lysates were quantified with BioRad DC Protein Assay kit and 10 μg of total proteins per lane were loaded on precast 10% polyacrylamide gels (BioRad) for SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). After transfer, immunoblots were probed with primary antibodies, anti-GLT-1 (1:1000) and anti-Actin (1:1000, Sigma), and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, GE Healthcare). Immunoblots were detected with SuperSignal West Pico chemiluminescent substrates (Thermo Scientific) and visualized using an ImageQuant LAS-4010 (GE). Images were captured using the “precision” setting and standard resolution/sensitivity. Band volumes were determined with ImageQuant TL analysis software. For mRNA analysis, total RNA from cultures cells was isolated by using RNeasy Kit (Qiagen), which was then reverse transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time polymerase chain reaction was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems; rnGLT1 = Rn00568080+\_m1; 18S

= 4319413E). Samples were normalized to 18S RNA expression.

#### *Primary Neuron-Astrocyte Coculture*

Primary astroglial cells were cultured from 2- to 3-day-old mouse pups. Cortices were dissected out and dissociated with papain and subsequently cultured on collagen-coated T75 flask in DMEM containing 10% FBS. At 14 DIV astroglial cells were seeded into collagen-coated 6-well plates at a concentration of  $7 \times 10^5$  cells/well. Primary cortical neurons were isolated from cortices of E16 embryonic mice. After dissociation with papain, 1 million neurons were seeded per well on top of the confluent astrocytes. Cocultures were first maintained in Neurobasal medium (Gibco/Invitrogen) supplemented with 5% FBS and 2% B27. After 4 days, half of the medium was changed into serum-free Neurobasal medium supplemented with 2% B27. Cells were treated with thiamphenicol at 5 DIV for 3–5 days accordingly. Glutamate transporter levels (protein and/or RNA) were determined as described above for 9L cells.

#### *Dose-Dependent Efficacy of Intracranial Delivered Thiamphenicol*

Fifty F344 rats received an intracranial injection of 9L gliosarcoma cells in accordance with previously published methods.<sup>11,46</sup> Briefly, animals were anesthetized by intraperitoneal injection of 0.4 ml per body weight of a stock solution containing ketamine and xylazine, with a dose of 75 mg/kg and 7.5 mg/kg, respectively, and their heads were shaved and prepared with ethanol and Prepoxyne. A midline incision was made and a bur hole drilled over the parietal lobe using a 2-mm drill bit and a dental drill. When the dura was exposed, the animal was transferred to the stereotactic frame (also draped and equipped with a sterile needle), and a needle containing the tumor cells was placed into the brain at a depth of 3.5 mm. The solution of cells (2  $\mu$ l) was delivered over 1 minute, the needle removed, and the area washed with sterile saline. The wound was then closed with sterile autoclips. The animals were then randomly divided into the following groups, with 10 animals/group: 1) saline treatment, 2) 10  $\mu$ M thiamphenicol delivered via Alzet pump, 3) 30  $\mu$ M thiamphenicol delivered via Alzet pump, 4) 100  $\mu$ M thiamphenicol delivered via Alzet pump, and 5) 5000  $\mu$ M thiamphenicol delivered via Alzet pump. All pumps (Durect) delivered drug over a 28-day period. Five days following 9L cell injection, animals were reanesthetized, and a subcutaneous subscapular pocket was made. The sterile pump was placed into the pocket and the cannula was placed into the intracranial bur hole and glued in place on the skull with Super Glue. The incision in the skin was then realigned and closed with sterile autoclips. The animal was then allowed to awaken and was returned to its cage until it fully recovered from the anesthetic.

Animals were observed daily and were killed when moribund. Brains were removed and either fixed in paraformaldehyde for measurements of tumor size and immunostaining or rapidly frozen on dry ice for biochemical analysis.

#### *Immunostaining*

Whole brains were fixed with 4% paraformaldehyde in PBS overnight at 4°C. After rinsing with PBS, tissue was cryoprotected in 30% sucrose in PBS until tissue sunk and embedded in tissue-freezing medium (Triangle Biomedical Sciences, Inc.) for cryostat sectioning. Thirty-micrometer sections were prepared for immunofluorescence staining. Sections were postfixed with 4% paraformaldehyde in PBS and incubated in blocking buffer (3% normal goat serum, 0.2% Triton X-100 in PBS) for 1 hour at room temperature, and then incubated with primary antibodies against GLT-1 (rabbit, 1:400) and NeuN (mouse, 1:500, Millipore) diluted in blocking buffer. Primary antibodies were incubated overnight at 4°C, washed, and then incubated for 1 hour at room temperature in Alexa 488/555 goat anti-mouse or anti-rabbit immunoglobulin (1:1000, Invitrogen) as a secondary antibody. Secondary antibody incubation was followed by nuclear counterstaining with Hoechst 33342 (2  $\mu$ g/ml, Invitrogen). Images were captured using Axiovision running a Zeiss Axio Imager.Z1 upright microscope. Signal intensity was measured using ImageJ (NIH). Peritumoral area is defined as the region surrounding the tumor where the Hoechst signal intensity is 50% of the tumor Hoechst signal intensity.

#### *Animal Care*

All animals were housed in standard facilities and given free access to food and water. They were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. On Day 120, efficacy studies were terminated with rats deemed as long-term survivors. They were then killed and specimens were collected and fixed in formalin after perfusion.

#### *Statistical Analysis*

In vitro results are reported as mean  $\pm$  SD. For the efficacy experiments, survival was the primary end point. Kaplan-Meier analysis was undertaken to compare survival using GraphPad Prism software, version 5.1. The Mantel-Cox log-rank test was used to compare groups, and group results were considered statistically different at  $p < 0.05$ . Probability analyses were 2 sided.

## **Results**

#### *Thiamphenicol Did Not Inhibit Proliferation of 9L Glioma Cells in Vitro*

As thiamphenicol is an antibiotic with unknown activities in glioma cells, we tested whether it would show any cytotoxic properties in 9L rodent gliosarcoma cells. The 9L cells were treated in vitro with increasing concentrations of thiamphenicol for 3 days, and cell viability was measured using a tetrazolium salt-based assay kit (see *Methods* section). Thiamphenicol increased cell proliferation of 9L cells in vitro at some concentrations but never showed inhibition of proliferation, not even at the highest concentration tested (5 mM). In comparison, known anticancer drugs such as taxol showed a dose-dependent

## Neuroprotective glutamate transporters in glioblastoma

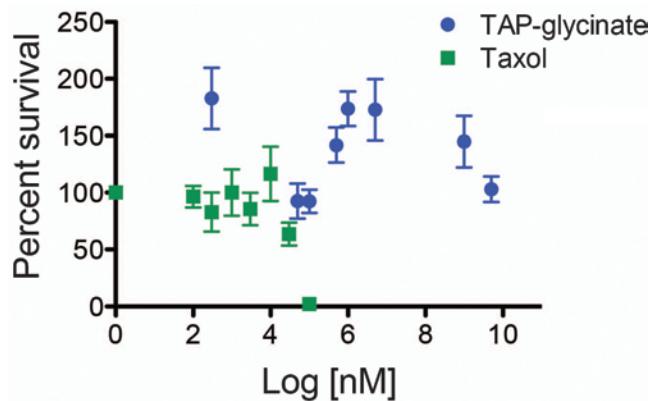
decrease in proliferation in vitro with a maximum effect at 100  $\mu\text{M}$  (Fig. 1).

### *Thiamphenicol Did Not Increase EAAT2/GLT-1 Levels in 9L Glioma Cells in Vitro*

Glioma cells are characterized by the loss of glutamate transporter expression, which explains why glutamate released by glioma cells can quickly reach excitotoxic levels and spill over into peritumoral tissue, causing neuronal cell death and thereby promoting malignant glioma progression.<sup>35</sup> While thiamphenicol has been shown to transcriptionally activate EAAT2/GLT-1 expression in healthy astrocytes,<sup>39</sup> it was not known whether it would be able to overcome the loss of transporter protein in malignant glioma cells. So 9L gliosarcoma cells were treated in vitro with increasing concentrations of thiamphenicol, and mRNA and protein levels of EAAT2/GLT-1 were measured at 3 and 5 days after treatment. Figure 2A shows that a 3-day treatment of 9L cells with 3, 10, or 100  $\mu\text{M}$  of thiamphenicol did not lead to increased GLT-1 mRNA levels in these cells. In comparison, levels of GLT-1 mRNA in primary mouse astrocyte cultures are about 15-fold higher and can be increased 2-fold when cocultured with primary cortical neurons (Fig. 2A and B, first and second bars). Similar results were obtained when cells were treated with thiamphenicol for 5 days (Fig. 2B). Glioma cells were also analyzed for EAAT2/GLT-1 protein levels, but, as expected, levels of transporter protein were undetectable in nontreated glioma cultures and were not increased with thiamphenicol (Fig. 2C). In comparison, equal amounts of thiamphenicol caused a significant increase in EAAT2/GLT-1 protein levels when applied to primary mouse cocultures (Fig. 2D) similar to what has been reported previously.<sup>39</sup> After normalization of GLT-1 protein levels to actin levels, thiamphenicol (TAP) in these cultures increased GLT-1 levels to an average of 300% over nontreated cells (ratio of GLT-1/actin: 0  $\mu\text{M}$  TAP = 100%; 3  $\mu\text{M}$  TAP = 297%; 10  $\mu\text{M}$  TAP = 300%; and 100  $\mu\text{M}$  TAP = 304%). These data suggest that any in vivo benefits observed with thiamphenicol treatment will most likely be due to increased glutamate transporter levels in the peritumoral tissue rather than direct effects on tumor tissue. Upregulated transporters in peritumoral tissues would clear glutamate released from the glioma cells, thereby reducing glutamate excitotoxicity and consequently decreasing tumor expansion.

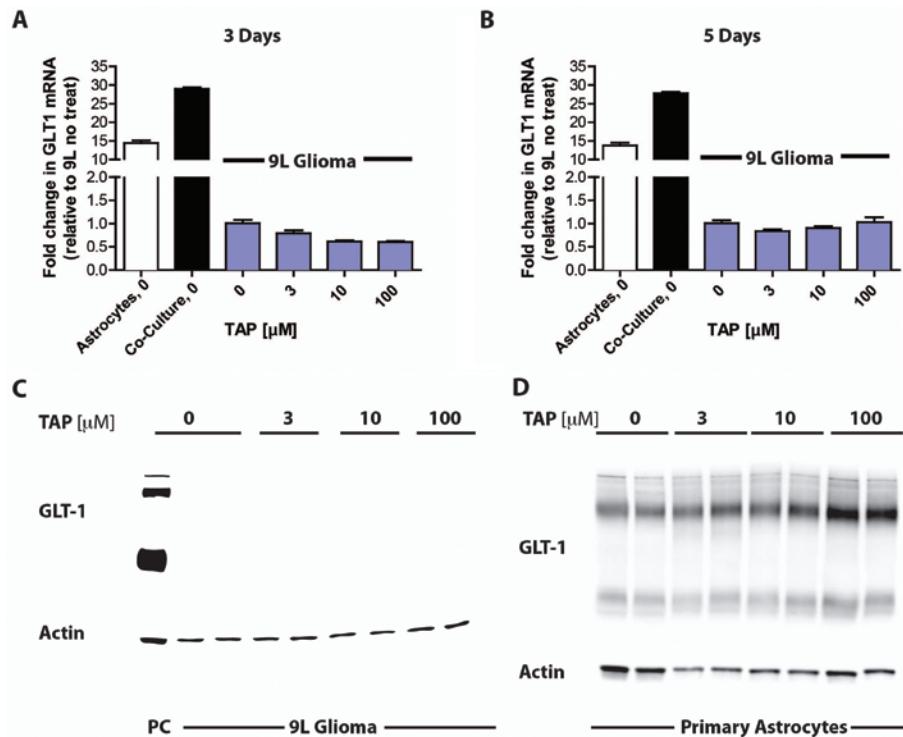
### *Thiamphenicol Dose Dependently Increased Survival and Decreased Tumor Size in Experimental Malignant Glioma Model*

Despite the known blood-brain barrier penetration of thiamphenicol and its effects on glutamate transporter levels in the CNS,<sup>2,12,30,39</sup> preliminary data of systemic thiamphenicol administration did not show significantly increased survival in our experimental malignant glioma model (data not shown). Based on the success of the Gliadel wafer (carmustine) technology, we decided to test thiamphenicol using local intrathecal delivery. We hypothesized that a positive result with a locally administered compound would still offer the possibility for the development of a therapeutic intervention using wafer implants



**Fig. 1.** Thiamphenicol (TAP) did not inhibit 9L cell proliferation in vitro. 9L gliosarcoma cells were tested for effects on cell proliferation by treating the cells for 72 hours with an 8-point dose-response curve (0.1–100  $\mu\text{M}$ ) of thiamphenicol as well as taxol, a well-known chemotherapeutic agent. Cell viability was determined using a commercially available proliferation kit. Thiamphenicol did not decrease 9L cell proliferation but increased cell proliferation at lower concentrations, which was normalized at concentrations (100  $\mu\text{M}$ ) known to increase glutamate transporter expression. Taxol showed significant cell growth inhibition as concentrations were increased.

for drug delivery. Therefore, to test the effects of thiamphenicol on survival and tumor growth in vivo, rats were pretreated with TAP for 4 days via intraperitoneal injections. On Day 0, rats received an intracranial implantation of 9L gliosarcoma and simultaneously an intracranial continuous delivery of TAP through a cannula connected to a surgically implanted mini-osmotic pump. Animals were divided into 5 groups, with 8 animals in each group receiving, respectively, a daily dose of 0, 10, 30, 100, and 5000  $\mu\text{M}$  thiamphenicol dissolved in saline. Daily survival analysis revealed that thiamphenicol-treated animals showed significant increases in survival in all treatment groups compared with saline-treated animals (median survival 21 days): 10  $\mu\text{M}$  (median survival 26 days,  $p = 0.004$ ), 30  $\mu\text{M}$  (median survival 23.5 days,  $p = 0.003$ ), 100  $\mu\text{M}$  (median survival 24.5 days,  $p = 0.011$ ), and 5000  $\mu\text{M}$  (median survival 28 days,  $p = 0.003$ ; Fig. 3A). At end stage, animals were sacrificed and brains were analyzed for tumor size. Figure 3B demonstrates a reduction in tumor size in animals that received either 100  $\mu\text{M}$  or 5000  $\mu\text{M}$  of TAP compared with control animals, although not statistically significant (median values for tumor size in saline = 46.2  $\text{mm}^2$ ; 10  $\mu\text{M}$  = 38.4  $\text{mm}^2$ ; 30  $\mu\text{M}$  = 37.8  $\text{mm}^2$ ; 100  $\mu\text{M}$  = 9.5  $\text{mm}^2$  [ $p = 0.1522$ ]; and 5000  $\mu\text{M}$  = 10.2  $\text{mm}^2$  [ $p = 0.0582$ ]). This reduction is further illustrated in representative images of brain slices obtained from treated rats (Fig. 3C). Note that the in vivo dose required to show decreased tumor size (100  $\mu\text{M}$  and 5000  $\mu\text{M}$ ) did not affect the proliferation of 9L cells in vitro (Fig. 1), suggesting that the diminished tumor growth was independent of TAP, acting as an antiproliferative agent. To test for tolerability of increasing concentrations of locally delivered thiamphenicol, control rats were sham-treated with thiamphenicol without receiving 9L cell implantations and monitored for survival. Figure 3D shows that none of the tested concentrations led to any significant animal weight loss, and no behavioral or health abnormalities were observed.



**Fig. 2.** Thiamphenicol did not increase glutamate transporter expression in 9L cells in vitro. 9L gliosarcoma cells were tested for thiamphenicol-dependent upregulation of glutamate transporter GLT-1. Neither 3-day treatment (**A**) nor 5-day treatment (**B**) of varying concentrations of thiamphenicol (0–100  $\mu$ M) increased mRNA levels of GLT-1. In comparison, GLT-1 mRNA levels in astrocytes were significantly higher than those in 9L cells and responded to neuronal activation as described before. Similarly, when TAP-treated cells were analyzed for GLT-1 protein levels, no detectable levels of GLT-1 were present in 9L cells, and TAP treatment failed to cause any increase in protein levels (**C**). Primary murine cortical astrocyte cell lysates were co-loaded as a positive control (PC). In comparison, when primary astrocytes were treated with equal concentrations of thiamphenicol, a dose-dependent increase in GLT-1 protein levels was observed, as previously described<sup>39</sup> (**D**). treat = treatment.

#### Thiamphenicol Increased GLT-1 Expression and Neuronal Survival in Peritumoral Tissue

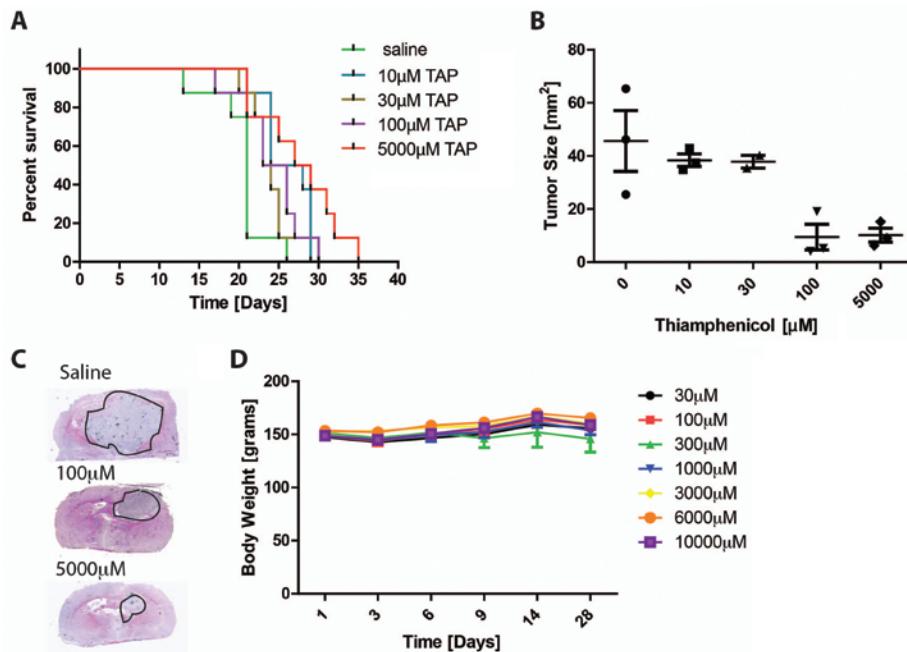
To confirm that thiamphenicol did increase glutamate transporter levels in peritumoral tissue, brains of control and TAP-treated animals were analyzed for levels of glutamate transporter protein by Western blot analysis and immunohistochemical analysis. Immunohistochemical analysis of peritumoral tissue confirmed higher levels of EAAT2/GLT-1 in the drug-treated animals (Fig. 4A), with more intense staining at the border between tumor and nontumor tissue. Quantification of the fluorescent signal revealed a significant difference between TAP- and vehicle-treated animals (controls vs TAP-treated animals, 100% vs 184% fluorescent pixel intensity,  $p = 0.0015$ ; Fig. 4B upper panel). Interestingly, co-staining of the tissue with the neuronal marker NeuN showed a significant increase in the number of neurons in the peritumoral tissue of the TAP-treated animals ( $p = 0.03$ ; Fig. 4B lower panel), suggesting that increased glutamate uptake in this tissue prevented neuronal damage. To further quantify the effects of TAP on glutamate transporter levels, multiple brain tissue samples in the peritumoral region were collected at different distances away from the tumor and analyzed separately for levels of EAAT2/GLT-1 protein. Western blot analysis confirmed the immunostaining results showing upregulation of glutamate transporter pro-

tein in the TAP-treated animals compared with saline-treated control groups ( $n = 3$ , Fig. 4B), although the finding was not statistically significant (Fig. 4C). These data suggest that the higher levels of glutamate transporter protein in the peritumoral region may contribute to the decrease in tumor size and subsequent increase in animal survival.

#### Discussion

The role of glutamate in brain cancer progression has long been discussed, but few therapeutic attempts targeting this pathway have been made to date. There are several different therapeutic targets for interfering with glutamate-mediated glioma growth, including glutamate receptors and the glutamate-cystine exchanger as well as glutamate transporters (reviewed by de Groot and Sontheimer<sup>7</sup>). Blocking glutamate receptors seems the most logical approach, given that this would target both the actual tumor cells and the surrounding neurons, thereby preventing glioma proliferation and invasion into the space vacated by dying neurons. While the use of glutamate receptor inhibitors has not been very successful in clinical trials of other neurodegenerative disorders, such as stroke, due to severe side effects in patients,<sup>13</sup> the noncompetitive AMPA receptor antagonist talampanel has been tested in 2 independent Phase II clinical trials for the treatment of

## Neuroprotective glutamate transporters in glioblastoma



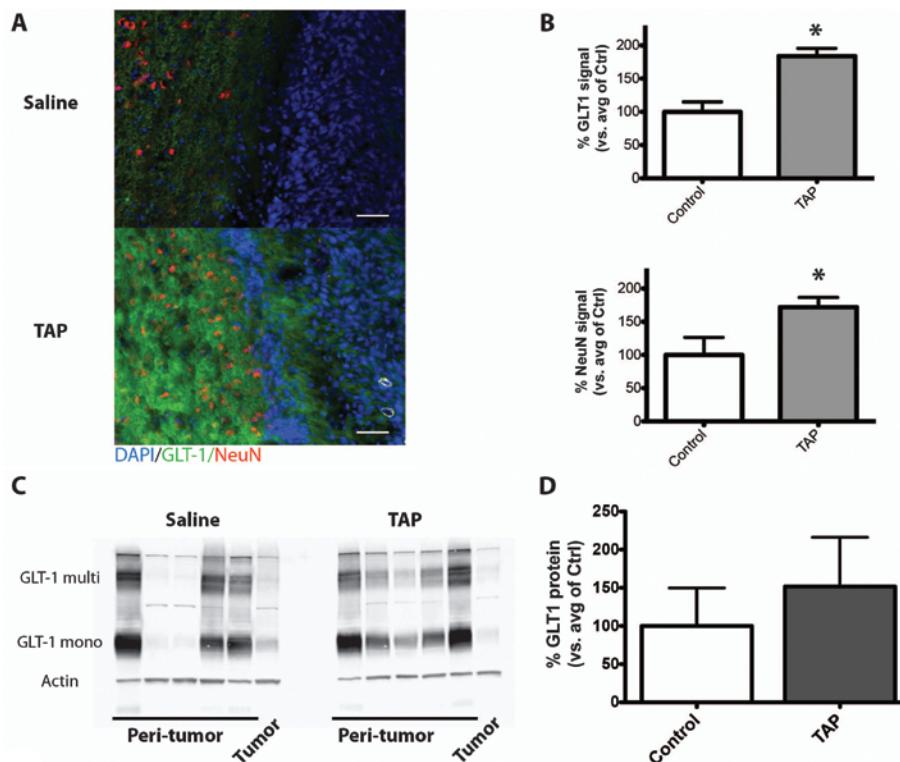
**Fig. 3.** Thiamphenicol dose dependently increased survival and decreased tumor size in experimental malignant glioma model. **A:** F344 rats were treated with intraperitoneal injections of thiamphenicol for 5 days. On Day 6, they received an intracranial injection of 9L gliosarcoma and were implanted with a mini-osmotic pump chronically delivering varying doses of thiamphenicol ( $n = 10/\text{group}$ ). Animals were monitored for survival, and data were plotted using a Kaplan-Meier survival analysis. The data indicated a dose-dependent increase in survival. **B:** Increasing doses of thiamphenicol greatly reduced the tumor size in the animals. **C:** Representative illustrations of brains obtained from animals treated with different doses of thiamphenicol (0, 100, and 5000  $\mu\text{M}$ ). At the end stage, animals were sacrificed and brain tissue was harvested for tumor size analysis ( $n = 3/\text{group}$ ). **D:** Control animals not receiving 9L cell injections were treated with increasing doses of locally delivered thiamphenicol (via minipumps) to test for toxicity and intolerance of drug treatment ( $n = 3/\text{group}$ ). Increasing doses of thiamphenicol maintained body weight and did not induce any toxicity in the animals.

malignant glioma.<sup>16,19</sup> When given in combination with radiation therapy and temozolomide, talampanel showed significant increase in survival in the absence of signs of unwanted side effects due to receptor inhibition.<sup>15,16</sup> In addition, the uncompetitive NMDA receptor blocker memantine, approved for the treatment of Alzheimer disease, and the mGluR II antagonist LY341495 showed decreased glioma proliferation in preclinical studies *in vivo* and *in vitro*.<sup>1,37</sup>

Preventing the inappropriate release of glutamate may also prohibit its tumorigenic activity. Glutamate release from glioma cells occurs via system  $x_c^-$ , a glutamate-cystine exchanger, releasing glutamate into the extracellular space in exchange for cystine.<sup>38,49</sup> Preclinical studies using system  $x_c^-$  inhibitors show that preventing glutamate release and thereby reducing extracellular glutamate levels reduces tumor growth *in vitro* and *in vivo*.<sup>4,40</sup> Sulfasalazine, a drug clinically used to treat chronic inflammatory bowel disease, has been found to inhibit system  $x_c^-$  and has been shown to not only prevent tumor growth but also peritumoral seizures caused by excessive glutamate stimulation.<sup>3-5,32</sup> Based on this, a small ( $n = 10$ ) Phase I trial was initiated with heavily pretreated patients with poor performance, and the trial was terminated early based on concerns of poor efficacy and brain swelling near the tumor.<sup>33,34</sup> A new Phase I study was opened recently, enrolling patients with newly diagnosed low-grade glioma

with the aim to measure glutamate levels before and after sulfasalazine treatment.<sup>7</sup>

Glutamate transporters have not been given much attention in this population of patients with gliomas compared with other neurodegenerative disorders. The data presented here suggest that increased glutamate transporter function is a valid target for therapeutic intervention in glioma patients. The interesting aspect of the neuroprotective potential of this therapy is that the drug benefits are observed primarily in the surrounding peritumoral tissue and not within the tumor tissue itself. Neuroprotection in the peritumoral environment led to significantly decreased tumor growth and expansion. Importantly, it could also potentially have effects on glioma-related seizures. Although tumor-induced seizure activity was not the focus of the current study, Buckingham et al.<sup>3</sup> reported that glutamate released from glioma cells via system  $x_c^-$  leads to hyperexcitability of surrounding extratumoral neurons and ultimately seizures, which could be prevented by inhibitors of system  $x_c^-$ . One would expect to see similar protection by increased astrocytic glutamate removal in this peritumoral environment. Indeed, ceftriaxone-induced increased EAAT2/GLT-1 expression provided significant protective effects against pentylenetetrazole-evoked generalized clonic convulsions, generalized clonic-tonic convulsions (GCTCs), and convulsion-induced death.<sup>20</sup> Similarly, ceftriaxone de-



**Fig. 4.** Thiamphenicol increased glutamate transporter expression and the number of NeuN-positive neurons in peritumoral tissue. **A:** Immunostaining of brain slices from TAP-treated xenografted animals showing increased GLT-1 (green) and NeuN (red) staining in close proximity to the tumor compared with saline-treated animals. **B:** Quantification of GLT-1 immunostaining in peritumoral tissue ( $p = 0.0015$ ,  $n = 3$  [biological replicates] and 9 [technical replicates]), as well as NeuN-positive cells ( $p = 0.03$ ,  $n = 3$  [biological replicates] and 9 [technical replicates]). **C:** Representative Western blot analysis of peritumoral tissue showing increased GLT-1 protein levels in TAP-treated peritumoral tissue versus saline-treated tissue. Note that in either group, tumor tissue itself shows low levels of GLT-1. **D:** Quantification of Western blot analysis revealing a near-significant increase in GLT-1 levels in the TAP-treated animals over saline-treated animals ( $p = 0.064$ ,  $N = 3$ ,  $n = 5$ ). Asterisks indicate statistically significant difference. Scale bar = 50  $\mu$ m. Ctrl = control.

creased seizures in a mouse model of tuberous sclerosis complex.<sup>52</sup> Furthermore, EAAT2/GLT-1-overexpressing transgenic mice showed reduced epileptogenic processes following pilocarpine-induced status epilepticus.<sup>21</sup> These studies suggest that there is a likelihood that combining treatments targeting the peritumoral tissue with traditional chemotherapeutic agents for brain tumors will have synergistic effects and thereby show improved outcomes over current therapeutic paradigms.

The glutamate transporter-activating agent thiamphenicol was only able to increase glutamate transporter levels in the surrounding peritumoral tissue, not in the actual tumor itself. The reasons for this are unknown, but given the data presented by de Groot et al.,<sup>9</sup> one could speculate that perhaps follow-up compounds with higher potency might be able to increase transporter levels inside the brain tumor tissue itself and thereby inhibit glioma cell proliferation in addition to protecting the peritumoral tissue, providing even more efficient neuroprotection and increased survival. It may also be possible that *in vivo* glioma cells are no longer capable of EAAT2 promoter activation, in part because of altered molecular regulatory mechanisms. In fact, a mechanism for decreased EAAT2/GLT-1 expression in glioma cells was proposed by Lee et al.,<sup>23</sup> suggesting that astrocyte elevated gene-1, or AEG-1,

regulates EAAT2/GLT-1 expression in glioma cells. Astrocyte elevated gene-1 was found to negatively correlate with levels of EAAT2/GLT-1 in tissue samples obtained from glioma patients. Follow-up *in vitro* studies in glioma cell lines confirmed that AEG-1 reduces EAAT2/GLT-1 expression on a transcriptional level by increasing YY1 binding to the EAAT2/GLT-1 promoter. These studies highlight AEG-1 as another potential therapeutic target for glioblastomas leading to increased glutamate transporter expression and consequent removal of excitotoxic levels of glutamate. These data could also explain why the transcriptional activation of EAAT2/GLT-1 using thiamphenicol was not effective in increasing the levels of EAAT2/GLT-1 in the glioma cells itself, but only in the surrounding astrocytes that express normal levels of AEG-1.

Removing glutamate from the peritumoral tissue could also lead to decreased glutamate levels within the tumor tissue and thereby reduced activation of AMPA receptors expressed in glioma cells. These AMPA receptors are thought to be involved in tumor signaling, proliferation, and tumorigenicity, as knockdown of the AMPA receptor GLUR1 subunit results in reduced glioma proliferation *in vitro* and reduced tumor growth *in vivo*.<sup>10</sup> It should be noted, however, that other studies showed no effect of cell proliferation when glioma cells were treated

# Neuroprotective glutamate transporters in glioblastoma

with AMPA receptor antagonists.<sup>41,47</sup> Despite these contradictory results, it is possible that protection from glutamate transporter overexpression in the peritumoral tissue could be due to decreased tumor AMPA receptor activation in addition to protection against AMPA and NMDA receptor-mediated excitotoxic cell death in noncancerous tissue. This hypothesis is further confirmed by the study described above showing increased survival in patients treated with AMPA receptor antagonist talampanel when combined with radiation therapy and chemotherapy (temozolomide).<sup>15,16</sup>

In the present study the use of local delivery of thiamphenicol, a derivative of chloramphenicol lacking any toxicity associated with aplastic anemia, was based on several considerations. Firstly, we picked thiamphenicol over ceftriaxone based on the completion of a Phase 1 biomarker study in healthy subjects confirming pharmacodynamic activity of thiamphenicol on glutamate transporter levels in human tissue.<sup>39</sup> While thiamphenicol does cross the blood-brain barrier and achieves significant concentrations in the CNS, we did not see efficacy in the current animal model with systemic delivery. We therefore decided to use a local delivery to obtain proof of principal data on the upregulation of glutamate transporter protein and function for neuroprotection in glioblastomas. In addition, we rationalized local delivery with the possibility of converting this treatment into a wafer-based slow release therapeutic based on the Gliadel wafer technology developed and validated in our laboratories.<sup>14,17</sup> At the same time, given the modest effects in this animal model, we have ongoing studies aimed at optimizing our lead “proof of principle compounds” for glutamate transporter upregulation. For example, we hope to develop an orally available ceftriaxone analog without any antibiotic activity. Similar efforts are ongoing for thiamphenicol as well as the β-carboline alkaloid harmine.<sup>24</sup>

## Conclusions

We demonstrated that increasing glutamate transporter EAAT2/GLT-1 expression in peritumoral tissue with thiamphenicol treatment effectively improved survival in a rodent model of glioblastoma when administered locally. Thiamphenicol did not affect glioma cell proliferation in vitro, but instead increased glutamate transporter levels and neuron counts in the tissue surrounding the 9L gliosarcoma and thereby increased survival in a dose-dependent manner. Furthermore, the increase in glutamate transporter levels in the peritumoral tissue reduced the actual tumor size significantly, probably due to the protection of surrounding neurons from excitotoxic glutamate levels and subsequent cell death. Additional studies should be performed to test the efficacy of this treatment in combination with traditional chemotherapy and/or with novel therapeutics targeting the glutamate-cystine exchanger system  $x_c^-$ , which could target both the glioma and the peritumoral tissue.

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*Address correspondence to:* Rita Sattler, Ph.D., Johns Hopkins University, John G. Rangos Sr. Building 2-223, 855 N. Wolfe St., Baltimore, MD 21205. email: rsattler1@jhmi.edu.