2012

Cystine/Glutamate Transporters As Prognostic & Therapeutic Markers Of Primary Brain Tumors

Stephanie Marie Robert
University of Alabama at Birmingham

CYSTINE/GLUTAMATE TRANSPORTERS AS PROGNOSTIC & THERAPEUTIC MARKERS OF PRIMARY BRAIN TUMORS

by

STEPHANIE M. ROBERT

HARALD SONTHEIMER, PH.D, COMMITTEE CHAIR
L. BURT NABORS, M.D
MICHELLE OLSEN, PH.D
PETER SMITH, PH.D

A THESIS

Submitted to the graduate faculty of the University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2012

DEDICATION

To Mom, for encouraging me to follow my dreams as far as they will take me;

To Dad, for teaching me the words ‘I can’t’ have no place in my vocabulary;

&

To Daniel, for his amazing support, patience, & understanding along this journey.

ACKNOWLEDGMENTS

I would like to thank all of the graduate students, post-doctoral fellows, faculty, and staff that I have worked with over the past year who have helped make this project and thesis a reality.

Foremost, I would like to express my sincere gratitude to my mentor, Dr. Harald Sontheimer. Without his continued support, inspiration, guidance, teaching, advice, and encouragement this thesis would not have been possible.

I would also like to thank my thesis committee, Dr. Peter Smith, Dr. Michelle Olsen, and Dr. Burt Nabors for their time and guidance. Additionally, I would like to thank the graduate students and post-docs in the Sontheimer lab for their support, guidance, and teaching over the past year. In particular, Dr. Toyin Ogunrinu and Dr. Sue Buckingham have contributed greatly to my success and to the completion of this work. Finally, I would like to express my appreciation to UAB's Center for Clinical and Translational Science (CCTS) for their support and for giving me the opportunity to complete this degree.

CYSTINE/GLUTAMATE TRANSPORTERS AS PROGNOSTIC & THERAPEUTIC MARKERS OF PRIMARY BRAIN TUMORS

STEPHANIE M. ROBERT

BASIC MEDICAL SCIENCES

ABSTRACT

Glioblastoma multiforme (GBM) are the most prevalent and aggressive malignant brain tumors. Current treatment – a combination of radiation, chemotherapy and resection – has limited effectiveness and offers poor prognosis. In this study, we examined the roles of system x_c^- (SXC) and excitatory amino acid transporters (EAATs), which transport the amino acids cystine and glutamate, on tumor growth, neurotoxicity, and peritumoral seizure activity. Tissue micro-arrays from 45 patients were examined by immuno-histochemistry, comparing tumor-bearing tissue and adjacent normal brain. Using a novel flank tumor propagation technique, we chose 3 glioma samples with varying SXC and EAAT expression levels to study their physiological differences using cell and tissue assays. Examination of patient tissues indicated glioma patients can be stratified into high or low level system x_c^- expressers, compared to normal brain. In low SXC expressing tumors, cystine uptake assays suggested an alternative transporter supplying intracellular cystine for GSH synthesis and redox protection; EAAT1 and EAAT3 were discovered to mediate this alternative transport. High SXC expression correlated with increased proliferation over 5 days, neurotoxicity in 48 and 72 hour glioma/neuron co-cultures, and a significant 67.8% increase in seizure prevalence upon intracranial implantation *in vivo*. Furthermore, system x_c^- inhibition using Sulfasalazine, an FDA approved drug, resulted in decreased proliferation of all tumor lines expressing SXC, and decreased cystine uptake and neurotoxicity in tumor lines expressing high

levels of SXC and no EAATs, during the same time points. Our results suggest that SXC should be further investigated as a prognostic marker for tumor growth and seizure activity as well as a therapeutic marker for glioblastoma patients, as our data argues for adjuvant Sulfasalazine administration in patients with glioblastomas expressing SXC.

TABLE OF CONTENTS

Page

ABSTRACT v

DEDICATION iii

ACKNOWLEDGMENTS iv

LIST OF TABLES viii

LIST OF FIGURES ix

INTRODUCTION 1

Central Hypothesis 3
Clinical Significance 4

METHODS 6

RESULTS 13

SUMMARY AND DISCUSSION 34

LIST OF REFERENCES 44

APPENDIX

A SUPPLEMENTARY AND PRELIMINARY DATA FIGURES 49

1 Western Blot Analysis of JX14 xCT Expression in Cell Culture 50
2 Radiation Sensitivity Differences between GBM22 and GBM14 51
3 Cortical Neuron Co-culture with GBM14, GBM22, and SAS 52
4 GBM22 Cell Proliferation with Nifedipine 53

B IACUC APPROVAL FORM 54

C IRB CERTIFICATION FORM 57

LIST OF TABLES

Table *Page*

1 xCT Immunoreactivity Score Sums in Grade IV Gliomas 14

2 xCT Expression Compared between Tumor Core, Edge, and Rim 17

3 xCT Expression in Tumor Core versus Normal Brain.....18

4 xCT Expression in Tumor Edge versus Normal Brain 18

LIST OF FIGURES

Figure *Page*

1 Low and High xCT Expression in Tissue Microarray Samples from Tumor Core, Edge, and Rim, in Matched Patient Samples 15

2 xCT Expression in Tissue Microarray Sample from Tumor Core, Edge, and Rim, in Single Patient Sample 16

3 xCT and CD98 Expression in Nine Xenograft Glioma Samples as determined by Western Blot 20

4 Western Blot & RT-PCR Determined xCT and EAAT Expression in Xenograft Glioma Samples Compared to Normal Human Brain 22

5 Immunofluorescence Staining of EAAT1Localization in GBM14 and GBM22 Tumor Cells.....24

6 ^{35}S-L-Cystine uptake in GBM22, GBM14, and GBM39 Xenograft Tumors with Either High or Low xCT Expression.....25

7 ^{3}H-L-Glutamate uptake in GBM22 and GBM14 Xenograft Tumors with Either High or Low xCT Expression 26

8 Basal Reduced Glutathione (GSH) levels in GBM22 and GBM14 Xenograft Tumors.....27

9 GBM22, GBM14, and GBM39 Xenograft Tumor Cell Proliferation with and without Sulfasalazine and/or TBOA Treatment.....29

10 Cortical Neuron Co-culture with GBM22, GBM14, and GBM39 Xenograft Tumors Quantified by Live/Dead Assay 31

11 Occurrence of Seizures and Average Day Post-Injection Euthanasia for Mice Implanted with GBM22, GBM14, and GBM39 Xenograft Tumors Intracranially.....33

INTRODUCTION

Gliomas are glial-derived tumors, which make up 80% of malignant brain tumors. Among them, Glioblastoma multiforme (GBM) is the most prevalent and aggressive type, leading to fatal outcomes despite expensive and intensive treatment (18, 46, 46). Current treatment options include a combination of radiation, chemotherapy and tumor resection. Despite advances in these modalities, the overall prognosis of patients with malignant brain tumors has remained virtually unchanged over the last 20 years (46). Today, the average survival rate is 9-12 months after diagnosis, with a 5 year survival rate of less than 4% (27).

Further complicating this picture is a lack of accurate and consistent prognostic markers to better predict disease progression and response in brain cancer patients. GBMs are heterogeneous tumors, and as they have the ability to diffusely invade the surrounding brain structure, complete resection is impossible (48). In addition, some glioma cells within a tumor population exhibit resistance to treatment, either to radiation therapy and/or chemotherapy (40) and as a result, patients inevitably develop recurrent disease within a few months of initial resection and treatment (44). When they do reoccur, the new tumors are refractory to treatment and ultimately lead to the patient's death within 6-9 months of reoccurrence (34). Furthermore, seizures are a common and early symptom in glioma patients, with up to 80% experiencing at least one seizure during the course of their disease (32).

Gliomas up-regulate a number of transport mechanisms to promote tumor growth and survival. System x_c^- is one of these transporters found to be increased in tumors, and it has been extensively studied in tumor growth and invasion (9, 10, 30, 42, 49). System x_c^- has also been linked to neuronal hyper-excitability and epileptic activity in a mouse model of glioma (5) and could be a source of these tumor-associated seizures.

Under physiological conditions, system x_c^- mediates the sodium independent, electro-neutral uptake of cystine in exchange for the release of glutamate (3, 4). System x_c^- has been shown to be involved in growth progression, metastasis, radiation and chemotherapy resistance of many types of tumors, including gliomas (1, 11, 15, 16, 20, 23, 31, 38, 43). Glutathione (GSH), the major antioxidant in the central nervous system (CNS), is synthesized from cysteine, glutamate and glycine. The main intracellular source of cysteine is through the influx of cystine through system x_c^- and its subsequent reduction to cysteine (8, 24). Cellular antioxidants, particularly GSH, are designed to protect cells from free radical damage; and as gliomas exhibit increased levels of free radical production (18), their survival becomes dependent upon GSH. Inhibition of system x_c^- leads to decreased cystine uptake, reduced intracellular GSH, and consequently slowed tumor growth (9).

Radiation therapy, a critical first line therapy for glioma patients, relies on the formation of double-stranded DNA breaks, ultimately leading to cell death. Radiation accomplishes this DNA damage either by directly interacting with DNA, or by creating hydroxyl radicals from surrounding water, that then interact with DNA to create strand breaks (6, 26). GSH effectively reduces these exogenously produced free radicals, and therefore serves to protect tumor cells from radiation-induced DNA damage and

ultimately cell death, conferring radiation treatment resistance to gliomas (42), leading to further treatment failure and tumor reoccurrence.

Additionally, glutamate, the main excitatory amino acid in the CNS, is released into the brain through system x_c^- in exchange for cystine uptake. Normally, extracellular glutamate is maintained at low micromolar concentrations through a class of sodium dependent glutamate transporters (13) known as Excitatory Amino Acid Transporters (EAATs) in the brain. However, in patients with glioma, it has been shown that through the release of glutamate, glioma cells expressing system x_c^- are neurotoxic (50) and show enhanced tumor growth as a result of the space clearing effect of the resulting neuronal death (50). Recently, glutamate release through system x_c^- has been shown to cause epileptic activity in a mouse model of glioma (5). It is not known what role the EAATs play in glioma neurotoxicity, excitotoxicity, or growth; however, as they remove glutamate from the extracellular space, they work in concert with system x_c^- in normal cells to prevent the extracellular accumulation of glutamate. We have found that system x_c^- and EAAT expression vary in patient tumor samples and in a xenograft model of glioma. It is possible that this variation in expression has direct consequences on individual glioma growth, malignancy, and resultant seizure activity of these tumors.

Central Hypothesis

System x_c^- is an important amino acid transporter in human cells and is up-regulated in many types of cancers. Gliomas are one of these tumors that seem to have increased their expression of system x_c^-, leading to growth and survival advantages over normal cells. However, gliomas are known for their heterogeneity, and as system x_c^- has

been determined to be an inducible transporter in cells maintained in culture, we wanted to investigate whether heterogeneity existed with respect to the expression of system x_c^- in the glioma patient population. Our hypothesis was that heterogeneity in system x_c^- expression would result in variable tumor biology, and as a result, may help explain the dramatically different tumor behavior seen clinically.

Preliminary data looking at system x_c^- expression in glioma patient surgical biopsy samples suggested system x_c^- expression did, in fact, vary both between patients, and within patient samples. With this knowledge, we hypothesized that gliomas expressing higher levels of system x_c^- would be more proliferative, have a greater ability to metastasize, show a higher degree of radiation resistance, be more neurotoxic, and cause more tumor-associated seizures in animals.

Additionally, we hypothesized that gliomas lacking system x_c^- use alternative transporters to satisfy cystine and glutamate transport, and that these may present with a very different biology; for example, slower tumor growth and/ or a lack of excitotoxicity or seizures. Our overarching hypothesis was that knowledge of which cystine/glutamate transporters individual tumors express would be beneficial in understanding and predicting the clinical progression and therapeutic options for individual patients.

Clinical Significance

The answers to the questions posed in this study have the possibility to impact patient treatment decisions and patient quality of life. For example, an FDA approved drug, Sulfasalazine (SAS), has been previously shown to inhibit system x_c^- and lead to tumor growth inhibition, both *in vitro* and *in vivo* (9). In our studies, we have found that

SAS inhibits the growth of tumors expressing both higher and lower levels of system x_c^-. This may indicate that even those tumors with low levels of system x_c^- may be treated with adjuvant SAS administration. Additionally, tumors expressing higher levels of system x_c^- may cause faster disease progression and more tumor-associated seizures than those with lower levels of system x_c^- and/or lower levels of EAATs, thereby leading to a worse prognosis for patients, and a decreased survival time, whereas those with lower system x_c^- expression may have a more favorable prognosis. This knowledge would be beneficial to physicians for patient and family counseling and for guiding treatment decisions. We therefore hypothesized that these cystine/glutamate transporters may be useful prognostic and therapeutic markers for glioblastoma patients.

METHODS

Details of Methods and Materials Used for Experiments

Drugs. All drugs and chemicals were purchased from Sigma Aldrich (St. Louis, MO), except (S)-4-Carboxyphenylglycine, which was purchased from Tocris Bioscience (Ellisville, MO).

Xenografts. Xenografts used in this study were established through subcutaneous injection of patient glioma tissue into the flanks of nude mice, as previously described (14). Tumors were maintained by serial passage via ectopic implantation into the flank of nude mice, and were harvested at defined time points based on tumor size. Tumors were mechanically minced and passaged by re-injection of 200µl of tumor subcutaneously into flanks of new nude mice.

***Glioma Cells*.** Xenograft flank tumors were harvested, mechanically and enzymatically disaggregated (enzymatic solution consisted of PBS (Phosphate Buffered Saline), 0.5% Trypsin, 1% Collagenase), and grown in flasks as spheroids in serum-free NeuroBasal medium (Invitrogen) supplemented with 10ng/ml FGF (Invitrogen), 10ng/ml EGF (Invitrogen), 2% B-27 Supplement w/o vitamin A (Invitrogen), and 260mM L-glutamine (Invitrogen) for up to one month, then flasks and medium were changed within 3 days of disaggregation , and was then changed weekly. For functional assays requiring a monolayer of cells, flasked cells were spun down, treated with Accutase for 5 min to

break up spheres, and plated for short-term cell culture (7-10 days) in DMEM/F-12 supplemented with 7% fetal bovine serum, 0.1% penicillin and streptomycin.

***Cell Proliferation*.** Proliferation was assessed by plating 150,000 cells per well of a 42-well plate (Fisher Scientific). After 3 days, the cell medium was replaced with medium containing either vehicle or drug. This treatment was repeated each day for four days. On Day 5, cells were treated with Accutase and re-suspended in 10ml bath solution consisting of the following (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1 $CaCl_2$, 1.6 Na_2HPO_4, 0.4 NaH_2PO_4, 10.5 Glucose and 32.5 HEPES acid. The pH was adjusted to 7.4 with NaOH and osmolarity was measured at ~310mOsm. On Day 0, Day 1, and Day 4, three readings were made with a Coulter-Counter Cell Sizer (Beckman-Coulter, Miami, FL) and cell number was recorded per 500µl. The mean cell number was normalized to Day 0.

***Neuron/Glioma Co-Culture*.** Cortical neurons harvested from Embryonic Day 18 (E-18) mice were plated either on coverslips or in 2-well chambered coverglass microscope plates (Lab-Tek). Neurons grown in NeuroBasal medium (Invitrogen) supplemented with 125µl/50ml L-glutamine (Invitrogen), 2% B-27 (Invitrogen), and 1% Penicillin/1% Streptomycin were used 7-14 days after harvesting. Glioma cells grown *in vitro* were spun down, re-suspended, and placed at 50,000 cells per filter on 12mm cell culture insert filters (Millicell). Filters (w/glioma cell suspensions) were transferred after 2 days to one well of the 2-well plate, while the 2^{nd} well contained control cells, or to 24-well plates containing neurons on glass coverslips. Using Time-Lapse Microscopy, plates were

imaged over 48 hours with an Axiocam MRm camera mounted on an Axiovert 200M inverted microscope equipped with a filter cube turret (Carl Zeiss, Thornwood, NJ) and motorized stage, enclosed in a 5% CO_2 incubator. 5-6 regions of interest (ROI) in each well of the 2-well plates were imaged. The 24-well plates were placed in the incubator for 72 hours, and then the filters were removed to conduct a Live/Dead® Assay (Molecular Probes™). The dyes Ethidium homodimer-1 and calcein AM were used at 2µM and 1µM, respectively. Cells were incubated in neuron medium (w/o phenol red) containing dyes for 30 min at 37ºC. Timelapse imaging was used to image neurons in previously determined ROIs. A Zeiss Axovert zoom microscope fitted with a 10x objective was used to image neurons in 24-well plates at randomly chosen ROIs. Five randomly chosen regions were imaged on each coverslip and images were taken blinded to the identity of the glioma cells co-cultured with the neurons.

***Immunohistochemistry*.** Glioma tissue microarray (TMA) consisting of 45 sets of WHO grade-IV glioma specimens from 10 institutes were collected and used for immunohistochemistry staining. TMA slides were baked at 65ºC for 1 hr, then de-paraffinized by 3 2-min xylene washes, and rehydrated by 20 dips each in 100% EtOH, 95% EtOH, 70% EtOH, and H_2O. Slides were then placed on a BondMax autostainer (Vision Biosystem, Norwell, MA), blocked, and antigen retrieval for xCT was performed for 20 min using an EDTA based buffer (pH 9.0). Slides were incubated in primary goat anti-xCT polyclonal antibody (Abcam; ab60171; 1:25 dilution) for 30 min, and then incubated in anti-goat secondary antibodies conjugated to HRP for 30 min followed with

a DAB (diaminobenzidine) substrate. Slides were counterstained with hematoxylin and put on cover slips for imaging.

Immunofluorescence microscopy. Glioma cells were plated on glass coverslips, grown to 50% confluence, then washed for 5 min with PBS and fixed with 4% paraformaldehyde for 10 min. This was followed by two 10 min washes in PBS. Cells were incubated in Blocking Buffer (PBS plus 5% donkey serum, 0.1% Triton X-100) for 30 min at RT. Cells were incubated overnight at 4ºC in anti-EAAT1 antibody (Santa Cruz; sc-15316) in blocking solution, diluted to 0.4µg/ml. Then the cells were rinsed 4 x 5-min in diluted blocking buffer (33% blocking buffer) and incubated with FITC-conjugated donkey anti-rabbit IgG for 2 hr at RT. Coverslips were washed in diluted blocking buffer 2 x 5-min each, and mounted on glass slides using Fluoro-mount mounting solution and dried in the dark for 2 hrs. A Zeiss Axiovert 200M microscope fitted with a 40x objective was used to image slides.

Western Blot. Glioma cells, or tissue, were lysed using Radioimmunoprecipitation assay buffer (RIPA), glioma cells (or tissue) were lysed as described (7). Blots were blocked with 10% milk for 1 hr and probed with primary antibody (goat anti-xCT [Abcam; ab60171], rabbit anti-EAAT1 [Santa Cruz; sc-15316], goat anti-EAAT2 [Millipore; MAB2262], or rabbit anti-EAAT3 [Santa Cruz; sc-2568]) overnight at 4ºC. For a loading control, blots were probed with mouse anti-GAPDH for 45 min at RT (0.05µg/ml, Abcam, Cambridge, MA). Then blots were washed 4 x 10-min each in TBST. Membranes were incubated in horseradish peroxidase (HRP)–conjugated secondary

antibodies (2µg/0.5ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hr at RT, washed (4 x 5-min in TBST) and blots developed using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) on a Kodak Image Station 4000MM (Kodak, New Haven, CT).

^{35}S-L-Cystine and ^{3}H-L-Glutamate Uptake. Na^+-dependent and Na^+-independent glutamate uptake was measured using ^{3}H-glutamate as previously described, with modifications (49). Na^+-dependent solution contained, in mM: 122 NaCl, 3 KCl, 1.3 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 10 Glucose, and 0.4 $MgSO_4$ (warmed to 37ºC and bubbled for 10 min with 5%CO_2/95% O_2). Na^+-independent solution contained, in mM: 122 Choline-Cl, 1.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 25 Triethylammonium bicarbonate, 10 Glucose, and 0.4 $MgSO_4$ (warmed to 37ºC and bubbled for 10 min with 5%CO_2/95% O_2). Cells were plated at 50,000 cells per well in 24-well plates and used within five days of plating. Cells were incubated with uptake solution (containing 0.1mM glutamate and 2µCi ^{3}H-glutamate (American Radiolabeled Chemical, St. Louis, MO)) for 3 min, washed 2x with ice cold PBS, and lysed using 0.3N NaOH for 30 min, then neutralized using 0.3N HCl. The Better Bradford Protein Assay (Thermo Fisher Scientific, Rockford, IL) was used to measure protein; uptake was normalized to protein from corresponding wells. L-cystine uptake assays were performed identically to L-glutamate uptake, with 2µCi ^{35}S-Cystine (American Radiolabeled Chemical, St. Louis, MO) and 100µM L-cystine in the uptake solution.

Glutathione Assay. QuantiChrom^{TM} Glutathione Assay Kit (DIGT-250) from BioAssay Systems (Hayward, CA) was used to measure reduced glutathione as described by the manufacturer. Cells were plated at a density of 200,000 per well in 6-well plates. When confluent (3-4 d), cells were placed on ice, washed once with ice cold PBS, collected, and subjected to on freeze/thaw cycle. Upon thawing, a solution containing 50mM NaH_2PO_4 and 1mM EDTA was added to the cells and samples were sonicated 2x for 5 sec; cells were cooled on ice between sonications. Cells were then centrifuged at 10,000*g* for 15 min at 4ºC. The supernatants were collected and used for the assays. The samples were read at OD_{450nm} and the following formula was used to calculate the GSH concentration:

$$(OD_{SAMPLE}\text{-}OD_{BLANK} / OD_{CALIBRATOR}\text{-}OD_{BLANK}) \times 100 \times n = GSH\ (\mu M)$$

where 'calibrator' = 100µM glutathione and the 'blank' is water. Protein was measured using the Bio-Rad DC protein assay kit, and GSH concentrations calculated were normalized to corresponding protein concentration.

Intracranial xenografts. Glioma cells were collected from flank xenografts and maintained as spheroids in NeuroBasal medium. For use, cells were pelleted and treated with Accutase. For intracranial implantation, 125,000 cells in 5µl of 5% (wt/vol) methylcellulose were stereotactically injected into the left hemisphere of 8-10 week old female C.B.17 severe combined immunodeficient (SCID) mice. Controls were injected with 5µl methylcellulose only.

EEG acquisition. To collect EEGs, intracranial recording electrodes (Plastics One) were placed on the right hemisphere (ground was placed on the left) of SCID mice five days

after intracranial tumor injections. Data was acquired at a 500Hz sampling rate, with Biopac Systems amplifiers, and collected and analyzed with AcqKnowledge 4.0 EEG Acquisition and Reader Software (Biopac Systems). Digitized files were analyzed by a blinded investigator and events were flagged for further analysis if they were 12-15Hz and ≥5x baseline amplitude. AcqKnowledge software was used to compute duration, amplitude, and frequency of events.

Data Analysis. Origin (v. 8.5.1, MicroCal Software, North Hampton, MA) software was used to graph results and Graphpad InStat 3.00 (GraphPad Software, San Diego, CA, USA Copyright 1992-1998 GraphPad Software, Inc) software was used to analyze results. Two-way ANOVA, followed by a Tukey post hoc test, was used to determine significance. For data sets with only two means tested, an unpaired t-test was used.

RESULTS

System x_c^-, a cystine/glutamate exchanger, is expressed at varying levels in human tumors

Commonly used and established glioma cells lines, including D54s, U251s, and STTG-1s are characterized by increased expression of system x_c^- (SXC) compared to non-malignant glia (9, 10). In order to investigate whether this increase in SXC is uniform across patient gliomas, samples from 45 patient biopsies were examined by immunohistochemistry for xCT protein expression on a Tissue Microarray (TMA). xCT is the catalytic subunit of SXC, and confers transporter specificity, therefore xCT is used as a marker of SXC. These samples were unique in that for each patient we had access to matching non-malignant samples as well as tumor core and tumor margin. Stainings of samples of matching tumor core (Core), tumor edge (Edge), and uninvolved brain (Rim) tissue were examined by a board certified pathologist, who was blinded to the tissue identification and location. Scores were based on intensity (0, 1, 2, or 3) multiplied by the percentage of tissue at the intensity determined (0 – 100%). Table 1 shows raw scores for each sample. Of the 45 biopsies obtained only 30 had complete sets consisting of core, edge and uninvolved brain tissue from the same patient (identified as 'good' in Table 1). Those missing one or more tissue samples (identified as 'FAIL' in Table 1) were included only in the appropriate groups (where both groups evaluated such as 'Core' versus 'Edge' were scored as 'good') for analysis. Scoring for xCT immunoreactivity varied between patient samples, with some samples receiving high

Table 1

xCT Immunoreactivity Score Sums in Grade IV Gliomas

ID#	Core		Edge		Rim	
	MT	Sum	MT	Sum	MT	Sum
1	good	0	good	0	good	0.2
2	good	1.1	good	0	good	0
3	good	1	good	1.5	good	0
4	good	0	FAIL	FAIL	good	0
5	good	0	good	0	good	0.75
6	FAIL	FAIL	good	0.5	good	0
7	good	0.1	good	0.25	good	0.2
8	good	0.05	good	0.5	FAIL	FAIL
9	good	0.1	FAIL	FAIL	good	0.05
10	good	0	good	0.1	good	0.2
11	good	1.5	good	1.5	good	0
12	good	0.3	good	1.5	good	0.25
13	good	1	good	0.5	good	0.3
14	good	2.25	good	2.45	good	0
15	good	0.1	good	0	good	0
16	good	0.5	good	1.5	good	1
17	good	0.5	good	1.05	good	0.75
18	good	1.8	good	0.3	good	0
19	good	0.5	good	0.5	good	0.1
20	good	0.2	good	0.5	good	0.5
21	good	0	good	0.25	good	1
22	good	0.1	good	0.6	good	0.6
23	good	0.9	good	0	good	0
24	good	0.1	good	1	FAIL	FAIL
25	good	0.9	good	1.4	good	1
26	good	2.25	good	2.25	FAIL	FAIL
27	good	0.2	good	0	FAIL	FAIL
28	good	0	FAIL	FAIL	good	0.25
29	good	0.3	good	0	good	0.2
30	good	0.5	FAIL	FAIL	good	0.1
31	good	0.1	good	0.1	good	0.1
32	good	0.15	good	0.2	good	0
33	good	0.5	good	1.55	good	0
34	good	1.5	good	2.5	good	0
35	good	2.7	FAIL	FAIL	good	0.5
36	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL
37	good	0.15	good	1.5	good	0.75
38	good	0.5	good	0.1	good	0
39	good	0.2	good	0.3	good	0
40	good	0.1	good	0.75	FAIL	FAIL
41	good	0	good	0	FAIL	FAIL
42	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL
43	good	1.8	good	0.05	good	0
44	good	2.25	good	1.5	good	0.5
45	FAIL	FAIL	FAIL	FAIL	FAIL	FAIL

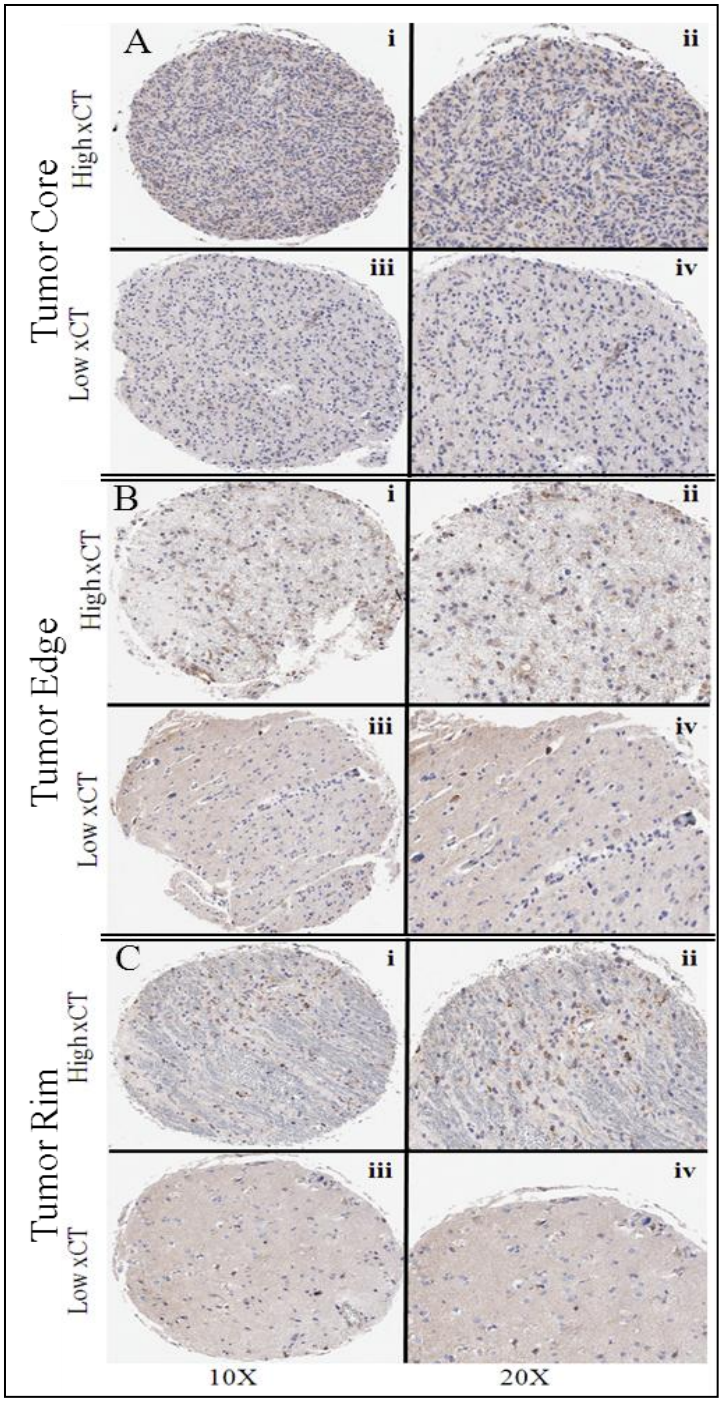


Figure 1. Low and High xCT Expression in Tissue Microarray Samples from Tumor Core, Edge, and Rim, in Matched Patient Samples. Five micron tissue sections from a glioma tissue microarray show heterogeneous xCT expression. High (i & ii) and low (iii & iv) xCT expression in tumor (**A**) Core, (**B**) Edge, and (**C**) Rim. Rim samples were taken from uninvolved brain tissue. 10X and 20X magnifications are shown.

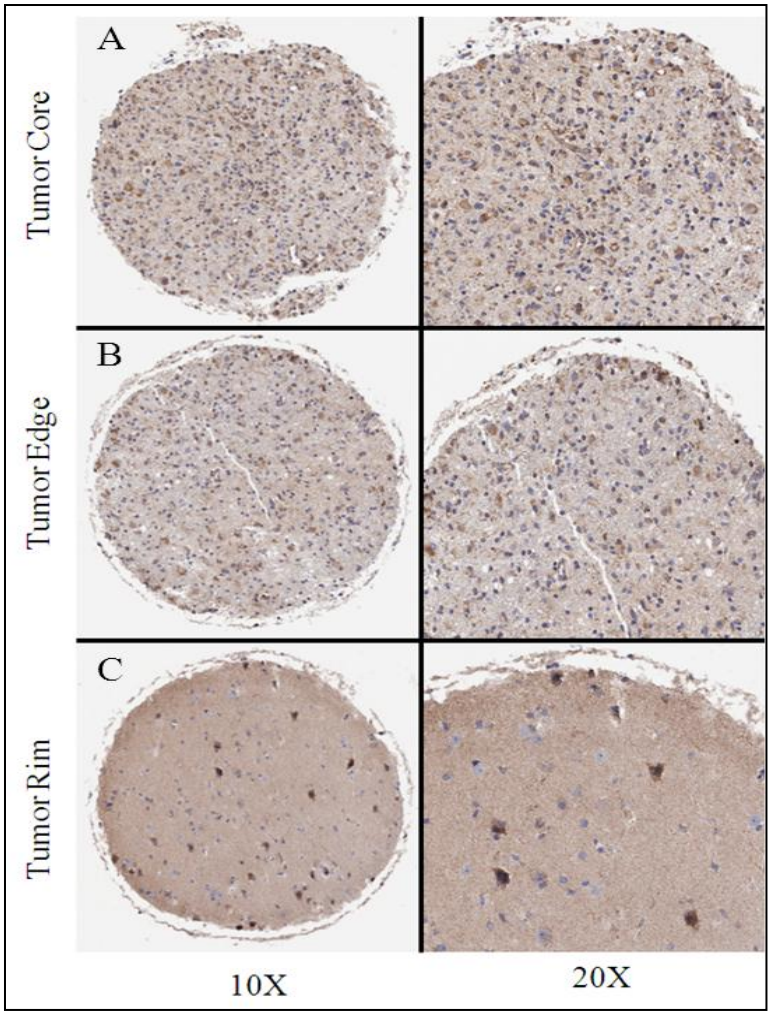


Figure 2. xCT Expression in Tissue Microarray Sample from Tumor Core, Edge, and Rim, in Single Patient Sample. Five micron tissue sections from a glioma tissue microarray show heterogeneous xCT expression within the same patient. (A) Tumor Core, (B) Tumor Edge, and (C) Tumor Rim samples show variable xCT expression. Rim samples were taken from uninvolved brain tissue. Samples shown at 10X and 20X magnifications.

scores, indicating high xCT expression levels (Fig. 1Ai & ii, 1Bi & ii, 1Ci & ii), and some samples receiving low scores, indicating low xCT expression levels (Fig. 1Aiii & iv, 1Biii & iv, 1Ciii & iv). Scores of zero indicate undetectable xCT levels (Table 1). Representative staining of samples from the tumor core, edge, and non-tumor brain (Rim) with high and low xCT expression are shown in Figure 1. Comparing the expression at the tumor core, tumor edge, and uninvolved brain in the same patient showed varying levels of xCT as well (Figure 2).

However, the difference in xCT expression between the core and edge of tumors within paired samples did not vary significantly ($p = 0.267$), unlike the expression between the Core and uninvolved brain (Rim) or the Edge and the uninvolved brain (Rim) – which did vary significantly ($p = 0.0349$ and $p = 0.0026$, respectively) (Table 2).

Table 2

xCT Expression Compared between Tumor Core, Edge, and Rim

Sample Region			¥p-value
Core	vs	Rim	0.0349*
Core	vs	Edge	0.267
Edge	vs	Rim	0.0026**

¥Wilcoxon Signed-Rank Test (P(2-tail); *p<0.05, **p<0.01

In order to compare xCT expression in the patient tumors to the xCT expression of normal brain, the 'normal' uninvolved brain (Rim) xCT expression was averaged and set as Baseline Expression, representing xCT expression in normal brain tissue. The Core and Edge xCT expressions of the tumors were compared to this baseline and identified as either being equal/below baseline expression or above baseline expression (Table 3 & 4, respectively). With a mean xCT expression of 0.076 ± 0.076, 46.3% of the Core samples

were equal/below baseline expression. The remaining 53.7% of samples, with a mean xCT expression of 1.125 ± 0.761, were above baseline expression (Table 3). The Edge samples showed similar results, with a mean xCT expression of 0.070 ± 0.094, with 40.5% of the Edge samples equal/below baseline expression, and the remaining 59.5% of samples above baseline expression, with a mean of 1.166 ± 0.681 (Table 4).

Table 3

xCT Expression in Tumor Core versus Normal Brain

Baseline Expression	# Samples	Mean xCT Expression	Percentage of Samples (%)
Equal or Below	19	0.076 ± 0.076	46.3%
Above	22	1.125 ± 0.761	53.7%

Only matched samples included; n=41

Table 4

xCT Expression in Tumor Edge versus Normal Brain

Baseline Expression	# Samples	Mean xCT Expression	Percentage of Samples (%)
Equal or Below	15	0.07 ± 0.094	40.5%
Above	22	1.166 ± 0.681	59.5%

Only matched samples included; n= 37

Calculating Total (Core + Edge) xCT expression compared to baseline gives 43.4% equal/below baseline and the remaining 56.6% above baseline. Significance was determined using Wilcoxon signed-rank test. Therefore, these results suggest that tumors can be divided into two sub classifications of gliomas – those that express higher than normal (physiological) levels of system x_c^- (high SXC) and those that express equal or lower than normal (physiological) levels of system x_c^- (low SXC).

Patient-derived xenograft tumors show high and low levels of system x_c^- expression, similar to patient biopsies

In order to study SXC function, we sought to identify suitable glioma cells that can be cultured and yet display similar differences in SXC expression as observed in the above TMA studies. Since essentially all established glioma cell lines show high SXC expression, these are not suitable for this purpose. Therefore we next examined xenografts of glioma tissue where patient-derived biopsy tissue is propagated and passaged by ectopic expression in the flanks of nude "breeder" mice (Fig. 3A). These cells essentially never see plastic or serum, as opposed to cell lines established and propagated in plates. This method of propagation and passaging of tissue has been shown to reduce cellular changes in gene and protein expression, which is frequently seen in established cell line models (37). Nine of these xenograft tumor samples were examined using Western blot for expression of xCT (Fig. 3B).

Like the patient microarrays examined by immunohistochemistry, these showed varying levels of the two SXC subunits, xCT and CD98. Five of the nine samples expressed high xCT (catalytic subunit) levels, and four expressed low or undetectable xCT levels. All of the samples expressed CD98, the regulatory subunit of SXC, but at differing levels as well (Fig. 3B). Hence these xenograft tissues appear to replicate SXC expression profiles observed across a larger patient sample in the TMAs. Based on protein expression data, we chose three xenografts – GBM 14 (low xCT), GBM 22 (high xCT), and GBM 39 (low xCT) – for further *in vitro* and *in vivo* studies (indicate by * in Fig. 3B). In order to preserve the integrity of the tumor cells, tumors were harvested from the nude mice flanks, disaggregated, and grown in NeuroBasal (serum-free) medium for

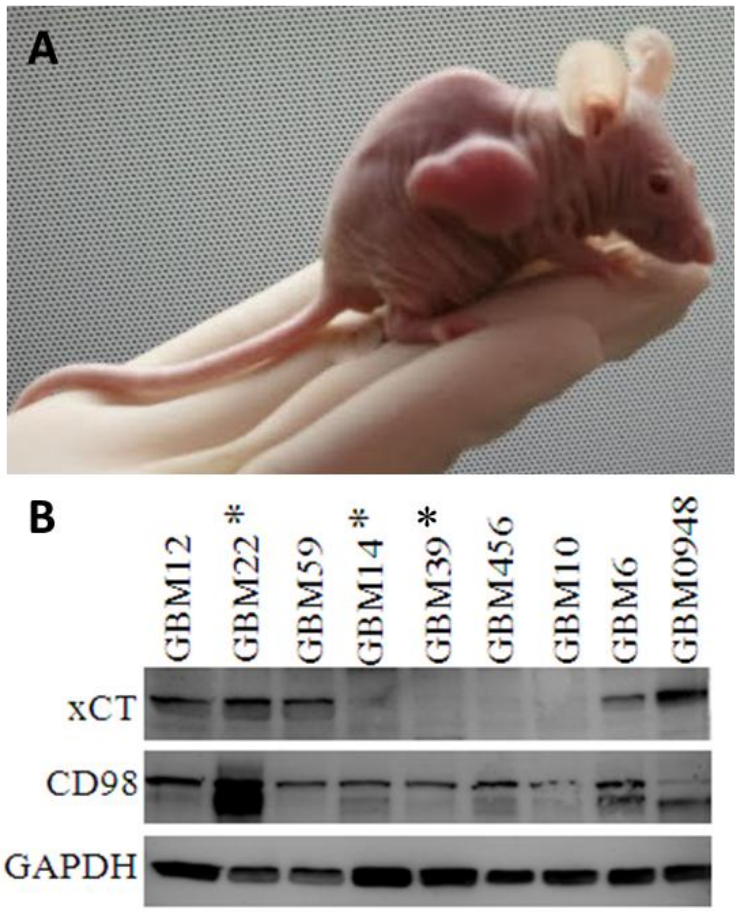


Figure 3. xCT and CD98 Expression in Nine Xenograft Glioma Samples as determined by Western Blot. Xenograft tumors show heterogenetic xCT expression. (**A**) Patient-derived xenografts were propagated and passaged in the flanks of nude mice. (**B**) Five out of nine Western blot examined xenograft tumors show high xCT expression, while the remaining show low expression or lack expression. *Indicates tumors chosen for use in this study.

up to one month. For specific assays requiring a confluent monolayer of cells, tumor cells were plated on dishes for a maximum of 7days and used for an individual experiment.

Low xCT tumors express glutamate transporters, specifically Excitatory Amino Acid Transporters 1 and 3

The near absence of SXC in some of the tumor samples is surprising since cystine uptake and subsequent GSH formation is critical to glioma cell survival (30) and SXC has been identified as a major player in cystine uptake in glioma cells (42). We therefore investigated whether other transporters may compensate for the absence of SXC and may serve to import cystine in those tumors with low xCT tumors. One class of glutamate transporters, the Excitatory Amino Acid Transporters, contains five important transporters for the release of glutamate in normal neurons and glia (13). These transporters have been previously shown to transport cystine, in addition to releasing glutamate (2, 19). Therefore, using Western blot and RT-PCR, we examined the expression of the glial transporters EAAT1, 2 and 3 (Glast, Glt-1, and EAAC1 in rodents, respectively) (Figure 4A & B). Expression of EAAT1 was seen in GBM14 (low xCT) and GBM39 (low xCT), but was not seen in GBM22 (high xCT). EAAT3 expression was seen in only GBM39 (low xCT). EAAT2 and 4 were not seen in any of the tumor lines. PCR confirmed the expression of EAAT1 in GBM14s, with levels comparable to normal brain (Fig. 4B).

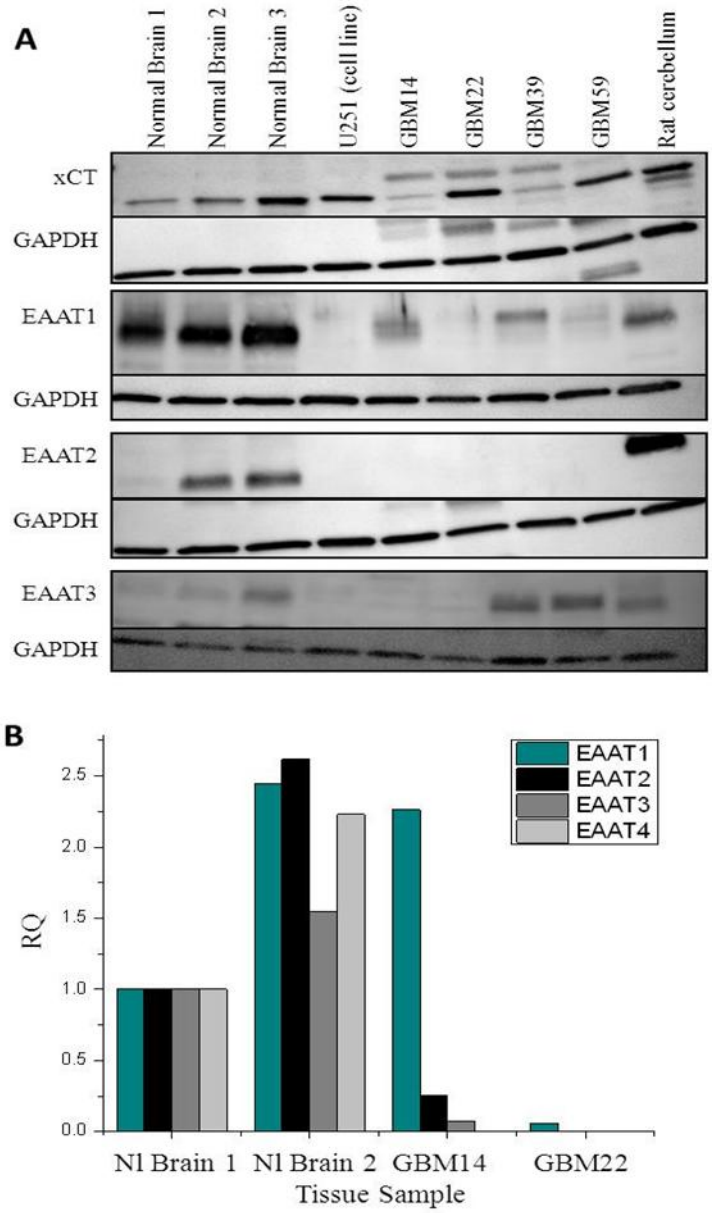


Figure 4. Western Blot & RT-PCR Determined xCT and EAAT Expression in Xenograft Glioma Samples Compared to Normal Human Brain. Varying expression of xCT, EAAT1, EAAT2, EAAT3 and EAAT4 in xenograft tumor samples was determined by Western blot and RT-PCR. (**A**) Western blot analysis showed heterogenous protein expression of xCT, EAAT1 and EAAT3. $n \geq 2$ (**B**) RT- PCR confirmed the presence of EAAT1 DNA in GBM14 tissue, comparable to normal brain levels. $n = 1$.

Previous studies have shown EAAT1 expression in glioma cell lines; however, it was found that the transporter was mis-localized to the nucleus, and was therefore not functional in those cells (47, 49). In order to determine whether these glutamate transporters were membrane localized – and therefore retain their function in the tumor cells – we used immunofluorescence to stain GBM14 and GBM22 cells for EAAT1 (Figure 5A & 5B, respectively). Unlike previously found in cell lines, EAAT1 was determined to be located on the membrane of the cells, as it co-localized with Phalloidin, a marker for cortical F-actin (Figure 5A). Interestingly, examination of GBM22 cells showed some EAAT1 protein (in agreement with PCR data in Figure 4B) that was indeed localized in the nucleus, and therefore non-functional in these cells (Figure 5B).

Cystine and Glutamate uptake correlates with SXC activity in high xCT tumors and with EAAT activity in low xCT tumors

In order to determine the functional role of SXC, EAAT1, and EAAT3 regarding cystine uptake we performed uptake assays using ^{35}S labeled cystine. As SXC is a sodium independent transporter, cystine uptake under sodium free conditions can be attributed to SXC activity. By contrast, the EAATs are sodium dependent transporters, and therefore increased uptake under sodium conditions can be attributed to EAAT activity. Total uptake under sodium and sodium-free conditions, with and without SXC and EAAT inhibitors was used to isolate the relative contribution of each to cystine uptake. The majority of cystine uptake in GBM22 (high xCT) occurred under sodium free conditions, and was decreased 36% by the SXC inhibitor Sulfasalazine, $p<0.05$ (Figure 6A). Interestingly, under sodium conditions, uptake in GBM22 cells increased by 40% ($p<0.05$), however, this uptake was not inhibited by the EAAT blockers DHK and

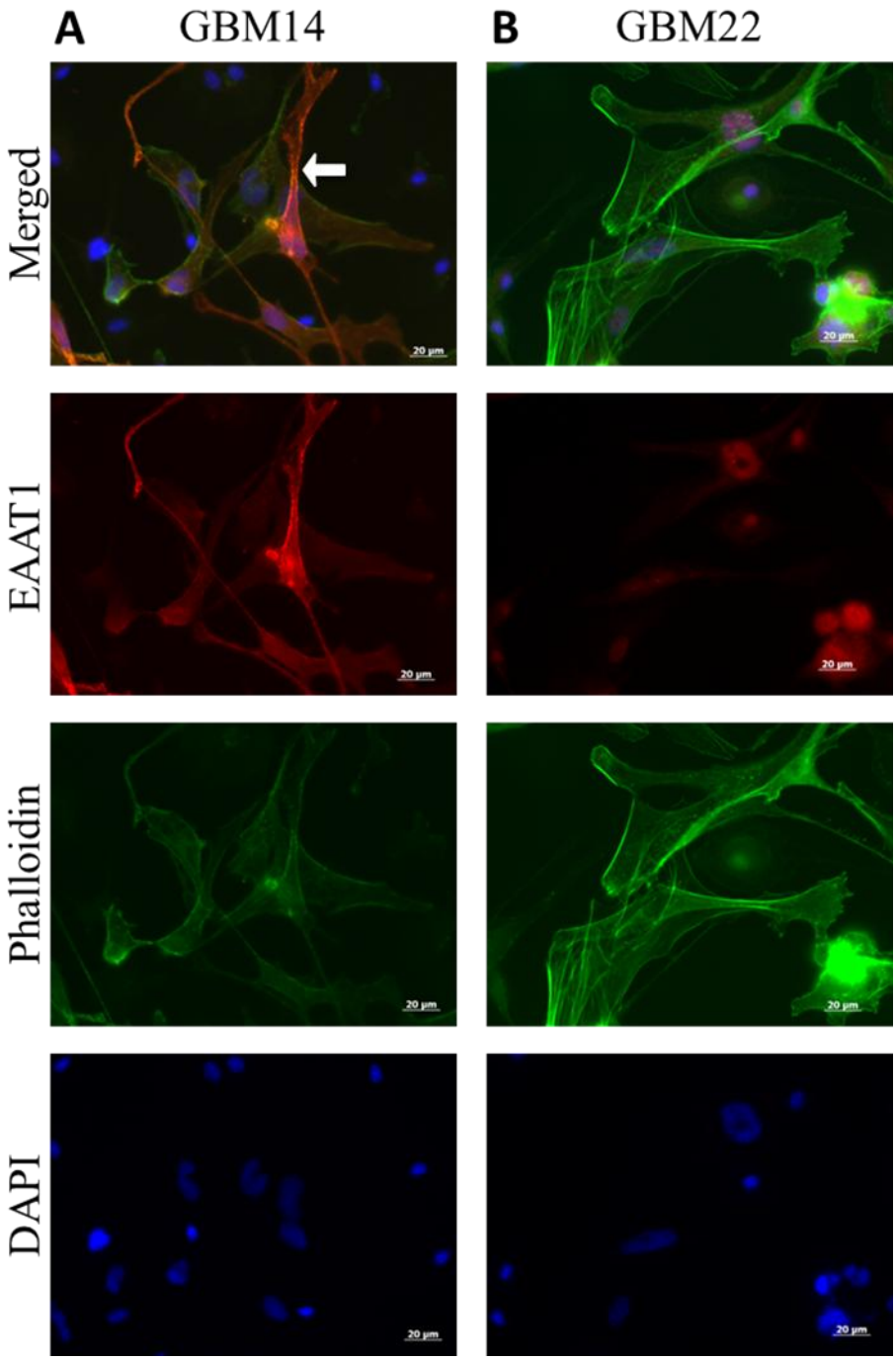


Figure 5. Immunofluorescence Staining of EAAT1Localization in GBM 14 and GBM22 Tumor Cells. EAAT1 is found on the membrane in GBM14 cells, unlike GBM22 cells, where it is nuclear. (**A**) EAAT1 staining in GBM14 cells, compared to actin-labeled Phalloidin and nuclear-labeled DAPI showing membrane localization. Arrow indicates membrane EAAT1. (**B**) EAAT1 staining in GBM22 cells, compared to Phalloidin and DAPI showing nuclear localization. 40X magnification.

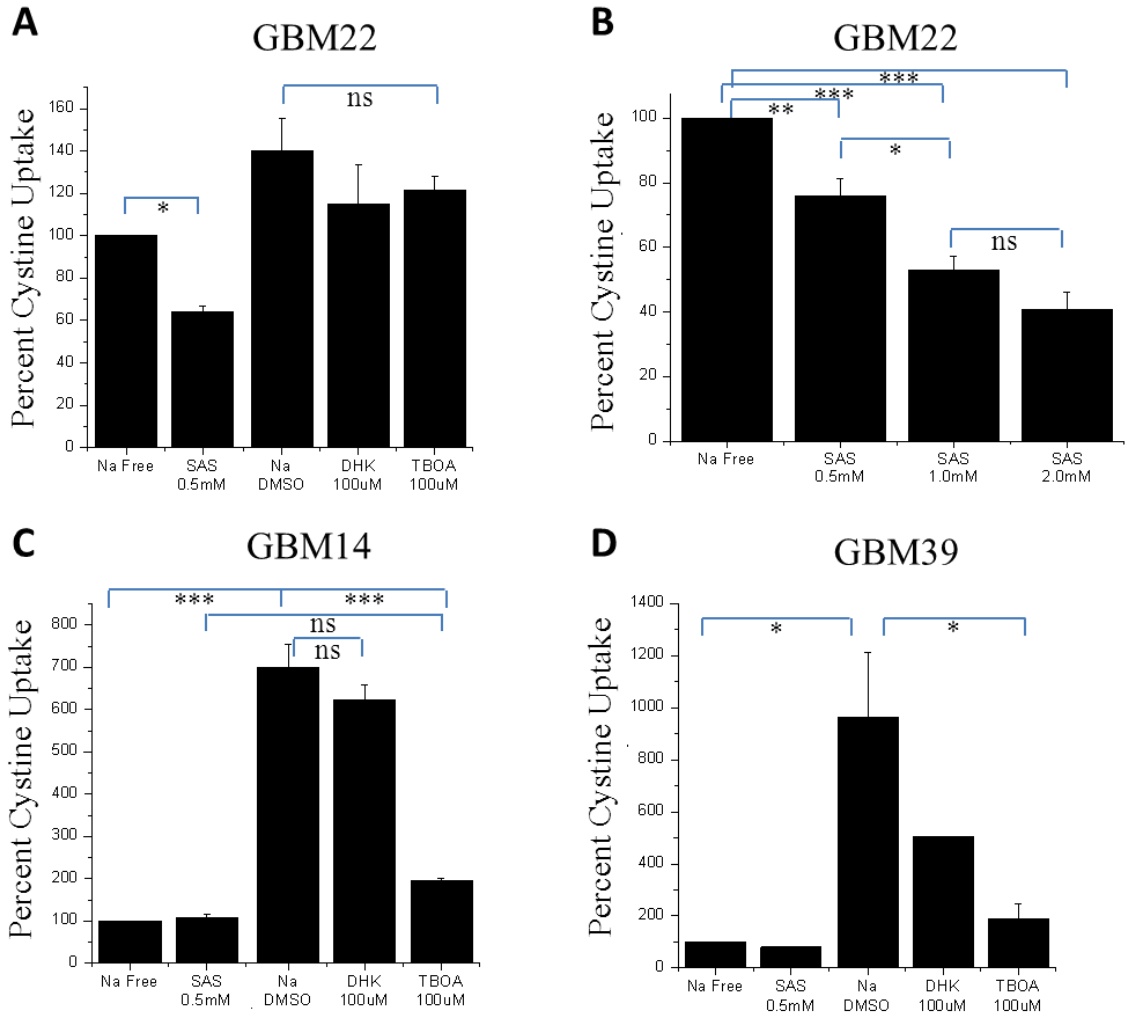


Figure 6. ^{35}S-L-Cystine uptake in GBM22, GBM14, and GBM39 Xenograft Tumors with Either High or Low xCT Expression. Cystine uptake differs between high and low system x_c^- expressing tumors. (**A**) GBM22 cells have Na^+-dependent, Sulfasalazine (SAS) inhibited cystine uptake, which is not sensitive to DHK or TBOA. (**B**) GBM22 cells are inhibited in a dose-dependent manner with SAS, with 1.0mM being the most effective concentration. (**C**) GBM14 cells and (**D**) GBM39 cells both show Na^+-dependent cystine uptake, which is inhibited by TBOA, but not DHK. Two-way ANOVA followed by Tukey post hoc was used to determine significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$; $n \geq 2$ (Note in (**D**) that DHK requires replication for statistical analysis).

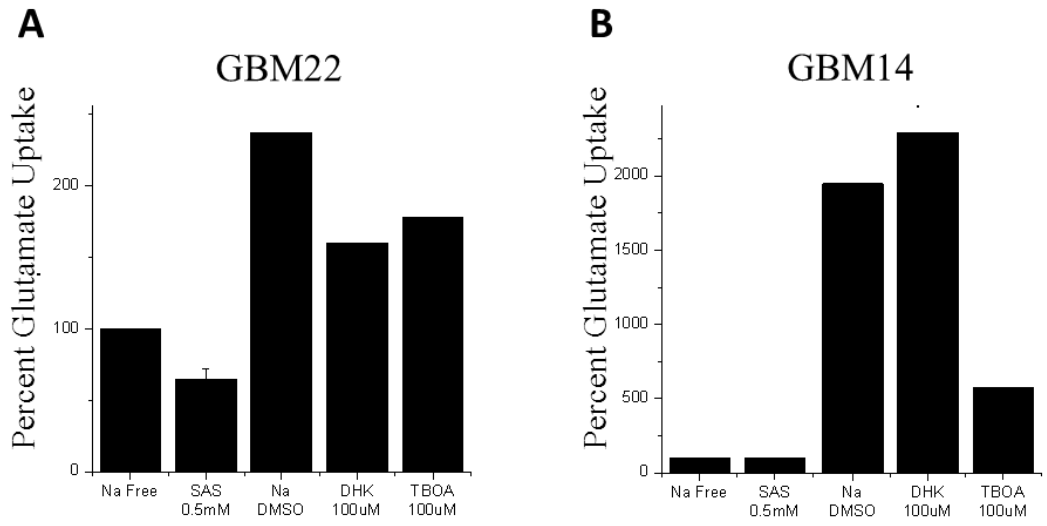


Figure 7. ^{3}H-L-Glutamate uptake in GBM22 and GBM14 Xenograft Tumors with Either High or Low xCT Expression. Glutamate uptake differs between high and low system x_c^- expressing tumors. (**A**) GBM22 cells have some Na^+-independent glutamate uptake that is decreased with SAS. (**B**) GBM14 cells have 100 fold greater Na^+-dependent glutamate uptake, which is decreased with TBOA. n=1-2 (Note further replication is needed for statistical analysis).

TBOA. The tumor lines expressing low xCT, GBM14 and GBM39, behaved as would be expected for the EAATs, relying on sodium for the majority of their uptake, with an 600% increase in uptake over sodium free conditions ($p<0.001$) in GBM14 cells (Figure 6C) and a 863% increase in GBM30 cells ($p<0.05$) (Figure 6D). Sodium dependent cystine uptake in both low xCT tumor lines was inhibited by TBOA, an EAAT1, EAAT2 & EAAT3 inhibitor, but not by DHK, and EAAT2 inhibitor, with a $p<0.001$ for GBM14 (Figure 6C) and $p<0.05$ for GBM39 (Figure 6D).

Glutamate uptake gave similar results, and agreed with the data from Western blots. Specifically, GBM22 (high xCT) exhibited sodium independent glutamate uptake, which was decreased by SAS (Figure 7A), and GBM14 demonstrated a much larger

sodium dependent glutamate uptake, which was decreased by TBOA (Figure 7B). Note that these data are preliminary and need to be repeated for statistical analysis.

Glutathione levels are similar in low and high SXC expressing tumors

Glutathione synthesis requires cystine uptake and reduction to cysteine, or direct cysteine uptake from the extracellular space. Cystine uptake for GSH synthesis has been linked to SXC function (30); therefore, we measured GSH levels in tumors with low SXC to determine if they had significantly lower levels. Interestingly, however, tumors with low SXC had equivalent levels of GSH to tumors with high SXC. Average GSH level in GBM22s was 1.63µmol/µg, compared to 2.29µmol/µg in GBM14s, values which are not considered significant using a paired t-test for analysis (Figure 8).

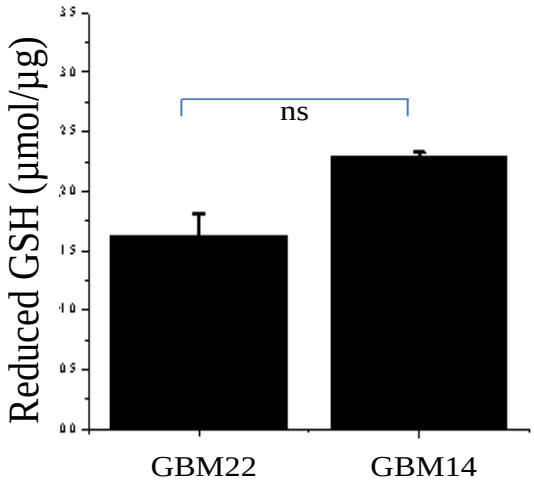


Figure 8. Basal Reduced Glutathione (GSH) levels in GBM22 and GBM14 Xenograft Tumors. GBM22 and GBM14 cells have similar basal levels of reduced glutathione. Paired t-test was used to compare samples. n=3.

Tumor cell growth *in vitro* is dependent upon SXC

Previous studies have shown that glioma cell growth is dependent on SXC, and that inhibition of this transporter leads to cell death by inducing caspase-3 mediated apoptosis, most likely due to a loss of intracellular GSH (9). In order to determine how differing levels of SXC affect cell growth in the tumor lines, we studied tumor cell growth using a proliferation assay over five days. Cells harvested from xenograft tumors were plated and two days later treated with either SAS (0.5mM, 1mM, 2mM), TBOA (100µM) or SAS (0.5mM) + TBOA (100µM) daily for five days, to determine the effect of inhibiting SXC, EAAT1/3, and both SXC & EAAT1/3 on the tumor cells. GBM22 (high xCT) cells showed a dose-response growth inhibition to SAS, with complete growth inhibition at 1.00mM SAS (Figure 9A). TBOA did not affect GBM22 cell growth (Figure 9B). GBM14 (low xCT, EAAT1) cell growth was inhibited by 45.1% ($p<0.05$) at 0.5mM SAS, but no inhibition was seen with 100µM TBOA. Similar inhibition was seen with SAS+TBOA treatment, with 48.1% inhibition (Figure 9C). GBM39 (low xCT, EAAT1, EAAT3) cell growth was inhibited 29.6% with 0.5mM SAS ($p<0.05$), 22.5% with 100µM TBOA ($p<0.05$), and 31.4% with the SAS + TBOA combination ($p>0.05$) (Figure 9D). Comparing the overall growth of the individual cell lines over 5 days shows a higher cell growth in the high xCT tumor line GBM22 ($p<0.001$), versus the two low xCT tumor lines, GBM14 and GBM39 showing an increased proliferation of 55.2% and 73.9%, respectively ($p<0.001$ for both) (Figure 9E).

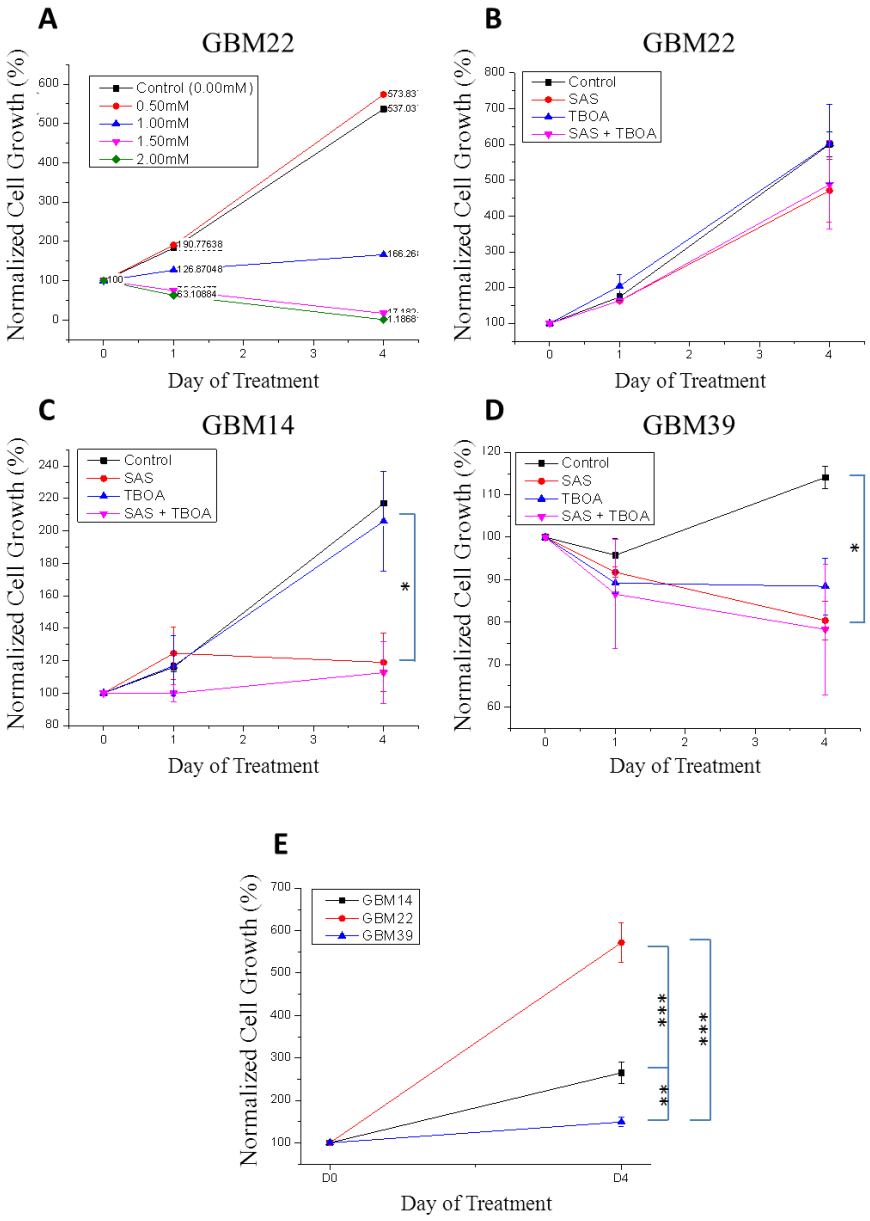


Figure 9. GBM22, GBM14, and GBM39 Xenograft Tumor Cell Proliferation with and without Sulfasalazine and/or TBOA Treatment. Xenograft cell proliferation differed depending on level of xCT expressed. (**A & B**) GBM22 cells showed growth inhibition with 1.0mM SAS and greater. No inhibition was seen with TBOA. (**C**) GBM14 cells showed growth inhibition with SAS and SAS + TBOA, but not with TBOA alone. (**D**) GBM39 cells showed growth inhibition with SAS, TBOA, and SAS+TBOA. (**E**) GBM22 cells showed a much higher growth rate than the GBM 14 and GBM39 cells. If not indicated, drug concentrations used are as follows: SAS = 0.5mM and TBOA = 100µM. Two-way ANOVA followed by Tukey post hoc was used to determine significance. *$p<0.05$, **$p<0.01$, ***$p<0.001$; $n \geq 3$.

High SXC expressing glioma cells exhibit neurotoxic due to glutamate release from SXC

In exchange for cystine uptake, SXC releases glutamate in the extracellular environment. Previous studies have linked glutamate release through SXC to epileptic activity in a mouse model of glioma (5). Glutamate, the most abundant neurotransmitter in the CNS and has been shown to be neurotoxic in numerous neurological diseases including stroke, ALS, and MS (42). We would predict that tumors that show high SXC expression but that lack EAATs would be releasing glutamate in the context of cystine uptake. By contrast, tumors that also express EAATs, which import glutamate, may not permit a buildup of extracellular glutamate. To investigate the effect of glutamate release from high xCT tumor cells versus glutamate uptake from EAAT expressing tumors, cortical neurons were co-cultured with the three tumor lines, so that the glioma cells and neurons shared medium, but did not come into contact with each other. After 72 hours of co-culture, a Live/Dead™ assay was performed on the co-cultured neurons, and the percentages of live versus dead cells were compared across tumor lines. Neurons co-cultured with low xCT tumor cells (GBM14 and GBM39), did not exhibit neurotoxicity, with average percentage of live cells being equal to that of the control. However, high xCT tumor cells (GBM22) did exhibit neurotoxicity compared to control (Figure 10A). In addition to the 72 hour co-culture experiment, a separate co-culture was performed using Timelapse microscopy to image the neurons every 2 hours during a 48 hour co-culture. During this time, a control well was imaged concurrently and a Live/Dead™ assay was performed at the end of the 48 hours. Figure 10B shows a representative figure of control neurons over 48 hours (left) compared to neurons co-cultured with GBM22 cells over 48 hours. The Live/Dead™ assay was performed without disturbing the position of the

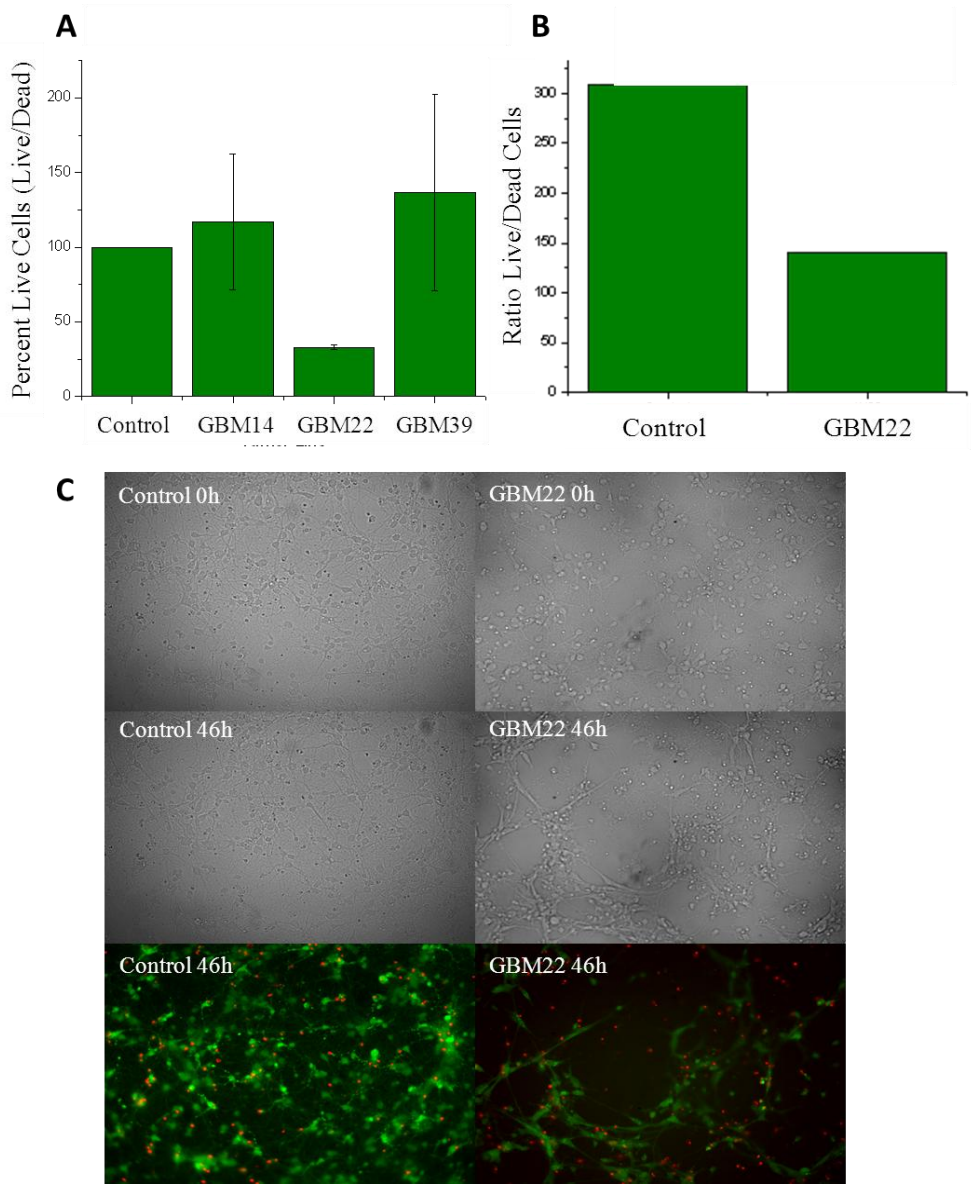


Figure 10. Cortical Neuron Co-culture with GBM22, GBM14, and GBM39 Xenograft Tumors Quantified by Live/Dead Assay. High xCT expressing cells showed neurotoxicity over 46 hours and 72 hours. Live/Dead™ Assay was performed and number of live (green) versus dead (red) cells were counted (**A**) GBM22 cells showed neurotoxicity when co-cultured over 72h. Live/dead ratio was determined and the percentage of live cells was calculated. n=3 (**B**) Quantification of live versus dead cells in (**C**). (**C**) Timelapse images taken over 46h of a control neuron well and GBM22 co-culture showing neuron death. Green cells indicating live neurons, red cells indicate dead neurons. n=1. (Note that replications are needed for statistical analysis due to large variability in experiment). 10X magnification.

wells, and the same fields of view were again imaged and the live (green) and dead (red) neurons were counted. Figure 10B shows a quantification of the ratio of Live/Dead cells in the corresponding image in Figure10C.

High xCT tumors provoke seizures when implanted intra-cranially in a mouse model of glioma

Previous data has shown that glutamate release through SXC causes epileptic activity in mice with intracranial glioma implantation using established glioma cell lines (5). In order to investigate the effect of high versus low xCT expression and EAAT expression on seizure activity *in vivo*, mice were implanted intracranially with low xCT tumors (GBM14 and GBM39) and with high xCT tumors (GBM22). Five days after intracranial implantation, EEGs electrodes were implanted and the mice were monitored for seizure activity. Based on the above data we would expect that GBM 22 tumors that cause glutamate excitotoxicity may be more likely to initiate seizures than GBM14 or GBM39 gliomas, which did not present with glutamate toxicity. Indeed, 92.8% of mice implanted with GBM22 tumor cells (high xCT) exhibited seizures (Figure 11A) compared with only 25% of GBM14 implanted mice and 12.5% of GBM39 implanted mice. In addition, GBM22 implanted mice reached the experimental endpoint (weight loss >7% of initial body weight & moribund condition) approximately 10 days earlier than the GBM14 and GBM39 implanted mice, and required euthanasia 20.4 days post injection, versus 28.5 and 32.2 days post-injection for the GBM14 and GBM39 implanted mice, respectively (Figure 11B).

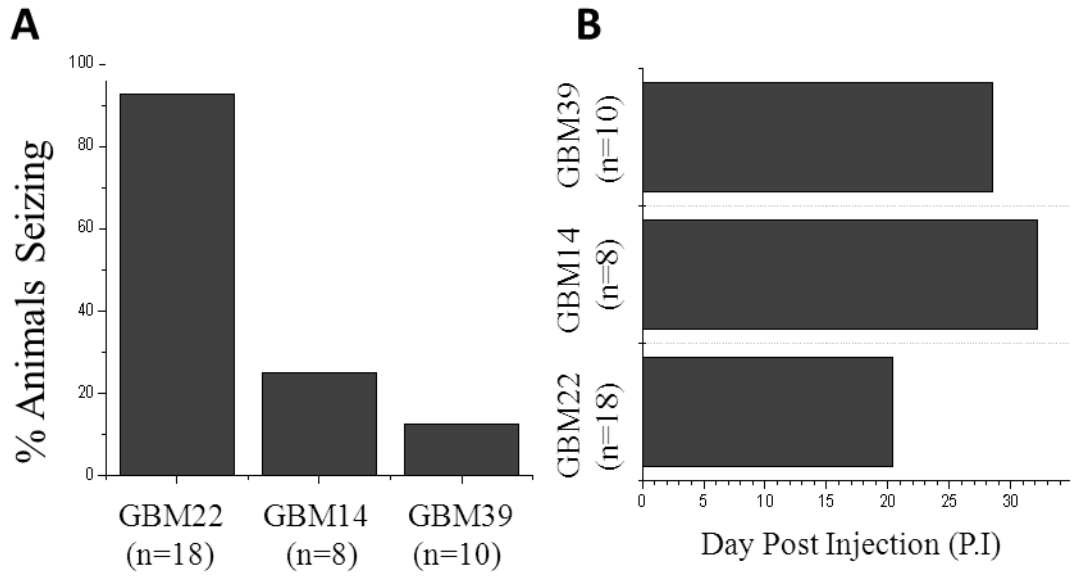


Figure 11. Occurrence of Seizures and Average Day Post-Injection Euthanasia for Mice Implanted with GBM22, GBM14, and GBM39 Xenograft Tumors Intracranially. High xCT tumors caused a greater number of seizures when implanted intracranially in mice. (**A**) 92.8% of mice implanted with GBM22 tumors showed seizures (n=18), compared to 25% of GBM14 (n=8) and 12.5% GBM39 (n=10) implanted mice. (**B**) GBM22 implanted mice reached required euthanasia endpoints an average of 20.5 days post injection, compared to 28.5 and 32.2 days for GBM14 and GBM39 mice, respectively.

SUMMARY & DISCUSSION

The purpose of this study was to investigate the expression of system x_c^- in glioma patient samples to determine whether system x_c^- is abundantly overexpressed in patient tissue and could be used as a tumor marker. We used tissue microarrays encompassing tumor and matching non-tumor tissue from 45 patients, and stained them for the catalytic subunit of system x_c^-, xCT, to quantitatively determine relative system x_c^- expression comparing tumor and control tissue. Approximately 60% of the patient samples showed increased system x_c^- levels while the remaining 40% had levels similar to, or less than, the matched normal brain. Short term primary cultures of patient-derived tissues with either high or low SXC expression were used to investigate the effect of low SXC expression on cell growth and survival. Interestingly, by western blot, tumors with low SXC showed compensatory up-regulation of the glutamate transporters EAAT1 and EAAT3, which were previously thought down-regulated, silenced, and/or mis-localized in gliomas. We discovered that tumors with high levels of system x_c^- proliferated faster *in vitro*, were more radiation resistant, and took up cystine and glutamate in a sodium independent manner. Sulfasalazine (an inhibitor of system x_c^-) decreased cell number, and cystine and glutamate uptake, pointing toward system x_c^- as the mediator of cell growth/survival and cystine uptake in tumors with high levels of system x_c^-. Additionally, high system x_c^- tumors exhibited neurotoxicity when co-cultured with cortical neurons, and 93% of mice have EEG-confirmed seizures as early as 12 days post-injection after intracranial implantation of these high system x_c^- expressing tumors. These mice reached

pre-defined endpoints (including weight loss and moribund condition) approximately ten days earlier than low xCT implanted mice, and therefore required euthanasia much earlier. Conversely, we found that tumors with low levels of system x_c^- proliferated slower *in vitro*, were less radiation resistant, and took up both cystine and glutamate in a sodium dependent manner. Interestingly, Sulfasalazine decreased cell number to a greater extent in low xCT tumors than in high xCT tumors, suggesting system x_c^-'s importance in cell growth and survival, even when expressed at low levels. Additionally, TBOA, a broad spectrum EAAT inhibitor, decreased cell number in low xCT tumors with both EAAT1 and EAAT3, but not in the low xCT tumors that expressed only EAAT1. Sodium dependent cystine and glutamate uptake were inhibitable by TBOA, but not DHK, an EAAT2 specific inhibitor, confirming the absence of this glutamate transporter in these cells. Low xCT tumors did not exhibit neurotoxicity, nor did they show a high percentage of seizures in mice, when implanted intracranially.

In this study, we found that, unlike in established cell lines, system x_c^- is not universally up-regulated in the glioblastoma patient population. Our findings support the use of the xenograft model, using patient tissues propagated and passaged in mice flanks, because it has furthered knowledge beyond that found in previous studies looking at system x_c^- and glutamate transporter expression in established cell lines (both *in vitro* and in mouse models of human glioblastoma). Cell lines change their properties over time in culture, as they adapt to their artificial environment; however, the genomic and protein expression patterns of cells in the flank tumors are preserved more readily due to their passaging and maintenance in a living host. This study further confirms this xenograft tumor model as a superior model for studying human cell behavior.

System x_c^- is an important cellular amino acid exchanger abundantly found in normal cells, including the brain, spinal cord, fibroblasts, and pancreas in humans, along with immune system cells in mice (25, 28). Up-regulation of xCT, the catalytic subunit of system x_c^-, has been seen in many types of cancers including glioma (9,30), lymphoma (15), pancreatic (29), breast (33), and ovarian (35). System x_c^- has been linked to enhanced cancer growth and survival through its ability to take up cystine and release glutamate. Cystine uptake through system x_c^- provides substrate for the synthesis of GSH, the most abundant antioxidant in the CNS (21). GSH neutralizes endogenously and exogenously produced free radicals and reactive oxygen species (ROS), to prevent cellular damage (48).

As cancer cells have increased cellular metabolism, and therefore create an excess of ROS, the function of GSH is important to protect these cells from oxidative cell death. In addition, GSH has been shown to be involved in both radiation therapy and chemotherapy resistance, by neutralizing free radicals to prevent radiation-induced cell death (41), enhancing the function of multi-drug resistance proteins (MRPs) to extrude pharmaceutical agents from the cell to prevent chemotherapy-induced cell death (36), and by conjugating certain xenobiotics, including cisplatin, doxorubicin, and melaphalan to neutralize their ability to cause cell death (12). Hence high SXC activity confers growth advantages to cancer cells through enhanced GSH synthesis.

As a cystine/glutamate antiporter, system x_c^- exchanges these amino acids in a 1:1 stoichiometric, sodium independent, electroneutral manner. The release of glutamate through system x_c^- has been implicated in neurotoxicity, epileptiform activity in a mouse model of glioma, glioma growth, and metastasis. In the rest of the body, the released

glutamate is inconsequential as serum concentrations of glutamate fluctuate widely and reach millimolar concentrations without being harmful. In the brain, however, glutamate acts as the major excitatory neurotransmitter in the CNS and is normally controlled at micromolar concentrations in the extracellular space of the brain. Through binding to post-synaptic neuronal glutamate receptors, glutamate mediates signal transmission (42). However, prolonged activation of post-synaptic glutamate receptors, specifically NMDA and AMPA receptors, leads to Ca^{2+}-dependent excitotoxicity (42, 48). This excitotoxicity has been found in other neurological diseases, including Alzheimer's disease, acute trauma, ischemic stroke, and amyotrophic lateral sclerosis (ALS) (42). In addition, glioma derived glutamate release through system x_c^- leads to neurotoxicity and neuronal death *in vitro* (50) and this glutamate release had also been linked to epileptiform activity in mice implanted with xCT expressing gliomas (5). Glutamate release resulting in neuronal death affords space for tumor growth within the tight confines of the skull (45) and acts through autocrine signaling to promote glioma cell invasion and metastasis (31).

Due to its upregulation in many types of cancer, system x_c^- is a promising clinical target for many types of cancers including glioma, pancreatic, breast, and lymphomas (9, 15, 29, 30, 33), and many others. However, system x_c^- can be induced in cultured cells *in vitro* (28) and therefore, although system x_c^- has been found to be generally up-regulated in commonly used, established glioma cell lines (9), little is known about the true expression of this transporter in the glioma patient population. Our present study focuses on experiments using patient biopsy tissue and patient-derived xenograft tissue propagated in nude mice flanks, which has been shown to retain its genetic and proteomic expression more readily than patient-derived cell lines that have adapted to culture over

many years (37). From our studies we have discovered that not all patient tumors express increased levels of system x_c^-. Some patient biopsies, when stained for xCT, actually had undetectable levels, suggesting either loss of system x_c^- expression or derivation from a cell type lacking system x_c^- expression. The majority (approximately 60%) of the patient biopsies, however, did in fact show increased xCT expression, which was higher than the normal, physiological levels of xCT expression in matched corresponding normal brain tissue. In light of this new finding, we argue that glioblastomas can be classified into two categories – those that express higher-than-physiological levels of system x_c^- and those that express equal or lower-than-physiological levels of system x_c^- . These differences in xCT expression point to i) a loss of system x_c^- expression in some gliomas and upregulation in others, determined by environmental conditions and proliferation activity, ii) differing cellular origins of the tumors, with tumors showing no xCT expression deriving from cells that do not normally express system x_c^-, and those showing xCT expression deriving from cells that do normally express system x_c^- and/or iii) a gain of system x_c^- expression in certain glioma populations.

With the knowledge that the glioma patient population is not uniform in regard to system x_c^- expression, we looked for other transporters that could replace system x_c^-'s role in cellular cystine uptake. Cystine uptake and subsequent conversion to GSH has been proven to be important for glioma cell growth and survival (9). It has previously been discovered that that Excitatory Amino Acid Transporters have the ability to take up cystine (19). Additionally, EAAT3 is also able to take up the reduced form cystine, cysteine (51), and therefore we investigated this class of transporters in our initial studies. We found expression of two glutamate transporters in the EAAT class, EAAT1 (GLAST

in rodents) and EAAT3 (EAAC1 in rodents), present in the xenograft tumor lines with low xCT.

Our uptake studies have revealed new and contrasting patterns of expression and function of these EAATs in brain tumor cells. High SXC cells showed a sodium independent cystine uptake, indicating normal system x_c^- function, with little glutamate uptake. However, low SXC cells showed sodium dependent cystine and glutamate uptake. Although cystine uptake has previously been defined in EAAT1, 2 & 3 in HEK cells, it was found that the sodium dependent cystine uptake was less than half that of sodium independent cystine uptake (19). Additionally EAAC1/EAAT3 was found to mediate neuronal cysteine uptake with a tenfold greater affinity than EAAT1 and EAAT2, and inhibition of EAAC1/EAAT3 reduces neuronal GSH levels therefore playing an important role in cysteine uptake in neurons (2). In normal, non-neural cells, system x_c^- mediates the majority of cystine uptake into cells. Our uptake studies show that in those tumors expressing EAAT1 and/or EAAT3, sodium dependent cystine uptake is 600-times greater than sodium independent uptake. Importantly, previous studies have found that glutamate transporters are either absent (49) silenced (17, 52) or mis-localized to the nucleus (47, 49) in human glioma tumors, and therefore do not contribute to glioma behavior. However, using the patient derived xenograft tissue, we found that in tumors expressing low levels of system x_c^-, EAAT1 and EAAT3 are present and are membrane localized, functional transporters that play a critical functional role in glioma physiology. Just as system x_c^- has been found to be induced under cell culture conditions, glutamate transporter expression may be altered under these artificial conditions as well, leading to

decreased expression, mis-localization, or silencing in established cell lines, which may not accurately reflect the expression in the glioma patient population.

To investigate how the varied expression of system x_c^- and EAATs affect cellular behavior we measured proliferation of the tumor lines and found that high SXC cells grow approximately 2 times faster than low SXC cells. Faster growing cells, due to increase metabolic processes, produce larger amounts of free radicals (39). Therefore, higher levels of system x_c^- may confer a growth advantage to these cells. As expected, cell growth in high SXC cells is attenuated by the application of Sulfasalazine to inhibit system x_c^-, but is not affected by TBOA. Inhibition of system x_c^- has been shown to decrease glioma cell growth in xCT expressing tumors, both *in vitro* and *in vivo* (9, 31). Interestingly, however, tumors with low SXC were inhibited by Sulfasalazine as well, suggesting that in slower growing cells, a lower level of system x_c^- may be sufficient for cell growth and survival. Low SXC cells expressing both EAAT1 and EAAT3 showed growth inhibition with TBOA application as well, however, the low SXC cells expressing only EAAT1 showed growth inhibition with only Sulfasalazine, and not TBOA. EAAT3 has a greater V_{max} for cystine uptake, compared to EAAT1 and preferentially transports cysteine (19), which could help explain why only the cells expressing EAAT3 were growth sensitive to TBOA, since cysteine can be directly incorporated into GSH, bypassing the need for cystine uptake. Furthermore, this finding could also be explained by the cooperation between system x_c^- and the glutamate transporters. In normal cells, cystine uptake through system x_c^- results in glutamate release, and excess glutamate is taken up through EAATs, back into the cell. The glutamate uptake through EAATs not only prevents accumulation of glutamate in the extracellular space, it also serves to

increase substrate for GSH (a tripeptide consisting of cysteine, glutamine, and glutamate), and to further increase cystine uptake through system x_c^- by providing an increased intracellular glutamate gradient (28). Therefore, it is conceivable that the main role of EAAT1 in the low SXC cells with only this transporter, may be to allow more efficient functioning of system x_c^- at low levels, and therefore why blocking system x_c^- only is sufficient to decrease proliferation and there is no effect when the EAATs are blocked under non-stressful conditions. This cystine/glutamate cycling could also explain why we find similar GSH levels in high and low SXC tumors. Further investigation into the consequences of system x_c^- and EAAT expression in glioma proliferation is needed to clearly define the roles of these transporters and their inhibition in low SXC tumors.

Cystine taken up through system x_c^- is rapidly reduced intra-cellularly to cysteine. This cysteine is incorporated into GSH, through the rate-limiting step in GSH synthesis (29). Intracellular GSH depletion can be accomplished by removing cystine from the medium, inhibiting system x_c^-, and by blocking GSH synthesis using buthionine sulfoximine (BSO) to inhibit gamma-glutamylcysteine synthetase, the enzyme required in the first step of GSH synthesis (22). Since cystine uptake and GSH synthesis has been previously linked to system x_c^- , we expected low SXC tumors to contain less GSH; however, we found that high and low SXC tumors have equivalent basal GSH levels. This finding can be explained by both EAAT-mediated cystine uptake, and the cystine/glutamate cycle created in cells expressing both classes of transporters.

GSH is not only important in normal cell and cancer cell survival from endogenously produced ROS, it is also important in cancer cell resistance to exogenously produced free radicals during radiation treatment. Radiation therapy is an important part

of treatment for glioma patients. It works by both direct DNA damage, and by creating hydroxyl free radical species that interact with cellular DNA, ultimately leading to cell death (26). Unfortunately, radiation resistance occurs in most cases, which leads to continued tumor growth and metastasis within the brain (40). System x_c^- may play an important role in development of radiation resistance in those cells with increased xCT expression, by allowing greater production of GSH and, as a result, more free radical neutralization in these cancer cells. In our preliminary studies we found that low SXC cells had a 2 times greater sensitivity to increasing doses of radiation than high SXC cells (Appendix Fig. 2). In fact, we could increase the sensitivity of high SXC cells by blocking system x_c^- function with Sulfasalazine. Although under normal, non-stress conditions the basal GSH levels are equivalent, high SXC tumors may be able to more readily increase their GSH levels, leading to a more radiation resistant phenotype. This finding also suggests that knowledge of system x_c^- expression may help guide treatment decisions for glioma patients. For example, starting Sulfasalazine treatment in combination with radiation therapy may help to decrease the radiation resistance of these tumors, and as a result prolong effective treatment.

Glutamate release, in exchange for cystine uptake is also important in glioma behavior. Our findings further support the role of glutamate release through system x_c^- in tumor growth, progression, and tumor-associated seizures. Using a co-culture experimental setup, in which cortical neurons and glioma cells share medium, but are separated by a filter so that they do not come into contact, we found that high SXC tumor cell medium becomes neurotoxic over 48 - 72 hours. Using a Live/Dead assay, we were able to quantify the neuronal cell death and compared to control, a higher percent of

neurons died when co-cultured with high SXC cells than with low SXC cells. Additionally, preliminary data suggests that addition of 0.5mM SAS to the medium decreases neuronal death in the GBM22 co-culture (Appendix Fig. 3) further suggesting system x_c^- involvement in the observed neurotoxicity. These findings agree with previous data showing neurotoxicity from system x_c^- expressing tumor cells (50). In agreement with the co-culture data, mice implanted intracranially with high SXC tumors showed a greater frequency and duration of seizures, as compared to mice implanted with low SXC tumors. Glutamate release from system x_c^- has previously been found to cause neuronal excitotoxicity and epilepiform activity in mice, due to glutamate release, which was inhibited with Sulfasalazine (5). These experiments were performed using established glioma cell lines, and all expressed high levels of system x_c^- . In this study, we have gathered more evidence connecting system x_c^- with glutamate induced glioma-associate seizures, and in addition have shown that low SXC tumors are less able to promote seizure activity *in vivo*. Additionally, it has been suggested that gliomas may be considered a neurodegenerative disease due to their similarity with other neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, with some of the main characteristics shared being seizures, increased oxidative stress, genomic alteration-mediated pathogenesis and glutamate-mediated neuronal excitotoxicity (48). These finding are important because they argue for the use of system x_c^- and EAATs clinically, as prognostic indicators and treatment markers to help guide patient care and treatment.

LIST OF REFERENCES

(1) Adamson C, Kanu OO , Mehta AI, *et al.* Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin Investig Drugs 2009;18:1061-83.

(2) Aoyama K, Suh SW, Hamby AM, *et al.* Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nat Neurosci 2006;9:119-26.

(3) Bannai S, Kitamura E. Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. J Biol Chem 1980;255:2372-6.

(4) Bannai S, Kitamura E. Role of proton dissociation in the transport of cystine and glutamate in human diploid fibroblasts in culture. J Biol Chem 1981;256:5770-2.

(5) Buckingham SC, Campbell SL, Haas BR, *et al.* Glutamate release by primary brain tumors induces epileptic activity. Nat Med 2011;17:1269-74.

(6) Bump EA, Brown JM. Role of glutathione in the radiation response of mammalian cells *in vitro* and *in vivo*. Pharmacol Ther 1990;47:117-36.

(7) Camacho A, Massieu L. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. Arch Med Res 2006;37:11-8.

(8) Cho Y, Bannai S. Uptake of glutamate and cysteine in C-6 glioma cells and in cultured astrocytes. J Neurochem 1990;55:2091-7.

(9) Chung WJ, Lyons SA , Nelson GM, *et al.* Inhibition of cystine uptake disrupts the growth of primary brain tumors. J Neurosci 2005;25:7101-10.

(10) Chung WJ, Sontheimer H. Sulfasalazine inhibits the growth of primary brain tumors independent of nuclear factor-kappaB. J Neurochem 2009;110:182-93.

(11) Cuddapah VA, Habela CW, Watkins S, Moore LS, Barclay TT, Sontheimer H. Kinase activation of ClC-3 accelerates cytoplasmic condensation during mitotic cell rounding. Am J Physiol Cell Physiol 2011;302:C527-C538.

(12) Dai Z, Huang Y, Sadee W, Blower P. Chemoinformatics analysis identifies cytotoxic compounds susceptible to chemoresistance mediated by glutathione and cystine/glutamate transport system xc-. J Med Chem 2007;50:1896-906.

(13) Danbolt NC. Glutamate uptake. Prog Neurobiol 2001;65:1-105.

(14) Giannini C, Sarkaria JN, Saito A, *et al.* Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. Neuro-oncol 2005;7:164-76.

(15) Gout PW, Kang YJ, Buckley DJ, Bruchovsky N, Buckley AR. Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells 9860. Leukemia 1997;11:1329-37.

(16) Guan J, Lo M, Dockery P, *et al.* The xc- cystine/glutamate antiporter as a potential therapeutic target for small-cell lung cancer: use of sulfasalazine. Cancer Chemother Pharmacol 2009;64:463-72.

(17) Guo H, Lai L, Butchbach ME, Lin CL. Human glioma cells and undifferentiated primary astrocytes that express aberrant EAAT2 mRNA inhibit normal EAAT2 protein expression and prevent cell death. Mol Cell Neurosci 2002;21:546-60.

(18) Guzy RD, Hoyos B, Robin E, *et al.* Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing 9837. Cell Metab 2005;1:401-8.

(19) Hayes D, Wissner M, Rauen T, McBean GJ. Transport of l-[^{14}C]cystine and l-[^{14}C]cysteine by subtypes of high affinity glutamate transporters over-expressed in HEK cells. Neurochemistry International 2005;46:585-94.

(20) Iglehart JK, York RM, Modest AP, Lazarus H, Livingston DM. Cystine requirement of continuous human lymphoid cell lines of normal and leukemic origin. J Biol Chem 1977;252:7184-91.

(21) Janaky R, Ogita K , Pasqualotto BA, *et al.* Glutathione and signal transduction in the mammalian CNS. J Neurochem 1999;73:889-902.

(22) Jia Y, Zhang W, Liu H, Peng L, Yang Z, Lou J. Inhibition of glutathione synthesis reverses Kruppel-like factor 4-mediated cisplatin resistance. Cancer Chemother Pharmacol 2012;69:377-85.

(23) Kaleeba JA, Berger EA. Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT. Science 2006;311:1921-4.

(24) Kato S, Negishi K , Mawatari K, Kuo CH. A mechanism for glutamate toxicity in the C6 glioma cells involving inhibition of cystine uptake leading to glutathione depletion. Neurosci 1992;48:903-14.

(25) Kim JY, Kanai Y, Chairoungdua A, *et al.* Human cystine/glutamate transporter: cDNA cloning and upregulation by oxidative stress in glioma cells. Biochim Biophys Acta 2001;1512:335-44.

(26) Komuro H, Rakic P . Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca^{2+} fluctuations. J Neurobiol 1998;37:110-30.

(27) Krex D, Klink B, Hartmann C, *et al.* Long-term survival with glioblastoma multiforme. Brain 2007;130:2596-606.

(28) Lewerenz J, Hewett S, Huang Y, *et al.* The Cystine/Glutamate Antiporter System xc- in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities. Antioxid Redox Signal 2012.

(29) Lo M, Ling V, Wang YZ, Gout PW. The xc- cystine/glutamate antiporter: a mediator of pancreatic cancer growth with a role in drug resistance. Br J Cancer 2008; 99:464-72.

(30) Lo M, Wang YZ, Gout PW. The x(c)- cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases. J Cell Physiol 2008;215:593-602.

(31) Lyons SA, Chung WJ, Weaver AK, Ogunrinu T, Sontheimer H. Autocrine glutamate signaling promotes glioma cell invasion. Cancer Res 2007;67:9463-71.

(32) Moots PL, Maciunas RJ, Eisert DR, Parker RA, Laporte K, Abou-Khalil B. The course of seizure disorders in patients with malignant gliomas. Arch Neurol 1995;52:717-24.

(33) Narang VS, Pauletti GM, Gout PW, Buckley DJ, Buckley AR. Sulfasalazine-induced reduction of glutathione levels in breast cancer cells: enhancement of growth-inhibitory activity of Doxorubicin . Chemotherapy 2007;53:210-7.

(34) Oehler C, Frei K, Rushing EJ, *et al.* Patupilone (Epothilone B) for Recurrent Glioblastoma: Clinical Outcome and Translational Analysis of a Single-Institution Phase I/II Trial. Oncology 2012;83:1-9.

(35) Okuno S, Sato H, Kuriyama-Matsumura K, *et al.* Role of cystine transport in intracellular glutathione level and cisplatin resistance in human ovarian cancer cell lines. Br J Cancer 2003;88:951-6.

(36) Rickert CH, Strater R, Kaatsch P, *et al.* Pediatric high-grade astrocytomas show chromosomal imbalances distinct from adult cases. Am J Pathol 2001;158:1525-32.

(37) Sarkaria JN, Carlson BL, Schroeder MA, *et al.* Use of an orthotopic xenograft model for assessing the effect of epidermal growth factor receptor amplification on glioblastoma radiation response. Clin Cancer Res 2006;12:2264-71.

(38) Savaskan NE, Heckel A, Hahnen E, *et al.* Small interfering RNA-mediated xCT silencing in gliomas inhibits neurodegeneration and alleviates brain edema. Nat Med 2008;14:629-32.

(39) Scatena R. Mitochondria and cancer: a growing role in apoptosis, cancer cell metabolism and dedifferentiation. Adv Exp Med Biol 2012;942:287-308.

(40) Segerstrom L, Stenerlow B, Eriksson V, Carlsson J. Effects of radiation on growth of two human tumour cell lines surviving a previous high dose, low dose-rate, radionuclide exposure. Int J Oncol 2008;33:341-9.

(41) Simone G, Tamba M , Quintiliani M. Role of glutathione in affecting the radiosensitivity of molecular and cellular systems. Radiat Environ Biophys 1983;22:215-23.

(42) Sontheimer H. A role for glutamate in growth and invasion of primary brain tumors. J Neurochem 2008;105:287-95.

(43) Stockhammer F, Von Deimling A, van Landeghem FK. Decreased expression of the active subunit of the cystine/glutamate antiporter xCT is associated with loss of heterozygosity of 1p in oligodendroglial tumours WHO grade II. Histopathology 2009;54:241-7.

(44) Stupp R, Mason WP , van den Bent MJ, *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-96.

(45) Takano T, Lin JH, Arcuino G, Gao Q, Yang J, Nedergaard M. Glutamate release promotes growth of malignant gliomas. Nat Med 2001;7:1010-5.

(46) Tang Y, Shah K, Messerli SM, Snyder E, Breakefield X, Weissleder R. *In vivo* tracking of neural progenitor cell migration to glioblastomas. Hum Gene Ther 2003;14:1247-54.

(47) Varini K, Benzaria A, Taieb N, *et al.* Mislocalization of the exitatory amino-acid transporters (EAATs) in human astrocytoma and non-astrocytoma cancer cells: effect of the cell confluence. J Biomed Sci 2012;19:10.

(48) Watkins S, Sontheimer H. Unique biology of gliomas: challenges and opportunities. Trends Neurosci 2012.

(49) Ye ZC, Rothstein JD, Sontheimer H. Compromised glutamate transport in human glioma cells: reduction- mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. J Neurosci 1999;19:10767-77.

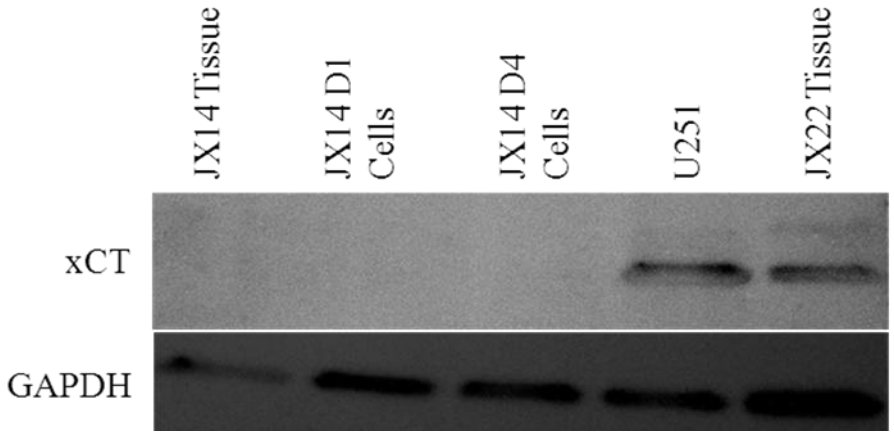
(50) Ye ZC, Sontheimer H. Glioma cells release excitotoxic concentrations of glutamate. Cancer Res 1999;59:4383-91.

(51) Zerangue N, Kavanaugh MP. Interaction of l-cysteine with a human excitatory amino acid transporter. Journal of Physiology 1996;493:419-23.

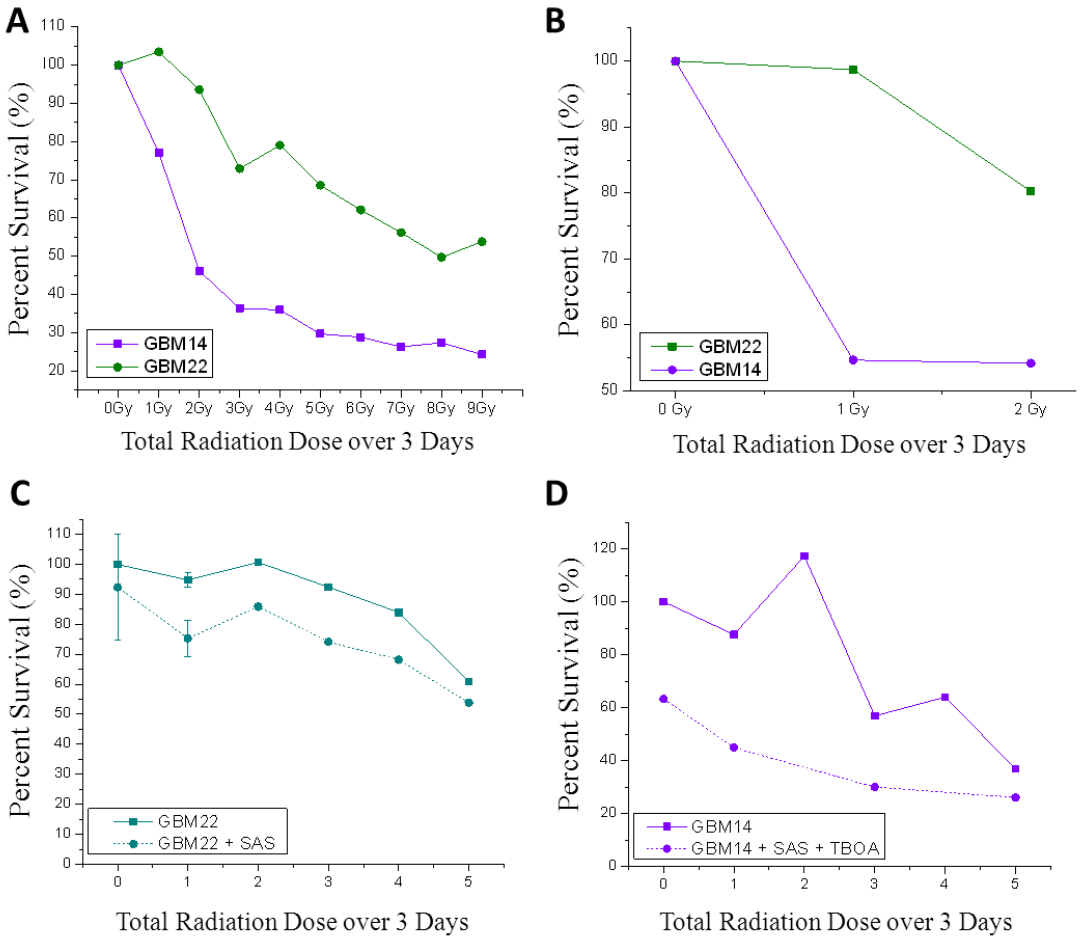
(52) Zschocke J, Allritz C, Engele J, Rein T. DNA methylation dependent silencing of the human glutamate transporter EAAT2 gene in glial cells. Glia 2007;55:663-74.

APPENDIX A

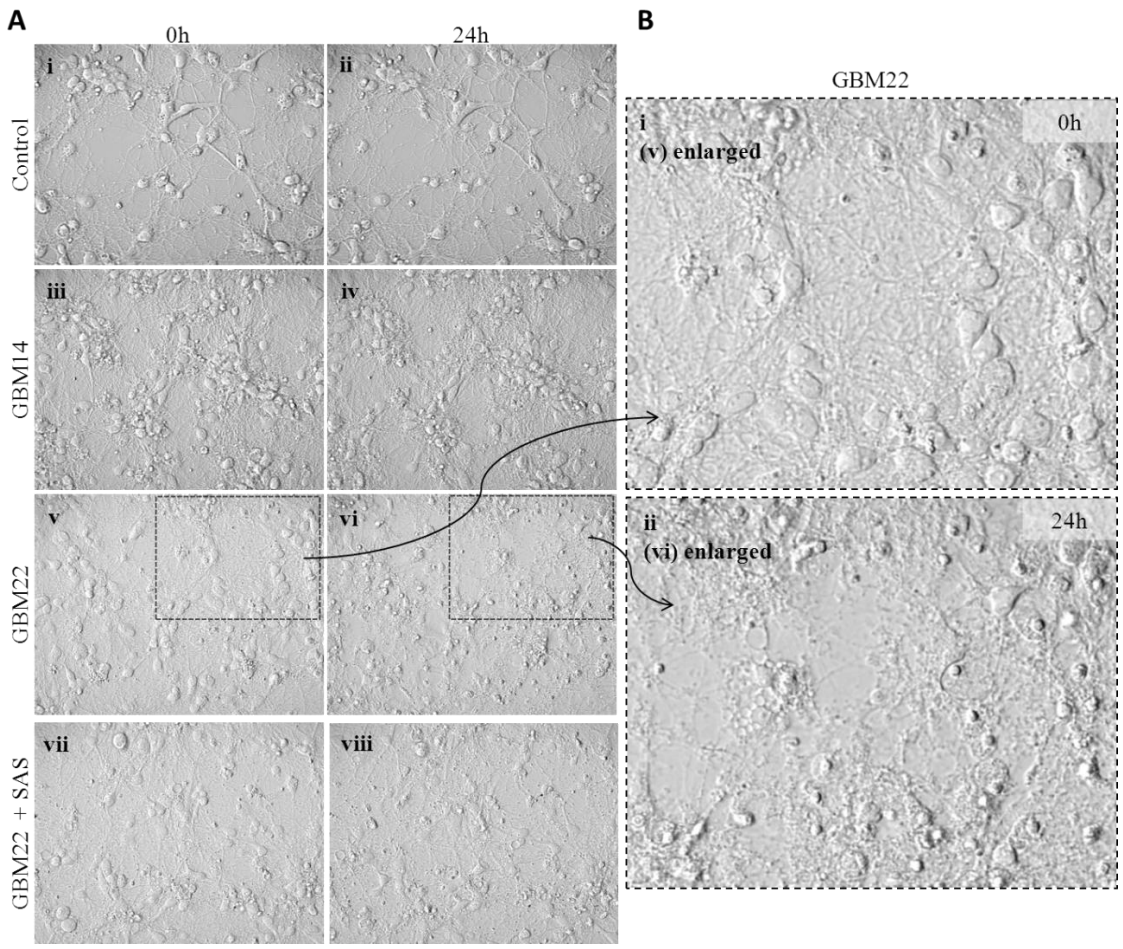
SUPPLEMENTAL & PRELIMINARY DATA



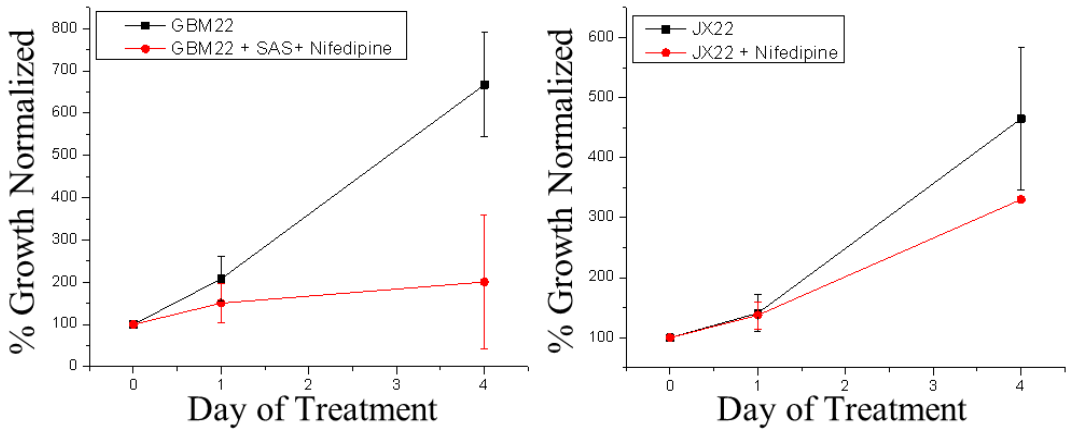
Appendix Figure 1. Western Blot Analysis of JX14 xCT Expression in Cell Culture. xCT is not up-regulated in JX14 cells over five days in cell culture. (Note GAPDH loading control needs to be repeated).



Appendix Figure 2. Radiation Sensitivity Differences between GBM22 and GBM14. GBM22 and GBM14 cells respond differently to fractionated radiation. (**A**) GBM14s are more sensitive to radiation than GBM22s. (**B**) The greatest difference in radiation sensitivity is between 1Gy and 2Gy fractionated radiation doses. (**C**) GBM22s are sensitized to radiation with 0.5mM SAS treatment. (**D**) GBM14s are sensitized to radiation with 0.5mM SAS + 100µM TBOA treatment. (Note this is preliminary data and needs replication before determining significance).



Appendix Figure 3. Cortical Neuron Co-culture with GBM14, GBM22, and SAS. GBM22 tumors show neurotoxicity that is decreased with 0.5mM SAS treatment. (**A**) Control, GBM14, GBM22, and GBM22 + SAS co-cultures at 0h and 24h after co-culture. Images between 0h and 24h are comparable for Control (A i & ii), GBM14 (A iii & iv), and GBM22 + SAS (A vii & viii). (**Bi**) Enlarged image of (A v) showing neurons co-cultured with GBM22 cells at 0h, (**Bii**) Enlarged image of (A vi) showing neurons co-cultured with GBM22 cells at 24h. 10x magnification.



Appendix Figure 4. GBM22 Cell Proliferation with Nifedipine. GBM22 cells, when treated with 0.5mM SAS and 100mM Nifedipine, show complete growth inhibition. Nifedipine alone has no effect. (**A**) GBM22 cells treated with SAS and Nifedipine. (**B**) Control showing Nifedipine treatment alone. n=3

APPENDIX B

IACUC APPROVAL FORM

Animal Use Request (AUR)
Institutional Animal Care and Use Committee (IACUC) of the
University of Alabama at Birmingham (UAB)

Volker Hall B10
1717 7th Avenue South
Birmingham, Alabama
35294-0019

Office: 205-934-7692
Fax: 205-934-1188
Email: iacuc@uab.edu
Website: www.uab.edu/iacuc

GENERAL INFORMATION

This Animal Use Request (AUR) is used to submit requests for new projects and third annual renewal of externally funded projects using vertebrate animals. Responses should be typed into the blank fields provided, beginning at the arrowhead (►). Questions with boxes may be answered by clicking on the appropriate box. If an item is not applicable, please indicate NA. Submit completed forms via email to iacuc@uab.edu.

Work proposed in the AUR form must match that described in the associated grant or contract, which must be submitted with the AUR as an attachment (PDF format is preferred). If the associated grant, progress report, or contract is ongoing, then the most recent progress report should be submitted with the AUR. If the work is internally funded, a study plan (1-2 page description) must be submitted. The grant, contract, or study plan must be submitted to the IACUC office before the AUR will be reviewed.

Any work proposing the use of potentially hazardous agents or materials in animals must be reviewed and approved by the appropriate UAB safety committee before the animal studies may begin. The project must also be registered with Occupation Health and Safety (OH&S). Registration information can be found at www.healthsafe.uab.edu.

The use of core facilities to conduct the work proposed must be appropriately documented. If the core facility used has an IACUC animal project number (APN) designated for patrons' usage, then this APN may be referenced in the AUR. Otherwise, all procedures that will be conducted by the core facility and the personnel involved must be included in this AUR. For additional information about the use of core facilities, contact the IACUC Director at 934-7847.

Regulations require that the attending veterinarian or a designee be consulted during the planning stages of any projects in which animals may experience pain or distress. The veterinarian consultation can be completed following the submission of the completed AUR or during a pre-review process. If the pre-review process is preferred, contact the veterinary staff at 934-7856.

The following research activities DO NOT require the submission of this IACUC AUR:

- The use of tissues from animals that have been euthanized under an approved IACUC protocol
- Commercial antibodies (except custom products) generated from live animals
- The use of nonviable embryos (e.g., unhatched eggs)
- The use of invertebrates (refer to www.uab.edu/iacuc for appropriate forms)

Animal Use Request (AUR)
Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham (UAB)

FOR OFFICE USE ONLY

Date Received: Date Approved: Animal Project Number (APN):

PROVIDE A COPY OF THE CORRESPONDING GRANT, CONTRACT APPLICATION, OR STUDY PLAN (PDF format is preferred), which should be consistent with the AUR but do not refer to it instead of responding to the questions below.

SECTION I: GENERAL INFORMATION (Questions 1-4)

1. Principal Investigator

Name	Harald Sontheimer	**Blazer ID**	hws
Department	Neurobiology	**Division**	
Office Address	1719 6th Ave. S. CIRC 410	**Office Phone**	5-5805
Email	hws@nrc.uab.edu	**FAX Number**	5-6330
Contact who should receive copies of IACUC correspondence (Optional)			
Name	**Kathryn Turner**	**Email**	**Kturner7@uab.edu**
Phone	**4-4455**	**FAX Number**	**5-6320**

2. Application

Project Title	Amino-acid Transport and the Biology of Human Gliomas
Sponsor	NINDS
Project Period (start date & end date)	09/30/11-07/31/16
Previous APN	110307391
If part of a Program Project, SCORE, Center, etc:	
Name of PI	
Title	

3. Will personnel other than the PI (e.g., faculty, staff, students, or fellows) be involved in the animal work being proposed? ☐ NO ☒ YES

If the response is YES, provide the name(s) below.

Name	Blazer ID	For Nonhuman Primate Users: Date of Confirmation of Negative TB Status
Susan Buckingham	sbucking	
Vishnu Cuddapah	cuddapah	
Michael McFerrin	mcferrm	
Stefanie Robel	Srobel	
Stephanie Robert	srobe25	
Stefanie Seifert	sseifert	
Katie Turner	Kturner7	
Stacey Watkins	swatki01	

4. Certification

By submission of this form, I certify that the information provided in this Animal Use Request (AUR) completely and accurately describes the work to be performed and all work proposed in the associated grant application, contract application, or study plan.

I further certify that

- **No personnel working under my direction will perform any animal procedures until their experience and training has been reviewed and approved by the IACUC.**
- **I will submit to the IACUC the names and qualifications of new or additional personnel including students and visiting faculty before they become involved in these studies,**
- **I will ensure that all personnel are enrolled in the institutional Occupational Health Program prior to their contact with animals or their entry into the animal facilities.**
- I will comply with the procedures described in the NIH Guide for the Care and Use of Laboratory Animals, the PHS Policy on Humane Care and Use of Laboratory Animals, the USDA Animal Welfare Act Regulations, applicable UAB policies, and Standard Operating Procedures as described by the Animal Resources Program and IACUC.
- I acknowledge responsibility for this project and all faculty, staff and students who participate in it.

I also understand that I must submit a modification to an approved protocol and obtain IACUC approval before I

- **Use additional animal species, increase the number of animals to be used, or increase the number of procedures performed on individual animals.**
- **Change procedures that in any way increase the pain/distress an animal might experience or that might be considered a significant departure from those described in this AUR.**
- **Perform procedures not described in this AUR.**
- **Use or allow to be used for other studies animals purchased, produced, or otherwise acquired for this project.**

I understand that I must request renewal of IACUC approval annually and on the third anniversary date of the original IACUC approval I am required to submit an AUR reflecting the work to be performed for the remainder of the grant/contract award.

APPENDIX C

IRB CERTIFICATION FORM

Project Revision/Amendment Form

Form version: October 28, 2010

In MS Word, click in the white boxes and type your text; double-click checkboxes to check/uncheck.

- Federal regulations require IRB approval before implementing proposed changes. See Section 14 of the IRB Guidebook for Investigators for additional information.
- Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.). See Item 4 for more examples.

1. Today's Date	January 23, 2012

2. Principal Investigator (PI)

Name (with degree)	Harald Sontheimer, PhD	Blazer ID	hws
Department	Neurobiology	Division (if applicable)	
Office Address	CIRC 410B, zip 0021	Office Phone	(205) 975-5805
E-mail	sontheimer@uab.edu	Fax Number	(205) 975-5518

Contact person who should receive copies of IRB correspondence (Optional)

Name	Faye Morrow	E-Mail	morrow@uab.edu
Phone		Fax Number	
Office Address (if different from PI)			

3. UAB IRB Protocol Identification

3.a. Protocol Number	X061218008
3.b. Protocol Title	Amino-acid Transporters and the Biology of Human Gliomas

3.c. Current Status of Protocol—Check ONE box at left; provide numbers and dates where applicable

- ☐ Study has not yet begun — No participants, data, or specimens have been entered.
- ☒ In progress, open to accrual — Number of participants, data, or specimens entered: 214
- ☐ Enrollment temporarily suspended by sponsor
- ☐ Closed to accrual, but procedures continue as defined in the protocol (therapy, intervention, follow-up visits, etc.)
  - Date closed:
  - Number of participants receiving interventions:
  - Number of participants in long-term follow-up only:
- ☐ Closed to accrual, and only data analysis continues
  - Date closed:
  - Total number of participants entered:

4. Types of Change

Check all types of change that apply, and describe the changes in Item 5.c. or 5.d. as applicable. To help avoid delay in IRB review, please ensure that you provide the required materials and/or information for each type of change checked.

- ☐ **Protocol revision (change in the IRB-approved protocol)**
  In Item 5.c., if applicable, provide sponsor's protocol version number, amendment number, update number, etc.
- ☐ **Protocol amendment (addition to the IRB-approved protocol)**
  In Item 5.c., if applicable, provide funding application document from sponsor, as well as sponsor's protocol version number, amendment number, update number, etc.
- ☒ **Add or remove personnel**
  In Item 5.c., include name, title/degree, department/division, institutional affiliation, and role(s) in research, and address whether new personnel have any conflict of interest. See "Change in Principal Investigator" in the IRB Guidebook if the principal investigator is being changed.
  - ☒ **Add graduate student(s) or postdoctoral fellow(s) working toward thesis, dissertation, or publication**
    In Item 5.c., (a) identify these individuals by name; (b) provide the working title of the thesis, dissertation, or publication; and (c) indicate whether or not the student's analysis differs in any way from the purpose of the research described in the IRB-approved HSP (e.g., a secondary analysis of data obtained under this HSP).
- ☐ **Change in source of funding; change or add funding**
  In Item 5.c., describe the change or addition in detail, include the applicable OGCA tracking number(s), and provide a copy of the application as funded (or as submitted to the sponsor if pending). Note that some changes in funding may require a new IRB application.
- ☐ **Add or remove performance sites**
  In Item 5.c., identify the site and location, and describe the research-related procedures performed there. If adding site(s), attach notification of permission or IRB approval to perform research there. Also include copy of subcontract, if applicable. If this protocol includes acting as the Coordinating Center for a study, attach IRB approval from any non-UAB site added.

☐ **Add or change a genetic component or storage of samples and/or data component—this could include data submissions for Genome-Wide Association Studies (GWAS)**
To assist you in revising or preparing your submission, please see the IRB Guidebook for Investigators or call the IRB office at 934-3789.

☐ **Suspend, re-open, or permanently close protocol to accrual of individuals, data, or samples (IRB approval to remain active)**
In Item 5.c., indicate the action, provide applicable dates and reasons for action; attach supporting documentation.

☐ **Report being forwarded to IRB (e.g., DSMB, sponsor or other monitor)**
In Item 5.c., include date and source of report, summarize findings, and indicate any recommendations.

☐ **Revise or amend consent, assent form(s)**
Complete Item 5.d.

☐ **Addendum (new) consent form**
Complete Item 5.d.

☐ **Add or revise recruitment materials**
Complete Item 5.d.

☐ **Other (e.g., investigator brochure)**
Indicate the type of change in the space below, and provide details in Item 5.c. or 5.d. as applicable.
Include a copy of all affected documents, with revisions highlighted as applicable.

▶

5. Description and Rationale

In Item 5.a. and 5.b, check Yes or No and see instructions for Yes responses.
In Item 5.c. and 5.d, describe—and explain the reason for—the change(s) noted in Item 4.

☐Yes ☒No **5.a. Are any of the participants enrolled as normal, healthy controls?**
If yes, describe in detail in Item 5.c. how this change will affect those participants.

☐Yes ☒No **5.b. Does the change affect subject participation, such as procedures, risks, costs, location of services, etc.?**
If yes, FAP-designated units complete a FAP submission and send to fap@uab.edu. Identify the FAP-designated unit in Item 5.c.
For more details on the UAB FAP, see www.uab.edu/cto.

5.c. Protocol Changes: In the space below, briefly describe—and explain the reason for—all change(s) to the protocol.

▶ We would like the following graduate student to be added to the protocol:

Stephanie Robert, other investigator
Stephanie is a Master's degree student working on the project as part of a thesis. Her thesis is entitled "Cystine/Glutamate Transporters as Possible Prognostic & Therapeutic Markers for Primary Brain Tumors." Gliomas contain varying levels of system xc-, a cystine/glutamate exchanger. Stephanie seeks to determine the effects of cystine uptake and glutamate release through system xc- in these gliomas. This project will use de-identified human glioma samples to study the role of this transporter.

5.d. Consent and Recruitment Changes: In the space below,
(a) describe all changes to IRB-approved forms or recruitment materials and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent, recruitment); and
(c) indicate either how and when you will reconsent enrolled participants or why reconsenting is not necessary (not applicable for recruitment materials).

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
- **a copy of the currently approved document (showing the IRB approval stamp, if applicable)**
- **a revised copy highlighting all proposed changes with "tracked" changes**
- **a revised copy for the IRB approval stamp.**

▶

Signature of Principal Investigator _____ Date 1/23/12

FOR IRB USE ONLY

☐ Received & Noted ☑ ~~Approved~~ Expedited* ☐ To Convened IRB

Marilyn Dass — 1-25-12

Signature (Chair, Vice-Chair, Designee) Date

DOLA 8/6-11

Change to Expedited Category Y / (N) / NA

*No change to IRB's previous determination of approval criteria at 45 CFR 46.111 or 21 CFR 56.111

RECEIVED
JAN 23 2012
OFFICE OF INSTITUTIONAL REVIEW BOARD