Published in final edited form as:

Int J Cancer. 2014 March 15; 134(6): 1300–1310. doi:10.1002/ijc.28465.

Triacetin-based acetate supplementation as a chemotherapeutic adjuvant therapy in glioma

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Abstract

Cancer is associated with epigenetic (i.e., histone hypoacetylation) and metabolic (i.e., aerobic glycolysis) alterations. Levels of N-acetyl-L-aspartate (NAA), the primary storage form of acetate in the brain, and aspartoacylase (ASPA), the enzyme responsible for NAA catalysis to generate acetate, are reduced in glioma; yet, few studies have investigated acetate as a potential therapeutic agent. This preclinical study sought to test the efficacy of the food additive Triacetin (glyceryl triacetate, GTA) as a novel therapy to increase acetate bioavailability in glioma cells. The growth-inhibitory effects of GTA, compared to the histone deacetylase inhibitor Vorinostat (SAHA), were assessed in established human glioma cell lines (HOG and Hs683 oligodendroglioma, U87 and U251 glioblastoma) and primary tumor-derived glioma stem-like cells (GSCs), relative to an oligodendrocyte progenitor line (Oli-Neu), normal astrocytes, and neural stem cells (NSCs) *in vitro*. GTA was also tested as a chemotherapeutic adjuvant with temozolomide (TMZ) in orthotopically grafted GSCs. GTA induced cytostatic growth arrest *in vitro* comparable to Vorinostat, but, unlike Vorinostat, GTA did not alter astrocyte growth and promoted NSC expansion. GTA alone increased survival of mice engrafted with glioblastoma GSCs and potentiated TMZ to extend survival longer than TMZ alone. GTA was most effective on GSCs with a mesenchymal cell phenotype. Given that GTA has been chronically administered safely to infants with Canavan disease, a leukodystrophy due to ASPA mutation, GTA-mediated acetate supplementation may provide a novel, safe chemotherapeutic adjuvant to reduce the growth of glioma tumors, most notably the more rapidly proliferating, glycolytic, and hypoacetylated mesenchymal glioma tumors.

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#Denotes equal contribution

Conflict of Interest Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Keywords

aspartoacylase; epigenetics; glioblastoma; glioma; glyceryl triacetate; metabolism; oligodendroglioma; Triacetin

Introduction

Median survival for patients with high-grade glioma (i.e., glioblastoma [GBM, WHO grade IV astrocytoma] and anaplastic oligodendroglioma without loss of heterozygosity of 1p and 19q) is approximately 14 months.^{1, 2} Despite multimodal therapeutic approaches, tumor recurrence is almost inevitable, with post-surgical persistence of chemoradioresistant glioma stem-like cells (GSCs) being a contributing factor.³ Therapies targeting GSCs, while sparing normal brain cells, are therefore of considerable interest.

Acetate supplementation may prove to be a novel efficacious therapeutic strategy for glioma since it acts at the intersection of epigenetics and metabolism, two hallmarks of aggressive tumor growth. The key component of this system is acetyl-coenzyme A (acetyl-CoA) which is required for protein acetylation reactions, including histone acetylation, and mitochondrial bioenergetics. In the human brain, N-acetyl-L-aspartate (NAA) is the most concentrated source of acetate (~ 10 mM).⁴ Aspartoacylase (ASPA) catalyzes the breakdown of NAA, its only known substrate⁵, to L-aspartate and acetate. L-aspartate is then used in protein synthesis and the Krebs cycle, while acetate is converted to acetyl-CoA via cytosolic/nuclear acetyl-CoA synthetase-1 (AceCS1) for lipid biosynthesis and histone/protein acetylation and mitochondrial AceCS2 for ATP production.^{6, 7} NAA levels are decreased in glioma⁸, thus reducing acetate bioavailability. NAA supplementation using mono-methyl NAA, which crosses membranes, is one possible approach. However, we recently demonstrated that treatment with physiological levels of NAA increased GSC proliferation *in vitro*⁹; thus, another acetate source which freely penetrates the blood-brain barrier is required.

Triacetin (glyceryl triacetate, GTA) is ideal for therapeutic acetate supplementation since it freely crosses the blood-brain barrier/plasma membrane and is hydrolyzed to glycerol and acetate by non-specific lipases and esterases in all cell types. This preclinical study sought to compare the *in vitro* growth effects of GTA on established glioma cells lines and tumor-derived GSCs relative to neural stem cells (NSCs), astrocytes, and an oligodendrocyte progenitor cell line (OPC, Oli-Neu).¹⁰ Since it has been reported that GTA can increase histone acetylation^{11, 12}, its growth effect was compared to the histone deacetylase inhibitor (HDACi) Vorinostat (suberoylanilide hydroxamic acid, SAHA) which is currently in glioma clinical trials.^{13, 14} GTA induced cytostatic growth arrest of glioma cells, but had no effect on NSCs or astrocytes, whereas SAHA negatively affected growth of all cells. In orthotopic xenografts, GTA enhanced temozolomide (TMZ) chemotherapeutic efficacy to reduce tumor volume and prolong survival relative to TMZ alone, suggesting that GTA may be an efficacious glioma chemotherapeutic adjuvant.

Materials and Methods

Detection of ASPA expression

ASPA mRNA expression in glioma relative to normal brain (pathologically normal tissue from patients undergoing surgery for epilepsy) was assessed by quantitative real-time PCR (Hs00163703_m1; Applied Biosystems; Carlsbad, CA) using ribosomal RNA control reagents according to manufacturer's instructions.

SDS-PAGE (25 µg protein from whole cell lysates) and western blotting¹⁵, immunohistochemical analysis of human tissue¹⁶, and immunocytochemistry¹⁷ were performed as described. The following antibodies were used: goat anti-human actin (1,000X, sc-1616 Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-human ASPA (500X; GTX13389 GeneTex; Irvine, CA), rabbit anti-mouse 2',3'-Cyclic-Nucleotide 3'-Phosphodiesterase (CNPase, 250X; sc-30158 Santa Cruz Biotechnology), mouse anti-porcine glial fibrillary acidic protein (GFAP, 5,000X; G3893 Sigma; St. Louis, MO), and rat anti-bovine myelin basic protein (MBP, 25X; ab7349 Abcam). Species-specific Cy3-conjugated (500X) and Cy2-conjugated (100X) secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Cell Culture

HOG cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Hs683, U87, and U251 cells were grown in DMEM with 10% FBS. The Oli-Neu cell line was grown on poly-L-lysine (10 µg/ml) coated dishes in SATO growth medium.¹⁰ Human cerebral cortical astrocytes (HA#1800 ScienCell; Carlsbad, CA) were cultured in basal medium with 2% FBS and astrocyte growth supplement (AM#1801 ScienCell). Mouse neural stem cells (NSCs) from postnatal day 4 cortex were prepared as described.¹⁸ GSCs were isolated from surgical specimens (detailed in Supplementary Methods) using previously described methodology.¹⁹ NSCs and GSCs were maintained in stem cell medium (SCM) consisting of DMEM/F12, 1X B27 supplement, 20 ng/ml epidermal growth factor and 20 ng/ml basic fibroblast growth factor on non-adhesive plastic. GSC differentiation was induced using DMEM with 10% FBS. All media contained 50 U/ml penicillin and 50 µg/ml streptomycin and was replenished every 48 hours. Cells were grown in the absence or presence of 0.25% GTA or 1 µM SAHA (both from Sigma). The GTA concentration was selected based on a dose response with GBM12 GSCs (Supporting Information Fig. S1). The SAHA concentration was selected because it is associated with increased histone acetylation, but not alterations in adhesion or cytotoxicity.^{20, 21} Growth dynamics were assessed using unbiased trypan blue exclusion based cytometry. Growth conditions are presented in greater detail in Supplementary Methods.

DNA Analysis

Cell line validation by STR DNA fingerprinting, single nucleotide polymorphism (SNP) mapping, reverse transcription PCR to characterize proneural versus mesenchymal antigenic profile, and methylation specific PCR to assess O⁶-methylguanine-methyltransferase (MGMT) promoter status were performed as detailed in Supplementary Methods.

Cell Cycle Analysis

Cell cycle profiles were visualized by propidium iodide (PI) staining as described²² with minor modifications (10⁶ cells/ml were incubated in low-salt PI at 37°C for 20 minutes, then an equal volume of high-salt PI was added and incubated at 4°C for 4 hours). Cell cycle profiles were recorded using the BD LSR II Flow Cytometer and analyzed using FACS Diva 7.0 software.

Orthotopic tumor model

GSCs were transduced with a lentiviral construct to express firefly luciferase as detailed in Supplementary Methods. All procedures were conducted in accordance with institutional guidelines for the humane care and use of animals. Orthotopic grafting was performed as described²³ and detailed further in Supplementary Methods. GTA (5.0 g/kg with 10% v/v Ora-Sweet SF) was administered intragastrically once per day starting on the third postoperative day. TMZ (20 mg/kg) was administered intragastrically in an oral suspension

vehicle containing 2.5 mg/ml Povidone K30, 0.013% citric acid, 50% Ora-Plus, 50% Ora-Sweet SF (Paddock Labs via Apotheca Inc.; Phoenix, AZ)²⁴ on days 5, 7, 9, 11, and 13. Mice receiving the “primed” combined GTA/TMZ therapy began GTA treatment on day 3 then received the TMZ in the morning and GTA in the evening on days 5, 7, 9, 11, and 13 with GTA alone given on days 6, 8, 10, 12 and day 14 onwards. For “concurrent” combination therapy, GTA and TMZ were both begun on day 5, while for “salvage” therapy GTA was administered daily after the completion of TMZ on day 13. Control mice received the oral suspension alone.

In vivo bioluminescent imaging was performed using the Xenogen IVIS 200 imaging system as detailed in Supplementary Methods. Mice were euthanized when they displayed neurological signs (e.g., altered gait, tremors/seizures, lethargy) or weight loss of 20% or greater of pre-surgical weight. Tumor volumetric measurements were performed using unbiased stereology as described²⁵ and detailed in Supplementary Methods.

Statistical Analysis

Analyses were conducted by individuals blinded to treatment group. Data are expressed as mean $\pm$ standard error of the mean. Significant differences were determined by either one-way or two-way ANOVA and Bonferroni multiple comparison tests using Prism software (GraphPad; San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

ASPA expression is decreased in glioma

Previously, we demonstrated that ASPA expression was decreased in neuroblastoma.¹⁶ Given the abundant ASPA expression in the central nervous system²⁶, we sought to assess whether ASPA expression was dysregulated in glioma. Quantitative PCR revealed decreased ASPA mRNA in glioma compared with normal brain (Fig. 1a). Microarray data from the REMBRANDT (Supporting Information Fig. S2a) and TCGA (Supporting Information Fig. S2b) databases corroborated decreased ASPA mRNA expression, which was independent of tumor subtype (Supporting Information Fig. S2c) or isocitrate dehydrogenase 1 mutation status (Supporting Information Fig. S2d). Western blot analysis confirmed decreased ASPA expression in glioma (Fig. 1b). In the rodent brain, ASPA is most prominently expressed by oligodendrocytes²⁶ and dual-label immunohistochemistry with CNPase confirmed this pattern of ASPA expression in normal human cortex (Fig. 1c). In contrast, ASPA expression was significantly reduced in oligodendroglioma tumors. Similarly, ASPA expression was detected in GFAP-positive astrocytes in normal human cortex, but not in GBM tumors. The net result of decreased ASPA and its substrate, NAA, in glioma is reduced acetate bioavailability.

GTA induces growth arrest of glioma-derived stem-like cells, but not neural stem cells

To determine whether differences in GTA responsiveness were correlated with chromosomal alterations, all cells used in this study, established oligodendroglioma cells (HOG, Hs683) relative to tumor-derived oligodendroglioma GSCs (grade II OG33, grade III OG35) and established GBM cells (U87, U251) relative to six tumor-derived GBM GSCs, were subjected to in-depth DNA analysis. The STR DNA fingerprint for the commercially available cells matched their known DNA fingerprint, while the profiles of the tumor-derived GSCs did not match known DNA fingerprints (Supporting Information Table S1). Surprisingly, the STR profiles for HOG, OG33, GBM9, GBM12, and GBM34 were identical even though each displayed distinct growth characteristics and morphology upon growth factor withdrawal (Fig. 4). Thus, DNA mapping for chromosomal copy variation using GeneChip® 250K Nsp arrays was undertaken (Fig. 2a, Supporting Information Figs.

S3A-E). Principal component analysis (PCA) with the SNP raw intensity data grouped OG33, OG35, and HOG cells together (Fig. 2a) and copy number analysis identified similar, but not identical, amplifications and deletions for these samples (Fig. S3). Although Hs683 cells were established from a GBM, they display oligodendroglioma features²⁷; yet, they failed to cluster either with oligodendroglioma or astrocytoma cells in the PCA plot. The GBM GSCs clustered into one group (GBM9, GBM12, GBM34) that expressed antigenic features indicative of a mesenchymal profile (i.e., BCL2A1, WT1, CD44, and CD44v6²⁸ expression) and a second group (GBM2, GBM8, GBM44) that exhibited a proneural profile (i.e., CD133, Notch1, SOX2, PDGFR- α , Nestin, and Olig2 expression) (Fig. 2b). U87 and U251 cells were more similar to proneural GSCs than mesenchymal GSCs. Thus, we propose that STR profiling and SNP karyotyping can be used to distinguish GSCs with proneural or mesenchymal GBM features.

First, the growth effects of 0.25% GTA and 1 μ M SAHA were assessed by flow cytometric analysis (Fig. 3a). GTA treatment for 24 hours induced cytostatic G₀ growth arrest which was more pronounced in GSCs than established cell lines and within GSCs was more pronounced in cells with a mesenchymal profile (GBM12, 34, 9). Continuous treatment was associated with reduced viability of SAHA treated, but not GTA treated cells (e.g., percent viable cells in GBM44 at 5 day: control 93.5 $\pm$ 1.89%; GTA 94.17 $\pm$ 1.49%, $p=0.78$; SAHA 78.8 $\pm$ 3.86%, $p=0.006$) (Fig. 3b).

Next, the long-term growth inhibitory effects of GTA and SAHA were assessed by unbiased cytometry over 5 days of treatment (Figs. 4a, b). In SCM, prolonged GTA treatment was associated with reduced growth of all glioma cells except the GBM GSCs with a proneural phenotype (Fig. 4a). In contrast, GTA had no effect on astrocytes and even increased NSC proliferation. The apparent GTA-mediated growth reduction of Oli-Neu cells may be due to decreased cell adhesion (Supporting Information Fig. S4). However, when treated in differentiation medium (DM), GTA, but not SAHA, reduced the growth of OG33 and OG35 GSCs (Fig. 4b). In addition, GTA more profoundly reduced cell growth than SAHA of the 3 mesenchymal GSCs and was as effective as SAHA in growth reduction of the 3 proneural GSCs. Interestingly, the growth rate of the 3 mesenchymal GSCs (GBM12, GBM34, GBM9) increased while the growth rate of the 3 proneural GSCs (GBM8, GBM44, GBM2) decreased in DM relative to SCM. Thus, the differentiation potential of the oligodendroglioma- (Fig. 4c) and GBM-derived (Fig. 4d) GSCs were examined after 3 days in DM. OG33 and OG35 GSCs are NG2-positive and PDGFR α -positive cells in SCM (not shown) that express a low level of CNPase, but not myelin basic protein in DM. The proneural GSCs (GBM8, GBM44, GBM2) differentiated into GFAP-positive astrocytes, strongly immunoreactive CNPase-positive oligodendrocytes (Fig. 4d), and class III β -tubulin-positive neurons (not shown). In contrast, the mesenchymal GSCs (GBM12, GBM34, GBM9) failed to express markers of differentiation (Fig. 4d) and were immunoreactive for the proliferation marker Ki67 (not shown) despite growth in differentiation permissive conditions. Taken together, these data support the use of GTA as a cytostatic agent for the treatment of the most aggressive mesenchymal GSCs and their differentiated progeny without affecting normal brain cells.

GTA enhances TMZ chemotherapeutic efficacy

Inasmuch as *in vitro* self-renewal is only one defining feature of GSCs, the tumorigenicity of the most aggressive GSCs (OG35, GBM12), as well as the growth inhibitory effects of GTA, was investigated. These GSCs exhibited MGMT promoter methylation (Supporting Information Fig. S5), suggesting chemosensitivity. Since this represents the first report of OG33 and OG35 GSC xenografting, OG33 tumorigenicity was also established (Supporting

Information Fig. S6). GTA treatment had no effect on the rate of bioluminescence increase or survival of mice engrafted with OG33 GSCs.

GTA increased the efficacy of TMZ in mice engrafted with OG35 GSCs (Fig. 5, previously published as an abstract²⁹). Because GTA provides metabolizable carbons and weight loss is a euthanasia criterion, blood glucose levels were monitored, but no differences were observed among treatment groups (Fig. 5a). GTA alone had no effect on survival or tumor volume relative to vehicle treated mice (Figs. 5a, c, d). However, when examined by a neuropathologist, reduced mitotic labeling was present in GTA, TMZ, and GTA/TMZ treated tumors (Fig. 5b), which was confirmed by Ki67 immunolabeling (not shown). As expected from the MGMT status, TMZ reduced tumor bioluminescence (Figs. 5a, c) and increased survival (Figs. 5a, d). The study was negatively biased by assigning mice with the greatest initial bioluminescence to the GTA/TMZ treatment group (Figs. 5a, c: *Initial Flux*). Even though GTA/TMZ treatment did not reduce end-point tumor volume relative to TMZ alone, it significantly reduced tumor bioluminescence (Fig. 5c) and increased survival (Fig. 5d), suggesting efficacy as a chemotherapeutic adjuvant. Moreover, survival is underestimated since the study was terminated at 40 days when 3 of 10 GTA/TMZ treated mice failed to redevelop flux. These mice also showed no histological signs of tumor at study termination. Based on the hypothesis that GTA acetylates histones to promote an open, euchromatic state^{11, 12}, GTA was administered for two days prior to TMZ (primed therapy). When compared to concurrent (i.e., GTA and TMZ initiated on day 5) and salvage (i.e., GTA administered after completion of TMZ) therapy, only the primed treatment regimen was associated with increased survival relative to TMZ alone (Fig. 5d), supporting the hypothesis that GTA should be administered prior to TMZ to exert maximal therapeutic effect.

Inasmuch as the mesenchymal GBM subtype is more treatment resistant, the anti-proliferative effect of GTA was assessed on the most aggressive GBM GSCs, GBM12 (Fig. 6). GBM12 vehicle treated mice possessed large tumors with invasive foci and hemorrhagic cores and only survived ~13 days. GTA alone did not alter blood glucose, bioluminescence, or end-point tumor volume; however, GTA increased survival (Fig. 6c). TMZ reduced bioluminescence (Fig. 6c) and end-point tumor volume (not shown). GTA did not enhance TMZ chemotherapeutic effect on tumor volume compared to TMZ alone (Fig. 6c). Nonetheless, GTA/TMZ significantly increased survival, with 2 of 8 mice never redeveloping measurable flux or displaying histological signs of tumor at study termination. In sum, GTA induces cytostatic growth arrest of oligodendroglioma-derived and GBM-derived GSCs *in vitro* comparable to that of SAHA, but, unlike SAHA, GTA had little to no effect on normal cells. More strikingly, when administered prior to TMZ, GTA enhances chemotherapeutic efficacy on orthotopic tumors and/or increases survival, suggesting efficacy as a chemotherapeutic adjuvant.

Discussion

This represents the first report detailing decreased ASPA expression in glioma tumors. Inasmuch as ASPA was previously thought to be an oligodendrocyte-restricted enzyme, it would not be unexpected to be decreased in astrocytoma tumors. However, we detected ASPA immunoreactivity in human cortical astrocytes and primary cultured human astrocytes. Moreover, ASPA was also decreased in oligodendroglioma tumors, not increased as would be expected for an oligodendrocyte mass. We propose that decreased ASPA expression primarily occurs within the tumor bulk, but is expressed by GSCs to maintain their undifferentiated state. Inasmuch as acetate serves as a substrate for lipogenesis, which promotes anabolic growth of tumor cells³⁰, and as a metabolic substrate for astrocytes, it is counter-intuitive that acetate supplementation decreases tumor growth.

We propose that GTA exploits the link between histone acetylation and cellular metabolism in glioma therapy and functions via an epigenetic mechanism. Promoter CpG hypermethylation is coupled to histone hypoacetylation and poorer clinical outcomes.³¹ Because AceCS1-dependent acetyl-CoA synthesis is energy-dependent, most nuclear acetyl-CoA for histone acetylation under normal nutrient conditions is derived from citrate via ATP-citrate lyase.³² However, in highly proliferative, glycolytically converted tumor cells, mitochondrial citrate is exported to the cytosol to support biomass accumulation for proliferation. GTA may permit citrate to remain within the mitochondria and promote oxidative phosphorylation, while GTA-derived acetate promotes AceCS1-dependent acetyl-CoA synthesis and histone acetylation. Studies by Rosenberger and colleagues have demonstrated that GTA promotes histone acetylation.^{11, 12} Preliminary mass spectrometry analysis of GTA treated GBM12 GSCs indicates increased H4K16 acetylation as well as acetylation of several other proteins involved in cell cycle regulation (Lam & Jaworski, unpublished observation). Notably, H4K16 acetylation is an important epigenetic mark of actively transcribed euchromatin and loss of H4K16 acetylation is a common hallmark of cancer.³³ We believe that GTA exerted the most profound growth inhibitory effects on GSCs with a mesenchymal phenotype because these cells exhibit the most glycolytic state³⁴ and, thus, display greater histone hypoacetylation. The superior effectiveness of GTA over calcium acetate³⁵ suggests that GTA has better absorption and/or GTA acts as an HDACi in its unhydrolyzed form. Similar to butyrate, GTA may exert both acetyl-CoA/histone acetyltransferase-dependent acetylation and HDAC inhibition based on the metabolic state of a cell.³⁶ Short-term GTA treatment (2 and 4 hours) was associated with a two-fold increase in HDAC activity¹¹; thus, raising the possibility that GTA acts as an HDACi. Preliminary studies demonstrate that GTA is more effective at growth inhibition than 36 mM sodium acetate (equivalent acetate to 0.25% GTA), again supporting the hypothesis that GTA may exert functions other than as an acetate source (unpublished observation). However, long-term GTA treatment did not alter HDAC activity¹²; thus, further *in vitro* studies will be needed to determine whether GTA functions directly as an HDACi. Interestingly, the mesenchymal GSCs display self-renewal and formation of aggressive orthotopic tumors, but exhibit reduced differentiation capacity relative to proneural GSCs (Figs. 4c, d). Hence, unlike SAHA which promotes differentiation, the anti-tumorigenic effect of GTA is likely not due to the promotion of GSC differentiation. Based on reports of mesenchymal differentiation of GSCs^{37–39}, we tested the adipogenic and osteogenic differentiation ability of the GBM9, GBM12, and GBM34 GSCs. This, as well as inhibition of PI3K/Akt, mTOR, and ERK signaling, either singly or in combinations, failed to promote differentiation (unpublished observation). This suggests that mesenchymal tumors may be more treatment resistant due to a bias toward self-renewal and sustained repression of differentiation genes.^{40–42}

The simplest explanation for the increased TMZ efficacy in GTA treated mice is that GTA functions as an excipient (i.e., a “Trojan horse” carrying TMZ). Although our GTA/TMZ therapy subjects mice to gavaging twice daily, we believe that the morning TMZ has undergone absorption prior to the evening GTA, reducing the possibility of GTA binding to TMZ and promoting its transport through the BBB. We acknowledge that GTA likely exerts pleiotropic effects. In addition to regulating protein acetylation and metabolism, GTA exerts anti-inflammatory effects¹² and increases plasma ketones and resting energy.⁴³ Thus, GTA may promote a metabolic state that is less conducive to glioma growth. In fact, the ketogenic diet has shown promising therapeutic results⁴⁴ and we do not exclude that some of the GTA-mediated survival is due to less weight loss. Hence, GTA may be an effective therapy for cancer cachexia.

GTA is an FDA approved food additive with “generally regarded as safe” status that has been tested for parenteral nutrition in a wide variety of species with no adverse effects.⁴⁵ It

may be necessary to chronically administer GTA since continuous histone hyperacetylation is critical for SAHA's effects.⁴⁶ Infants with Canavan disease have been chronically treated with high dose GTA (4.5 g/kg/day, similar to the dose administered in our orthotopic model) and showed no hepatotoxicity or significant side effects.⁴⁷ In contrast, SAHA is associated with cardiotoxicity, anemia, and thrombocytopenia.^{13, 48} While GTA alone increased survival of GBM12 mice, it is GTA's ability to enhance TMZ's effects that is most clinically relevant. Unfortunately, resistance limits the therapeutic benefit of TMZ. Our GSCs exhibit MGMT methylation and, thus, are TMZ responsive. However, DNA methylation does not stably lock gene expression since continuous histone hyperacetylation potentiates acquired TMZ resistance via up-regulation of MGMT expression without altering promoter methylation.^{49, 50} If GTA does not similarly promote TMZ resistance, it may prove more effective than SAHA. We are highly encouraged that GTA showed growth arrest of the more aggressive mesenchymal GSCs and that its effects positively correlated with proliferation rate. Moreover, that the growth inhibitory effect was not dependent of GSC differentiation. Hence, we assert that further investigations of GTA as a chemotherapeutic adjuvant are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by R01NS045225 co-funded by NINDS and NCRR, and Pilot Project grants from the Vermont Cancer Center/Lake Champlain Cancer Research Organization, Neuroscience COBRE (NIH NCRR P20 RR016435), and UVM College of Medicine (DMJ). Facilities and equipment supported by the Neuroscience COBRE Molecular Core Facility (NIH NCRR P20 RR016435), Vermont Cancer Center DNA Analysis Facility (NIH P30 CA22435), Vermont Genetics Network Bioinformatics Core and Microarray Facility (NIH NIGMS 8P20GM103449), and The Penelope and Sam Fund of the Vermont Cancer Center were instrumental to the completion of the study.

The corresponding author wishes to thank Professor Dylan R. Edwards and Dr. Caroline J. Pennington (University of East Anglia School of Biological Sciences, Norwich UK) for the fabulous sabbatical experience performing the TLDA-based degradome profiling that identified ASPA dysregulation in glioma, Drs. William C. Broaddus and Helen L. Fillmore (Virginia Commonwealth University Division of Neurosurgery) for providing the necessary surgical samples, and Dr. John R. Moffett (Uniformed Services University of the Health Sciences Department of Anatomy, Physiology & Genetics) for insightful discussions regarding NAA metabolism and therapeutic uses of GTA. We acknowledge Dr. Glyn Dawson (University of Chicago Department of Pediatrics) for kindly providing the HOG cell line, Dr. Antonio Chiocca (Brigham and Women's Hospital Department of Neurosurgery) for kindly providing the GSCs, and Dr. Bin Hu (Ohio State University Department of Neurological Surgery) for determining the GSC MGMT methylation status.

Abbreviations

acetyl-CoA	acetyl-coenzyme A
AceCS1	acetyl-coenzyme A synthetase-1
AceCS2	acetyl-coenzyme A synthetase-2
ASPA	aspartoacylase
DM	differentiation medium
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
GBM	glioblastoma

GFAP	glial fibrillary acidic protein
GSC	glioma stem-like cell
GTA	glyceryl triacetate
MGMT	O ⁶ -methylguanine-methyltransferase
NAA	N-acetyl-L-aspartate
NSC	neural stem cell
OPC	oligodendrocyte progenitor cell
PCA	principal component analysis
PCR	polymerase chain reaction
PDGFRα	platelet-derived growth factor receptor alpha
REMBRANDT	Repository for Molecular Brain Neoplasia Data
SAHA	suberoylanilide hydroxamic acid
SCM	stem cell medium
SNP	single nucleotide polymorphism
TMZ	temozolomide

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What's new?

Cancer is associated with global hypoacetylation and aerobic glycolysis; yet, studies have not investigated acetate supplementation as a therapeutic approach. We demonstrate that aspartoacylase, the enzyme that catabolizes N-acetyl-L-aspartate, the primary storage form of acetate in the brain, is reduced in glioma tumors. Furthermore, using oligodendroglioma- and glioblastoma-derived glioma stem-like cells (GSCs), we show that the food additive Triacetin (glyceryl triacetate) induces GSC growth arrest *in vitro* and potentiates the chemotherapeutic effects of temozolomide in orthotopic grafts. These pre-clinical data warrant the further examination of Triacetin as a chemotherapeutic adjuvant.

Figure 1.

ASPA expression is decreased in glioma tumors. (a) Quantitative real-time PCR revealed decreased ASPA mRNA expression in recurrent grade III oligodendroglioma, anaplastic astrocytoma and GBM. $n = 4$. Refer to Supplementary Fig. 2 for analysis of REMBRANDT and TCGA datasets. (b) Western blot (25 μg crude protein homogenate, normalized to actin) densitometric analysis revealed that ASPA expression was decreased in grade II (OII) and grade III (OIII) oligodendroglioma, anaplastic astrocytoma (AA) and glioblastoma (GBM) tumors, but similar to ASPA mRNA, ASPA protein was most significantly decreased in recurrent grade III (ReO) oligodendroglioma relative to normal (N) brain (pathologically normal tissue from patients undergoing surgery for epilepsy). $n = 6$ normal, 10 GBM, and 4 all others, with 2 representative protein samples shown. (c) Dual-label immunohistochemistry using normal human cerebral cortex (i.e., post-mortem brain) revealed that ASPA was more abundantly expressed in CNPase-positive oligodendrocytes within the corpus callosum (WM) than the overlying isocortex. ASPA expression was also detected within the cortical grey matter (GM, arrowheads) by GFAP-positive astrocytes. Immunohistochemistry using two independent tissue samples confirmed the western blot results that GBM and grade III (GIII) oligodendroglioma tumors possess significantly fewer ASPA immunoreactive cells. Scale bar = 100 μm (left panel), 50 μm (right panel). * $p < 0.05$, # $p < 0.001$, ## $p < 0.0001$.

Figure 2.

Characterization of GSC genetic profile by whole genome cytogenetic analysis and PCR. (a) Principal component analysis (PCA) of SNP raw intensity data from GeneChip® Human Mapping 250K Nsp Arrays revealed that the established GBM cell lines U87 and U251 share similar gene amplifications/deletions to the proneural GSCs (GBM44, GBM8, and GBM2). The oligodendroglioma-derived cells (grade II OG33 and grade III OG35 GSCs and the HOG established oligodendroglioma cell line) were more similar to mesenchymal GBM GSCs (GBM12, GBM9, and GBM34). The Hs683 cell line, which was derived from a GBM tumor, but shares features of oligodendroglioma tumors, failed to cluster with either tumor type. (b) PCR was performed with a panel of well-accepted markers of proneural (e.g., CD133, Notch1, SOX2, PDGFR α , Nestin, and Olig2) and mesenchymal (e.g., BCL2A1, WT1, CD44, and CD44v6) glioma phenotypes. Although this analysis is non-quantitative, these markers display distinct bimodal expression patterns. Similar to STR profiling (Supplementary Table 1), PCR profiling confirms that GBM12, GBM34, and GBM9 GSCs exhibit a mesenchymal signature, while GBM8, GBM44, and GBM2 GSCs exhibit a proneural signature. In keeping with their oligodendroglial origin, OG33 and OG35 GSCs express PDGFR α and NG2 (not shown), but otherwise exhibit a mesenchymal signature.

Figure 3.

GTA induces G_0 growth arrest of established glioma cell lines and primary tumor-derived GSCs *in vitro*. (a) Cell cycle profile of PI-labeled cells in growth/stem cell medium after 24 hours of 1 μM SAHA or 0.25% GTA treatment. GTA induced G_0 growth arrest of all glioma cells, except U87, U251 and GBM8 GSCs, without affecting Oli-Neu OPCs or astrocytes and promoted neural stem cell (NSC) expansion. In contrast, SAHA significantly reduced proliferation of glioma and normal cells equally. (b) GSCs (50,000 cells per well of 24 well plate) were cultured in SCM in the absence or presence of 0.25% GTA or 1 μM SAHA for 5 days with medium replenished every 48 hours. While GTA-mediated growth reduction was largely cytostatic, SAHA-mediated growth reduction did not promote differentiation (except in GBM8 GSCs), but was more cytotoxic. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$, ## $p < 0.0001$. n = 3 independent experiments. Scale bar = 200 μm.

Figure 4.

GTA-mediated growth reduction of established glioma cell lines and primary tumor-derived GSCs *in vitro* is not due to the promotion of differentiation. GSCs were dissociated and plated (10,000 cells per well of 24 well dish) in the absence or presence of 0.25% GTA or 1 μ M SAHA in SCM (a) or DM (b). Growth dynamics were assessed using unbiased trypan blue exclusion based cytometry over 5 days, with medium replenished every 48 hours. (a) GTA reduced cell growth dynamics comparable to that of SAHA, except that proneural GBM GSCs (GBM8, GBM44, GBM2) were unresponsive in SCM. (b) When treated in DM, GTA was as or more effective than SAHA, particularly on oligodendroglioma-derived GSCs. (c, d) GSCs were cultured in DM for 3 days, fixed, and stained for markers of mature oligodendrocytes (CNPase, myelin basic protein [MBP]) and astrocytes (GFAP). Oli-Neu

cells were used as a positive control. (c) OG33 and OG35 cells expressed CNPase, but failed to express MBP. (d) The proneural GSCs (GBM8, GBM44, GBM2) differentiated into GFAP-positive astrocytes, CNPase-positive oligodendrocytes, and Tuj1-positive neurons (not shown). In contrast, the mesenchymal GSCs (GBM12, GBM34, GBM9) failed to express GFAP, CNPase, or TuJ1 even when cultured for up to 7 days. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$, ## $p < 0.0001$. Scale bar = 100 μm .

Figure 5.

GTA enhances TMZ chemotherapeutic efficacy on orthotopically engrafted oligodendroglioma-derived GSCs. (a) Images and photon flux (p/cm²/s/sr) of representative mice imaged longitudinally throughout the study. OG35 GSCs (2,500 cells) expressing luciferase were engrafted in the striatum of athymic mice. After 3 days, mice were injected with luciferin (150 mg/kg, i.p.), imaged using the Xenogen imaging system, and randomized to a treatment group: 1) vehicle treated mice received daily oral suspension, 2) daily GTA (5.0 g/kg) with 10% Ora-Sweet to mask GTA's bitterness, 3) TMZ (20mg/kg) on days 5, 7, 9, 11, 13 with oral suspension on alternate days, 4) GTA/TMZ with GTA administered daily starting at day 3 (2 days prior to TMZ) and TMZ on days 5, 7, 9, 11, 13. Treatment was administered by oral gavage until mice displayed neurological signs or weight loss of 20% the pre-surgical weight. Days when imaging failed to detect photon flux are indicated by a negative sign (e.g., 10-). Mean glucose levels were not different between the treatment groups. (b) Low and high magnification hematoxylin and eosin (H & E) stained sections of representative orthotopic tumors from each treatment group failed to reveal oligodendroglioma histological features, rather a preponderance of undifferentiated cells was observed. Immunohistochemical analysis of tumors failed to detect discernible differences in ASPA expression in the four treatment groups (not shown). Scale bar = 1 mm (low mag), 100 μ m (high mag). (c) The study was negatively biased by assigning mice with the greatest flux on day 3 to the GTA/TMZ group (Initial Flux). Although GTA/TMZ treated mice started with greater flux, the rate of bioluminescence increase was reduced in GTA/TMZ treated mice relative to TMZ alone treated mice (Flux Slope). Terminal tumor volume (i.e., day of euthanasia), determined by unbiased stereology was only reduced in GTA/TMZ treated mice relative to vehicle treated mice (left bar graph). However, when taking into account the increased survival of TMZ and GTA/TMZ treated mice (i.e., tumor volume/survival day), the tumor volume of TMZ alone treated mice was reduced relative to vehicle treated mice and the tumor volume of GTA/TMZ treated mice was reduced relative to GTA alone treated mice (right bar graph). GTA/TMZ tumor volume did not differ from TMZ alone tumor volume ($p = 0.068$). (d) Kaplan-Meier analysis showed that GTA alone did not increase survival, but TMZ increased survival relative to vehicle and GTA/TMZ

survival was greater than TMZ alone (upper panel). Survival of mice administered GTA for 2 days prior to TMZ (i.e., primed, Figs. 5a–c) was compared to GTA and TMZ both starting on day 5 (i.e., concurrent) and GTA administered after termination of TMZ (i.e., salvage). Only the primed therapy was associated with increased survival relative to TMZ alone, suggesting that GTA should be presented prior to TMZ to exert its maximal therapeutic effect (lower panel). * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ unless otherwise indicated symbols represent significance relative to vehicle treated mice. $n = 6$ vehicle, 6 GTA, 10 TMZ, 10 GTA/TMZ.

Figure 6.

GTA alone increases survival of mice orthotopically engrafted with GBM-derived GSCs. (a) Images and photon flux ($\text{p/cm}^2/\text{s/sr}$) of representative mice engrafted with GBM12 GSCs (2,500 cells) imaged longitudinally throughout the study. Mice were treated with the “primed” combination GTA/TMZ therapy where GTA was started on post-surgical day 3 and TMZ started on post-surgical day 5. Days when imaging failed to detect photon flux are indicated by a negative sign (e.g., 10-). (b) Low and high magnification H & E stained sections of representative orthotopic tumors from each treatment group.

Immunohistochemical analysis of tumors failed to detect discernible differences in ASPA expression among the four treatment groups (not shown). Scale bar = 1 mm (low mag), 200 μm (high mag). (c) Mean glucose levels were not different between the treatment groups. Bioluminescent flux and end tumor volume (not shown) of TMZ and GTA/TMZ treated mice were reduced relative to vehicle and GTA treated mice. Although GTA/TMZ did not reduce bioluminescent flux or end tumor volume greater than TMZ alone, GTA alone increased survival relative to vehicle treated mice and, in conjunction with TMZ, increased survival greater than TMZ alone. ## $p < 0.0001$. $n = 7$ vehicle, 7 GTA, 6 TMZ, 8 GTA/TMZ.