Malate-aspartate shuttle inhibitor aminooxyacetic acid leads to decreased
intracellular ATP levels and altered cell cycle of C6 glioma cells
by inhibiting glycolysis

Caixia Wang ^{1,*}, Heyu Chen ^{1,*}, Mingchao Zhang¹, Jie Zhang¹,
Xunbin Wei¹, Weihai Ying^{1,2,#}

¹*Med-X Research Institute and School of Biomedical Engineering, Shanghai Jiao
Tong University, Shanghai 200030, P.R. China;* ²*Institute of Neurology, Ruijin
Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200030,
P.R. China*

*: *Contributed equally*

#: Corresponding author

Weihai Ying, Ph.D.

Professor, Med-X Research Institute and School of Biomedical Engineering

Shanghai Jiao Tong University

1954 Huashan Road

Shanghai, 200030, P.R. China

E-mail: weihaiy@sjtu.edu.cn

Tel: 86-21-6293-3075

Fax: 86-21-6293-2302

Running Title: MAS mediates glioma cell survival by affecting glycolysis

Abstract

NADH shuttles, including malate-aspartate shuttle (MAS) and glycerol-3-phosphate shuttle, can shuttle the reducing equivalents of cytosolic NADH into mitochondria. It is widely accepted that the major function of NADH shuttles is to increase mitochondrial energy production. Our study tested the hypothesis that the novel major function of NADH shuttles in cancer cells is to maintain glycolysis by decreasing cytosolic NADH/NAD⁺ ratios. We found that AOAA, a widely used MAS inhibitor, led to decreased intracellular ATP levels, altered cell cycle and increased apoptosis and necrosis of C6 glioma cells, without affecting the survival of primary astrocyte cultures. AOAA also decreased the glycolytic rate and the levels of extracellular lactate and pyruvate, without affecting the mitochondrial membrane potential of C6 cells. Moreover, the toxic effects of AOAA were completely prevented by pyruvate treatment. Collectively, our study has suggested that AOAA may be used to selectively decrease glioma cell survival, and the major function of MAS in cancer cells may be profoundly different from its major function in normal cells: The major function of MAS in cancer cells is to maintain glycolysis, instead of increasing mitochondrial energy metabolism.

Keywords: Cell cycle; Pyruvate; Mitochondria

1. Introduction

NADH, the reduced form of nicotinamide adenine dinucleotide (NAD), plays significant roles in not only the redox reactions in energy metabolism, but also calcium homeostasis and antioxidation [1]. Because the inner mitochondrial membrane is impermeable to cytosolic NADH, NADH shuttles, including malate-aspartate shuttle (MAS) and glycerol 3-phosphate shuttle [1], act to transfer the reducing equivalents of cytosolic NADH into mitochondria thus enhancing mitochondrial ATP production.

MAS is considered the major NADH shuttles in the brain [2]. Previous studies have suggested that MAS plays important roles in multiple biological processes, including glial synthesis of glutamate and glutamine [3], calcium-dependent regulation of mitochondrial respiration in intact cortical neurons [4], glucose-stimulated insulin secretion from β -islet cells [5], and proliferation of breast cancer cells [6]. Aspartate aminotransferase (AAT), a pyridoxal phosphate-dependent transaminase, is the rate-limiting enzyme of MAS which functions in tandem with malate dehydrogenase [7]. It has been reported that AOAA, a most widely used AAT inhibitor of MAS [2, 8], can selectively inhibit the proliferation of breast cancer cells and suppress the growth of breast adenocarcinoma in athymic mice, suggesting that AAT and MAS is required for breast cancer cell proliferation [6]. It is warranted to further investigate the roles of MAS in the biological functions of other types of cancer cells. More importantly, future studies are necessary to investigate the mechanisms underlying the roles of MAS in the biological functions of cancer cells.

It is widely accepted that the major function of NADH shuttles is to increase mitochondrial energy production by shuttling the reducing equivalents of cytosolic NADH into mitochondria [1, 9, 10]. So far there has been no study suggesting that the NADH shuttles may have differential biological roles in normal cells and cancer cells. For the following reasons, we proposed the hypothesis that the major function of NADH shuttles in cancer cells may be prevention of glycolytic inhibition produced by high levels of cytosolic NADH/NAD⁺ ratios, instead of increasing mitochondrial ATP production: There are Warburg effects in cancer cells, in which glycolysis becomes a much more significant pathway for ATP production compared to its role in normal cells [11-13]. Because the much greater glycolytic rate in cancer cells can lead to much higher levels of cytosolic NADH/NAD⁺ ratios that may inhibit glycolysis, NADH shuttles may play significant roles in maintaining glycolysis by decreasing the cytosolic NADH/NAD⁺ ratios in cancer cells.

Glioma represents 80% of all malignant brain tumors, while the pathological mechanisms of the cancer remain unclear [14]. Due to the critical roles of glucose metabolism in cancer biology [11-13], it is necessary to investigate the mechanisms of the regulation of glucose metabolism of glioma cells. In our current study, we investigated the roles of MAS in the glucose metabolism, cell cycle and cell death of C6 glioma cells, with the aim of testing our above-mentioned hypothesis regarding the novel roles of MAS in cancer cell metabolism. Our study has not only suggested that AOAA may be used to selectively decrease glioma cell survival, but also provided evidence suggesting that MAS may have the novel function of maintaining

glycolysis in cancer cells.

2. Materials & methods

2.1 Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted.

2.2 Cell cultures

C6 glioma cells were plated into 24-well or 12-well cell culture plates at the initial density of 1×10^6 cells/ml in Dulbecco's Modified Eagle Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (PAA, Germany). The cells were maintained in a 5% CO₂ incubator at 37 °C.

Primary rat cortical astrocyte cultures were prepared as described previously [15]. In brief, cortices were harvested from 1-day-old rat (SLAC, Shanghai, China). The cortices were dissociated with trypsin, then plated in 24-well culture plates in Dulbecco's Modified Eagle Medium containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate (Thermo Scientific, Waltham, MA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum (PAA, Germany), and maintained in a 5% CO₂ incubator at 37 °C. After 12 to 15 days, the cells became confluent, which were treated for 48 hrs with 10 μM

cytosine arabinoside. The cell cultures could be used two days after removal of cytosine arabinoside.

2.3 Intracellular lactate dehydrogenase (LDH) assay

Cell survival was quantified by measuring the intracellular LDH activity of the cells. Briefly, cells were lysed for 15 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES and 0.01% bovine serum albumin (pH 7.5). Then 50 μ L cell lysates were mixed with 150 μ L 500 mM potassium phosphate buffer (pH 7.5) containing 0.34 mM NADH and 2.5 mM sodium pyruvate. The $A_{340\text{nm}}$ changes were monitored over 90 sec. Percentage of cell survival was calculated by normalizing the LDH values of samples to LDH activity measured in the lysates of control (wash only) culture wells.

2.4 Flow cytometry-based Annexin V/7-AAD assay

The flow cytometry assay was performed to determine the levels of early-stage apoptosis, late-stage apoptosis, and necrosis by using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. In brief, cells were digested with 0.25% trypsin-EDTA, washed by cold PBS one time and resuspended in cold 1 X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2 , 0.1% BSA) at concentrations between 1×10^6 and 1×10^7 cells/ml. Then 5 μ L of labeled Annexin V was added into 100 μ L of the cell suspension. After incubation on ice for 15 min in dark, 200 μ L 1 X binding buffer and 5 μ L 7-AAD

solution were added into the cell suspensions. The number of stained cells was assessed immediately by a flow cytometer (FACSAria II, BD Biosciences).

2.5 Cell cycle analysis

The C6 glioma cells were collected by trypsinisation, washed with PBS and assessed for cell cycle by flow cytometry as described with a modification [16]. In brief, the cells were fixed with 70% cold ethanol at 4 °C overnight. Then the fixed cells were washed with PBS, treated with 100 µg/ml RNase A for 30 mins at 37 °C, and stained with 50 µg/ml propidium iodide (PI) in the dark. Subsequently, the cells were analyzed by flow cytometry (BD FACSCalibur). At least 10,000 cells in each sample were analyzed to obtain a measurable signal. The cell debris and fixation artifacts were gated out and the cell populations that were at the G0/G1, S and G2/M phases were quantified using the Modfit LT 4.0 software (BD Biosciences).

2.6 ATP assay

Intracellular ATP levels were quantified using an ATP Bioluminescence Assay Kit (Roche Applied Science, Mannheim, Germany) following the standard protocol provided by the vendor. Briefly, the cells were lysed with the Cell Lysis Reagent on ice, and 50 µl of the lysates was mixed with 50 µl of the Luciferase Reagent. Then the chemiluminescence of the samples was measured by using a plate reader (Biotek Synergy 2). The ATP concentrations of the samples were calculated using an ATP standard, and normalized to the protein concentrations of the samples, which were

determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

2.7 FACS-based determinations of mitochondrial membrane potential ($\Delta\Psi_m$)

A molecular probe, JC-1 (5, 5', 6, 6'-tetrachloro-1, 1',3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) (Enzo Life Sciences, Plymouth Meeting, PA, USA) was used to measure mitochondrial membrane potential [17]. In healthy cells that have higher $\Delta\Psi_m$, JC-1 is accumulated in the mitochondria matrix, forming J-aggregates that emit red fluorescence at the wavelength of 590 nm. When cells have decreased $\Delta\Psi_m$, JC-1 monomers are generated, which emit green fluorescence at the wavelength of 529 nm. Briefly, C6 glioma cells were digested with 0.25% trypsin-EDTA, washed by PBS one time and resuspended in PBS. Then 10 $\mu\text{g/ml}$ JC-1 was added to the PBS. After 15 mins incubation in dark at 37 °C, the cells were analyzed immediately by flow cytometer (FACS Aria II, BD Biosciences). FACS was used to determine both the red and green fluorescence of a cell, and the ratio between the red fluorescence to the green fluorescence was calculated as a measurement of $\Delta\Psi_m$.

2.8 Determination of mitochondrial membrane potential ($\Delta\Psi_m$) by JC-1-based confocal imaging

A molecular probe, JC-1 (5, 5', 6, 6'-tetrachloro-1, 1',3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) (Enzo Life Sciences, Plymouth Meeting, PA,

USA), was used to measure mitochondrial membrane potential using confocal microscopy. In healthy cells that have relatively high $\Delta\Psi_m$, JC-1 is accumulated in the mitochondria matrix, forming J-aggregates that emit red fluorescence. When cells have decreased $\Delta\Psi_m$, JC-1 monomers are generated, which emit green fluorescence. Briefly, the cells were incubated with 0.1 $\mu\text{g/ml}$ JC-1 at 37 °C for 15 min, then washed with PBS and analyzed immediately by confocal laser scanning microscopy (TCS SP5, Leica, Germany). Confocal images were taken with excitation at 488 nm and emission at (585 ± 21) nm for red fluorescence or (530 ± 15) nm for green fluorescence. The ratio between the red fluorescence to the green fluorescence was used for indicating the levels of $\Delta\Psi_m$. The levels of red and green fluorescence were evaluated in a blinded fashion.

2.9 Glucose uptake assays in cells

For glucose uptake assays in C6 glioma cells, cells were incubated for 60 minutes at 37 °C in a growth medium containing 100 μM 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)(Invitrogen) [18], a fluorescent glucose analog, which typically displays excitation/emission maxima of ~ 465/540 nm. Then the cells were digested by 0.25% trypsin-EDTA, washed by PBS one time and suspended in PBS. The fluorescence of the samples was immediately measured by a flow cytometer (FACSAria II, BD Biosciences).

2.10 Extracellular pyruvate and lactate assay

Extracellular pyruvate and lactate were measured by NADH-coupled lactate dehydrogenase (LDH) spectrophotometric assay [19]. Briefly, for pyruvate assay, 100 μ l supernatant samples were mixed with 56 μ l 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM NADH and 4 unit/ml LDH. The values of $A_{340\text{nm}}$ were monitored immediately. After 15 mins at RT, The values of $A_{340\text{nm}}$ were monitored again. The pyruvate concentrations of the samples were calculated using the difference of the two values of $A_{340\text{nm}}$ and the pyruvate standard, and normalized to the protein concentrations of the samples, which were determined using the Pierce® BCA Protein Assay Kit. For lactate assay, 100 μ l samples (containing 50 μ l supernatant samples and 50 μ l DMEM medium) were mixed with 100 μ l reaction buffer (pH 9.5) containing 1 M glycine, 0.56 M hydrazine sulfate and 0.1 M NAD^+ . Five mins later, 1 Unit/ μ l LDH was added. The values of $A_{340\text{nm}}$ were monitored immediately. After incubation 60 mins at 37 °C, The values of $A_{340\text{nm}}$ was monitored again. The lactate concentrations of the samples were calculated using the difference of the two values of $A_{340\text{nm}}$ and the lactate standard, and normalized to the protein concentrations of the samples, which were determined using the Pierce® BCA Protein Assay Kit.

2.11 Senescence-associated- β -gal (SA- β -gal) staining

Cell staining of SA- β -gal was performed using a Senescence- β -Galactosidase Staining Kit according to the standard protocol provided by the vendor (Beyotime, Jiangsu Province, China). Then the cells were photographed under a Leica inverted

fluorescence microscope. A cell is considered SA- β -gal-positive when the cytoplasm of the cell is in blue color. The number of SA- β -gal-negative cells and SA- β -gal-positive cells was counted in each field, with five randomly picked fields counted for each well. The results are presented by the ratios of the number of SA- β -gal-positive cells to the total cell number which are then normalized.

2.12 DAPI staining

C6 glioma cells were washed with PBS one time, fixed with 4% paraformaldehyde for 20 min, and then stained with 10 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) solution (Beyotime, Jiangsu Province, China) for 5 min in dark. After washed with PBS for three times, the cells were photographed under a Leica fluorescence microscope. A cell is considered in cell division when the cell has obviously broken nuclei [20].

2.13 Statistical Analyses

All data are presented as mean $\pm$ SEM. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls *post hoc* test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1 Differential effects of AOAA treatment on the survival of C6 glioma cells and

primary astrocyte cultures

We determined the effect of AOAA treatment on the survival of C6 glioma cells by intracellular LDH assay, showing that AOAA dose-dependently decreased the survival of C6 glioma cells (Fig. 1A). In contrast, treatment of primary astrocyte cultures with the same concentrations of AOAA did not affect the survival of the cells (Fig. 1B).

3.2 AOAA treatment led to significant alterations of the cell cycle of C6 glioma cells

Cell cycle analysis was conducted to determine if the AOAA treatment may produce the decreased C6 cell survival by altering the cell cycle of the cells. Our study showed that AOAA treatment produced a significant increase in the percentage of the cells arrested in the stage of G0/G1, as well as a significant decrease in the percentage of the cells at S phase and G2/M phase (Fig. 2A and Fig. 2B). We also used DAPI to stain the nuclei of the cells, showing that AOAA treatment led to an obvious decrease in the number of the cells in the phase of cell division (Supplemental Fig.1).

3.3 AOAA treatment induced both early-stage apoptosis and necrosis of C6 glioma cells

We further applied FACS-based Annexin V/7-AAD assay to determine the effects of AOAA on the apoptosis and necrosis of C6 cells, showing that AOAA significantly increased the percentage of the cells in both early-stage apoptosis

(Annexin V⁺/7-AAD⁻) and necrosis (Annexin V⁺/7-AAD⁺) (Fig. 3A and Fig. 3B).

We also conducted SA- β -gal assay – a widely used assay for cellular aging [21, 22] - to determine the effect of AOAA treatment on the aging state of C6 glioma cells. We found that treatment of the cells with 1 mM or 5 mM AOAA led to decreased levels of aging of the cells (Supplemental Fig.2). Therefore, our study has provided evidence arguing against the possibility that AOAA led to cell death by promoting the cells into aging state.

3.4 AOAA treatment induced significant ATP decreases without affecting the mitochondrial membrane potential of C6 glioma cells

Because NADH plays important role in ATP production of cells [1], we determined the effect of AOAA treatment on the intracellular ATP levels of C6 cells. We found that AOAA treatment led to significant decreases in the intracellular ATP levels of C6 glioma cells at both 3 hrs and 5 hrs after AOAA treatment (Fig. 4).

We also found that AOAA significantly decreased the pyruvate/lactate ratios of C6 cells, suggesting that AOAA inhibits the MAS leading to increased cytosolic NADH/NAD⁺ ratios. It has been indicated that the major side effect of AOAA is to inhibit GABA aminotransaminase (GABA-T) [2]. Because GABA-T are present in both astrocytes and microglia [23], we determined the effects of Vigabatrin [23-25], a specific GABA-T inhibitor, on the cell survival and the intracellular ATP levels of C6 glioma cells. Our study did not show any significant effects of Vigabatrin at various concentrations on the cell survival and ATP levels of the cells (Supplemental Fig. 3),

thus arguing against the possibility that AOAA influences C6 glioma cells by affecting GABA-T receptors.

It is widely accepted that the major function of NADH shuttles is to increase mitochondrial energy production. Therefore, we conducted FACS-based JC-1 assay to determine the mitochondrial membrane potential of the cells, so as to determine if the AOAA-produced decrease in the intracellular ATP levels results from its capacity to induce mitochondrial depolarization of C6 glioma cells. We found that treatment of C6 glioma cells with 5 mM AOAA did not affect the mitochondrial membrane potential of the cells (Fig. 5A), while 10 μ M carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), a mitochondrial uncoupler, produced an approximately 60% decrease in the mitochondrial membrane potential of the cells (Fig. 5A). Treatment of the cells with AOAA plus FCCP produced the same level of decrease in the mitochondrial membrane potential as treatment of the cells with FCCP alone (Fig. 5A). By using confocal microscopy to determine the mitochondrial membrane potential of the cells, we obtained experimental results showing that AOAA treatment was incapable of altering the mitochondrial membrane potential of C6 glioma cells (Supplemental Fig.4).

We further found that treatment of C6 glioma cells with either 5 mM AOAA or 10 μ M FCCP significantly decreased the intracellular ATP levels of the cells (Fig. 5B). Treatment of the cells with AOAA plus FCCP produced a significantly greater decrease in the intracellular ATP levels of the cells, compared with treatment of the cells with FCCP alone (Fig. 5B).

3.5 AOAA treatment led to decreased glycolytic rate of C6 glioma cells

In order to determine the effects of AOAA treatment on glycolysis, we used 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose), a fluorescent analog of glucose, to assess the effect of AOAA on the glycolysis of C6 cells. We found that treatment of the cells with AOAA for 1 hr led to a significant decrease in the glycolysis of the cells (Fig. 6A). We also found that AOAA treatment significantly decreased the extracellular lactate levels (Fig. 6B), pyruvate levels (Fig. 6C) and (pyruvate + lactate) levels (Fig. 6D) of C6 glioma cells. Because pyruvate is the major end-product of glycolysis and lactate is mainly generated from pyruvate by a LDH-catalyzed reaction, the AOAA-induced decreases in both pyruvate and lactate have further supported the notion that AOAA produced glycolytic inhibition.

Our study also showed that AOAA treatment led to a significant decrease in the ratios of extracellular pyruvate / extracellular lactate (Supplemental Fig. 5), suggesting that AOAA was capable of decreasing the ratios of cytosolic NAD^+ / cytosolic NADH.

3.6 Pyruvate treatment prevented the AOAA-induced toxic effects on C6 glioma cells

We further determined if pyruvate supplement may affect the AOAA-induced toxic effects on C6 glioma cells. We found that treatment of the

cells with 2.5 to 5 mM pyruvate led to complete prevention of the AOAA-induced toxic effects on C6 glioma cells, including decreased levels of cell survival (Fig. 7A), intracellular ATP (Fig. 7B), and extracellular lactate (Fig. 7C).

4. Discussion

Our current study has the following major observations: 1) The MAS inhibitor AOAA can dose-dependently decrease the intracellular ATP levels and the surviving C6 glioma cells, without affecting the survival of primary astrocytes cell cultures. 2) AOAA led to significant alterations of the cell cycle of C6 cells. 3) AOAA induced both early-stage apoptosis and necrosis of C6 glioma cells. 4) AOAA significantly decreased the glycolytic rate and the extracellular levels of pyruvate and lactate, without affecting the mitochondrial membrane potential of C6 cells. 5) The toxic effects of AOAA on C6 cells can be prevented by pyruvate treatment.

Our study has suggested that AOAA may be used to selectively decrease glioma cell survival: AOAA treatment can significantly decrease C6 glioma cell survival by not only increasing early-stage apoptosis and necrosis, but also increasing the cells that are arrested at the cell cycle of G0/G1 phase. Moreover, AOAA does not impair the survival of primary astrocyte cultures.

Another major goal of our study is to test our hypothesis that the major function of NADH shuttles in cancer cells may be prevention of glycolytic inhibition produced by high levels of cytosolic NADH/NAD⁺ ratios, instead of

increasing mitochondrial energy production. Our study has provided several lines of evidence supporting this hypothesis: In the cells treated with FCCP, AOAA can further decrease the intracellular ATP levels, while not affecting the mitochondrial membrane potential. This observation has suggested that AOAA produces its effects on the intracellular ATP levels by a mitochondria-independent pathway. Indeed, we found that treatment of 5 mM AOAA did not decrease the mitochondrial membrane potential of C6 cells. Because glycolysis is the other major pathway for ATP production, our observation has implicated that AOAA may produce its effects on the intracellular ATP levels by inhibiting glycolysis. Indeed, our 2-NBDG-based assay showed that AOAA treatment significantly decreased the glycolytic rate of C6 cells. In addition, AOAA treatment also significantly decreased the levels of extracellular pyruvate and lactate, which has further suggested that AOAA treatment decreased the glycolytic rate of C6 cells. The key roles of glycolytic inhibition and the consequent decrease in pyruvate levels in the AOAA-produced decrease in the intracellular ATP levels of C6 cells was further indicated by our finding that the toxic effects of AOAA were completely prevented by pyruvate treatment. Collectively, our study has provided first evidence suggesting that the major function of MAS in cancer cells may be profoundly different from its major function in normal cells: The major function of MAS in cancer cells is to maintain glycolysis, instead of increasing mitochondrial energy metabolism. Our study has also suggested that the decreased pyruvate level, as one of the major consequences of glycolytic inhibition,

plays a key role in the toxic effects of AOAA on C6 glioma cells.

AOAA is a most widely used specific inhibitor of MAS, that can inhibit MAS by inhibiting aspartate aminotransferase - a limiting enzyme of the shuttle [6]. The major side effect of the drug is that it can also inhibit GABA aminotransaminase (GABA-T) [2]. Because our study have indicated that Vigabatrin, a specific GABA-T inhibitor [23-25], did not affect the cell survival and the ATP levels of C6 glioma cells (Supplemental Fig. 3), the effects of AOAA on C6 glioma cells does not appear to result from its side effect as a GABA-T receptor inhibitor.

Collectively, our current study has suggested that the major function of MAS in cancer cells is profoundly different from its major function in normal cells: The major function of MAS in cancer cells is to maintain glycolysis, instead of increasing mitochondrial energy metabolism. Our study has also suggested that MAS inhibitor may be used to selectively decrease glioma cell survival.

5. Conflict of interest statement

The authors declare that they have no conflict of interests.

6. Acknowledgments

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FIGURE LEGENDS:

Fig.1 Aminooxyacetic acid (AOAA) treatment dose-dependently decreased surviving C6 glioma cells, while not affecting the survival of primary astrocyte culture. (A) AOAA dose-dependently decreased the survival of C6 glioma cells, assessed 24 hrs after AOAA treatment by intracellular LDH assay. $N = 11-12$. Data were collected from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$. (B) AOAA treatment did not decrease the cell survival of primary astrocyte culture, assessed 24 hrs after AOAA treatment by intracellular LDH assay. $N = 8$. Data were collected from two independent experiments.

Fig.2 AOAA treatment significantly altered the cell cycle of C6 glioma cells. (A) Representative histograms depicting cell cycle profiles of control cells and the cells treated with AOAA for 24 hrs. (B) Quantifications of the histograms suggested that the AOAA treatment led to a significant increase in the percentage of cells in G0/G1 phase, as well as significant decreases in the percentage of cells in S phase and G2/M phase, indicating that AOAA treatment inhibited the cell cycle of C6 glioma cells. $N = 9 - 11$. Data were collected from three independent experiments. ***, $p < 0.001$.

Fig.3 AOAA treatment induced both early-stage apoptosis and necrosis of C6 glioma cells. (A) After C6 glioma cells were treated with 1 or 5 mM AOAA for 24 hrs, FACS-based Annexin V/7-AAD assays were conducted to determine the AOAA-induced apoptosis and necrosis of C6 cells. In the four fields of the original

images from the FACS-based study, the number of the dots of Annexin V⁻/7-AAD⁻ (the bottom-left field), Annexin V⁺/7-AAD⁻ (the bottom-right field), Annexin V⁻/7-AAD⁺ (the top-left field), and Annexin V⁺/7-AAD⁺ cells (the top-right field) indicate the number of normal cells, cells in early-stage apoptosis, necrotic cells, and cells in late-stage apoptosis, respectively. (B) Quantifications of the results from the FACS-based study showed that the AOAA treatment induced significant increases in the number of both Annexin V⁺/7-AAD⁻ and Annexin V⁻/7-AAD⁺ cells. $N = 9$. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

Fig.4 AOAA treatment can significantly decrease the intracellular ATP levels of C6 glioma cells. AOAA can dose-dependently decrease the intracellular ATP levels of C6 glioma cells, assessed 3 or 5 hrs after the AOAA treatment. $N = 11-12$. Data were collected from three independent experiments. ***, $p < 0.001$.

Fig.5 AOAA treatment led to a significant decrease in the intracellular ATP levels without affecting the mitochondrial membrane potential of C6 glioma cells. (A) AOAA treatment did not affect the mitochondrial membrane potential of C6 glioma cells with or without carbonyl cyanide-(trifluoromethoxy)phenylhydrazone (FCCP) treatment. The cells were pre-treated with or without 10 μ M FCCP for 30 min, and then co-treated with 5 mM AOAA for 5 hrs. The mitochondrial membrane potential of the cells was determined by FACS-based assay using JC-1 as probe. $N = 9$. Data were pooled from three independent experiments. ***, $p < 0.001$. The values were

normalized to baseline JC-1 aggregate /JC-1 monomer ratios (red fluorescence/green fluorescence ratios). (B) FCCP treatment significantly decreased the intracellular ATP levels of C6 glioma cells, and AOAA treatment further decreased the intracellular ATP levels of FCCP-treated cells. The cells were pre-treated with or without 10 μ M FCCP for 30 min, and then co-treated with 5 mM AOAA for 5 hrs. $N = 12$. Data were collected from 3 independent experiments. ***, $p < 0.001$.

Fig.6 AOAA treatment led to decreased glycolysis of C6 glioma cells. (A) AOAA treatment significantly decreased the glucose uptake of C6 glioma cells. The cells were co-treated with 100 μ M fluorescent glucose analog 2-NBDG and 5 mM AOAA for 1 hr, and then glucose uptake was determined by flow cytometry. $N = 12$. Data were collected from three independent experiments. ***, $p < 0.001$. (B) AOAA treatment significantly decreased the extracellular lactate levels of the cells. $N = 10$. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. (C) AOAA treatment significantly decreased the extracellular pyruvate levels of the cells. $N = 10$. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. (D) AOAA treatment significantly decreased the extracellular (lactate + pyruvate) levels of the cells. The cells were treated with 1 or 5 mM AOAA for 5 hrs, which were subsequently collected for the analyses. $N = 10$. Data were collected from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

Fig.7 Pyruvate treatment prevented the toxic effects of AOAA on C6 glioma cells.

Treatment of the cells with pyruvate prevented the AOAA-induced decreases in cell survival (A), intracellular ATP levels (B), and extracellular lactate levels (C). The cells were pre-treated with 1 mM AOAA for 30 min, and then co-treated with 2.5 mM pyruvate. Cell survival assay was conducted at 24 hrs after the pyruvate treatment, and ATP assay and lactate assay were conducted at 5 hrs after the pyruvate treatment. $N = 9$. Data were collected from 3 independent experiments. ***, $p < 0.001$.

Supplemental Figures:

Supplemental Fig.1 AOAA treatment led to decreased cell division of C6 glioma cells. (A) Representative images showing DAPI staining of the nuclei of control cells and the cells treated with 1 mM and 5 mM AOAA for 12 hrs. $N = 9$. Images were representative of three independent experiments. Arrows indicate the dividing cells. (B) Representative images showing DAPI staining of the nuclei of control cells and the cells treated with 1 mM and 5 mM AOAA for 24 hrs. $N = 9$. Images were representative of three independent experiments. Arrows indicate the dividing cells. Scale bar = 20 μm . $N = 9$. Images were representative of three independent experiments.

Supplemental Fig.2 AOAA treatment led to decreased cellular senescence of C6 glioma cells. (A) Representative images showing SA- β -gal staining of control cells and the cells treated with 1 mM and 5 mM AOAA for 12 or 24 hrs. Arrows indicate the senescence cells. Scale bar = 50 μm . (B) Quantifications of the percentage of

SA- β -gal-positive cells indicated that AOAA treatment led to decreased cellular senescence of C6 glioma cells. $N = 9$. Data were collected from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Supplemental Fig.3 Treatment of the cells with Vigabatrin, a specific GABA-T inhibitor, did not decrease the cell survival and the intracellular ATP levels of C6 glioma cells. (A) The cells were treated with 150 or 300 μ M Vigabatrin for 24 hrs. Subsequently the intracellular LDH levels of the cells were determined. $N = 7 - 8$. Data are representative of two independent experiments. (B) The cells were treated with 150 or 300 μ M Vigabatrin for 5 hrs. Subsequently the intracellular ATP levels of the cells were determined. $N = 6 - 7$. Data are representative of two independent experiments.

Supplemental Fig.4 Confocal imaging of JC-1-stained cells did not show that treatment of C6 glioma cells with 5 mM AOAA for 5 hrs affect either green or red fluorescence of C6 glioma cells, suggesting that AOAA did not affect the mitochondrial membrane potential (indicated by the ratios of red fluorescence / green fluorescence) of the cells. In contrast, as a positive control, treatment of the cells with 0.5 mM H_2O_2 for 0.5 hr markedly decreased the red fluorescence of the cells. The images were acquired by using X40 oil objective. Scale bar = 20 μ m. The images are representatives of three independent experiments with 6 wells per treatment group in each experiment.

Supplemental Fig. 5 AOAA treatment significantly decreased the extracellular pyruvate/lactate ratios of C6 glioma cells. The cells were treated with 1 or 5 mM AOAA for 5 hrs. Subsequently the extracellular pyruvate and lactate levels were assessed. $N = 10$. Data were collected from three independent experiments. ***, $p < 0.001$.

Figure 1

A

B

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7