Enhanced Mitochondrial Reductive Stress and Cell Death Observed Via the Synergistic Effect of Glucose Starvation and Ceftriaxone/N-acetylcysteine Treatment on Human Glioma Cells

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Introduction

Background on Gliomas:

Gliomas derived from glial cells are the most common primary malignant tumor in the brain and spinal cord. These cancers are aggressive, destructive, and incurable tumors; they are considered among the most deadly human cancers (Figure 1).[1] "Glioma" is a general term used to describe any tumor that arises from glial cells.[2] Malignant glial tumors account for roughly 80% of malignant intracranial tumors, and as of 2016 affect over 20,000 patients within the United States annually.[3,4] In addition to glioma accounting for majority of human central nervous system malignancies, studies have shown poor prognosis for glioma patients despite treatment.[5] To date, the only treatment options that exist for glioma patients include surgery, radiation therapy and chemotherapy.[6] Novel therapeutic drugs that effectively kill tumors have yet to be discovered, and are in demand, considering the increasing incidence of glioma worldwide.[7]

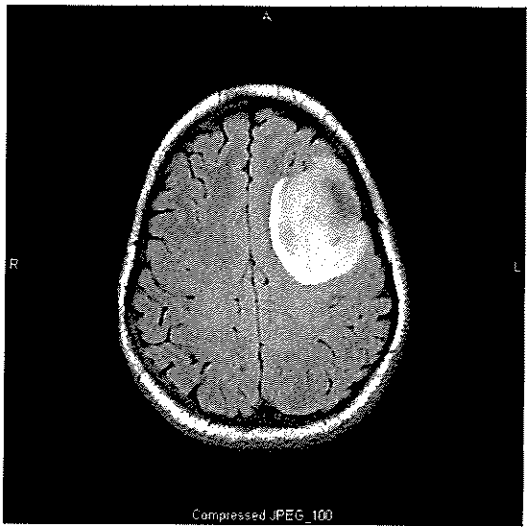

Figure 1: MRI Scan showing a low grade glioma. Source: https://www.merckmanuals.com/professional/neurologic-disorders/intracranial-and-spinal-tumors/gliomas

Necessity of Cysteine for Glioma Survival:

It has been widely recognized that tumor cells exhibit a dysregulated form of metabolism due to the extreme need for nutrients as an effect of their increased proliferation.[8] As a result of this higher rate of metabolism found in tumor cells, antioxidants become a necessity for tumor survival due to the need to alleviate associated stress.[9] For this reason, it has long been

hypothesized that this vulnerability be exploited in order to treat cancers. A hallmark of tumors is their ability to redesign their metabolism in order to satisfy their metabolic needs.[10] Furthermore, many tumors are able to utilize amino acids to enable viability, whether it be through sustaining metabolism or alleviating stress; gliomas are no different.[11] Oxidative stress is primarily caused by mitochondrial respiration, wherein a shortage of antioxidant species and an increase in Reactive Oxygen Species (ROS) leads to cellular and mitochondrial damage. Despite many cancers being caused by oxidative stress-related damage, this increase in oxidative stress as a result of increased metabolic activity still forces cancer cells to rely on antioxidants to alleviate stress to prevent further damage.[12] Recently, the amino acid cysteine has been found to be necessary for glioma proliferation and surivival.[13] Cysteine, a non-essential amino acid, is used for a variety of mechanisms within the cancer cell, including catalysis, protein formation, and aiding in the oxidative stress response.[14,15] Mediating the oxidative stress response through the use of this non-essential amino acid is of particular interest, since as previously mentioned, this metabolic stress may be exploited to terminate cancer cells.

N-Acetylcysteine as a Precursor to Cysteine:

N-Acetylcysteine, also known as NAC, has long been utilized as an antidote for acetaminophen overdose.[16] However, NAC may also be utilized for its antioxidant and reducing qualities.[17] Through the use of the Acylase I enzyme, NAC is deacetylated to form cysteine.[18] As a known precursor to the amino acid cysteine, NAC can therefore be used by tumorous cells to mitigate oxidative stress.

Cysteine in the Alleviation of Oxidative Stress:

As previously described, cysteine can be utilized in the reduction of oxidative stress. This is accomplished due to the fact that it is a precursor to the known antioxidant glutathione (GSH); because of its antioxidant properties, glutathione is then utilized by the cell to blunt the negative impact of ROS.[19] GSH acts as one of the key antioxidants within cells, allowing for cell viability. In addition to cysteine, glutathione is also composed of both glutamate and glycine. To form GSH within the cell, glutamate cysteine ligase (GCL) first attaches glutamate and cysteine before glycine is attached via glutathione synthetase (Figure 2).[20] Although GSH is a necessary antioxidant, its importance within cancer cells has yet to be exploited, considering recent studies showing the negative impact of increased GSH concentrations within cells.

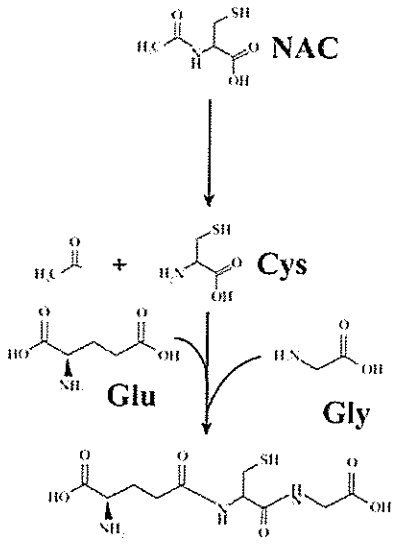

Figure 2: Process with which glutathione is formed, beginning first with the deacetylation of NAC to form cysteine, then followed by the combination of cysteine and glutamate by glutamate cysteine ligase (GCL), before finally the addition of glycine by glutathione synthetase to form glutathione. Source: https://jcbmr.com/index.php/jcbmr/article/view/13/28

Ceftriaxone on Glutamate, a Necessary Component of Glutathione:

As previously mentioned, GSH is composed of three major components: cysteine, glycine, and glutamate. Glutamate, also known as Glu, is primarily found within the brain, and is used as a neurotransmitter.[21] Glial cells reuptake glutamate through the use of Glutamate transporter protein 1, also known as GLT-1. GLT-1 accounts for over 90% of glutamate uptake in glial cells, and is therefore a primary target for the increase of intracellular glutamate concentrations.[22] Recent studies have shown that beta-lactam antibiotics, such as ceftriaxone (CTX), are involved in upregulation of the function of GLT-1, thus

increasing intracellular glutamate concentration within glial cells. The use of CTX and NAC to increase glutathione abundance within the cell may be accomplished through increases of the precursors of glutathione, thereby creating an opening for exploitation of this antioxidant.

xCT within Glial Cells:

In addition to GLT-1, xCT, a portion of system x_c^-, is also a major transporter of glutamate within glial cells. However, unlike GLT-1, xCT functions as a glutamate-cystine antiporter, wherein xCT facilitates the transport of glutamate into the extracellular environment and cystine into the cytosol.[23] Once cystine has been imported into the cell by xCT, it is then reduced by thioredoxin (TXN) to form cysteine.[24] This cysteine, as previously described, can be used to form glutathione (Figure 3). Thus, xCT may also contribute to increased glutathione concentrations within the cell.

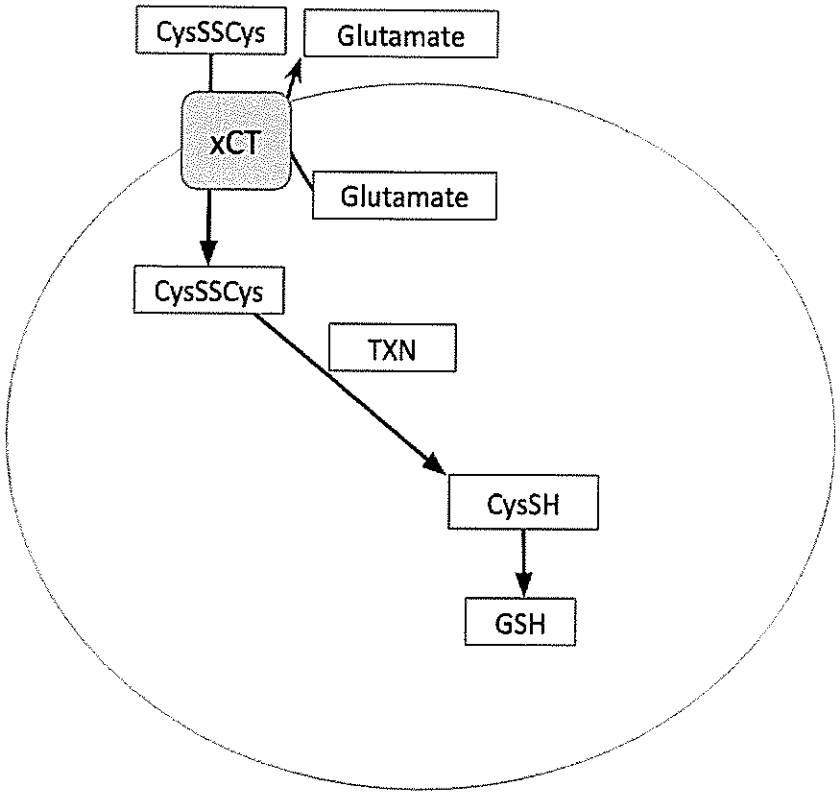

Figure 3: Depiction of the acquisition of cystine (CySSCys) from the extracellular environment through xCT export of glutamate. Thioredoxin (TXN) then reduces cystine to form cysteine. Cysteine is subsequently used for the synthesis of a variety of molecules, of which glutathione (GSH) is included.
Source: Self constructed diagram

CTX/NAC, xCT, and Mitochondrial Reductive Stress:

Due to increased intracellular concentrations of glutamate by CTX as well as increased cysteine concentrations through xCT activity and NAC, intracellular glutathione levels can be increased (Figure 4). These increased levels of glutathione within the cell may very well cause damage to the cell itself through mitochondrial reductive stress (MRS). MRS refers to the paradoxical process through which an increase in intracellular antioxidant species leads to increased mitochondrial oxidative stress.[25] Normally, antioxidants such as glutathione scavenge for ROS, preventing cellular damage because of oxidative stress. However, when ROS concentrations become depleted and there is an excess of antioxidant species, such as GSH, the cell will undergo reductive stress due to the

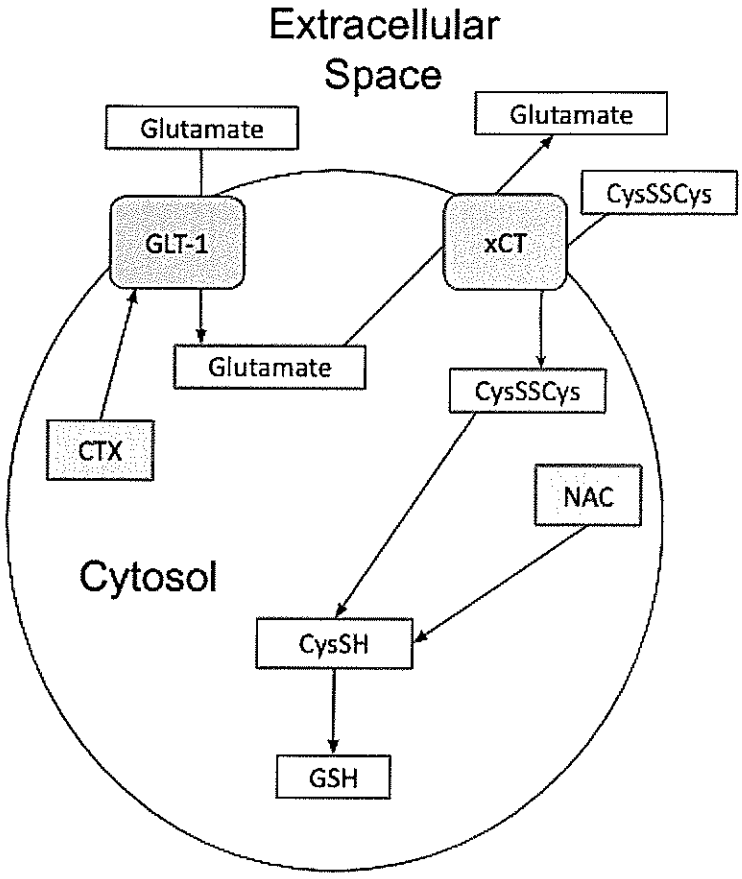

Figure 4: Depiction of the acquisition of glutamate from the extracellular environment through enhanced GLT-1 stimulation by ceftriaxone (CTX) (Left). Glutamate is then utilized by xCT to transport cystine into the cell (CysSSCys). *N*-Acetylcysteine (NAC) as well as imported cystine are then reduced to form cysteine (CysSH). Produced cysteine is then utilized in synthesis of glutathione.
Source: Self constructed diagram

donation of electrons to O_2 by reducing agents, thus creating more ROS.[26] This MRS may be further enhanced through glucose starvation, as glucose starvation will lead to further decreased mitochondrial activity. This further decreased activity may lead to an even greater disparity between the concentrations of ROS and antioxidant species, thus enhancing MRS and damage to cellular function, which may possibly lead to cell death. Therefore, one of the glioma's greatest necessities can be turned against it.

Objective:

The goal of this project is to observe whether or not an increase in intracellular glutathione concentration through CTX/NAC administration in conjunction with glucose starvation would lead to mitochondrial reductive stress and cell death, and thus a new route for glioma treatment and therapeutic drug development. We hypothesized that glucose deprivation (GD) and CTX/NAC will work synergistically to cause mitochondrial reductive stress within gliomas due to an increase in antioxidant species and ROS species, leading to cell death.

Methodology:

To test this hypothesis, three experiments were performed. First, a Seahorse Assay would be utilized to visualize rates of Oxygen Consumption and Extracellular Acidification. Next, a Western Blot would be performed to view changes in Glucose transporter 1 (Glut1) localization at the membrane. Lastly, a Lactate Assay would be carried out to confirm increases in glycolysis.

Seahorse Assay:

Seahorse Assays use an array of sensors to measure extracellular fluxes of oxygen consumption (OCR) and extracellular acid release (ECAR) from cells in microplates. The sensors are found on the ends of the 96 plastic probes of the sensor cartridge, which fit over the 96 wells of the cell culture microplate. The sensor cartridge detects depletion of oxygen and decreases in pH every 14 seconds for a period of ~6 minutes for 55 minutes. Four injection ports surround each sensor probe, and can be used to automatically deliver experimental compounds (Figure 5). In our experiment, the compounds used were CTX, NAC, CTX/NAC, and a permeabilization reagent. 25µL of each reagent (1mM CTX, 10mM NAC) were added to the sensor cartridges. Reagent mixture was made through the use of each compound and the mitochondrial assay buffer included in the Seahorse XFe96 Extracellular Flux Analyzer kit to create a 1:1000 dilution of compound in mitochondrial assay buffer. Beneath the sensor cartridge lies the cell culture microplate. Optimal cell seeding density for the microplate is within $5x10^3$ to $5x10^4$ cells per well; our procedure seeded $5x10^4$ cells per well. After the reagent mixture was added, the sensor

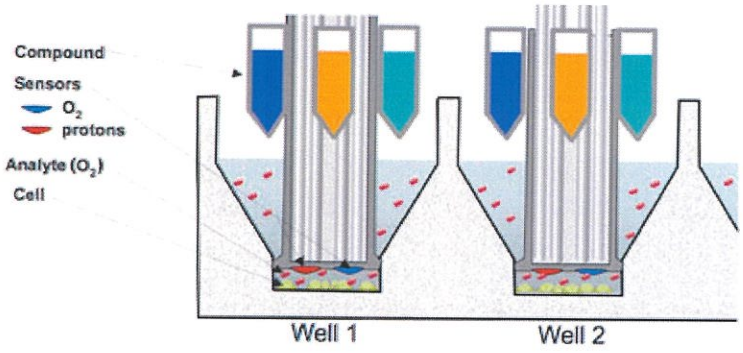

Figure 5: View of the mechanism with which the Seahorse XF96 Analyzer acts on. Sensors found on the ends of probes of the sensor cartridge (top) sense changes in pH and O_2 concentration. The sensor cartridge has four wells that automatically inject compounds into the cell culture microplate, which resides just below. Within the cell culture microplate are the cells, ready for treatment. Source: https://www.genengnews.com/magazine/80/assay-measurement-of-mitochondrial-function/

cartridge and microplate were loaded into the XFe96 Analyzer and allowed to run for its length. Results were then recorded and visualized.

Western Blot:

Western Blots are able to visualize protein abundance within the cell utilizing a series of mechanisms, which include protein extraction, gel loading, protein transfer, and treatment with primary and secondary antibodies. These blots are able to utilize antibodies to perform a semi-quantitative analysis of relative abundance. In our experiment, cells were treated with CTX/NAC for four different time intervals: 30 minutes, 1 hour, 8 hours and 24 hours. 667 Glioma cells were treated with 1mM CTX and 10mM NAC. After treatment, $1x10^6$ cells were harvested by centrifugation at 1000 RCF for 3 minutes before being permeabilized using 75µL permeabilization buffer solution (PBS). Cells were then centrifuged at 16000 RCF for 15 minutes, separating cytosolic and membrane proteins. Supernatant containing cytosolic proteins was then transferred to a new tube, and remaining pellet was solubilized using 50µL solubilization buffer (CST). This solubilized pellet was then centrifuged at 16000 RCF for 15

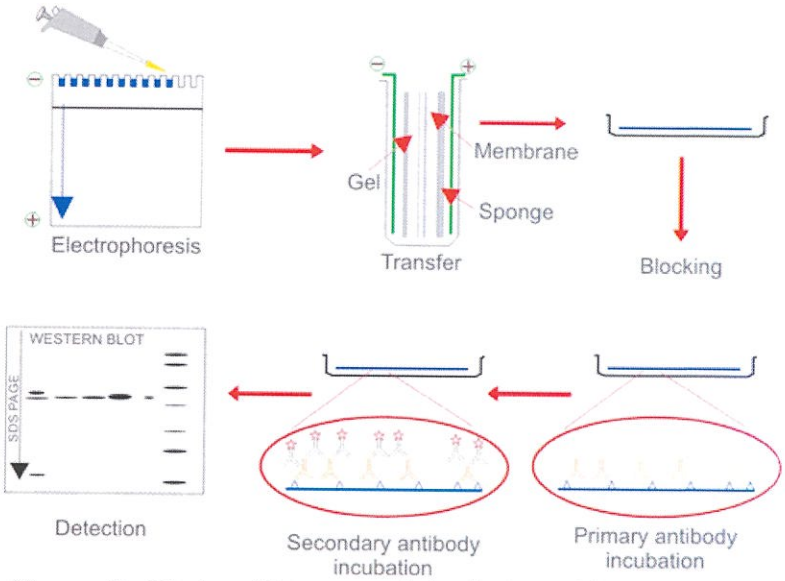

Figure 6: Western blot protocol beginning with transfer of proteins onto gel using gel electrophoresis, followed by transfer of proteins from gel onto membrane, blocking of membrane to prevent nonspecific binding, primary antibody (Glut1) incubation, secondary antibody (rabbit) incubation before finally imaging (Image Lab). Source: https://www.cusabio.com/m-244.html

minutes. Supernatant containing solubilized membrane and membrane proteins was then transferred to a new tube. Standard Western Blot protocol as shown in Figure 6 was then followed, and resulting blot was blotted with a Glut1 primary antibody and a rabbit secondary antibody before imaging in Image Lab software.

Lactate Assay:

Lactate Assays function on the principle that L-lactate is converted to pyruvate by L-lactate dehydrogenase (L-LDH) in the presence of NAD^+. The amount of NADH formed in this process is then measured spectrophotometrically at 340 nm. An increased level of NADH indicates a greater lactate concentrations. In our experiment, the Spectramax spectrophotometer was employed to determine the concentration of NADH. A 96 well plate was used, with samples that had been treated with CTX/NAC (1mM CTX, 10mM NAC) from 0 to 60 minutes (n=7). 20μL of each sample was added to each well, along with 25μL NAD, 2.5μL L-LDH, and 250μL buffer solution. A well with 20μL of water instead of sample was added to serve as a blank . The wells were then incubated at 25 °C for 1 hour inside the Spectramax before optical density was measured at 340 nm. Once optical density had been measured, results were quantified to determine lactate concentration.

Results and Discussion:

CTX/NAC and Glucose Deprivation Increases Mitochondrial Stress:

Our findings results confirm our hypothesis that mitochondrial reductive stress will occur and damage the mitochondria, impairing the cell's ability to perform mitochondrial respiration. This was shown through the elevated extracellular acidification rate (ECAR) and drastically

lowered oxygen consumption rate (OCR) found in CTX/NAC treated samples, as shown in Figure 7. In Figure 7, the OCR of CTX/NAC treated samples is shown to be drastically lower than that of the vehicle, confirming decreased mitochondrial function upon CTX/NAC administration.

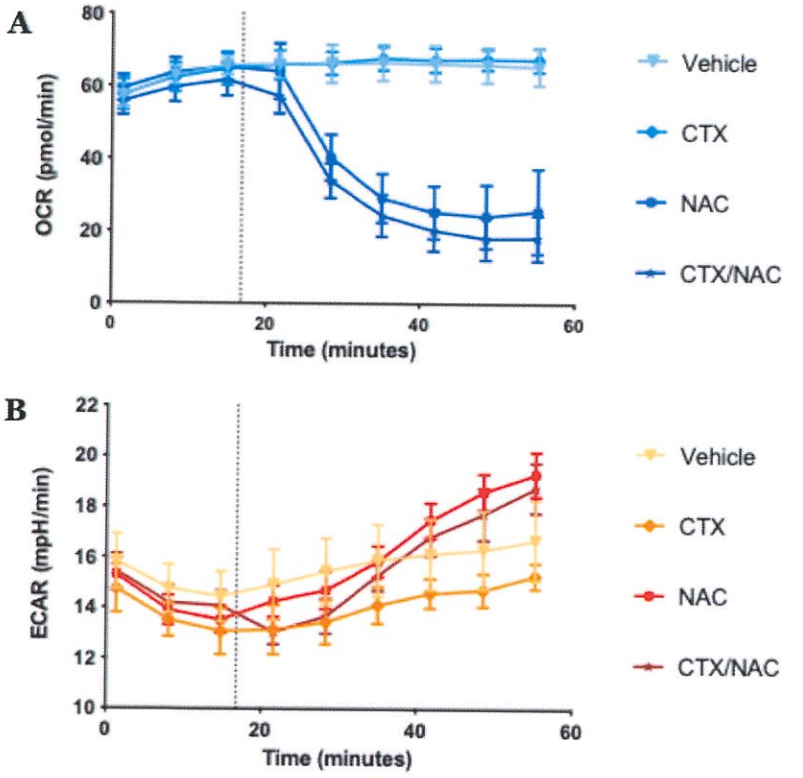

Figure 7: Seahorse Assay data displaying the effect of CTX, NAC, and the two in conjunction on 667 Glioma cell lines. A) Oxygen Consumption Rate decreased dramatically ~ 18 minutes after both NAC and CTX/NAC administration. CTX/NAC OCR plateaued at ~20 pmol/min 50 minutes after administration. B) Extracellular Acidification Rate increased for all samples excluding CTX/NAC by ~18 minutes. However, at ~20 minutes after administration ECAR for CTX/NAC increased rapidly, and by ~55 minutes had surpassed all samples excluding NAC.

Additionally, results confirmed the synergistic effect of CTX/NAC. This is especially shown when viewing OCR, as after 50 minutes of treatment NAC began to show increasing levels of OCR, whereas in comparison OCR resulting from CTX/NAC administration failed to increase, plateauing at a lower OCR of 20pmol/min after 50 minutes. Furthermore, NAC alone had a lowest OCR of roughly 22pmol/min whereas CTX/NAC had a lowest OCR of 20pmol/min, thus indicating further decreased mitochondrial respiration due to CTX/NAC administration. Additionally, ECAR results confirmed the hypothesized increase in glycolysis of

CTX/NAC treated samples. Our results indicate increased glycolysis as an effect of decreased mitochondrial function, suggesting mitochondrial damage. CTX/NAC samples showed a sharp increase in ECAR after ~21 minutes, rising to a maximum of roughly 19mpH/min by 55 minutes; in comparison the vehicle rose to a maximum of ~16.5 mpH/min over the same duration. Additionally, ECAR resulting from CTX/NAC administration rose higher than any other substance administration, with the exception of NAC. However, this difference in ECAR between NAC and CTX/NAC treated samples is negligible, as is shown by their largely overlapping standard deviations. Our results also suggest that CTX/NAC may induce mitochondrial respiration failure through a targeted mechanism, since MRS caused failure to the mitochondria alone, as shown through functioning glycolysis.

CTX/NAC and Glucose Deprivation Increases Glut1 Localization at the Membrane:

Glucose uptake by glial cells is facilitated predominantly by Glucose transporter protein 1.[27] Additionally, gliomas often express higher levels of Glut1 in order to maintain higher levels of metabolism.[28] Therefore, a decrease in mitochondrial respiration and ATP formation will lead to increased glycolysis in an attempt to increase ATP formation. Thus, Glut1 abundance at the membrane should theoretically increase as the cell's need for glucose increases. Our results confirm this theory, as

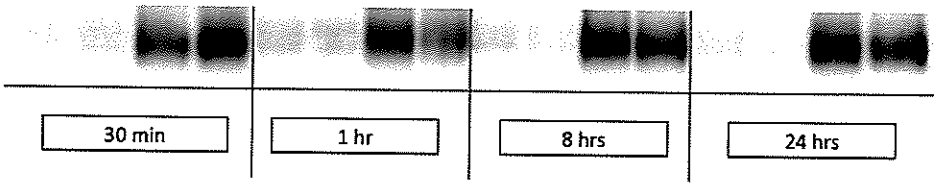

Figure 8: Western Blot displaying membrane and cytosolic proteins for both Control and CTX/NAC administered samples. Four different incubation times are indicated, beginning with 30 minutes, and followed by 1 hour, 8 hours, and 24 hours. Bands are ordered with first being Ctrl cytosolic proteins, followed by CTX/NAC cytosolic proteins, Ctrl membrane proteins, and CTX/NAC membrane proteins.

shown in Figure 8, where Glut1 abundance increases at the membrane within 30 minutes of CTX/NAC administration with GD, which is indicated by the greater band intensity of the CTX/NAC membrane protein in comparison to the band intensity of the control membrane protein. This increase in abundance clearly displays the cell's need for glucose, as an increase in Glut1 membrane abundance suggests that the cell is attempting to increase glycolysis. The increased need for glucose due to increased glycolysis is easily explained by our hypothesis, where mitochondrial reductive stress leads to cellular damage and thus a greater need for ATP. Additionally, the need for glycolysis as implicated by our results suggest that CTX/NAC may also hinder ATP formation through mitochondrial damage. The lesser band intensity of the CTX/NAC membrane protein abundance in comparison to that of the control membrane protein abundance following 30 minutes of treatment may suggest that CTX/NAC administration may hinder Glut1 membrane localization in the long term. This implication may contribute to further glioma cell damage due to lesser Glut1 membrane abundance as an effect of drug administration, as gliomas regularly express increased Glut1 membrane localization. In addition, the similarity of the band intensity for the control and CTX/NAC cytosolic proteins throughout the time interval is not surprising, considering Glut1 is recognized as a membrane protein. Our results indicate that CTX/NAC and GD may lead to decreased Glut1 membrane localization in the long term following increased abundance in the short term, as well as increased mitochondrial damage, as explained by the cell's greater need for glucose.

CTX/NAC and Glucose Starvation Has No Effect on Lactate Concentration:

Quantifying lactate concentration within the cell may indicate changes in glycolysis, especially when mitochondrial respiration fails. This is due to lactic acid fermentation, wherein

the cell utilizes lactate to form ATP rather than performing mitochondrial respiration. We expected our lactate assay to further confirm increases in glycolysis by displaying increased intracellular lactate concentrations. However, our results did not show increased lactate concentrations but rather concentrations similar to that of the control, as shown in Figure 9. The lactate concentration spike at 30 minutes in the CTX/NAC and GD treated sample may be attributed to a human pipetting error, which would explain the much larger standard deviation at this time point in comparison to the other samples. In a repeat of the lactate assay conducted after that shown in Figure 9, the results were almost the same, where the CTX/NAC sample had similar lactate concentrations to that of the control. These results suggest that CTX/NAC and GD have no effect on lactate concentration.

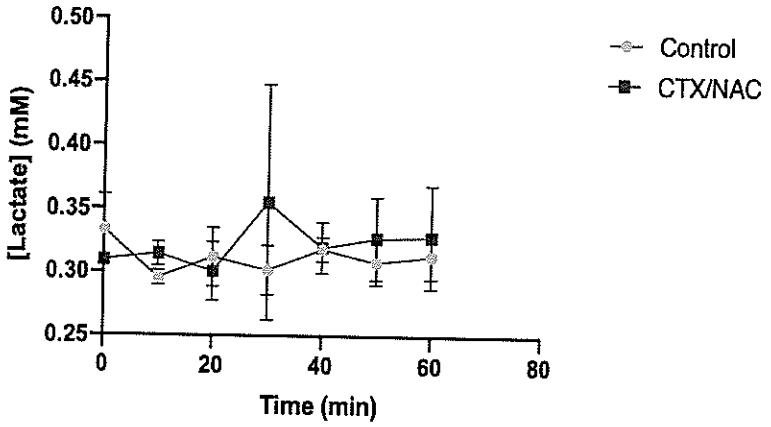

Figure 9: Lactate Assay indicating lactate concentrations in both control and CTX/NAC samples. Lactate concentrations were recorded at 7 intervals, beginning with 0 minutes and subsequently increasing by 10 minutes per recording.

CTX/NAC and Glucose Deprivation Causes Cell Death:

Quantifying cell growth after 24 hours of CTX/NAC administration and GD would confirm cell death through mitochondrial reductive stress. As shown in Figure 10, CTX/NAC and GD will cause cell death within 24 hours of administration when cells are treated with 1mM CTX and 10mM NAC. This data also emphasizes the necessity for glioma cells to be treated with CTX/NAC in conjunction with GD, since sole administration of CTX/NAC does not

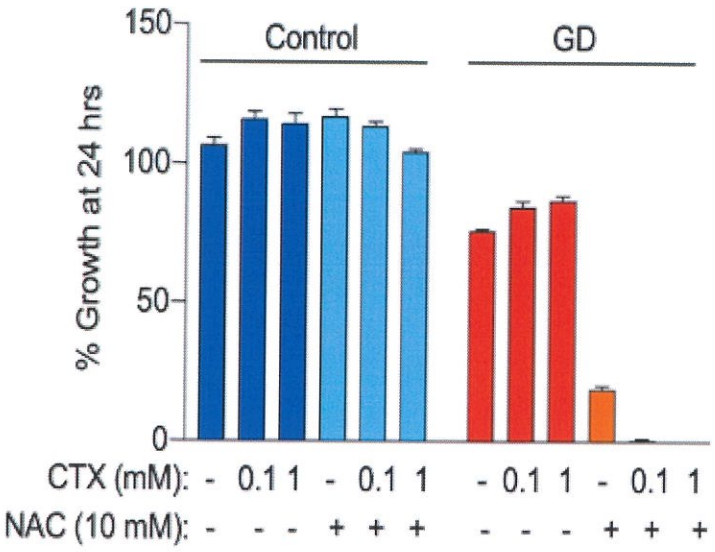

Figure 10: Graph depicting the effect of CTX/NAC on glucose-starved (GD) and non glucose-starved (Control) cells. As shown here, % Growth for glioma cells treated with CTX at 1mM and NAC at 10mM within 24 hours is zero, indicating that GD is essential for mitochondrial reductive stress induced cell death to be effective in CTX/NAC treated samples. Source: Property of mentor (Dr. Evan Noch), and provided for student use by mentor.

considerably lower % growth after 24 hours of treatment. Additionally, quantified growth further demonstrates the need for CTX/NAC to work synergistically to cause cell death, since although NAC will considerably decrease % growth after 24 hours of treatment when cells are under GD, CTX in conjunction with NAC has shown to further decrease this % growth to zero.

Conclusion:

The use of CTX/NAC in combination with glucose starvation on to cause cell death in gliomas presents a novel therapeutic approach towards the growing incidence of human glioma worldwide. Here we have demonstrated how the synergistic effect of CTX/NAC on increasing mitochondrial reductive stress causes cell death within glioma. This project opens a new pathway for cancer research and drug development, as to date the prognosis for glioma patients remains poor.

Our results clearly demonstrate how CTX/NAC causes mitochondrial reductive stress while simultaneously increasing glycolysis within the glioma cell to ultimately cause cell death.

Results from this study may be applied towards drug development, and provide promising pathways for drug development for a number of reasons. CTX and NAC are widely used as an antibiotic for infection and an antidote for acetaminophen overdose, respectively, which contributes to the accessibility of these drugs.[29,30]

Furthermore, CTX has been shown in a study conducted in 2010 to have some ability to cross the blood brain barrier.[31] Although NAC has not been shown to have this ability, *N*-Acetylcysteine amide (NACA) has demonstrated blood brain barrier permeability and has presented therapeutic potential in recent studies.[32] The ability of NACA to permeate the blood brain barrier opens up the possibility for drug development utilizing both NACA and CTX as an alternative to CTX/NAC. Additionally, due to NACA's greater bioavailability in comparison to NAC (67% versus 15%, respectively), NACA may also be just as if not more cost efficient.[33]

Future Work:

Although CTX/NAC has proven to have promise as a novel cancer therapeutic, the lack of blood brain barrier permeability by NAC raises an issue. Fortunately, as previously described, NACA may prove to be useful as an alternative to NAC. Furthermore, NACA may serve as a precursor to glutathione, similar to NAC. This possibility of an alternative to NAC that may have both the antioxidant-producing abilities of NAC and the ability to permeate the blood brain barrier shows promise for replication of this study with NACA instead of NAC. However, although CTX/NAC and GD have created new pathways in cancer research, the issue of how this potential drug combination would differentiate between cancerous and healthy cells remains.

Aside from research into how CTX/NAC would differentiate between cancerous and healthy cells, this study raises questions concerning intracellular activity of CTX/NAC on glioma cells. As shown, the mitochondrial reductive stress and decreased mitochondrial respiration caused by CTX/NAC and GD treatment suggests that CTX/NAC has a targeted mechanism against glioma cell mitochondria. Future research on this topic may include modifying the Seahorse Assay to view ECAR and OCR through individual complexes on the electron transport chain, instead of as the mitochondria as a whole, as performed in this study. In this alternative Seahorse Assay, complex inhibitors such as rotenone and oligomycin could be used to determine individual ECAR and OCR.

In addition to experimentation of the effect of CTX/NAC and GD on individual mitochondrial complexes, further experimentation on lactate concentration should be conducted to confirm the conclusion that CTX/NAC and GD have no effect on intracellular lactate concentration. Additional research may be conducted in order to determine alternative sources of extracellular acidification.

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