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Chapter 18

# The Thiol Redox System in Glioma Biology: Clinical Target and Significance

# in Resistance to Glioma Chemotherapy

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## Abstract

Sulfur-containing compounds play an essential role in maintaining redox balance in glioma cells. Chief amongst these are the reduced and oxidised (disulfide) forms of cysteine (cysteine/cystine) and glutathione (GSH /GSSG) as well as thioredoxin and glutaredoxin, members of the thiol-disulfide oxidoreductases. GSH is also important as an antioxidant, as a ‘safe’ storage form of cysteine and for detoxification reactions involving the GSH sulfur transferase family of enzymes.

Glioma cells contain a high concentration of GSH, compared to normal astrocytes, which renders these cells particularly resistant to chemotherapeutic agents. The rate of synthesis of GSH is controlled by the availability of cysteine, which is imported in its oxidised form, cystine, through specialized channels in the plasma membrane. These channels, known as the cystine-glutamate xc- exchanger, operate by taking up cystine in exchange for glutamate, which is released into the extracellular medium. Glioma cells export large quantities of glutamate by this mechanism, which, if unchecked, causes damage and eventual death of surrounding neurones. Thus, GSH synthesis in glioma cells fuels resistance to chemotherapy and, at the same time, kills off surrounding neurones thereby providing space for tumor cell growth.

Much is now known of the molecular mechanism of cystine import and of GSH synthesis in glioma cells. In particular, a number of therapeutic strategies that target the cystine-glutamate exchanger have been proposed on the basis that this would inhibit synthesis of GSH and remove the source of glutamate release.

This review describes new developments in the field of glioma cell redox balance and evaluates the potential for clinical intervention at the level of cysteine and GSH biosynthesis that may prove effective in combating brain tumor growth and development.

**Keywords:** Glioma, astrocyte, cysteine, GSH, oxidoreductase, xc-cystine-glutamate exchanger, transsulfuration

## 1. Introduction

Regulation of thiol redox balance is crucially important for multiple metabolic, signalling and transcriptional processes in mammalian cells. Thiol groups, whether in proteins or small molecules, are highly reactive and susceptible to oxidation that may cause significant loss of biological activity. In proteins, oxidation of the thiol group of cysteinyl residues produces modifications that, depending on the location of the cysteine(s), will impact on the structure, catalytic activity or ability to engage in protein-protein interactions. Such modifications include formation of inter- or intra-molecular disulfides between protein thiols (Protein (P)-S-S-P) or small molecular weight thiols such as GSH (P-S-SG), or successive oxidation to sulfenic (P-SOH), sulfinic (P-SO2H) or sulfonic (P-SO3H) acids and nitrosylation (P-S-NO). Cell-based thiol redox buffering systems are designed to protect thiol groups from oxidation and to repair those that may have become oxidised as a result of either normal or aberrant cellular metabolism. The key components of the thiol redox system are the cystine (Cys)/ cystine (CySS) and glutathione (GSH) / glutathione disulfide (GSSG) redox pairs, and the thiol disulfide oxidoreductases.

The brain is highly susceptible to oxidative damage, due to its high metabolic load and dependency on oxidative metabolism. Astrocytes play a central role in the brain’s defence from oxidative stress, particularly in the case of thiol-based antioxidants, and are the primary source of antioxidant molecules for neurones. Astrocytes synthesise the major cytosolic sulfur-based antioxidant, GSH, and actively export cysteine and GSH to manage extracellular redox balance and to supply neurons with cysteine for neuronal GSH synthesis.

Glioma cells are noted for their resistance to oxidative stress and apoptosis. In many instances, this relates to upregulation of several components of the thiol redox buffering systems, with the consequence that standard anti-cancer therapies are frequently ineffective in the treatment of gliomas. This is particularly true in the case of glioblastoma multiforme (GBM), which is the most common and highly aggressive primary malignant brain tumour in adults. Survival time of patients with GBM is still only about one year and therapeutic approaches that have a high success rate in other cancers are largely ineffective in GBM. Not only is accessibility limited, due to the blood brain permeability barrier, but GBM are unresponsive to all but a small number of drugs and CNS-related side effects are common [1]. Improved and effective treatment strategies are urgently required. Amongst these, the thiol-based redox buffering system is seen as having the potential for development of more effective and specific therapies. Significant differences in this system in gliomas compared to normal astrocytes signals the potential for therapies directed exclusively at glioma cells.

This review introduces the biochemistry and biological activity of the major classes of thiol-based antioxidants in astrocytes and describes the abnormal activity of the thiol-based redox system in glioma cells. It provides an account of the basis of chemotherapeutic resistance that is characteristic of glioma cell biology and describes new approaches to targeting the thiol-based redox system in cancer therapy.

## 2. The Components of the Thiol Redox System

### 2.1. Cysteine

The sulfur amino acid, cysteine, plays an important role in maintaining cellular redox potential and is the essential amino acid constituent in the antioxidant, GSH and in the functional (CXXC) motif of the major cellular antioxidant families that include the glutaredoxins, thioredoxins and peroxiredoxins [2]. Cysteine is highly reactive and readily oxidises to the disulfide form, cystine, with concomitant loss of electrons (Figure 1). Extracellular oxidising conditions favour cystine, whereas cysteine is the dominant form inside the cell. Even so, the intracellular concentration of free cysteine is kept in the low micromolar range, which minimises the risk of auto-oxidation to cystine and loss of redox balance.

Astrocytes accumulate cystine more readily than cysteine and, due to the relative abundance of cystine in the extracellular fluid, transport of cystine is the dominant mechanism for importing the amino acid. In astrocytes and glioma cells, the majority of cystine is taken up by the xc- cystine-glutamate exchanger. Lesser amounts can be imported as a substrate of the membrane-associated enzyme -glutamyltranspeptidase, which is a component of the -glutamyl cycle involved in GSH synthesis [3]. Experiments with cultured strocytes and glioma cells have shown that cystine is a low affinity substrate for the high affinity glutamate transporters, but this is unlikely to make a significant contribution to intracellular cystine in physiological conditions, because of the considerably higher affinity of these transporters for glutamate [3-6].



Figure 1. The cysteine / cystine and GSH/GSSG redox pairs.

As an alternative to direct transport of cystine from the extracellular medium, cysteine can also be derived from the indispensible amino acid, methionine, by transsulfuration via homocysteine. In mammalian liver, approximately 50% of cysteine in GSH is derived from methionine via transsulfuration [7], but, until recently, this pathway was not thought to have any significance in astrocytes. It has lately been recognised that transsulfuration provides between one quarter and one third of cysteine for GSH synthesis in astrocytes. It is believed that this pathway is particularly important during oxidative stress or conditions in which the import of cysteine from the exchanger may be limited ([8, 9] and references therein).

#### 2.1.1. The xc- Cystine-Glutamate Exchanger

The xc- cystine-glutamate exchanger is a sodium independent, chloride-dependent, electro-neutral antiporter that releases glutamate in exchange for cystine [6, 10-13]. The antiporter is composed of a heavy chain subunit, 4F2, that is common amongst amino acid transporters and a light chain, xCT, that confers substrate specificity [12, 13]. Several structural analogues of glutamate and/or cystine have been identified as substrate-inhibitors of the exchanger. These include L-homocysteate, L-quisqualate, L-aminoadipate and L--N-oxalylamino-L-alanine [8, 14-16]. Non substrate inhibitors, such as 4-carboxyphenylglycine and sulfasalazine ((3E)-6-oxo-3-[[4-(pyridin-2-ylsulfamoyl)phenyl]hydrazinylidene] cyclohexa-1,4-diene-1-carboxylic acid) have also been identified [17].

The directionality of cystine-glutamate exchange, first identified by Bannai and co-workers in the 1980s, is governed by the respective concentration gradients of the transported species: intracellular glutamate is up to a thousandfold higher in the cytosol than outside the membrane. In contrast, the extracellular concentration of cystine is significantly higher than in the cytosol, because it is reduced to cysteine once inside the cell [10, 12, 13, 18]. In short, intracellular glutamate provides the driving force for cystine uptake by the exchanger and is continuously released into the medium.

In normal astrocytes, glutamate is re-cycled back into the cytosol by the high affinity sodium-dependent glutamate transporters (SLC 1 transporter family). This is essential for ensuring that the extracellular concentration of glutamate does not rise to a level that will cause excitotoxicity due to hyperactivation of NMDA receptors that would threaten the viability of surrounding neurones [19]. Furthermore, impaired glutamate uptake will result in an elevation in the extracellular concentration of glutamate that will compete with cystine for entry via the exchanger, resulting in a deficiency in cysteine for GSH synthesis, leading to oxidative stress [19].

Until now, it has been the assumption that glutamate was merely the counter-ion and had no physiological role beyond enabling cystine import by the exchanger. However, interesting new evidence suggests that extracellular glutamate from the exchanger has a specific function in the tonal regulation of extrasynaptic metabotropic glutamate receptors and the control of neurotransmitter release [20].

#### 2.1.2. The Transsulfuration Pathway

Synthesis of cysteine *in situ* from methionine provides an alternative to importation of cystine. This process begins with the adenosine-mediated methylation of methionine to homocysteine, which can either be re-methylated back to methionine (transmethylation), or directed towards transsulfuration via the intermediate cystathionine (Figure 2). The catalysts of the transsulfuration pathway are two pyridoxal-phosphate containing enzymes, cystathionine--synthase (L-serine hydro-lyase (adding homocysteine); EC 4.2.1.22) and cystathionine--lyase (L-cystathionine cysteine lyase; EC 4.4.1.1) that catalyse the formation and metabolism of cystathionine, respectively. Regulation of metabolic flux through the transsulfuration pathway occurs at several levels. S-adenosylmethionine is an allosteric activator of cystathionine--synthase that channels excess sulfur towards cysteine when methionine levels are high. The enzyme is redox-sensitive and is two-fold less active in the reduced form, i.e., when the heme group is in the ferrous state [7]. Cysteine is a competitive inhibitor of cystathionine--lyase, thereby preventing an accumulation of cysteine that would otherwise threaten intracellular redox balance. Mild oxidative stress causes up-regulation of the enzyme in primary astrocytes and glioma cell cultures [8], whereas severe oxidative stress results in inhibition of the enzyme.

A deficiency in the transsulfuration pathway leads to excessive homocysteine production, loss of cellular redox homeostasis and reduced levels of GSH [21]. Genetic defects in cystathionine--synthase are the most common hereditary causes of hyperhomocysteinemia in humans.

Cystathionine -synthase is abundant throughout brain and is particularly highly expressed in Purkinje cells, the hippocampus, cerebellar Bergmann glia and astrocytes [22, 23]. Cystathionine--lyase is expressed much more restrictedly than cystathionine--synthase, and until recently was thought to be entirely absent from brain, giving rise to the view that the transsulfuration pathway was not functional in this tissue [24]. It is now known that the enzyme localises to astrocytes, including retinal Müller cells, and microglia [8, 25-28]. Expression and functional activity of the enzyme has also been recorded in rat C6 glioma cells [8].

The functional significance of the transsulfuration pathway in astrocytic sulfur metabolism is only beginning to be understood, but there are indications that it operates as a reserve pathway that may be upregulated in response to mild oxidative stress [8]. It is hypothesised that, under conditions in which the availability of cystine via the exchanger is compromised, the transsulfuration pathway provides a crucial back-up system to ensure continuing supply of cysteine for synthesis of GSH [9]. Additional research is required for understanding the full significance of this pathway in glioma metabolism and to evaluate the extent of interplay between transsulfuration and cystine uptake in management of GSH.

### 2.2. Glutathione

GSH (-L-glutamyl-L-cysteinyl-glycine) is the major non-protein thiol-containing antioxidant in mammalian biology and has a crucial role in maintaining redox balance in the brain. GSH is a strong reducing agent, as the free sulfhydryl group of the cysteine residue readily oxidises forming an intermolecular disulfide bridge (GSSG; Figure 1) that is reduced back to GSH by GSH reductase using NADPH as electron donor. The cytosolic concentration of GSH is typically 2-3 mM, an order of magnitude higher than the cytosolic concentration of free cysteine (0.1-0.3 mM). Since GSH is non-toxic, it is an important storage and carrier form of cysteine. GSSG accounts for less than 1% of total GSH in normal cells, although the ratio shifts in favour of GSSG in aged animals and is accompanied by lower antioxidant efficiency [29]. Similarly, in oxidative stress, the GSH:GSSG ratio can fall to as low as 50. The bulk of GSH is localised to the cytosol, with small quantities in the mitochondria and endoplasmic reticulum. Mitochondrial GSH makes up approximately 10% of the intracellular concentration of the tripeptide.

As an antioxidant, GSH is effective by a number of different mechanisms. It reacts non-enzymatically with reactive oxygen and nitrogen species, including superoxide (O2**.** **-**), nitric oxide (NO**.**), hydroxyl radical (HO**.-**) and peroxynitrite (ONOO**.-**). GSH is more effective than cysteine in scavenging free radicals and is the only molecule able to detoxify hydroxyl radicals [30]. GSH is both cofactor and electron donor for GSH peroxidase that detoxifies hydrogen peroxide, with concomitant oxidation to GSSG. GSH also functions in the detoxification of xenobiotic compounds by GSH-S-transferases and in the protection of free sulfydryl groups on proteins in association with the glutaredoxins.

#### 2.2.1. The -Glutamyl Cycle

Synthesis of GSH occurs in the cytosol by two consecutive, ATP requiring reactions that form the first steps of the -glutamyl cycle first described by Meister and co-workers more than 40 years ago [31]. In the first step, which is catalysed by L--glutamate cysteine ligase (-glutamylcysteine synthase; EC 6.3.2.2; -GCL), the dipeptide -glutamylcysteine is formed from glutamate and cysteine (Figure 2). Thereafter, addition of glycine, catalysed by GSH synthase (EC 6.3.2.3), generates GSH. In the second half of the cycle, GSH is exported and the -glutamyl moiety transferred to an acceptor amino acid in a reaction catalysed by the membrane-bound enzyme, -glutamyltranspeptidase. A variety of amino acids can function as the acceptor, but cystine is one of the best [32]. In this case, the net result of the -glutamyltranspeptidase-catalysed reaction is the import of one cystine in exchange for cysteine, which equates to a gain of one cysteine per enzyme cycle. Overall, this reaction allows GSH to continuously provide a source of cysteine. The final steps of the -glutamyl cycle see glutamate and glycine regenerated via oxo-proline.

#### 2.2.2. Factors that Regulate GSH Synthesis

Cysteine is the rate-limiting precursor for GSH synthesis. The cytosolic concentration of this amino acid is close to the Michaelis constant (Km) value for GCL of 0.1 -0.3 mM, whereas the intracellular glutamate concentration may be as much as an order of magnitude higher than its Km value of 1.8 mM. Thus, regulation of GSH synthesis occurs at the level of GCL, which is also controlled by feedback inhibition by GSH. GCL is comprised of two subunits, each encoded by different genes. The heavy (catalytic) subunit, GCLc, has a relative molecular mass of 73,000 D, whereas the light (modifier) subunit, GCLm, has a relative molecular mass of 30,000 D. GCLm is inactive in catalysis, but increases the affinity of the catalytic subunit for glutamate and raises the Ki to approximately 2.3 mM for inhibition by GSH. GSH synthase is not a regulatory enzyme, indicating that -glutamylcysteine does not accumulate in cells.

Astrocytes are the site of GSH synthesis in the brain and the intracellular concentration of the tripeptide exceeds that of neurons [33]. Export of GSH from astrocytes can occur at a rate of up to 10% per h and follows the lines described above. Importantly, however, astrocytic release of GSH has the additional function of providing the component amino acids for GSH synthesis in neurons. Neurones do not readily take up cystine and preferentially transport cysteine as a substrate of the EAAC1 /EAAT3 member of the SLC 1 high affinity glutamate transporter family. Extensive analysis of the transfer of the components of GSH from astrocytes to neurones has been undertaken by Dringen and co-workers [26, 34], who identified that, whilst astrocytes in culture accumulate cystine in preference for cysteine, neurones have a reduced capacity for import of cystine, and rely on provision of cysteine from degradation of cys-gly that is a product of -glutamyltranspeptidase (Figure 2). Ultimately, therefore, neurones rely on GSH synthesised in astrocytes for protection from oxidative stress. In fact, astrocytes increase synthesis of GSH in response to oxidative stress [35].



Figure 2. Cystine (CySS), taken up via the xc- cystine-glutamate exchanger is reduced to cysteine (Cys). Cys can also be synthesised intracellularly from methionine by the trans-sulfuration pathway, as shown. The rate-limiting step in GSH (GSH) synthesis is formation of -glutamylcysteine (-Glu-Cys), catalysed by -glutamate cysteine ligase (GCL). Conversion of methionine to cysteine in the transsulfuration pathway is catalysed by cystathionine--synthase (CBS) and cystathionine--lyase (CSE). These enzymes also catalyse formation of hydrogen sulfide (H2S) from cysteine.

### 2.3. Thiol-Disulfide Oxidoreductases

The thiol-disulfide oxidoreductases (TDOR) include the thioredoxin (Trx) and glutaredoxin (Grx) systems that catalyse complementary reactions involved in protection of vulnerable sulfhydryl groups on proteins from oxidative damage. The peroxiredoxins (Prx) are a third member of the family that are involved in detoxification of H2O2. Thioredoxins catalyse reduction of intramolecular and intermolecular disulfides and sulfenic acids. Glutaredoxin specifically reduces protein-GSH mixed disulfides. All of these enzymes share the common Cys-X-X-Cys motif, located at the end of a -strand and beginning of an -helix in the protein.

Extensive immunohistochemical analysis of thioredoxin proteins in mouse brain has shown all members of the thioredoxin family localised in the hippocampus, substantia nigra, cerebral cortex, striatum, cerebellum and spinal cord. Subtypes of Trx and Grx showed strong staining in neurons, whereas specific subtypes of Prx (Prx3 and Prx6) were localised in astrocytes in all regions studied [36].

#### 2.3.1. Thioredoxins

The thioredoxins are a small family of redox-active proteins (10-12 kDa) that mediate the reversible oxidation-reduction of sulfhydryl groups on proteins. The two principal forms of thioredoxin in mammalian cells are thioredoxin 1 (Trx-1) and thioredoxin 2 (Trx-2). Trx-1 (104 aa) is a cytosolic protein, whereas Trx-2 (166 aa) localizes to the nucleus. Thioredoxins have a number of additional roles, including acting as co-factor in DNA synthesis by providing electrons for reduction of ribose by ribonucleotide reductase and inhibition of apoptosis [37]. These proteins are effective antioxidants, in which the thiol groups operate as an electron source for scavenging reactive oxygen species (ROS), due to formation of homo – and hetero-dimers linked by disulfide bonds of the conserved cystiene residues. Thioredoxins increase the DNA-binding activity of a number of transcription factors, for example, Nf-kB, AP-1, AP-2 and nuclear receptors for oestrogen and glucocorticoid. They also bind (causing inactivation of) a number of kinases, including protein kinase C (PKC) and apoptosis signal regulating kinase 1 (ASK1) that regulates activation of the JNK and p38MAPK pathways. During oxidative stress, thioredoxin is oxidised by ROS, which causes its release from ASK-1, leading to activation of JNK and p38MAPK and a pro-apoptotic response [37], and references therein.

The redox activity of the thioredoxins is due to the presence of a highly-conserved catalytic site with the sequence Trp-Cys-Gly-Pro-Cys-Lys. The negative redox potential of the two cysteine residues provides a strong reducing environment that, with electrons provided by NADPH / glucose-6-phosphate dehydrogenase in the hexose monophosphate shunt, maintains the free sulfhydryl group of cytosolic proteins in the reduced state.



Figure 3. Schematic diagram of the thioredoxin (Trx)/ thioredoxin reductase-mediated reduction of oxidised sulfhydryl groups in protein (Pr).

Recycling of oxidised Trx occurs via Trx reductase, by the mechanism illustrated in Figure 3. The mammalian Trx reductases are a family of selenium-containing flavoproteins with the conserved sequence Cys-Val-Asn-Val-Gly-Cys at the catalytic site. They exist as a homodimer and activity is regulated by NADPH. They function in conjunction with Trx in the transfer of electrons to oxidised proteins.

#### 2.3.2. Glutaredoxins

One of the major functions of GSH is S-glutathionylation, a process in which free sulfhydryl groups in proteins are reversibly oxidised by GSH as a protective mechanism to prevent damaging irreversible oxidation. This process is catalysed by a GSH-S-transferase family of enzymes. S-glutathionylation occurs in response to a number of external factors, including hormones and cytokines, and as an integral part of signal transduction cascades initiated by ROS and RNS. Glutaredoxins, on the other hand, catalyse the de-glutathionylation of proteins, which restores the protected cysteinyl residues to the reduced state.

There are several forms of glutaredoxin in mammalian cells: glutaredoxin 1 (Grx1) and glutaredoxin 2 (Grx2) are the principal forms, but others, Grx3 (also known as ‘protein kinase C-interacting cousin of thioredoxin’; PICOT), and Grx5 have also been identified [38]. Grx1 is the most abundant form, occurs in a micromolar concentration and is localised primarily in the cytosol. It contains the sequence Cys-Pro-Tyr-Cys at the catalytic site. The protein is also found in the mitochondrial inter-membrane space although its function here is unclear. Grx2 localises to the mitochondria and shares 30% sequence homology with Grx1 and has an active site motif of Cys-Ser-Tyr-Cys. To date, two human clones of Grx2 have been discovered: Grx2a and Grx2b that differ in their N-terminal sequences. The catalytic activity of Grx2 is approximately ten-fold lower than Grx1. Whilst glutathionylation of proteins is a protective mechanism that increases in response to oxidative stress, there are instances where de-glutathionylation has a regulatory role. For example, Grx-mediated de-glutathionylation of the small GTPase RAS is believed to be a factor in regulation of cell proliferation and hypertrophy [39].

In brain, most research on the glutatredoxins to date has been centered on investigating their neuroprotective function. For example, Grx2 is constitutively expressed in both neurons and glia in mouse and human brain [40].

It has a neuroprotective role in maintaining mitochondrial integrity in neurodegenerative diseases, such as Parkinson’s disease. In Alzheimer’s disease brains, an increase in Grx1 and a reduction in Trx1 has been observed [41], which is believed to be a direct effect of amyloid beta neurotoxicity and signals that deregulation of Grx1 and Trx1 antioxidant systems may be important events in the pathogenesis of AD. Increased de-glutathionylation (i.e., reduction) of DJ-1, a protein that functions as an antioxidant, transcriptional co-activator and molecular chaperone, may be the cause of decreased proteolysis of the protein and may contribute to the pathogenesis of sporadic Parkinson’s Disease [42]. Specific references to Grx activity in astrocytes and/or glioma cells are absent in the literature.

#### 2.3.3. Peroxiredoxins

The peroxiredoxins (Prx) represent a third branch of the Trx family of proteins, of which up to 6 subtypes have been identified (Prx1- Prx6). These enzymes specifically reduce hydrogen peroxide and, based on their catalytic mechanism, are divided into three categories: (1), 2-cysteine Prxs, (2) atypical 2-cysteine Prxs and (3) 1-cysteine-Prxs.

During the peroxidase reaction, a sulfenic acid intermediate is formed on one of the cysteinyl residues close to the N-terminal region of the protein. A second cysteinyl residue, known as the resolving cysteine, which is located at the C-terminal region, reacts with the sulfenic intermediate, resulting in an inter-subunit disulfide bond that is subsequently reduced by Trx.

## 3. Redox Buffering Systems in Gliomas:

## Resistance to Therapy and Oxidative Stress

### 3.1. Cysteine and GSH in Glioma Cells

Gliomas have elevated intracellular GSH, compared to normal astrocytes, which is considered largely responsible for their noted resistance to oxidative stress and chemotherapeutic agents [43]. Samples of primary human glioma show increased expression of the xCT subunit of the xc- exchanger, compared to peritumoural regions and human brain tissue without malignant transformation [44]. Correspondingly , experimental cell lines that are resistant to oxidative stress have a significantly up-regulated xc- exchanger, as indicated by increased mRNA encoding the xCT subunit and greater functional activity of the protein, and elevated GSH [19].

One of the most remarkable features of glioma cell biology is the failure to express functional high affinity glutamate transporters and consequent loss of 95% or more of glutamate uptake observed in normal astrocytes [17, 45]. Moreover, glioma cells actually release glutamate via the xc- exchanger, the complete opposite of the role of normal astrocytes in sequestering glutamate.

This is illustrated in Figure 4. Due to overexpression of the xc- exchanger combined with an absence of high affinity uptake, a significant and prolonged increase in the extracellular concentration of the amino acid has been recorded [17, 46]. Direct sampling of peritumoural extracellar fluid in Grade IV GBM patients by microdialysis revealed glutamate concentrations in excess of 100 µM at the tumour margin, whereas non-malignant tissue had normal glutamate levels in the submicromolar range [47]. It is well established that hyperactivation of glutamate receptors, particularly the NMDA sub-type, triggers a sustained increase in intracellular calcium ions, that exceeds the intracellular calcium buffering capacity, leading ultimately to cell death.

This process, known as ‘excitotoxicity’ was first described 40 years ago and is now known to represent the final common pathway of neuronal cell death in a variety of neurodegenerative disorders [48, 49]. Thus, glutamate released from tumour cells causes widespread destruction of peritumoural neurones. Experts agree that this strategy allows the tumour to expand into the space vacated by dying neurones [17, 46]. Furthermore, evidence suggests that released glutamate acts as a trophic factor that promotes tumour invasion and formation of tumour metastasis [17, 50]. Overall, glioma cells display a remarkable adaptation that ensures a survival advantage over surrounding neurones and generates space for tumour expansion (Figure 4).

A

B

C

Figure 4. Transport of cysteine (Cys), cystine (CySS) and glutamate (Glu) in (A) normal astrocytes, (B) glioma cells and (C) in response to oxidative stress. CySS import via the xc- cystine glutamate exchanger (i) is matched by release of Glu that, in normal astrocytes, is taken up by the high affinity glutamate transporters (ii). In glioma cells, outflow of glutamate via the exchanger in the absence of high-affinity uptake increases the extracellular concentration of the amino acid that may compete with import of CySS and insufficient Cys for synthesis of GSH unless provided by an alternative source, such as the trans-sulfuration pathway (TS). Oxidative stress causes an increase in uptake of CySS and export of Cys (iii), potentially diverting Cys away from GSH synthesis.

Other disruptions in glutamate metabolism are evident in gliomas, compared to normal astrocytes. Gliomas do not express glutamine synthase, an enzyme that converts cytosolic glutamate to glutamine, which is then exported back to neurones to replenish the neurotransmitter pool of glutamate (the glutamate-glutamine cycle). Whilst not directly related to redox buffering systems, the absence of this enzyme highlights the far-reaching consequences that disruption of high affinity uptake may have on glutamate metabolism. In the absence of glutamine, neurones may contribute to glutamate-mediated neuronal dysfunction, which would exacerbate the effects of increased extracellular glutamate due to extrusion via the xc- exchanger. Glioma cells display heightened uptake of methionine, compared to normal astrocytes, that correlates with the proliferative activity of the tumours [51]. Whilst acknowledging that methionine is required to support DNA synthesis in rapidly proliferating cells, the question arises as to whether glioma cells upregulate the transsulfuration pathway as a mechanism to support heightened demand for GSH in growth and development and for enhanced ability to withstand oxidative stress. In particular, if one considers that gliomas must maintain an elevated concentration of GSH in face of reduced import of cystine due to an increased extracellular concentration of glutamate, then there must be an alternative source of cysteine for GSH synthesis. Clearly, there is a need for further experimentation to determine the full significance of this pathway in glioma cell biology.

### 3.2. Oxidoreductases in Glioma Cells

To date, few investigations on thio-oxidoreducatases have been conducted in brain, particularly in the context of glioma biology. What reports there are tend to concentrate on the link between neurodegeneration and dysregulation of apoptosis in neurodegenerative disorders. For instance, there is accumulating evidence that dysregulation of apoptosis through enhanced glutathionylation of p53 may be linked to the inhibition of apoptosis that is observed in Alzheimer’s disease [52]. In cultured astrocytes, carbon monoxide triggers glutathionylation of adenine nucleotide translocase, again resulting in inhibition of apoptosis [53]. Nonetheless, continued investigation into redox buffering in neurodegenerative disease may provide important insights into how the system could be targeted in glioma therapy. So far, there is a lack of consensus in the literature in regard to the link between expression of oxidoreducateses and tumour growth and progression in human gliomas.

Increased levels of Trx and Trx reductase in glioblastoma/astrocytoma cells signify aggressive tumor growth, resistance to chemotherapy and poor patient prognosis. Normal cells, including astrocytes, have low levels of Trx, but overexpression has been noted in several malignancies. Increased Trx expression is linked to prevention of apoptosis in diffuse astrocytomas [54] and oligodendroglial tumors [55], which increases viability and the chance of survival of the tumour, but signifies poor patient prognosis. Trx overexpression increases that of HiF-1, which provides cancer cells with a survival advantage over normal cells. This is particularly important in the case of gliomas, as growth and expansion of brain tumors cannot occur unless space is provided as a result of death of surrounding neurons. Expression of other redox proteins, like superoxide dismutase, also increases in line with the severity of the tumour [56]. The opposite may be true of ependymomas, wherein decreased expression of antioxidant enzymes is associated with more aggressive tumors [57].

In a recent study on Prx expression in malignant glioma cells, Prx I and Prx II expression decreased significantly according to increasing tumour grade. Higher expression of these Prxs was associated with younger patient age and with a significantly better survival rate. Prx I expression was associated with a lower rate of tumour cell proliferation [58]. On the other hand, expression of Prx was increased in pilocytic astrocytomas, with Prx VI expression having a positive association with patient survival [59].Human glioma cells overexpress Prx II, and lowering Prx II expression decreased intracellular GSH and resulted in a significant decline in GSH reductase activity, which may have accounted for the observed shift towards a more oxidising environment in these cells. It was suggested that decreasing Prx II expression in glioma cells might reduce clonogenic cell survival following exposure to ionising radiation and H2O2 [60].

There is a scarcity of reports of changes in glutaredoxin activity in glioma cells, compared to normal astrocytes. However, this is an area ripe for investigation, since glutathionylation is anti-apoptotic and one would expect that there would be a greater level of protein glutathionylation in gliomas.

## 4. The Thiol Redox System As a Therapeutic Target

### 4.1. Targetting the Cystine-Glutamate Exchanger

Several investigators have examined the hypothesis that targeting the xc- exchanger may provide an effective treatment for glioma, on the basis that limitation of incoming cystine would deplete GSH and render the cell more vulnerable to oxidative stress, leading to enhanced effectiveness of chemotherapeutic strategies. There is no doubt that targeting the xc- exchanger provides a mechanism for limiting glutamate efflux and improves patient outcome by lessening glutamate-mediated neurodegeneration. However, evidence for depletion of GSH following xc- exchanger knock-out is less clear-cut. Absence of a functional xc- exchanger has a bigger impact on extracellular glutamate than on intracellular GSH, suggesting that xc- inhibitors may have greater potential for treatment of CNS conditions linked to excessive release of glutamate than for achieving glioma cell death [61]. Research indicates that the xc- exchanger is geared towards facilitating a cystine-cysteine redox cycle, rather than feeding cysteine into GSH for export of the tripeptide [61]. In this study, overexpression of xCT in Burkitt’s lymphoma cells did not translate into an increase in GSH. Rather, the extracellular cysteine concentration was elevated, from which it was concluded that the xc- exchanger was driving a highly efficient cysteine/cystine redox cycle, consisting of cystine import, intracellular reduction of cystine to cysteine, cysteine excretion and re-oxidation to cystine in the extracellular environment (Figure 4c). Additional evidence has come from the experiments of Mandal et al [62], who showed that forced expression of the xc- exchanger in GCL-knock-out cells *in vitro* led to increased uptake of cystine and augmented intracellular and extracellular cysteine concentrations. More recent support for this view has come from experiments in which a mathematical model was used to examine the dynamics of cysteine, GSH and their disulfides in the medium of cultured astrocytes.

The indications are that astrocytes export cysteine, not GSH to provide antioxidant protection of neurones [63]. Mice lacking the xCT subunit of the exchanger do not have lower GSH than normal controls and dysfunction of xc- does not induce oxidative stress *in vivo* [64]. The implication is that regulation of GSH and cystine-cysteine operate much more independently of each other than was previously supposed. Upregulation of the exchanger may drive resistance to oxidative stress because of increased cycling between cystine-cysteine. This information raises the question of how GSH levels are maintained (and up-regulated) if cystine import is channelled to cysteine export. The transsulfuration pathway is an obvious candidate for channelling cysteine to GSH and the demonstration that the transsulfuration pathway is upregulated in astrocytes and glioma cells in response to oxidative stress and inhibition of the xc- exchanger suggests that astrocytes have the capacity to adapt in favour of maintaining cysteine supply for GSH synthesis [8]. As stated, inhibition of glutamate efflux from gliomas is important for limiting tumour development. Sontheimer and co-workers make a compelling argument for targeting excess glutamate in development of treatments for glioma patients [17, 46]. Accordingly, a number of xc- inhibitors have been assessed for their efficacy as pharmacological agents to target gliomas [65, 66]. The search was necessarily confined to non-substrate inhibitors, as substrate inhibitors, whilst limiting GSH synthesis, would promote release of glutamate and contribute to peritumoural neuronal injury [67]. (S)-4-carboxyphenylglycine and sulfasalazine have been evaluated for their anti-tumour potential [17, 80, 65, 66]. As expected, the rate of tumour growth in mice was slowed by treatment with sulfasalazine [68, 69]. However, although already approved by the FDA, a recent Phase 1 clinical trial of sulfasalazine in glioma patients had to be curtailed due to investigator concerns about the efficacy of the drug [70]. Moreover, sulfasalazine is less than ideal, as its half-life in circulation is only approximately 80 min and its ability to cross the blood brain barrier is unknown. Nonetheless, there is a demand for sulfasalazine analogues with improved capacity to penetrate the brain and with greater effectiveness *in vivo*.

Other approaches to modification of xc- exchanger activity include ablation of xCT expression in glioma cells with small interfering RNAs (siRNAs), which produces a considerable reduction in glutamate secretion and delays neurodegeneration [44].

Similarly, rats implanted with xCT-silenced gliomas show a delayed onset and progression of neurological deficits compared to controls. In cystine/glutamate exchanger knock-out mice, there is a doubling in the plasma concentration of cystine and a drop in GSH. The plasma cystine/cysteine redox potential value (Eh) shows an oxidative shift from -100 mV in normal mice to -89 mV in xCT-/- mice [71]. The plasma concentration of cysteine is unchanged, but the GSH concentration falls by 50%.

Other approaches to glioma treatment that rely on targeting GSH have been proposed. *In vivo* experiments with mice have shown that elevated levels of GSSG in mouse brain induces neuronal cell death in a 12-lipoxygenase pathway-dependent manner, which is believed to be a result of increased glutathionylation of the enzyme. Based on these observations, it has been proposed that a strategy aimed at arresting cellular GSSG clearance may be effective in killing tumour cells [72].

### 4.2. Targetting Thioreductases

The Trx system has been heralded as an ‘attractive target for cancer therapy’ [73], particularly in cells that do not contain Grx reductase, as treatment would not require parallel depletion of GSH. Proposals include directly targeting Trx, or indirectly by inhibition of the hexose monophosphate shunt and loss of reducing power in the form of NADPH [73]. Cisplatin, for example, is a metallodrug the irreversibly inhibits Trx reductase via covalent modification of the selenocysteine at the active site, but has no effect on glutaredoxin [37].

Second-generation platinum complexes (compounds with a platinum (II) and (IV) core) are also viewed as potential drug candidates and have been the subject of scientific and clinical investigations. These complexes, for example terpyridineplatinum(II) complexes (TPCs), target the C-terminally located Sel-Cys redox pair of thioredoxin reductase, causing specific and irreversible inhibition of the enzyme and are considerably more potent than cisplatin, with IC50 values in the low nanomolar range.

It has been reported that TPCs inhibit proliferation of glioblastoma cells *in vitro* and inhibit tumor growth in C6-glioma-containing rats without producing severe side effects [1, 56]. Other drugs that target Trx/Trx reductase systems include carmustine (BCNU), dehydroepianrdrosterone (DHEA) and motexafin gadolinium (MG) [37, 74]. However, none of these has proven suitable for use in glioma patients.

## Conclusion

In summary, the various components of the thiol-based redox system are closely associated with the resistance of glioma cells to oxidative stress and remain an attractive target for glioma cell therapy. However, much remains to be understood in relation to the interaction between the components of this system and how the cell adapts to resist oxidative stress before truly meaningful therapies can be designed. Indeed, Mandel et al. [75] caution that cancer therapies that are aimed at depletion of intracellular GSH and consequent cell death may not be effective due to cross-talk between different redox buffering systems.

GSH deficiency in cancer cells was rescued by upregulation of the Trx / Trx reductase system that is used by the cell to counteract the accumulation of excessive quantities of intracellular cystine. The upshot of these observations is that a combined strategy, targeting both the Trx / Trx reductase and GSH systems, may be required for effective treatment of cancer cells [62]. Glioma therapy has the additional challenge of limiting the neurotoxicity that arises from glutamate efflux. Targeting the xc- exchanger remains an attractive option, although additional information is needed before we can fully understand the relationship between cysteine, GSH and antioxidant enzymes in glioma biology.

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