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Antioxidant responses related to temozolomide resistance in glioblastoma

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ABSTRACT

Glioblastoma remains one of the most challenging and devastating cancers, with only a very small proportion of patients achieving 5-year survival. The current standard of care consists of surgery, followed by radiation therapy with concurrent and maintenance chemotherapy with the alkylating agent temozolomide. To date, this drug is the only one that provides a significant survival benefit, albeit modest, as patients end up acquiring resistance to this drug. As a result, tumor progression and recurrence inevitably occur, leading to death. Several factors have been proposed to explain this resistance, including an upregulated antioxidant system to keep the elevated intracellular ROS levels, a hallmark of cancer cells, under control. In this review, we discuss the mechanisms of chemoresistance -including the important role of glioblastoma stem cells-with emphasis on antioxidant defenses and how agents that impair redox balance (i.e.: sulfasalazine, erastin, CB-839, withaferin, resveratrol, curcumin, chloroquine, and hydroxychloroquine) might be advantageous in combined therapies against this type of cancer.

1. Introduction

Glioblastoma (GBM) is the most common and devastating malignancy in the central nervous system. It is characterized by a great cellular pleomorphism with nuclear atypia, high mitotic activity, prominent microvascular proliferation, diffuse infiltration and central necrotic areas, combined with a profuse genomic instability and intense resistance to apoptosis (Furnari et al., 2007; Louis et al., 2016a). These hallmarks finally translate into the destruction of normal brain tissue and resistance to therapeutic agents, leading to death. Defined as grade IV astrocytoma according to the traditional World Health Organization (WHO) classification, the majority of GBM are primary tumors without a previous history of low-grade disease, rapidly arising *de novo* in elderly patients from normal glial cells, while a small percentage corresponds to secondary gliomas that progress from lower-grade tumors preferentially in younger patients (Ohgaki and Kleihues, 2007).

Over the last two decades, molecular studies have revealed a broad constellation of epigenetic and genetic (mutations, deletions, amplifications and translocations) abnormalities in GBM, leading to significant changes in core signaling pathways, namely receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K), p53 and retinoblastoma, as well as in chromatin organization/remodeling and transcriptional regulation (The Cancer Genome Atlas (TCGA), 2008; Verhaak et al., 2010; Brennan et al., 2013; Ceccarelli et al., 2017). Although some alterations are not limited to one type over another, it has become evident that both primary and secondary GBM evolve through different molecular pathogenetic routes (Maher et al., 2006). Primary GBM typically show overamplification and mutation of the tyrosine kinase epidermal growth factor receptor (*EGFR*), often expressed as a truncated variant, EGFRvIII, that exhibits a constitutive activity and confers enhanced tumorigenicity. Activated EGFR triggers PI3K/protein kinase B (AKT), RAS/RAF/MAPK and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, central mechanisms for controlling cell survival, metabolism and proliferation (An et al., 2018). In addition, the tumor suppressor phosphatase and tensin homolog (*PTEN*) and cyclin-dependent kinase inhibitor A (*CDKN2A*) genes, encoding specific inhibitory proteins of the PI3K/AKT pathway and the cyclin-dependent kinases 4 and 6 (CDK4/6), respectively, are frequently mutated and/or deleted in these tumors (Knobbe et al., 2002; Vital et al., 2010). In contrast, common alterations in secondary GBM include *TP53* and isocitrate dehydrogenase 1/2 (*IDH1/2*) mutations. In particular, the identification of *IDH1* mutations (mainly R132H at the substrate binding site of IDH1) as diagnostic molecular markers of secondary GBM has allowed to unequivocally separate these two types as distinct tumor entities, otherwise histopathologically indistinguishable, thus suggesting a link between specific metabolic alterations and glioma

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development (Parsons et al., 2008; Nobusawa et al., 2009; Yan et al., 2009). *IDH* mutations, which correlate with an increased overall survival, have been incorporated in the classification of glioblastomas (Louis et al., 2016b).

Although much progress has been made in the elucidation of molecular events involved in the pathogenesis and malignancy of GBM, recent strategies against specific activated pathways in these tumors have shown no significant benefit in the clinical setting. Currently, numerous clinical trials with new proposed drugs and targets are underway (Delgado-Martín and Medina, 2020). Therefore, most of the patients with newly diagnosed GBM will receive identical treatment, consisting of maximal feasible surgical resection followed by ionizing radiation plus concomitant and maintenance temozolomide (3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide; TMZ) chemotherapy (Fisher and Adamson, 2021; Tan et al., 2020). The addition of this alkylating agent to surgery and radiation has been shown to modestly increase the survival of these patients compared with postoperative radiotherapy alone. Despite this aggressive treatment, the median survival is 12–16 months, with a 5-year survival of merely 5.5% after therapy (Stupp et al., 2005). Unfortunately, virtually all of them will inevitably experience tumor progression and recurrence due to intrinsic and acquired resistance of cancer cells to radiation and alkylating agents (Sarkaria et al., 2008).

Emerging evidence suggests a crucial role of cancer stem cells (CSC) in GBM resistance and recurrence. According to the CSC hypothesis, the cellular hierarchy of the tumor is maintained by a fraction of cells (referred as CSC or tumor-initiating cells) which exhibit the main features of stem cells: self-renewal and differentiation potential, that allow them to recapitulate the heterogeneity of the parental tumor from which they derive when injected orthotopically into mice (Hale et al., 2013; Singh et al., 2004). Glioblastoma stem cells (GSC) show greater resistance to current radiotherapy and chemotherapy than their more differentiated progeny and the ability to repopulate the whole tumor after these treatments (Bao et al., 2006; Chen et al., 2012). Several mechanisms have been identified that may confer that resistance: besides upregulated DNA repair capacity (Liu et al., 2006) and high EGFR activity (Pang et al., 2017), GSC exhibit enhanced drug efflux by ATP-binding cassette (ABC) transporters (Hirschmann-Jax et al., 2004), elevated levels of anti-apoptotic members of Bcl-2 family (Fanfone et al., 2020) and reduced intracellular reactive oxygen species (ROS) accumulation associated to higher expression of ROS scavenging systems (Kim et al., 2014). Various studies suggest that antioxidant capacity may be beneficial to cancer cells in order to minimize DNA, lipid membrane and protein damage, in a similar fashion to normal cells (Cockfield and Schafer, 2019). In this context, targeting redox homeostasis to tip the balance toward oxidative stress and induce cell death in cancer cells, which already have raised levels of ROS (Fig. 1), could be an effective anticancer approach (Pelicano et al., 2004; Reczek and Chandel, 2017). This review will focus on recent findings documenting the antioxidant systems that contribute to TMZ resistance in glioblastoma and the development of new combinatorial pro-oxidant therapies with TMZ that may serve to surpass this acquired resistance.

2. Temozolomide and ROS generation

TMZ is a small methylating imidazotetrazinone prodrug (molecular mass 194 Da) that is administered orally and, due to its lipophilic nature, it effectively crosses the blood-brain barrier (BBB), with a concentration in cerebrospinal fluid 30% of its plasma levels (Patel et al., 2003; Ostermann et al., 2004). Unlike other triazene compounds, it does not require hepatic metabolism, but spontaneously undergoes a hydrolysis cascade at physiologic pH to the unstable intermediate form 5-(3-methyl)1-triazen-1-yl-imidazole-4-carboxamide (MTIC), which then breaks down into a highly reactive methyldiazonium cation –the active alkylating agent- and an inactive molecule, 5-aminoimidazole-4--carboxamide (Darkes et al., 2002). The most studied cytotoxic effects of

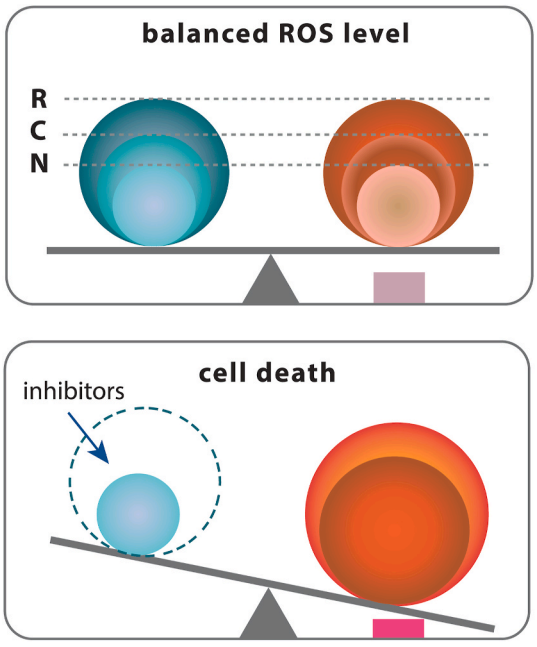

Fig. 1. Targeting redox homeostasis in chemoresistant GBM could be an effective anticancer approach. Dashed lines represent the balance between ROS elimination (left) and production (right) in normal (N), cancer (C) and chemoresistant cancer (R) cells. When ROS elimination is attenuated by inhibition of antioxidant defense mechanisms, higher oxidative stress will result in cell death.

TMZ derive from the instantaneous reaction of methyldiazonium cation with accessible nucleophilic sites within the DNA molecule, causing its methylation at N^7 and O^6 positions of guanine and N^3 position of adenine bases (Denny et al., 1994).

In addition to DNA alkylation, oxidative stress induced by TMZ plays a decisive role in its cytotoxic effects (Fig. 2). It is well recognized that ROS has a "two-faced" character: although they act as key second messengers in intracellular signaling pathways, which induce and maintain the oncogenic phenotype of cancer cells when their levels are moderately increased, excessive ROS production damages proteins, lipids and DNA, triggering cellular senescence and apoptosis. Oxidative stress refers to the condition experienced by cells when ROS levels are increased or ROS scavenging capacity is diminished (Matés et al., 2008). There are three major types of ROS: superoxide anion radical ($O_2^{\bullet -}$), produced by the one-electron reduction of molecular oxygen via the mitochondrial electron transport chain or by NADPH oxidases; hydrogen peroxide (H_2O_2), generated in the superoxide dismutation reaction catalyzed by superoxide dismutase (SOD); and the very reactive hydroxyl radical ($^{\bullet}OH$), formed by the Fenton reaction (Schieber and Chandel, 2014). Most chemotherapeutics contribute to oxidative stress in cancer cells through amplification of ROS levels or inhibition of antioxidant systems (Yang et al., 2018).

Zhang and coworkers demonstrated that GBM cells incubated with TMZ exhibited enhanced ROS production associated with DNA damage, leading to strong AMP-activated protein kinase (AMPK) upregulation, that could be inhibited with the antioxidant N-acetylcysteine (NAC) (Zhang et al., 2010). AMPK is a central metabolic sensor that becomes activated by the tumor suppressor liver kinase B1 (LKB1) and determines cell fate through activation of pro-apoptotic p53 (Okoshi et al., 2008). In response to TMZ treatment, LKB1-activated AMPK positively regulated p53 by its Ser-15 phosphorylation, inducing pro-apoptotic proteins Noxa and Bax. It also inhibited mammalian target of rapamycin complex 1 (mTORC1), which caused downregulation of anti-apoptotic protein Bcl-2. The inhibitor O^6-benzylguanine, which prevents O^6-methylguanine-DNA methyltransferase (MGMT) from

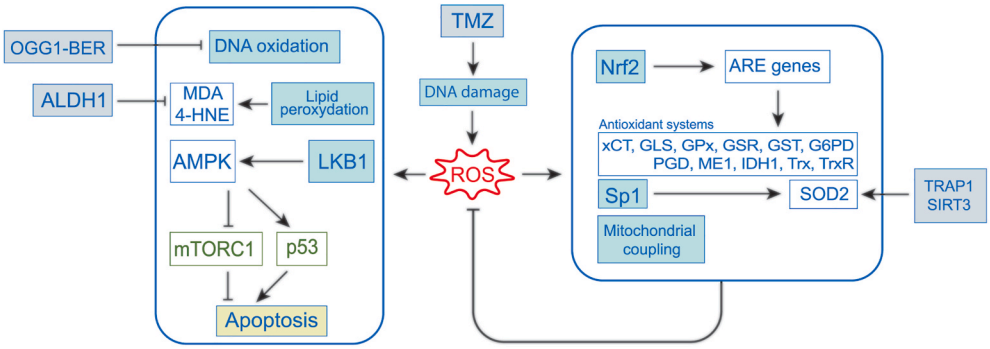

Fig. 2. The double-edged sword effect of TMZ. The alkylating agent TMZ triggers apoptosis through a ROS burst associated to DNA damage but, eventually, induces the overexpression of antioxidant and DNA repair systems and a tighter mitochondrial coupling, leading to the acquisition of chemoresistance and to tumor proliferation. ALDH1: aldehyde dehydrogenase 1; AMPK: AMP-activated protein kinase; ARE: antioxidant response element; BER: base excision repair; GLS: glutaminase; G6PD: glucose-6-phosphate dehydrogenase; GPx: glutathione peroxidase; GSR: glutathione reductase; GST: glutathione S-transferase; 4-HNE: 4-hydroxy-2-nonenal; IDH1: isocitrate dehydrogenase 1; LKB1: liver kinase B1; MDA: malondialdehyde; ME1: malic enzyme 1; mTORC1: mammalian target of rapamycin complex 1; Nrf2: nuclear factor erythroid 2-related 2; OGG1: 8-oxoG-DNA glycosylase 1; PGD: phosphogluconate dehydrogenase; SIRT3: sirtuin 3; SOD2: superoxide dismutase 2; TRAP1: TNF receptor-associated protein 1; Trx: thioredoxin; TrxR: thioredoxin reductase; xCT: subunit of the antiporter Xc^-.

demethylating O^6-methylguanine, the most lethal alkylation lesion in DNA, induced higher ROS generation and downstream AMPK activation. Thus, TMZ-mediated lesions act as upstream signals for apoptosis (Zhang et al., 2010).

Oxidation of DNA bases, such as 8-oxo-7,8-dihydroguanine (8-oxoG) and 8-oxo-7,8-dihydroadenine, is another effect of TMZ (Lopes et al., 2013). Reaction of ROS with DNA predominantly generates 8-oxoG. This lesion is recognized and excised primarily by 8-oxoG-DNA glycosylase 1 (OGG1), which initiates the base excision repair (BER) mechanism (Ba and Boldogh, 2018). In order to identify genes that confer sensitivity to TMZ when silenced, Sobol laboratory performed a synthetic lethal small interfering RNA (siRNA) screen against more than 5000 genes in a TMZ-resistant GBM cell line. Several oxidative DNA glycosylases, including OGG1, were discovered to be involved in the resistance to TMZ, likely the result of an increased ROS production after incubation with the alkylating drug (Svilar et al., 2012).

Oxidative stress causes lipid peroxidation, especially of polyunsaturated fatty acids, that may induce cell death. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) are two major lipid peroxidation by-products that can covalently modify proteins of critical functional importance in the cell (Ayala et al., 2014). Enzymes of the aldehyde dehydrogenase (ALDH) 1 family catalyze the oxidation of intracellular aldehydes to their corresponding carboxylic acids (Muzio et al., 2012) and have been suggested as markers of therapy-resistant CSC, including GSC (Rasper et al., 2010). ALDH1A1 and ALDH1A3 have been proposed as new components of resistance to TMZ. ALDH1A1 inhibition or knock down restored the sensitivity of GBM cells to the alkylating drug. Furthermore, patients with GBM whose levels of ALDH1A1 were high had worse clinical outcomes compared with those with lower levels (Schäfer et al., 2012). An explanation of the role of ALDH1A3 in TMZ resistance has been recently presented. In this study, MDA and 4-HNE, resulting from lipid peroxidation, were increased in GBM cells after TMZ treatment and detoxified by ALDH1A3. Knocking down ALDH1A3 rendered these cells more sensitive to TMZ and addition of ROS scavengers restored their viability. Again, levels of this enzyme were significantly upregulated in recurrent GBM compared with primary tumors and higher expression correlated with poor clinical outcome (Wu et al., 2020). Two subtypes of GSC, expressing a different set of genes and signaling pathways, have been characterized by mRNA profiling: compared with proneural subtype ($CD133^+$), GSC of mesenchymal (Mes) subtype ($CD133^-$) showed a more aggressive behavior *in vitro* and as intracranial xenografts, and higher resistance to radiation. The inhibition of ALDH1A3 clearly diminished the growth of Mes GSC, indicating that the upregulation of this enzyme is critical for the maintenance of the Mes phenotype. In addition, the transition from proneural to Mes phenotype was induced by radiation and this process could be reversed by inhibition of ALDH1A3 (Mao et al., 2013). The aberrant expression of ALDH1A3 in Mes GSC is maintained by USP9X, a member of the ubiquitin-specific proteases family that deubiquitinates and protects ALDH1A3 from proteasomal degradation, leading to the preservation of GSC features (Chen et al., 2019).

3. Antioxidant mechanisms and TMZ chemoresistance

A hallmark of most cancer cells is their deregulated redox homeostasis, a consequence of increased metabolic rates, genetic alterations and harsh tumor microenvironment (Panieri and Santoro, 2016). The elevated levels of ROS are a common feature of cancer cells that contribute to tumor development and progression, but at the same time can have a harmful effect, ultimately leading to cell death (Liou and Storz, 2010; Wu, 2006). Cancer cells must rely on efficient antioxidant defense mechanisms for survival, making them more susceptible to further oxidative damage induced by exogenous agents that promote disproportionate ROS production or inhibit antioxidant systems (Gorrini et al., 2013; Trachootham et al., 2009).

Both normal and cancer cells depend on the activation of the nuclear factor erythroid 2-related 2 (Nrf2) transcriptional network to neutralize the oxidative insult. The transcription factor Nrf2, a member of the cap 'n' collar/basic leucine zipper (CNC/bZIP) protein family, is considered a master controller of redox homeostasis that drives the expression of an array of antioxidant defense genes and, as such, it is tightly regulated (Ma, 2013). According to its crucial protective role against ROS, it is not surprising that its activity is enhanced in GBM cells following treatment with TMZ. Thus, in recurrent tumor tissues after TMZ and irradiation, nuclear Nrf2 immunostaining was markedly increased compared with primary tumors and correlated negatively with the time to tumor recurrence (Cong et al., 2014). Nrf2 and related antioxidant response genes, detected in a CRISPR activation screen in GBM cancer cells, showed a robust negative correlation with GBM patient survival rates (Rocha et al., 2020). It has been shown that GSC require Nrf2 to maintain their proliferation and self-renewal capacities. When Nrf2 was downregulated by short hairpin RNA (siRNA), the expression levels of the pluripotency-associated markers polycomb complex protein BMI1, Sox2 (sex determining region Y-box 2) and Cyclin E were reduced and the proportion of cells in G2 phase increased. The self-renewal and tumorigenicity of GSC were also diminished *in vivo* after Nrf2 depletion (Zhu et al., 2013). Under normal conditions, Nrf2 is kept as an inactive cytoplasmic form through its binding to Kelch-like ECH-associated protein1 (Keap1), an adaptor for the Cullin-3-dependent E3 ubiquitin ligase complex, which ubiquitinates Nrf2 and targets it for proteolysis by 26S proteasome. This constant mechanism of ubiquitination and degradation maintains Nrf2 at low levels (Cullinan et al., 2004). Upon

exposure to ROS or electrophilic chemicals, Keap1 is inactivated by modification of reactive cysteine thiols and the sequestration of Nrf2 is disrupted. This allows the accumulation of Nrf2 in the nucleus, where it heterodimerizes with the small musculoaponeurotic fibrosarcoma (sMAF) proteins and activates the transcription of cytoprotective genes containing ARE (antioxidant response element) regulatory sequences in their promoter regions (Yamamoto et al., 2018).

Nrf2 tightly regulates the expression of enzymes involved in the biosynthesis of the tripeptide glutathione (γ-L-Glutamyl-L-cysteinylglycine, GSH), the most important intracellular antioxidant molecule that plays a critical function in ROS detoxification (Dringen, 2000). In cancer cells, increased levels of GSH have been associated with drug resistance (Bansal, 2018). Nrf2 drives the expression of the catalytic (Gclc) and modifier (Gclm) subunits of glutamate/cysteine ligase, the enzyme that catalyzes the rate-limiting step –the ATP-dependent ligation of glutamate and cysteine to form γ-glutamilcysteine- in the synthesis of GSH (Wild et al., 1999). The system X_c^-, a cystine-glutamate antiporter that imports cystine into the cell, which is then reduced to cysteine in the cytosol, is essential for GSH biosynthesis (Lin et al., 2020). The gene *Slc7a11* (solute carrier family 7 member 11), that encodes the xCT subunit of the antiporter X_c^-, is a Nrf2 target (Sasaki et al., 2002). The glioblastoma TMZ-resistant cell line U-138MG showed a higher expression level of Nrf2 than the TMZ-sensitive cell line U-87MG. Because of Nrf2 induction after TMZ treatment, GSH levels were increased in GBM cells. Nrf2 silencing or GSH depletion by L-buthionine-sulfoximine (BSO), an inhibitor of GSH synthesis, greatly sensitized these cells to TMZ (Rocha et al., 2016). Disruption of X_c^- function by knocking down xCT expression in U-251MG GBM cells resulted in low levels of GSH, enhanced sensitivity to TMZ and pronounced apoptosis. Conversely, overexpression of xCT increased the survival of cells under oxidative stress (Polewski et al., 2016). In addition, strong xCT expression in GBM patients correlated with shorter progression-free survival and overall survival (Takeuchi et al., 2013). GSH homeostasis also depends on the activity of glutaminase isoenzymes (GLS and GLS2), that catalyze the hydrolytic deamidation of glutamine to glutamate in the first step of glutaminolysis (Matés et al., 2020). Besides their contribution to nucleotide and α-ketoglutarate biosynthesis, these isoenzymes are essential for GSH synthesis not only because the tripeptide contains glutamate, but also because this amino acid is equally necessary for the uptake of cystine (Matés et al., 2013). GLS is another target whose expression is upregulated by Nrf2 (Agyeman et al., 2012). Higher expression of GLS, together with other two amino acids-metabolizing enzymes, asparagine synthetase and branched-chain-amino-acid aminotransferase (BCAT), was found to be associated with recurrence of GBM, shorter patient survival and resistance to TMZ (Panosyan et al., 2016). Furthermore, GBM cells *in vitro* and *in vivo* may induce neuronal death in peritumoral regions through the release of neurotoxic concentrations of glutamate, facilitating the tumor expansion (Ye and Sontheimer, 1999; Takano et al., 2001). Glutamate also acts as an autocrine or paracrine signal that stimulates the migration of GBM cells mediated by Ca^{2+}-permeable α-amino-3--hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Lyons et al., 2007).

Several enzymes that utilize GSH to neutralize ROS and electrophiles, and generate oxidized GSH (GSSG), are encoded by Nrf2 target genes (Chanas et al., 2002; Thimmulappa et al., 2002). Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides to water or the corresponding alcohols, respectively, via oxidation of GSH, thus preventing the formation of the very reactive and damaging hydroxyl radical (Lubos et al., 2011). Glutathione S-transferases (GSTs) inactivate electrophilic compounds by conjugation to GSH (Allocati et al., 2018). The reduction of GSSG back to GSH is catalyzed by another Nrf2 target, glutathione reductase (GSR), that uses reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing factor. In GBM cell lines, GSR activity has been positively correlated with MGMT expression and, by regulating GSH levels and redox state balance, mediates TMZ resistance in a MGMT-independent manner. Knocking down GSR decreased GSH levels and resensitized TMZ-resistant cells to the alkylating drug, and the addition of GSH attenuated this effect. Conversely, overexpression of GSR dramatically lowered ROS levels and BSO reversed the resistance to TMZ (Zhu et al., 2018). The generation of NADPH is also modulated by Nrf2 through several targets: glucose-6-phosphate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD), both oxidative enzymes of the pentose phosphate pathway (PPP), malic enzyme 1 (ME1) and IDH1 (Mitsuishi et al., 2012). In addition to the regulation of the GSH antioxidant system, Nrf2 controls the thioredoxin system (Hayes and Dinkova-Kostova, 2014), a critical antioxidant defense pathway composed of thioredoxin (Trx), NADPH and thioredoxin reductase (TrxR). Trx, with a dithiol-disulfide active site, acts as the major protein disulfide reductase and as an electron donor to peroxiredoxins for the reduction of H_2O_2. Then, TrxR reduces oxidized Trx back to its active dithiol form using NADPH as reduction equivalents (Arnér and Holmgren, 2000). Trx and TrxR are elevated in a variety of human cancers (Jia et al., 2019). Inhibition of Trx with the small-molecule inhibitor 1-methylpropyl 2-imidazolyl disulfide (PX-12) or by Trx-interacting protein (TNIP) overexpression sensitized TMZ-resistant GBM cells to cisplatin and TMZ and triggered apoptosis (Haas et al., 2018). In line with these findings, high cytoplasmic TxrR expression was associated with poor overall survival in patients with GBM (Yao et al., 2020). Interestingly, Trx binds directly to apoptosis signal-regulating kinase 1 (ASK1), a member of mitogen-activated protein (MAP) kinase family that plays a crucial role as a sensor stress involved in apoptotic signaling. Under oxidative stress, ASK1 dissociates from Trx and activates c-Jun N-terminal kinase (JNK) and p38-MAP kinase, thus resulting in induction of apoptosis (Saitoh et al., 1998).

Another mechanism of chemoresistance independent of MGMT is mediated by specificity protein 1 (Sp1), a transcription factor that is highly expressed in a variety of cancers and associated with poor prognosis (Vizcaíno et al., 2015). Experiments in which MGMT-deficient GBM cells were incubated with TMZ, to make them resistant to this drug, showed a high increase in Sp1 levels and lower levels of intracellular ROS compared to those of their parental cells. Mitochondrial enzyme superoxide dismutase 2 (SOD2), a target of Sp1, was found to be upregulated in the resistant cells. Treatment with Sp1 inhibitor mithromycin or SOD2 inhibitor diethyldithiocarbamate strongly sensitized these cells to TMZ and induced a significant accumulation of ROS. Similar results were obtained with a TMZ-resistant cell xenograft model (Chang et al., 2017). Among significant mitochondria-related genes differentially expressed between parental and resistant GMB cells, SOD2 was a crucial factor in resistance and patient survival. SOD2 expression was also positively correlated with CSC features. Thus, its downregulation by siRNA decreased the expression levels of BMI1 and Sox2 (Chien et al., 2019). The interplay between the mitochondrial proteins TNF receptor-associated protein 1 (TRAP1) and NAD^+-dependent protein deacetylase sirtuin-3 (SIRT3), both overexpressed in GSC, reduced production of ROS by activating SOD2 and increased mitochondria respiratory capacity, essential for maintaining the stemness of GSC. Inhibiting either SIRT3 or TRAP1 led to ROS overproduction and cell death (Park et al., 2019).

Mitochondrial DNA (mtDNA) and bioenergetics are also impacted by treatment with TMZ. In response to TMZ-induced stress, a mitochondrial adaptive response that contributes to chemoresistance, consisting of alterations in mtDNA and a dramatic remodeling of electron transport chain, is generated in glioblastoma cells. A significantly lower activity of respiratory complexes I and V and increased activity of cytochrome *c* oxidase (CcO) and complexes II-III were observed in a TMZ-chemoresistant stable GBM cell line. Suppression of CcO activity by pharmacological or genetic inhibition reversed chemoresistance to TMZ. Most importantly, these changes were recapitulated in TMZ-resistant GBM xenografts and recurrent GBM biopsies (Oliva et al., 2010). These resistant cells overcome TMZ-induced damage with a more

efficient mitochondrial coupling: decreased activity of complex I combined with higher activity of CcO confer more efficient electron flux through the electron transport chain for ATP generation, resulting in low ROS production (Oliva et al., 2011).

4. Combined pro-oxidant therapies against TMZ chemoresistance

Numerous strategies have been developed to reverse the resistance to TMZ in GBM. One of the mechanisms of radiotherapy and chemotherapy is the excessive generation of ROS, to which tumor cells adapt by increasing their antioxidant defense systems. The combination of TMZ and agents with pro-oxidant properties that increases ROS production and override the intracellular antioxidant systems could improve therapeutic outcomes in GBM treatment. In this section, we have focused on small molecules and natural compounds (Fig. 3), some of them tested in clinical trials, for which a molecular mechanism that causes enhanced oxidative stress has been described. Table 1 summarizes their main effects on GBM cell lines.

System X_c^- appears to be the only feasible mechanism for cystine uptake in GBM cells, that depend on its overexpression for the synthesis of GSH (Chung et al., 2005). After disruption of xCT function, significant anti-cancer effects have been observed in different types of tumors, making this molecule a target for GBM therapy (Liu et al., 2020). Sulfasalazine (2-hydroxy-5-[[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl] benzoic acid; SAS), a conjugate of 5-aminosalicylic acid and sulfapyridine that has been approved for the treatment of several inflammatory diseases, is a potent xCT inhibitor (Gout et al., 2001) that depletes intracellular GSH levels in GBM cells -but not in astrocytes or neurons- and retards tumor growth *in vitro* and *in vivo* (Chung et al., 2005; Sehm et al., 2016). Although SAS also targets the nuclear factor kappa B (NF-κB) signaling pathway, several studies have demonstrated that its growth inhibitory effects in different cancer types are entirely attributable to the blocking of cystine uptake via system X_c^- (Chung and Sontheimer, 2009). SAS synergistically intensified the cytotoxic effect of TMZ on GBM cell lines and their association altered both cell cycle and antioxidant pathways (Ignarro et al., 2016). However, it should be noted that no apparent benefit was noticed in a phase I study of SAS without radiotherapy or chemotherapy (Robe et al., 2009), but these results are difficult to interpret due to the advanced disease of the patients enrolled (Sontheimer and Bridges, 2012). Again, a second clinical trial with SAS administered continuously with TMZ did not show positive outcome in progression-free survival and overall survival and was discontinued due to side effects (Takeuchi et al., 2014). Nevertheless, a study carried out with GBM xenografts found that SAS sensitized cancer cells to gamma knife radiosurgery (Sleire et al., 2015).

Erastin (eradicator of RAS and small T antigen-expressing cells; 2-[1-[4-[2-(4-chlorophenoxy)acetyl]piperazin-1-yl]ethyl]-3-(2-ethoxyphenyl)quinazolin-4-one), an oncogenic RAS-selective lethal small molecule (Dolma et al., 2003) that binds to voltage-dependent anion

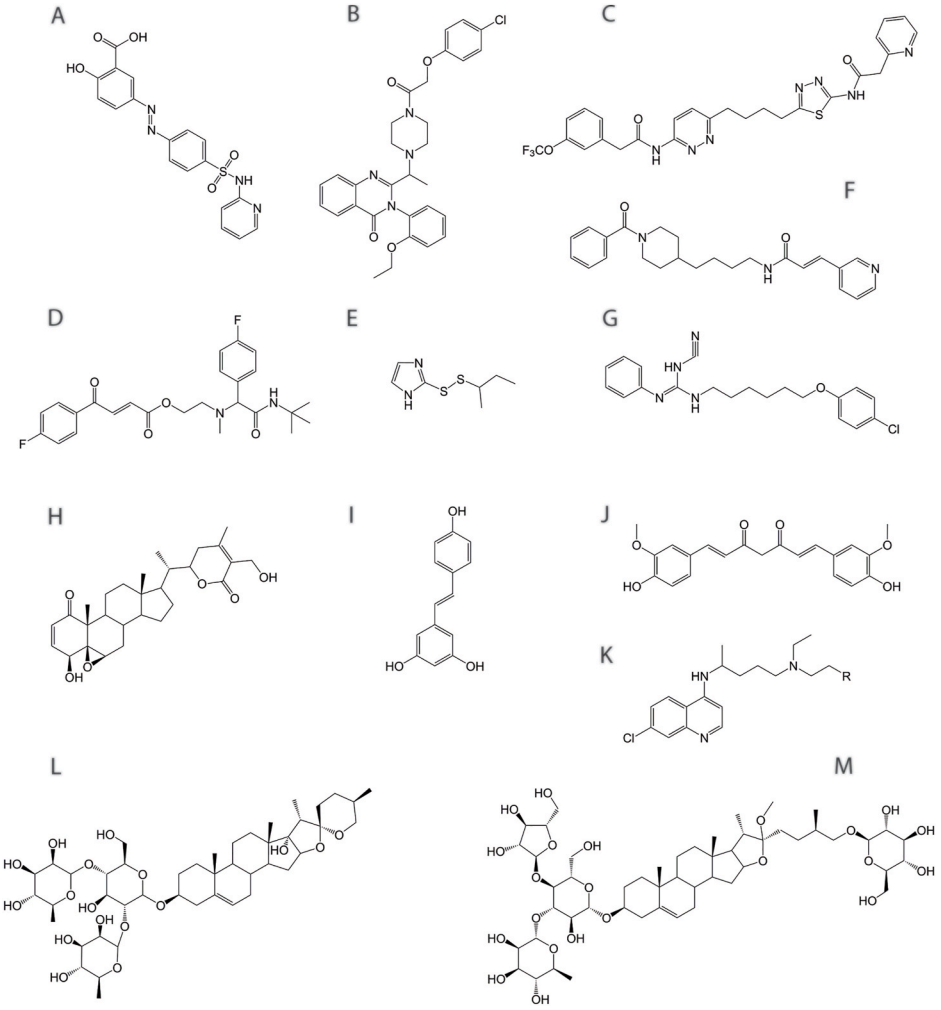

Fig. 3. Some inhibitors of antioxidant systems and compounds that synergize with TMZ in ROS generation. A: sulfasalazine; B: erastin; C: CB-839; D: DVD-445; E: PX-12; F: FX866; G: CHS-828; H: withaferin A; I: resveratrol; J: curcumin; K: chloroquine (R: CH3) and hydroxychloroquine (R: CH2OH); L: compound N45; M: polyphillin VII.

Table 1
Main effects of pro-oxidant compounds on GBM cell lines.

Compound	GBM cell lines	[C] (μM)	Main effects	References
Sulfasalazine	D-54MG, D-65MG, U-87MG, U-251MG, STTG-1 U-251MG	250–1000 400–1000	xCT inhibitor: depletion of GSH after inhibition of cystine uptake induces apoptosis. ER stress and ferroptosis.	Chung et al. (2005) Sehm et al. (2016)
Erastin	A-172, U-87MG, T98G	5	xCT and CTH inhibitor: depletion of GSH.	Chen et al. (2015)
CB-839	IDH1-mutant NHA and HOG cells	0.1	GLS inhibitor: depletion of GSH in IDH-mutant cells.	McBrayer et al. (2018)
PX-12	U-87MG, U-251MG, U-373MG	10	Trx inhibitor: induction of apoptosis.	Haas et al. (2018)
DVD-445	U-87MG	8	Antiproliferative effect through inhibition of TxrR and P-gp.	Jovanović et al. (2020)
FX866 CHS-828	U-251MG, T98G	0.005 0.01	NAMPT inhibitors: enhanced apoptosis, activation of JNK pathway.	Feng et al. (2016)
Chloroquine	U-87MG, U-251MG, LN229 U-87MG	50 10	Inhibition of autophagosome clearance and induction of ER stress-mediated apoptosis. Inhibition of mitochondrial autophagy.	Golden et al. (2014) Hori et al. (2015)
Resveratrol	SHG-44	10	G2/M arrest, cell differentiation and apoptosis through inhibition of AKT/mTOR activity	Yuan et al. (2012)
Curcumin	U-87MG	3.4	Apoptosis through disruption of AKT/mTOR signaling.	Yin et al. (2014)
Withaferin A	U-87MG, U-251MG, U-138MG, T98G	0.5–6	G2/M arrest and apoptosis through modulation of AKT/mTOR and MAPK pathways; depletion of MGMT resentitizes resistant cells to TMZ.	Grogan et al. (2014)
Compound N45	U-87MG, U-251MG	1.9–3.8	Mitochondrial apoptosis through ROS-mediated inhibition of AKT/mTOR pathway; downregulation of MGMT expression.	Zhang et al. (2020)
Polyphyllin VII	U-87MG, U-251MG	0.4–6	Apoptosis and autophagy through inhibition of AKT/mTOR activity; downregulation of MGMT expression.	Pang et al. (2019)

CTH: cystathionine γ-lyase; GLS: glutaminase; GSH: glutathione; ER: endoplasmic reticulum; IDH: isocitrate dehydrogenase; MGMT: O6-methylguanine-DNA methyltransferase; NAMPT: nicotinamide phosphoribosyltransferase; P-gp: P-glycoprotein 1; Trx: thioredoxin; TrxR: thioredoxin reductase; xCT: subunit of the antiporter X_c^-.

channels, has a stronger inhibitor effect on xCT than SAS. It induces an oxidative iron-dependent cell death mechanism distinct from apoptosis, necrosis or autophagy, known as ferroptosis. This mechanism is associated to accumulation of lipid ROS by impaired cysteine-dependent synthesis of GSH and is prevented by iron chelation or inhibition of iron uptake (Dixon et al., 2012). GSH depletion by erastin also inactivates GPx-4, an essential regulator of ferroptosis that specifically neutralizes lipid peroxidation (Yang et al., 2014). The transsulfuration pathway, which is activated by treatment with TMZ, is another target of erastin. In addition to its uptake by system X_c^-, cysteine can be synthesized from homocysteine by the successive reactions of cystathionine β-synthase and cystathionine γ-lyase (CTH). In a study with several GBM cell lines treated with TMZ, a significant reduction of intracellular cysteine level (together with enhanced ROS levels) was observed following the inhibition of xCT by siRNA or SAS, although this was not accompanied by a marked decrease of GSH level. However, a stronger cytotoxic effect of TMZ was achieved by co-treatment with erastin, which suppressed cysteine uptake and CTH activity simultaneously (Chen et al., 2015).

The biosynthesis of GSH can also be impaired by inhibition of GLS. GBM cells that harbor IDH mutations accumulate 2-hydroxyglutarate, an oncometabolite primarily derived from glutamine (Dang et al., 2009). When compared to IDH-wild type cells, IDH-mutant cells treated with the GLS inhibitor BPTES showed a slower growth rate that could be restored by adding exogenous α-ketoglutarate (Seltzer et al., 2010). 2-hydroxyglutarate inhibits the synthesis of glutamate by α-ketoglutarate-dependent BCATs and, hence, the dependence of these cells on GLS isoenzyme for glutamate and GSH generation increases. Inhibition of GLS with CB-839 (N-[6-[4-[5-[(2-pyridin-2-ylacetyl)amino]-1,3,4-thiadiazol-2-yl]butyl]pyridazin-3-yl]-2-[3-(trifluoromethoxy)phenyl]acetamide) caused GSH depletion and made IDH-mutant cells more susceptible to ROS-induced cytotoxicity (McBrayer et al., 2018). Based on these findings, a combination of CB-839 with radiation and TMZ is now being tested in patients with IDH-mutant gliomas (ClinicalTrials.gov Identifier: NCT03528642). In IDH-wild type GBM cells, the pharmacological blockade of Notch pathway reduced the levels of GLS mRNA, which was reflected in reduced intracellular glutamate concentration, suggesting that targeting GLS could be a feasible therapeutic strategy against GBM (Kahlert et al., 2016).

As previously mentioned, the thioredoxin system seems to be another important player in chemoresistance. Besides Trx inhibition by PX-12, which sensitizes GBM cells to TMZ (Haas et al., 2018), novel inhibitors of TrxR1, such as DVD-445 (2-[[2-(tert-butylamino)-1-(4-fluorophenyl)-2-oxoethyl]-methylamino]ethyl (E)-4-(4-fluorophenyl)-4-oxobut-2-enoate), have been developed (Jovanović et al., 2019) that show a synergistic effect in combination with TMZ. Treatment with these molecules potentiated the effect of TMZ through ROS accumulation and disruption of mitochondrial membrane potential, significantly suppressing proliferation and invasion, and promoting cell death. Interestingly, they inhibited the P-glycoprotein 1 (P-gp), an ABC transporter highly expressed in the endothelial cells of the BBB and in cancer cells. Due to its important role in excreting drugs and xenotoxins and, consequently, in multidrug resistance (MDR) in GBM, P-gp inhibition by TrxR1 inhibitors could suppress MDR phenotype and evade BBB (Jovanović et al., 2020).

Nicotinamide phosphoribosyltransferase (NAMPT) is another promising therapeutic target in cancer (Sampath et al., 2015). Its overexpression in GBM tissues is correlated with poor prognosis (Guo et al., 2019). This enzyme catalyzes the rate-limiting step in the salvage pathway of nicotinamide adenine dinucleotide (NAD^+) biosynthesis (Garten et al., 2015), that serves as a source of NADPH and hence of reducing equivalents for the GSH system (Heske, 2020). In human GBM cell lines (U-251MG and T98G), inhibition of NAMPT by drugs such as FK866 ((E)-N-[4-(1-benzoylpiperidin-4-yl)butyl]-3-pyridin-3-ylprop-2-enamide) and CHS-828 (2-[6-(4-chlorophenoxy)hexyl]-1-cyano-3-pyridin-4-ylguanidine) enhanced ROS and superoxide anion, synergistically activating the JNK signaling pathway, and increasing the TMZ-induced apoptosis through a caspase-1, caspase-3, and caspase-9 mechanism. Thus, FK866 and CHS-828 sensitize GBM cells to TMZ treatment (Feng et al., 2016).

The currently used antimalarial drug chloroquine (4-N-(7-chloroquinolin-4-yl)-1-N,1-N-diethylpentane-1,4-diamine; CQ) is an

inhibitor of autophagic flux and sensitizes GBM cells to TMZ by preventing autophagosome clearance dependent of glucose-regulated protein 78/binding-immunoglobulin protein (GRP78/BiP) and increasing the expression of one of the components of the endoplasmic reticulum stress-mediated apoptotic pathway, the CCAAT enhancer-binding protein (C-EBP) homologous protein/growth arrest and DNA damage 153 (CHOP/GADD-153) (Golden et al., 2014). GRP78 acts as a prosurvival arm of the unfolded protein response (UPR) which maintains endoplasmic reticulum integrity, assists in autophagosome formation and promotes chemoresistance by interfering with apoptotic pathways. Its expression is elevated in GBM and inversely correlated with patient survival (Lee et al., 2008). CQ also enhanced TMZ cytotoxicity by increasing mitochondrial ROS and inhibiting mitochondrial autophagy (Hori et al., 2015). Both CQ and its derivative hydroxychloroquine have been tested in several clinical trials (Rosenfeld et al., 2014; Abbruzzese et al., 2017).

In addition, several natural compounds, such as curcumin ((1E, 6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione), resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol), compound N45 (pennogenin-3-O-α-L-rhamnopyranosyl-(1–4)-[α-L-rhamnopyranosyl-(1–2)]-β-D-glucopyranoside), polyphyllin VII (pennogenin-3-O-α-L-rhamnopyranosyl(1–4)-α-L-rhamnopyranosyl(1–4)-[α-L-rhamnopyranosyl(1–2)]-β-D-glucopyranoside) and withaferin A ((1*S*,2*R*,6*S*,7*R*,9*R*,11*S*,12*S*,15*R*,16*S*)-6-hydroxy-15-[(1*S*)-1-[(2*R*)-5-(hydroxymethyl)-4-methyl-6-oxo-2,3-dihydropyran-2-yl]ethyl]-2,16-dimethyl-8-oxapentacyclo[9.7.0.0^{2,7}.0^{7,9}.0^{12,16}]octadec-4-en-3-one), have been tested in GBM cells in combination with TMZ. Curcumin (an inexpensive and well-tolerated natural bioactive compound found in the spice turmeric, *Curcuma longa* L.) and TMZ caused a synergistic effect on ROS generation, enhancing TMZ-induced apoptosis in U-87MG GBM cells. Mechanistically, curcumin increased TMZ-disrupted AKT/mTOR signaling. Noteworthy, the combined treatment of curcumin and TMZ significantly diminished the phosphorylation of AKT, Bcl2-associated agonist of cell death (BAD) and mTOR in the extracts derived from U-87MG xenograft tumor tissues (Yin et al., 2014). Resveratrol, a naturally occurring polyphenolic molecule, abundant in grapes, peanuts and red wine, showed additive effect when combined with TMZ in human GBM cell line SHG44. Resveratrol and TMZ synergistically inhibited cell migration, induced the G_2/M arrest and cell differentiation, enhancing ROS generation, and activating AMPK signaling pathway. Of note, resveratrol also increased TMZ-induced inhibition of the growth of orthotopic implanted GBM in nude mice (Yuan et al., 2012). Compound N45, a steroidal saponin isolated from the rhizomes of *Paris vietnamensis* (Takht.) H.Li, demonstrated oxidative cytotoxicity and suppressed the proliferation of U-87MG GBM cells and TMZ-resistant U-87MG GBM (U-87R) cells by evoking mitochondrial apoptosis through ROS/PI3-K/AKT signal pathway. Additionally, N45 decreased the drug-resistance by downregulation of NF-κB p65 to constrict MGMT in U-87R cells (Zhang et al., 2020). Another steroidal saponin, polyphyllin VII from *Paris polyphylla* Sm., induced apoptosis and autophagic cell death in U-87MG and U-251MG cells, through inhibition of AKT/mTORC1 activity via ROS generation. Combined treatment with TMZ showed a synergistic cytotoxic effect. Furthermore, polyphyllin VII reduced TMZ resistance by suppressing the expression of MGMT (Pang et al., 2019). Similarly, the steroidal lactone withaferin A, isolated from several genera of *Solanaceae* plants, elevated ROS levels and prevented cell proliferation in several GBM cell lines by G_2/M arrest and apoptosis largely through modulation of AKT/mTOR and MAPK pathways. Addition of the ROS scavenger NAC abrogated these effects. In combination with TMZ, it resensitized TMZ-resistant cells by MGMT depletion (Grogan et al., 2014).

5. Conclusions

Effective therapies against GBM, one of the most challenging cancers, remain elusive. Despite combined action of TMZ and inhibitors of antioxidant systems showing excellent results in GBM cell lines and *in vivo* models, only a few of them have been assayed in clinical trials with no substantial progress. This may be due to several factors: 1) the extensive GBM heterogeneity, both intratumoral and intertumoral; 2) the presence of GSC as crucial drivers of recurrence and resistance; 3) the activation of efficient drug efflux systems; 4) the dynamic interaction with a complex microenvironment; 5) the poor permeability to drugs due to BBB. Additional efforts will be necessary to test known pro-oxidant compounds and find new molecules that synergistically increase therapeutic effect of TMZ combinatory treatments against GBM.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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