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Review

Cellular stress responses as modulators of drug cytotoxicity in pharmacotherapy of glioblastoma

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ABSTRACT

Despite the extensive efforts to find effective therapeutic strategies, glioblastoma (GBM) remains a therapeutic challenge with dismal prognosis of survival. Over the last decade the role of stress responses in GBM therapy has gained a great deal of attention, since depending on the duration and intensity of these cellular programs they can be cytoprotective or promote cancer cell death. As such, initiation of the UPR, autophagy or oxidative stress may either impede or facilitate drug-mediated cell killing. In this review, we summarize the mechanisms that regulate ER stress, autophagy, and oxidative stress during GBM development and progression to later discuss the involvement of these stress pathways in the response to different treatments. We also discuss how a precise understanding of the molecular mechanisms regulating stress responses evoked by different pharmacological agents could decisively contribute to the design of novel and more effective combinational treatments against brain malignancies.

1. Introduction

According to the classification of the World Health Organization (WHO), glioblastomas (GBM) are a grade IV diffuse astrocytic and oligodendroglial tumors, which represent the most frequent and malignant form of brain neoplasms [1,2]. The incidence of GBM ranges from 0.59 to 5 per 100,000 people and it is on the rise in many countries [2]. One of the hallmark features of GBM is high intra- and inter-tumoral heterogeneity, which can be attributed mainly to the aberrations on genetic and transcriptional levels [3]. The most frequent genetic alterations of primary GBM include loss of heterozygosity of chromosome 10, which bears the phosphatase and tensin homolog (*PTEN*) gene; deletion or mutation of the $p16^{INK4}$ gene; amplification of the epidermal growth factor receptor (*EGFR*) gene; and enhanced activity of telomerase reverse transcriptase [3]. On the other hand, secondary GBM holds mutations in isocitrate dehydrogenase gene (*IDH1/2*) and displays loss of function in the platelet-derived growth factor receptor-α (PDGRA/PDGR-α) and the tumor suppressor protein 53 (p53) [3]. Moreover, recent studies demonstrate that the presence of a subpopulation of self-renewing and pluripotent GBM stem-like cells (GSCs) in tumor mass may be an essential factor responsible for GBM formation, maintenance, invasiveness, and recurrence [4–7]. These characteristics together with the aggressiveness and highly infiltrative nature of this tumor result in systematic failure of current therapeutic strategies [1,8,9].

Thus far, the standard of care treatment for GBM relies on maximal possible resection, followed by concomitant radio- and chemotherapy with the use of temozolomide (TMZ). Nevertheless, current clinical regimens include the use of several other chemotherapeutic agents, such as cisplatin, lomustine, and carmustine in order to slow down the progression of this incurable cancer [1,10–17]. Unfortunately, despite certain therapeutic improvements resulting mainly from the development of second-line treatments such as anti-angiogenic agents, the overall prognosis for GBM patients remains dismal [14,15]. The updated survival statistics in the United States report that only 10% of patients subjected to combinational treatment lived up to 5 years after diagnosis in oppose to merely 2% for those who were treated with the radiation alone [18]. Sadly, the median survival of patients with GBM still ranges from 15 to 18 months [1,2,18,19]. Due to these obstacles constant efforts are undertaken to identify molecular pathways engaged in tumorigenesis of GBM and signal transduction cascades involved in response to pharmacotherapeutic agents.

Indeed, current therapies against GBM are ultimately ineffective

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mainly due to glioma cells' ability to adapt to the treatments and activate mechanisms of resistance. As such, a major therapeutic challenge is to combat the adaptability of tumor cells, which to a large extent relies on the activation of stress-dependent signaling. The most common inducers of cellular stress are nutrient deprivation, protein misfolding and aggregation, overproduction of reactive oxygen species (ROS), and mitochondrial damage. These stresses trigger the activation of endoplasmic reticulum (ER) stress/the unfolded protein response (UPR), oxidative stress and autophagy, three cellular mechanisms that play a critical role in the regulation of cancer generation and progression [20,21]. Thus, the signaling pathways that regulate the activation of these stress responses contribute decisively to determine whether the cancer cell survives the different (stressing) challenges that faces during the progression of the disease or undergoes a cell death program. Accordingly, a large body of evidence supports the idea that the sensitivity or resistance to many anticancer agents relies on the activation of autophagy and oxidative stress [1]. As such, understanding the precise mechanisms by which activation of these cellular processes contributes to promote cancer cell death or to evade the action of anticancer agents would be of pivotal importance to design more efficient antineoplastic therapies.

On these premises, this review will focus on the involvement of key cellular stress responses: oxidative stress, ER stress, and autophagy in the mechanism of action of pharmacological agents used as chemotherapeutics or that are being investigated as potential drug candidates for GBM treatment.

2. Crucial stress pathways activated during gliomagenesis

Along the neoplastic process cancer cells must withstand numerous internal and environmental challenges such as genomic instability, replicative stress, exposure to increased oxidative stress, exposure to hypoxic environments, lack of nutrients, etc. To adapt to these unfavorable situations and progress to more advanced stages of the disease they activate specific stress-response programs that allow them to survive and generate a supportive tumor microenvironment. Cellular stress responses are highly conserved pathways activated upon exposure to both intrinsic factors (including increased metabolic burden, genomic instability, and oncogene expression) and extrinsic factors (such as nutrient deprivation, lactic acidosis and hypoxia) [1,22,23]. There are several different types of stress responses and the way in which cells deal with these insults depends on the intensity of the damaging factor and the time of exposure. In general, stress-dependent pathways guard the balance between the activation of cytoprotective and pro-death mechanisms in the stressed cell. Overall, stress-evoked protective responses are based on enhancing protein folding capacity through an increase in chaperone protein activity; and on the removal of damaged organelles [24,25]. However, if the stress is severe and insuperable, these survival strategies are unsuccessful and the cell death programs are activated in order to eliminate damaged cells [23–27].

Activation and propagation of the pro-oncogenic signaling cascades requires elevated metabolism rates, high demands for protein synthesis and folding, and dealing with the deprivation of essential nutrients. These molecular and physiological circumstances also apply to the pathogenesis of brain tumors, making these cells prone to develop various stresses. To date, activation of the three critical types of cellular stress mechanisms have been identified in pathogenesis of GBM. Therefore, ER stress, autophagy, and oxidative stress have been found to play a significant role in the development and progression of this malignancy (Fig. 1) [1,5,19–21,23,28,29].

Studies show that adaptive stress responses contribute to the oncogenesis of GBM. Moreover, stress-activated pathways intercross, overlap and merge with each other to orchestrate an integrated response that contributes to glioma generation and progression (Fig. 1). As such, it is well documented that ROS-mediated oxidative stress can evoke cellular responses that involve ER stress and autophagy [30]. Likewise, ER stress can lead to the initiation of autophagy through the UPR, and activation of the oxidative stress via stimulation of protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin-1 (ERO1) (Fig. 1) [31]. Thus, a deeper knowledge of the mechanisms activated in response to stresses is essential not only to understand the biology of gliomas, but also to design novel therapeutic approaches based on the pharmacological modulation of these stress responses in a manner that contributes to maximize current or future anti-GBM treatments.

2.1. ER stress in glioblastoma

In comparison to nonmalignant cells, cancer cells require a high rate of protein synthesis and folding to maintain their tumorigenic properties and the activity of oncogenic signaling pathways [1]. Additionally, transformed cells need to withstand constant exposition to multiple insults including intrinsic stresses e.g. increased metabolic burden, genomic instability, or oncogene expression, as well as extrinsic stresses such as nutrient deprivation, oxidative stress, and hypoxia [23,26,27]. This altogether, may lead to protein misfolding/unfolding and perturbation of protein homeostasis. The organelle in charge of controlling the biogenesis of secretory and transmembrane proteins is the endoplasmic

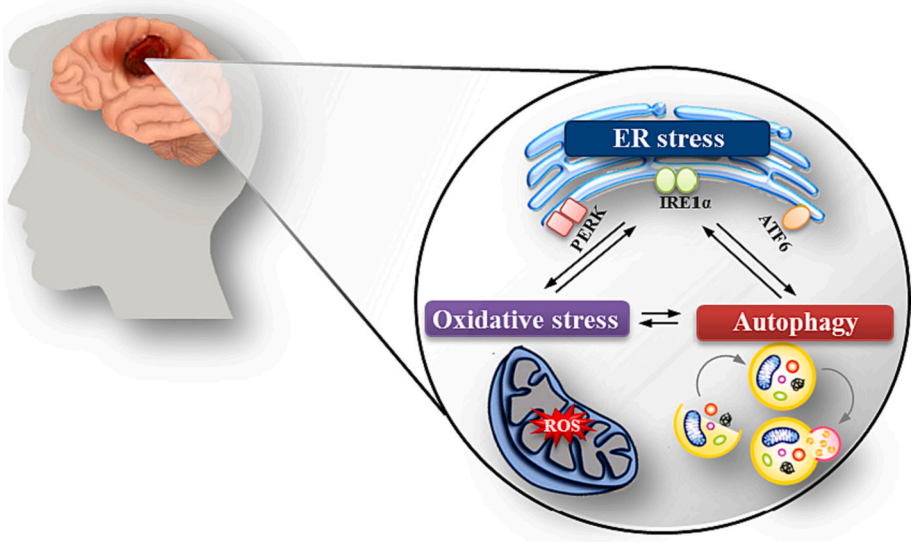

Fig. 1. Schematic representation of key stress responses activated in glioblastoma cells during gliomagenesis and the way they interact with each other.

reticulum (ER). The ER is a cellular compartment where many processes crucial for cell functioning take place, including protein synthesis and folding, lipid synthesis, and calcium storage. Within the ER lumen, a set of enzymes and chaperones facilitate protein folding, therefore preserving ER-dependent proteostasis. However, if the equilibrium between folding capacity and enhanced folding demands is disrupted, improperly folded proteins accumulate in the ER lumen causing ER stress [23,26]. In order to deal with these challenges, cells activate a mechanism orchestrated from the ER called the unfolded protein response (UPR). The mechanisms of ER stress and the UPR have been thoroughly studied and numerous excellent comprehensive reviews have been published [1,23,24,26,32]. Therefore, herein only a brief overview of the crucial regulatory mechanisms of the UPR will be delineated. Regulation of the UPR is dependent on the activation of the three transmembrane sensors: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and the inositol-requiring enzyme 1 α (IRE1α) [23]. Under basal conditions, these sensors are kept in an inactive state by binding to the ER-resident chaperone glucose regulated protein 78 (GRP78). The UPR signaling is initiated when GRP78 dissociates from these sensors to rescue misfolded proteins accumulated in the ER lumen. Once released from the inhibitory interaction of GRP78 each of these sensors triggers a specific "arm" of the UPR aimed at activating various adaptive mechanisms that will help the cell to cope with the unfavorable conditions generated in the ER lumen. The activated IRE1α stimulates the unconventional splicing of the x-box binding protein 1 (XBP1) mRNA. A GRP78-free ATF6 moves into the Golgi apparatus where it is cleaved by serine proteases S1P and S2P to form its active 50-kDa subunit ($_{p50}$ATF6). Active PERK phosphorylates the eukaryotic translation initiation factor 2 α (eIF2α) thereby suppressing general translation. This event prevents a further accumulation of misfolded proteins, and at the same time favors the translation of genes carrying ER stress response elements, which leads to a selective increase in the expression of several genes and specifically of the transcription factor ATF4. XBP1-s, $_{p50}$ATF6, and ATF4 are translocated to the nucleus where they promote the expression of a whole set of genes encoding proteins involved in restoring protein homeostasis. Therefore, a primary aim of the UPR is to reinforce protein folding and activate ER-associated degradation (ERAD). However, if the stress is prolonged or too severe for the restoring capacity of the UPR, proapoptotic mechanisms, mostly connected with the activity of transcription factor CHOP, are activated (see Fig. 4) [23,24,26,27,32].

The concept of disruption of ER proteostasis has been proposed as a mechanism promoting malignant cell transformation and tumor growth in glioma. In line with this idea, GRP78 overexpression has been repeatedly reported in GBM patients where this event is associated with reduced survival [19,28,32–34]. Furthermore, chronic activation of the prosurvival branch of the UPR in GBM cells correlated with apoptosis resistance, insensitivity to chemo- and radiotherapy and worse patients' prognosis [35,36]. Moreover, studies on clinical samples have shown that elevated levels of GRP78 are associated with higher GBM malignancy and recurrence rate [19,34,37]. Further studies evidenced that the expression of GRP78 increased together with the pathologic grade of primary astrocytomas and therefore the expression of this protein had prognostic value in these tumors [37]. In line with these observations, mRNA and protein levels of GRP94, another ER-resident chaperone, were shown to be significantly upregulated in GBM in comparison to normal brain tissue [38]. Increased expression of GRP94 was also associated with a worse overall survival of GBM patients [38]. Moreover, GRP78 has also been found to indirectly influence tumor malignant potential via stimulating AKT/extracellular signal-regulated kinase (ERK) signaling and inhibiting caspase-dependent apoptosis, thus promoting cell proliferation [37]. Of note, levels of GRP78 were also found to be higher in the vasculature derived from human glioma specimens than in the surrounding normal brain tissues and blood vessels [39,40]. Considering the essential role of tumor vasculature in GBM growth and survival, it has been proposed that this enhanced expression of GRP78 might play a critical role in the regulation of tumor angiogenesis thereby leading to enhanced tumor progression and resistance to chemotherapy [39,40]. Recently, it has been discovered that a siRNA-mediated knockdown of *PDIA4* and *P4HB* (two ER-resident enzymes belonging to the PDI family) resulted in significant suppression of the proliferative capacity, migration and clonogenicity of glioma cell lines, suggesting that inhibition of ER stress might attenuate glioma progression [41]. Moreover, bioinformatic and mechanistic approaches led to the identification of specific ER stress-related gene signatures serving as relevant prognostic markers for GBM patients' outcome [41–44]. These analyses revealed significant differences in the overall survival rate of glioma patients with different ER stress-related risk signatures. Furthermore, the ER stress-related risk score identified in these studies correlated with the clinicopathological characteristics of tumors, which indicated that this value could serve as an independent prognostic indicator and supported the idea that selective modulation of ER stress could be a therapeutic strategy to develop individualized treatments against GBM [41,44]. Altogether, these findings suggest that targeting GRP78/GRP94 would mitigate the adaptive capacity of tumor cells thereby sensitizing GBM to antiangiogenic therapeutics. Nonetheless, a better mechanistic understanding of the signaling mechanisms that are activated in response to ER stress during GBM generation and progression in different GBM subtypes is still required to identify a precise set of ER stress-related biomarkers that have prognostic value and can be implemented in the clinical setting.

2.2. *Autophagy in glioblastoma*

Macroautophagy, hereafter named autophagy, is a highly conserved cellular process that facilitates the degradation and clearance of cellular components in the lysosomes. Autophagy is frequently stimulated in response to various stressing conditions, such as accumulation of aggregated proteins or dysfunctional organelles that can disturb cellular homeostasis [25]. This cellular process comprises of a complex signaling network, which has been analyzed in detail in numerous excellent comprehensive reviews [23,25,45–48]. Thus, only a brief description of this mechanism will be provided here. Autophagy is directed by a series of essential autophagy related (ATG) proteins such as Beclin 1, PI3KC3, UVRAG, LC3 and p62 [48]. These proteins act in a concerted manner to orchestrate the formation of double membrane vesicles called autophagosomes. Mature autophagosomes eventually fuse with lysosomes thereby leading to the degradation of its content [48]. Autophagy can be divided into several phases: induction, phagophore nucleation, elongation, fusion with the lysosomes, degradation of the autophagosome content, and recycling [45]. In response to nutrient deprivation and other autophagy-triggering stimuli the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is inactivated, which leads to the stimulation of the Unc-51-like kinase 1 (ULK1, also named ATG1) complex. This ATG1/ULK1 complex subsequently initiates the formation of the phagophore by stimulating the activity of the phosphoinositide 3-kinase (PI3K) class III-complex 1 (PI3KC3-C1) containing Vps34, UVRAG, ATG14L1, Beclin 1, and p150. Specifically, Vps34 (PI3KC3) catalyzes the phosphorylation of phosphatidylinositol to phosphatidylinositol-3-phosphate on the growing autophagosomal membrane during the nucleation process [48,49]. The elongation and maturation of the autophagosome requires engagement of two ubiquitin-like conjugation systems involving the ubiquitin-like proteins ATG12 and ATG8 (also named microtubule-associated protein 1 A light chain 3 MAP1LC3 or LC3). The first one permits the formation of the Atg12-Atg5-Atg16L-complex, whereas the second one leads to the generation of the phosphatidylethanolamine (PE)-modified and autophagosomal membrane-associated form of LC3 (named LC3-II) [50]. Autophagosomes then fuse with lysosomes to form an autophagolysosome (or autolysosome) in which the degradation process occurs. The products of this degradation are further recycled back to the cell providing nutrients and restoring energy supplies to support cell growth (see Fig. 5) [4,23,46]. Despite the

role of autophagy as a self-digestion mechanism, this process is mainly activated to protect cells from cell death [23,46]. Nevertheless, similarly to the UPR, in certain circumstances promotion of autophagy may be required to initiate or execute cell death [23,46].

Numerous recent reports show that autophagy is one of the processes disrupted during development and progression of many types of cancer including brain tumors [6,50–59]. Since autophagy is driven by various stress stimuli such as hypoxia, nutrient deprivation or chemotherapy, GBM cells are prone to develop highly active autophagy signaling to survive and surpass the given treatment. In contrast, GBM cells are genetically predisposed to block autophagy via the AKT/mTORC1 pathway. The AKT/mTORC1 cascade is up-regulated due to inactivation of PTEN, which usually serves as a negative regulator of AKT signaling [59]. Indeed, it has been demonstrated that the expression of *ATG* genes was correlated with clinicopathological features of GBM and served as good prognostic model for glioma patients [51–53]. As such, overexpression of Beclin 1, LC3, or p62 was found to be significantly more frequent in high-grade than in low-grade gliomas and was correlated with poorer prognosis [50,53]. Wen et al. demonstrated that the median overall survival time of patients with high expression of ATG4C was significantly reduced, and up-regulation of *ATG4C* mRNA was correlated with increasing glioma grade [10]. On the contrary, it has been found that the expression of Beclin 1, but not LC3, was lower in high-grade astrocytic tumors than in low-grade tumor samples, suggesting that decreased autophagic capacity might be associated with higher progression of astrocytic tumors [54,56]. Moreover, it has been reported that in glioblastoma patients, expression levels of ULK1 and ULK2, were markedly lower in GBM tissues in comparison to normal brain samples [55]. Therefore, this down-regulation of autophagy-related genes such as *ULK1*, *ULK2*, and *FIP200* in GBM suggests that impaired autophagy promotes astrocytic transformation [56]. Notably, in GBM patients with poor performance scores the increased levels of Beclin 1 were correlated with improved patients' outcomes and survival [6,57]. On the contrary, Cj et al. demonstrated that patients with low LC3/Beclin 1 expression showed better progression free survival than those with high expression of LC3/Beclin 1 in their tumors [58]. Other findings have shown that autophagy was enhanced in human gliomas as compared to normal brain, however this up-regulation was unrelated to the grade of malignancy and patient survival [60].

Overall, the role of autophagy in the development and progression of GBM is still obscure and contradictory. However, it seems undeniable that modulation of autophagy may control tumor growth and progression and may serve as an important prognostic biomarker and potential target of pharmacotherapy in GBM.

2.3. Oxidative stress in glioblastoma

Cancer cells including GBM are characterized by the overproduction of ROS, which leads to the activation of molecular and biochemical pathways determining tumor progression and resistance to chemotherapy [61]. Detailed oxidative stress-dependent signaling in cancer cells has been thoroughly analyzed in many excellent reviews [3,5,20,62,63], therefore, only a brief description of these mechanisms will be presented here. Growing body of evidence shows that due to the enhanced cell metabolism and mitochondrial dysfunction cancer cells are often exposed to high levels of ROS and imbalanced redox status in comparison to the normal counterparts [64]. The primary source of ROS within the cells is the energetic aerobic metabolism [20]. The electron transport chain located on the inner mitochondrial membrane is responsible for generation of ROS in the process of oxidative phosphorylation. Leakage of electrons from the electron transport chain results in partial reduction of oxygen, first to the O_2^- and subsequently to H_2O_2, both of which are considered as mitochondrial ROS [5,62]. Except for the mitochondria, increased metabolic requirements and accompanied up-regulation of glucose transport together with anaerobic metabolism may also be responsible for ROS production. Therefore, the Warburg effect may act as a major player in controlling ROS levels in cancer cells. The Warburg effect can increase steady-state ROS levels by producing lactate that is extruded through monocarboxylate transporters to the microenvironment of cancer cells [5,62]. Moreover, the NADPH oxidases, peroxisomes as well as ER stress may generate ROS. The NADPH oxidases transfer the electrons from NADPH to the molecular oxygen producing ROS [5]. After diffusing across the membrane, the H_2O_2 produced this way may affect signaling pathways connected with cancer progression mainly by increasing cell survival and inducing genomic instability [5,62]. Peroxisomes are organelles capable of producing and scavenging H_2O_2, O_2^- and ·OH, which allows them to regulate dynamic changes in ROS levels [5,65]. Finally, ER stress can lead to the increase in ROS production through the catalytic processes of NADPH oxidase and oxidoreductase ERO1 [62]. Persistent ER stress can cause the redox-dependent imbalance in the ERO1/PDI electron flow and in consequence enhance ROS generation in the ER [62,66]. The role of ROS generated during gliomagenesis seems to be crucial considering that low/moderate levels of ROS may act as mediators of normal cell functioning including cell survival, proliferation, and migration, which can finally lead to progression of GBM cells [5,20,25,61,67]. A summary of the main ROS-generating mechanisms in GBM cells is presented in Fig. 2.

The scavenging of ROS is guarded by the highly coordinated activity of antioxidant defense systems e.g. superoxide dismutases (SOD), peroxidases (PXD), catalases (CAT), glutathione (GSH) or glutathione reductases (GR), and the imbalance between ROS production and the efficiency of these protective systems results in oxidative stress [20]. Whereas mild ROS production can benefit cancer cell survival and proliferation the overload of ROS causes detrimental injuries to the cell structure [5]. Oxidative stress damages the molecules critical for cell functioning, such as proteins, nucleic acids, lipids, and carbohydrates [63]. ROS are able to react with polyunsaturated fatty acids (PUFA) causing peroxidation of membrane lipids and subsequent disruption of membrane integrity. The DNA can also be a target of ROS attacks, resulting in single- and double-strand breaks as well as modifications of pyrimidine and purine bases. Moreover, ROS can alter side chains of the amino acids and damage the backbone of the proteins causing disruption of cellular proteostasis [20]. This duality of ROS nature makes ROS-dependent signaling and oxidative stress an important factor in determining cell fate and a promising target for the development of novel therapeutic agents in GBM.

Oxidative stress is considered one of the major contributors to the pathogenesis of GBM leading to oxidation of proteins, peroxidation of lipids in cellular membranes, and DNA damage, which in turn results in genetic mutations, alterations in chromosome structure, and dysregulation of cell growth [3,68–70]. In normal conditions redox mechanisms act against cell oxidative damage, however during gliomagenesis and in glioma cells the cellular antioxidant systems are strongly impaired. Recent studies investigated the correlation of oxidative stress with tumor grade to assess the prognostic value of oxidative stress markers in predicting glioma progression [71,72]. In the study of Lu et al., 14 pivotal oxidative stress-related genes were identified to be associated with prognosis of glioma patients. The risk score model created using these genes was found to have good predictive ability, giving hope for future application of oxidative stress-related biomarkers in clinical settings [71]. Moreover, Hardiany et al. measured the levels of carbonyl and 8-hydroxy-2′-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and MnSOD in brain tissue of GBM patients [72]. They showed that all the measured markers of oxidative stress were significantly elevated in tumor tissue in comparison to normal brain samples. Moreover, an increase in the level of stress markers was correlated with tumor grade [72]. Likewise, Iida et al. demonstrated stronger accumulation of 8-OHdG and overexpression of human MutT homolog protein 1 (hMTH1) an enzyme hydrolyzing 8-OHdG, in high-grade gliomas in comparison to low-grade tumors indicating correlation of oxidative stress markers with tumor grade [69]. Accordingly, studies of Faraji-Rad

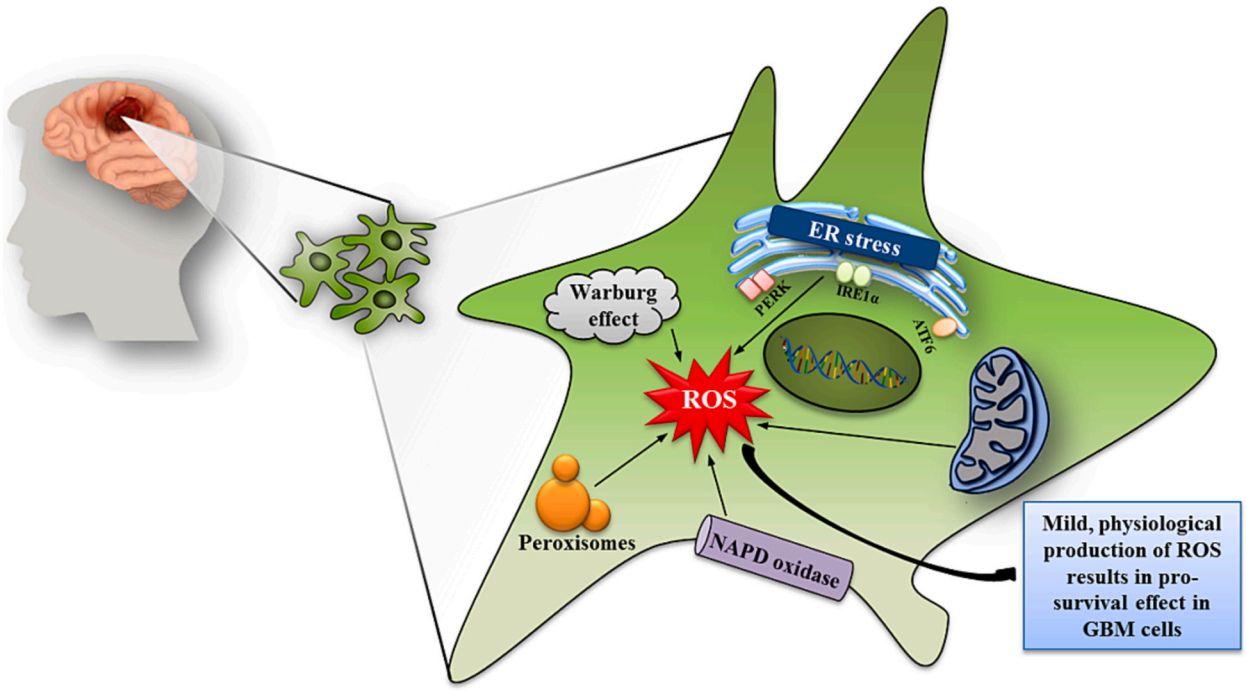

Fig. 2. Major intracellular sources of ROS in GBM cells. The meaning of ROS in cell functioning depends on their concentrations. Low/moderate levels of ROS can act as signaling molecules regulating wide variety of biological processes including cell proliferation, migration and survival, which in consequence leads to progression of GBM.

et al. showed increased levels of oxidative stress in sera of GBM patients in comparison to the healthy individuals, indicating that imbalanced redox status spreads way beyond the brain tissue [73]. These results suggest that oxidative stress plays a role in tumor progression and mediators of oxidative stress could hold a promising prognostic value predicting the course of GBM in patients.

Given this, understanding the mechanisms of oxidative stress in GBM may help in designing novel therapeutic options for patients. The elevation of ROS levels exceeding the antioxidant capacity may be considered a strategic opportunity to target GBM cells. As such, a great deal of attention should now be concentrated on potential anticancer drugs effectively killing transformed cells and overcoming drug resistance through ROS overproduction and destabilization of cellular antioxidant defense mechanisms.

3. Pharmacotherapeutic strategies in glioblastoma

The gold-standard adjuvant treatment for malignant gliomas is ionizing radiation (IR) [8,74,75]. IR preceded by maximal safe resection has been used for patients with newly diagnosed GBM as the standard of care since 1978. Vast majority of radiation utilized in clinical practice relies on low energy transfer IR, which is mainly responsible for indirect cell damage induced by the overproduction of ROS [76]. Although these free radicals can interact with various cellular targets, the primary mechanism of IR-induced cell death is associated with the DNA damage [76]. However, considering the radioresistance and common in-field recurrence in patients with GBM, novel approaches improving the efficiency of IR are indispensable. Thus, the co-treatment with IR and potential cytostatic drugs would favor the improvement of the therapeutic index for GBM patients. Although numerous therapeutic strategies for GBM have been investigated, so far only several drugs have been approved by Food and Drug Administration (FDA) and entered clinical usage in GBM-suffering individuals. The most often used chemotherapeutic agent for GBM is an alkylating pro-drug temozolomide (TMZ) [12,77–79]. However, novel alkylating agents such as lomustine and carmustine have been FDA-approved as treatment modalities for GBM lately [14–17]. Unfortunately, the main drawback of using these agents is that GBM cells often acquire resistance to these drugs, typically due to the activity of the DNA repairing enzyme O^6-methylguanine methyltransferase (MGMT). The MGMT reverses the DNA-alkylating effects of these drugs, while its epigenetic inactivation (methylation of the promoter) results in loss of the DNA-repairing function and better treatment efficiency. The principal cytotoxic effect of TMZ consists of the instantaneous reaction of its secondary metabolite - methyldiazonium cation with the accessible nucleophilic sites of the DNA, resulting in methylation of guanine at N^7 and O^6 sites, and adenine at N^3 site [80]. Likewise, one of the most relevant molecular effects of lomustine is the chloroethylation of guanine at the O^6 position, which generates O^6-chloroethylguanine. Notably, the therapeutic efficiency of lomustine was stated only for oligodendrogliomas or MGMT-deficient GBM. On the other hand, carmustine was found to stimulate formation of interstrand crosslinks of guanines and cytosines in the DNA and is usually applied for both initial glioma diagnosis and prevention of tumor recurrence. Nevertheless, acquired resistance of tumors together with restricted dosing regimens resulting from debatable safety and considerable adverse effect of these agents, are the factors limiting their effective use in monotherapy [81,82]. As such, other therapeutic options have been quested. Bevacizumab is an FDA-approved humanized therapeutic antibody binding to the vascular endothelial growth factor (VEGF) to suppress angiogenesis and vascular permeability in tumor microenvironment. Moreover, recent advances in GBM therapy resulted in FDA approval of the tumor treating fields (TTF), which are an alternating electric fields of low intensity (1–3 V/cm) and intermediate frequency (~100–500 kHz) aimed at disrupting cell division [81,83]. However, despite extensive efforts in developing effective treatment regimens, current therapeutic strategies have not delivered significant improvements for the survival of patients with GBM. Hence, novel emerging therapeutic approaches are being quested to test as potential drug candidates for this malignancy. Given this, recent studies have focused on engineering more effective drug delivery systems, optimizing pharmacotherapeutic interventions, and implementing personalized care based on the genomic profile of the tumor [84,85]. In consequence, latest

experimental and clinical strategies in GBM treatment include: radiotherapy [75,86,87], physical therapy [81,83], immunotherapy [15,88], stem cell therapy [7], gene therapy [11,89–91], nanomedicine [92–94], alkylating agents [10,12,95], and natural compounds [4,96,97] (Fig. 3).

Moreover, multi-targeted therapeutic approaches are currently under investigation in GBM treatment (NCT02270034, NCT03529448). Monotherapy is often insufficient due to resistance triggered by the multiple compensatory feedback loops and relatively low number of specific predictive biomarkers [84,85]. Combination therapy allows simultaneous targeting of several molecular pathways crucial for cancer cell survival and eradicates cellular mechanisms connected with adaptive resistance. Therefore, novel treatment regimens combining classical chemotherapeutics together with adjuvant therapy and innovative drug delivery systems may bring hope for preventing tumor recurrence and increasing patients' survival. However, the selection of specific therapeutic options requires in-depth knowledge of their functioning at the molecular level in order to maximize their curative potential.

Hence, progress in the development of pharmacotherapeutic strategies for GBM entails the urgent need for identification of molecular mechanisms underlying therapeutic effect of cytostatic agents. In line with this notion, in recent years signaling pathways engaged in adaptation to cellular stresses are emerging as relevant drivers of initiation, growth, and chemoresistance in brain tumors. In this respect, increasing amount of data reports disruption of cellular homeostasis and activation of stress pathways not only during progression of GBM but also upon treatment with various pharmacological agents [4,8,10,43,44,64,70,77,93,96,98–101]. As such, contribution of key stress responses such as oxidative stress, ER stress, or autophagy to the functioning and effectiveness of anti-GBM drugs is gradually gaining more and more recognition of the scientific community. To date, in spite of undisputable progress in the development of novel treatment regimens in preclinical models, most widely clinically used therapeutic approach for GBM comprises of the application of TMZ and/or bevacizumab with radiotherapy [9,88,102,103]. Nevertheless, recent innovations in optimizing chemotherapy of GBM include administration of other cytostatic agents such as cisplatin, lomustine or carmustine as treatment regimens for brain tumors [12–17]. Additionally, numerous experimental data from in vitro and in vivo studies reports good efficiency of a wide variety of other potential drug candidates including nanoparticles [101,104,105] or natural phytochemicals [4,64,96,97,106,107] in suppressing proliferation of GBM. Notably, despite having a different primary mode of action, in the case of all these agents stress responses were reported to be entangled in drug functioning at the molecular level. This happened either by hindering therapeutic efficiency of the drug via initiation of cytoprotective response or by potentiating cytostatic or cytotoxic effects. These observations strongly suggest that the pharmacological targeting of these stress responses could be a strategy to fight GBM or to enhance the therapeutic activity of the treatments that are currently used for the management of these tumors.

3.1. *ER stress as modulator of drug functioning in glioblastoma*

Constitutive UPR signaling and ER stress is activated in multiple types of tumors and is also known to be a hallmark of GBM cells [33,41,44]. The prosurvival branch of the UPR promotes proliferation of tumor cells, however an overload of the ER machinery seems to be beneficial in evoking ER-stress mediated apoptosis. Given this, ER stress is emerging as relevant driver not only of tumorigenesis and tumor growth, but also chemoresistance in GBM [1,33,44]. Therefore, a great deal of attention is currently shifted towards the involvement of ER stress and the UPR into the functioning of anti-GBM pharmacological agents.

To date, many standard and experimental anti-GBM treatments have been demonstrated to interfere with ER stress signaling pathways. Various research on the involvement of ER stress into cytotoxicity of pharmaceutics against GBM have shown that activation of the UPR may have both prosurvival and cell death promoting implications. Thus, ER stress and the UPR have been identified as important modulators of therapeutic effectiveness of the most widely applied GBM treatment, which comprises of the application of IR and TMZ [8,32,77,108]. Studies have shown that induction of ER stress signaling by irradiation

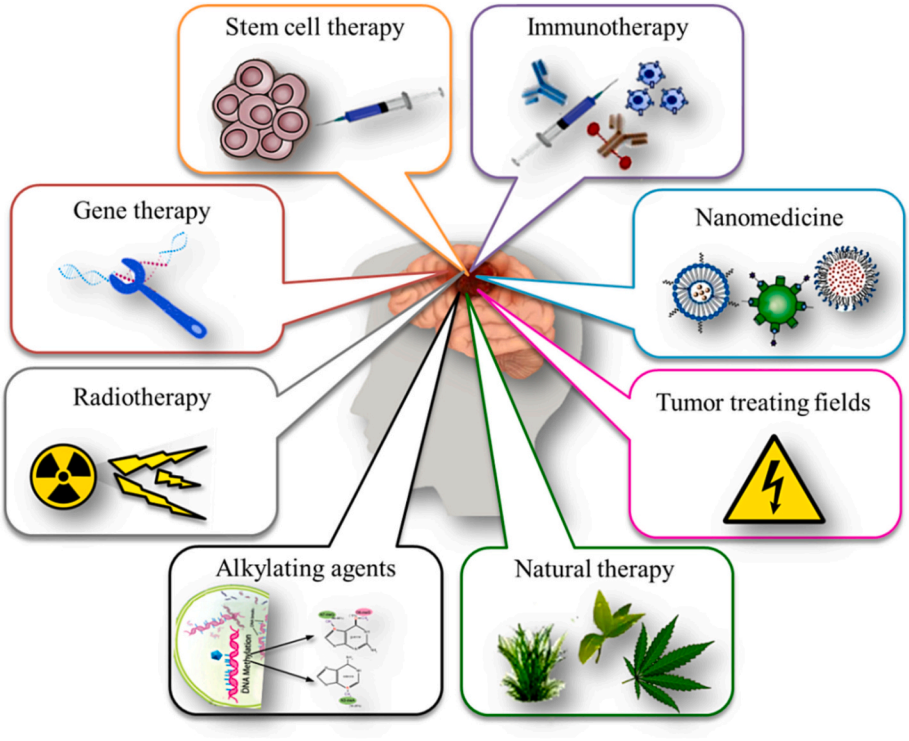

Fig. 3. A summary of key clinical and experimental therapeutic approaches in glioblastoma.

contributed to the development of adaptive prosurvival mechanisms during radiotherapy of GBM [8,108]. This effect was demonstrated to depend on both the molecular signaling of the ATF6-mediated pathway and the PERK/eIF2α/ATF4 axis of the UPR [8,108]. The ATF6 was found to up-regulate GRP78 and NOTCH1 as downstream molecular targets resulting in enhanced survival of GBM cells, whereas PERK-mediated signaling stimulated the expression of VEGFA suggesting the connection between therapy-triggered ER stress and a proangiogenic response in GBM (Fig. 4) [8,108]. On the contrary, in cells treated with salubrinal – an inhibitor of eIF2α phosphatase, irradiation enhanced the pro-death component of PERK signaling [8]. Given this, ATF6 and PERK might be considered as potential therapeutic targets to enhance the efficacy of IR-based therapy. As such, chemical/genetic modulation of ER stress might be a helpful tool in designing novel combined therapeutic regimens directed at potentiating antitumor properties of ionizing radiation. Moreover, an increasing body of evidence has shown that the UPR signaling may influence the therapeutic efficiency of TMZ. Although the direct influence of TMZ on ER stress is questionable, several in vitro studies reported that TMZ activated the UPR in GBM cells as evidenced by increased levels of GRP78 and CHOP proteins (Fig. 4) [32,77]. In fact, drug sensitivity analysis using colony survival assays showed that upon repression of GRP78, GBM cells were significantly more sensitive not only to TMZ but also to other chemotherapeutic agents including etoposide, irinotecan, 5-fluorouracil, and cisplatin [32,36]. Furthermore, modulation of the UPR response by hyperoxia or IRE1α inhibitors sensitized GBM cells to TMZ [78,98]. This sensitization was most probably connected with activation of the proapoptotic branch of the UPR as highlighted by the up-regulation of CHOP [78]. Likewise, the co-treatment of GBM cell lines with TMZ and the ER stress inducing drug - salinomycin resulted in increased expression of GRP78, p-PERK, and ATF4, which was accompanied by a decreased expression of MGMT, *N*-methylpurine DNA glycosylase (MPG), and DNA repair protein RAD51

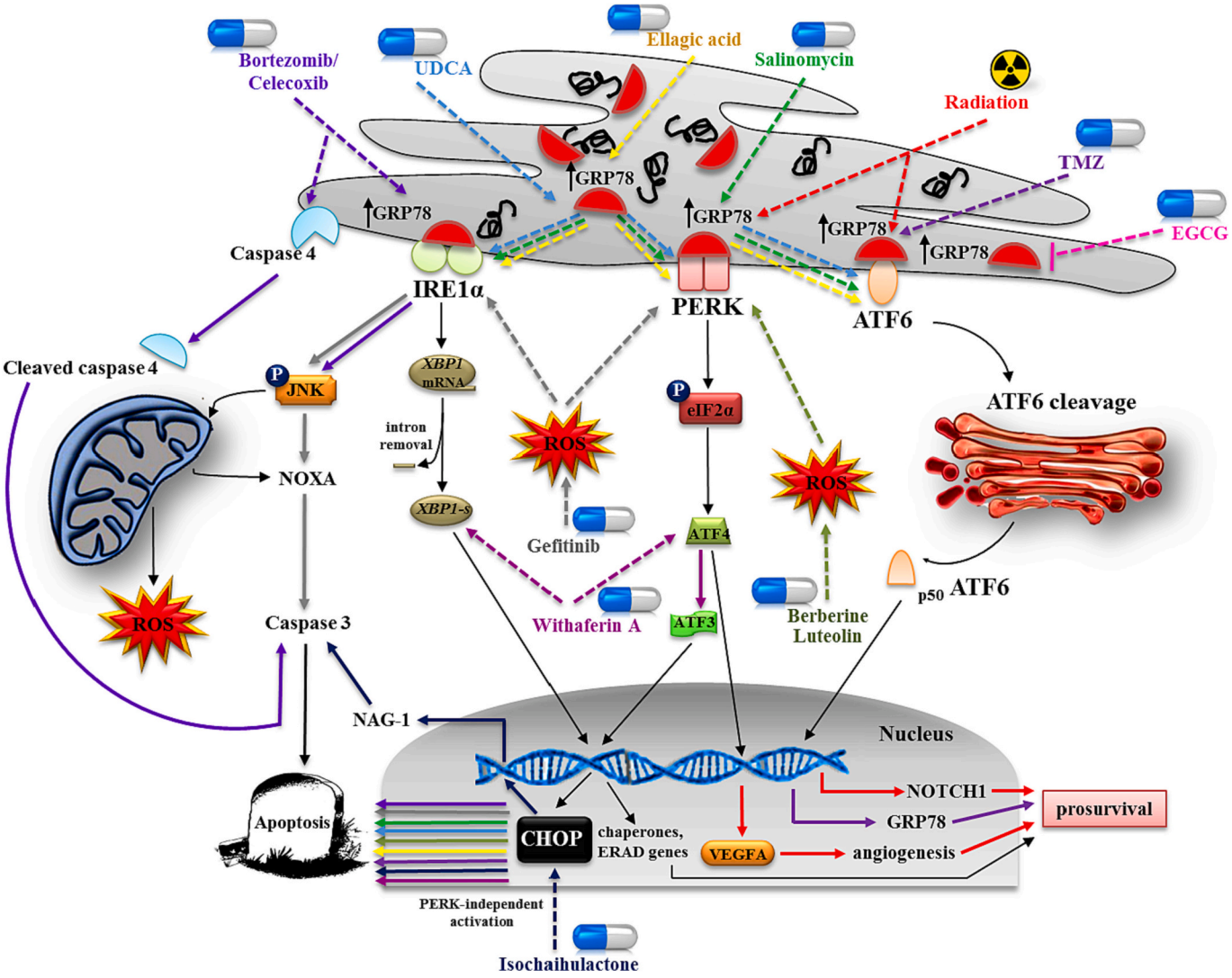

Fig. 4. Representation of the core three branches of the classic UPR in GBM cells together with the modulatory effect of various drugs on ER stress-dependent cell survival and cell death. Ionizing radiation stimulates ATF6- and PERK/eIF2α/ATF4-dependent up-regulation of NOTCH1 and VEGFA resulting in enhanced angiogenesis and survival of GBM cells. TMZ promotes up-regulation of both GRP78 and CHOP showing prosurvival and proapoptotic implications respectively. Salinomycin and ellagic acid promote CHOP overexpression via activation of all three branches of the UPR. Bortezomib and celecoxib cause activation of CHOP and caspase 4 resulting in proapoptotic effect. Gefitinib causes generation of ROS, which results in activation of IRE1α-mediated phosphorylation of JNK, and overexpression of Noxa and caspase 3. Likewise, berberine and luteolin promote ROS-mediated activation of PERK-dependent CHOP expression. Withaferin A upregulates XBP1-s together with ATF4 and ATF3 followed by CHOP induction. EGCG inhibits GRP78 function preventing the UPR signaling. Isochaihulactone induces GRP78- and PERK-independent overexpression of CHOP followed by up-regulation of NAG-1 and caspase 3. Short-term stimulation with UDCA activates three branches of the UPR resulting in CHOP overexpression. Dashed colorful arrows indicate the ER stress-dependent targets of anti-GBM drugs; continuous colorful arrows indicate the effects of drug functioning. Color patterns of arrows match the drug-effect scheme.

homolog 1 (RAD51), thereby sensitizing cells to the cytotoxic effect of TMZ and increasing apoptotic cell death (Fig. 4) [79]. Although direct influence of ER stress on the expression of MGMT, MPG, and RAD51 was not clear, restoration of ER homeostasis with a chemical chaperone 4-phenylbutyrate resulted in subsequent re-expression of these proteins and de-sensitization to TMZ, confirming the essential role of ER stress in the modulation of TMZ effectiveness [79].

The essential role of ER stress prompted a quest for pharmacological agents directly targeting this cellular mechanism. In this respect, a proteasome inhibitor bortezomib has been tested as potential drug candidate for GBM. Studies have shown that bortezomib alone induced overexpression of crucial mediators of the UPR cascade including GRP78, CHOP and cleaved caspase 4. However, this effect was insufficient to induce apoptosis neither in the glioma cell line U87MG nor in xenografts generated with these cells in mice [109]. Of note, when bortezomib was combined with celecoxib, another ER stress-inducing drug, the expression of GRP78, c-Jun NH(2)-terminal kinase (JNK), CHOP, and caspase 4 was highly potentiated and resulted in significantly enhanced apoptotic cell death (Fig. 4). Furthermore, siRNA-mediated knockdown of *GRP78* sensitized GBM cells to the drug combination up to the lethal effect confirming the pivotal role of this chaperone in modification of the effectiveness of anti-GBM pharmacotherapy [109]. Additionally, the modulatory effect of the UPR signaling on cytotoxicity of drugs primarily targeting ER stress-independent pathways was also investigated. Chang et al. demonstrated that gefitinib, an inhibitor of EGFR, caused activation of the Ire1α, ATF6, and PERK together with intracellular mobilization of free Ca^{2+} in human U87 and H4 glioma cell lines [110]. Specifically, gefitinib-mediated effects included enhanced phosphorylation of IRE1α, Ask1, and JNK as well as increased expression of Noxa, resulting in up-regulation of caspase 3 and augmented apoptosis. However, this effect was reversed by the application of the antioxidant *N*-acetylcysteine (NAC), which suggested that ER stress induction was secondary to ROS overproduction (Fig. 4) [110]. Nevertheless, these findings support the idea that targeting ER stress could be a promising strategy in EGFR inhibitor-based therapy and that it may contribute to enhance the efficiency of drugs such as gefitinib.

The urgent need for novel active cytostatic agents against GBM is currently shifting the attention of the scientific community towards natural compounds [18,48,64,106,111–114]. Interestingly, activation of stress responses seems to be a common mode of action for multiple phytochemicals. As such, withaferin A was found to up-regulate the expression of XBP1-s as well as ATF4, which was followed by overexpression of ATF3 and then CHOP (Fig. 4) [18]. The crucial role of ATF4, ATF3, and CHOP was confirmed by siRNA-mediated down-regulation of each of these molecules, which resulted in diminished withaferin A-induced decrease of mitochondrial membrane potential ($\Delta\Psi_m$), unblocked cell cycle arrest and decreased apoptosis [18]. Furthermore, Chen et al. found that the green tea component epigallocatechin 3-gallate (EGCG) inhibited GRP78 function and brought benefit to the TMZ treatment in preclinical models of GBM [114]. Immunohistochemical analysis revealed that the overexpression of GRP78 occurred in xenografts of TMZ-treated animals, while this effect was alleviated when TMZ was combined with EGCG [113]. Studies have demonstrated that GRP78-inhibitory activity of EGCG was accomplished via interaction with the nucleotide-binding domain of GRP78 (Fig. 4) [115]. Hence, EGCG was found to act as competitive inhibitor to ATP that suppresses the ATPase activity of this chaperone simultaneously converting GRP78 into its inactive dimeric and/or oligomeric form [115,116]. Moreover, EGCG could prevent the formation of the anti-apoptotic GRP78-caspase 7 complex in the ER, thus enhancing cancer cell death [115,116]. These results suggest that polyphenolic agents alleviating the cytoprotective capacity of the UPR signaling might serve as co-therapeutic pharmaceutics increasing the cytotoxic efficacy of TMZ. Interestingly, Tsai et al. demonstrated that isochaihulactone induced GRP78-independent and PERK-independent expression of CHOP, which was followed by an up-regulation of the non-steroidal anti-inflammatory drug-activated gene (NAG-1) resulting in enhanced apoptosis of GBM cells in vitro and in vivo [111]. Of note, CHOP was reported to up-regulate the expression of *NAG-1* upon isochaihulactone treatment via transcriptional activation and stabilization of *NAG-1* mRNA. This effect was confirmed by the siRNA-mediated knockdown of *CHOP*, which was followed by over 40% reduction of NAG-1 expression and restored viability of GBM cells (Fig. 4) [111]. Additionally, several reports indicated more complex activity of natural substances involving simultaneous activation of several stress pathways. Thus, Wang et al. reported that ellagic acid caused conjoined oxidative stress and ER stress response in U251 cells promoting overexpression of GRP78 and CHOP and subsequent apoptotic cell death [112]. Likewise, luteolin and berberine were also demonstrated to promote ROS generation and cause ER stress [64,106]. However, here activation of the UPR seemed to be the secondary mechanism activated as a downstream effect of ROS signaling as evidenced by reversed ER stress upon pretreatment with an antioxidant NAC [64,106,117]. In the case of these phytochemicals enhanced ER stress response resulted in activation of PERK-dependent CHOP expression followed by apoptotic death of T98, U87, U251 and C6 cells (Fig. 4) [64,106,117]. Interestingly, although plant-derived chemicals are usually tested as anticancer agents of natural origin, Yao et al. investigated another natural agent – ursodeoxycholic acid (UDCA) as potential anti-GBM substance [118]. UDCA is an endogenous bile acid existing in human bile. Unlike hydrophobic bile acids considered as carcinogens, UDCA is hydrophilic, but despite this hydrophilicity it tends to cross the blood brain barrier and suppress tumor progression [118]. Amongst various deleterious effects of UDCA in GBM cells, it has been shown to induce ER stress, which was accompanied by an overexpression of GRP78, IRE1α, ATF6, p-PERK, ATF4, and CHOP. However, the prolonged incubation time resulted in alleviated expression of p-PERK, ATF4, and CHOP suggesting that UDCA was insufficient to generate a continuous proapoptotic effect (Fig. 4). Interestingly, the cell death-inducing activity of UDCA was reactivated by co-stimulation of LN229 and A172 cells with bortezomib, showing predominance of combined therapies over single-agent treatments [118]. An overview of the influence of therapeutic compounds, alone or in combination with co-therapeutic agents, on stress responses together with main cellular effect triggered by the applied treatment is summarized in Table 1.

Taking into account the dual role of ER stress and the UPR signaling in mediating cytoprotective/cytotoxic switch upon drug stimulation, investigation of ER stress pathways should be performed while establishing the therapeutic profile of pharmacological agents. To date, a growing body of evidence has reported the existence of a significant role of ER stress-dependent pathways on the efficacy of already available chemotherapeutics and potential drug candidates. Yet, no standard ER stress-modulatory co-therapeutic agents have been established and recommended as treatment regimen in clinics. Given this, the constant quest for novel therapeutic compounds and treatment strategies regarding ER stress response is ongoing and opens the way for future studies.

3.2. *Autophagy as modulator of drug functioning in glioblastoma*

Recently, it has been shown that autophagy is an essential process activated in GBM cells as a clearance mechanism for damaged proteins [25]. Furthermore, in response to chemotherapy and radiotherapy, GBM patients show up-regulated autophagic pathway, giving cancer cells an advantage to survive. Considerable amount of clinicopathological data reports that enhanced autophagy is correlated with poor patient prognosis, a more aggressive clinical picture and drug resistance [10,25,50,53,119]. However, the dual role of autophagy, prosurvival on one hand, and cell death-promoting on the other, has attracted the attention of many scientists wanting to unravel its impact on oncogenesis and pharmacotherapy of cancer. Due to the defective mechanisms of apoptosis in GBM cells, promotion of cell death by autophagy may be an

Table 1
An overview of the influence of therapeutic compounds alone or in combination with co-therapeutic agents on stress responses together with main cellular effect triggered by applied treatment. ↑ - stimulation of the effect, ↓ - alleviation of the effect, ↑↑ - potentiation of the effect of the single drug.

Compound	Co-therapeutic agent	ER stress	Oxidative stress	Auto-phagy	Cellular effect	References
TMZ		↑			Drug-resistance, prosurvival/ cytoprotective	[32,77]
	–		↑ROS		Proapoptotic	[103]
			↑antioxi-dant response		drug-resistance, prosurvival/ cytoprotective	[147,148]
				↑	Cytoprotective/ prosurvival	[99,125,126,128,130]
	Lovastatin			↑↑	Cytotoxic, pro-death	[107]
	Simvastatin			↓	Pro-death	[133]
	EGCG	↓			Pro-death	[114]
	IRE1 inhibitors (methotrexate, cefeperazone, folinic acid, fludarabine phosphate)	↓ IRE1 ↓ XBPs			TMZ-sensitization	[98]
	Salinomycin	↑↑			Proapoptotic	[79]
	Tubastatin A	↑↑			Proapoptotic	[77]
	Resveratrol		↑	↓	Proapoptotic	[125,126,154]
	Rotenone, sodium azide, oligomycin, cyclosporine A			↓	pro-death	[130]
	Chloroquine, BAFA1, 3-MA, biochanin A, PX-866			↓	Pro-death	[119,126,129,131]
	Chloroquine		↑	↓	Pro-death	[153]
	Thioridazine			↓	Pro-death	[132]
	everolimus			↑↑	pro-death	[59]
	Momelotinib			↑↑	Pro-death	[134]
	Steroidal saponins		↑	↑	Pro-death	[156,157]
	Withaferin A		↑		proapoptotic	[158]
	curcumin		↑↑		Proapoptotic	[155]
	NAMPT inhibitors		↑		Proapoptotic	[141]
	THC	↑		↑↑	Pro-death	[135]
	THC:CBD (1:1)	↑	↑	↑↑	Pro-death (proapoptotic)	[135–137]
Ionizing radiation		↑			Prosurvival	[8,108]
			↑		Proapoptotic	[108]
	–		↑antioxi-dant response		Drug-resistance, prosurvival/ cytoprotective	[150]
	TMZ		↑antioxi-dant response		Drug-resistance prosurvival/ cytoprotective	[95,148]
	Salubrinal	↑			Proapoptotic	[8]
TTF	–			↑	Prosurvival	[83]
SAHA	–			↑	Prosurvival	[138]
Bevacizumab	–			↑	Drug-resistance, prosurvival	[139]
THC	–	↑		↑	Pro-death (proapoptotic)	[4,143]
CBD	–	↑	↑	↑	Pro-death	[135,142,159]
THC:CBD (1:1)	–	↑	↑	↑	Pro-death (proapoptotic)	[135–137]
Cucurbitacin I	–			↑	Prosurvival	[141]
Bortezomib	–	↑			No effect	[109]
	Celecoxib	↑↑			Proapoptotic	[109]
Gefitinib	–	↑			Proapoptotic	[110]
Withaferin A	–	↑			Proapoptotic	[18]
Isochaihulacto-ne	–	GRP78- and PERK-indepen-dent expression of CHOP			Proapoptotic	[111]
Ellagic acid	–	↑	↑		Proapoptotic	[112]
Luteolin	–	↑	↑		Proapoptotic	[64]
Kaempferol	–		↑		Proapoptotic	[97]
Resveratrol	–		↑		Proapoptotic	[96]
Brevilin A	–		↑		Proapoptotic	[100]
Berberine	–	↑	↑		Proapoptotic	[106,117]
8-azaKR, 7-deazaKR, dihydroartemisinin	–		↑		Proapoptotic	[61,155]
Ursodeoxycho-lic acid	–	(short-term)↑ (long-term)↓			Proapoptotic, No effect	[118]
	Bortezomib	↑↑			Proapoptotic	[118]
PUFAs	–		↑		Proapoptotic	[161]
Nanoparticles	–		↑		Proapoptotic	[94,101,104,105]

alternative way to remove tumor cells [120,121]. The regulation of autophagy as a death mechanism has prompted investigation of both autophagy inducers and inhibitors as potential anti-GBM drugs. Thus, the manipulation of autophagy may improve or aggravate the efficacy of anticancer agents emerging as new therapeutic target in GBM treatment [25,75,122–124].

Since TMZ is a standard chemotherapy for GBM, it seems necessary to understand the way in which autophagy influences functioning of this chemotherapeutic drug. Indeed, it has been known that TMZ evokes autophagy by a multitude of mechanisms occurring mainly as a downstream effect of the DNA damage [99]. Given this, activation of autophagy in TMZ-treated GBM cells was demonstrated to occur via several molecular routes including: (I) accumulation of ROS and subsequent activation of the mitogen-activated protein kinases (MAPKs)/ERK1/2 signaling pathway [125,126] (II) inhibition of the Akt/mTOR pathway via diminished phosphorylation of these proteins [127], and (III) activation of the ATM/AMP-activated protein kinase (AMPK)/ULK1 cascade [99,128,129] (Fig. 5). Moreover, Lin et al. showed that TMZ-dependent autophagy may also be activated as a result of ER stress-mediated activation of JNK and Ca^{2+} influx [130]. These findings were confirmed by suppression of autophagy upon co-stimulation with ER-stress-alleviating chemical chaperone 4-phenylbutyrate [130]. The precise mechanism of Ca^{2+} and JNK involvement in the process of autophagy has not been discovered therein, however it is known that Ca^{2+} release can stimulate autophagy via activation of AMPK and the death associated protein kinase 1 (DAPK)-mediated phosphorylation of Beclin 1, while JNK may act through the regulation of expression and function of Beclin 1 (Fig. 5) [23]. In most cases, autophagy was demonstrated to hinder the proapoptotic potential of TMZ facilitating survival of GBM cells [99]. Abrogation of this process using pharmacological inhibitors e.g., 3-methyladenine (3-MA), bafilomycin A1 (BAFA1), PX-866, and chloroquine or by knocking out key autophagy-related genes such as *Becn1*, *Atg4C*, *Atg5* or *Atg7* resulted in enhanced apoptotic cell death in TMZ-treated GBM cells [10,119,126,129,131]. As such, silencing of *Atg4C*

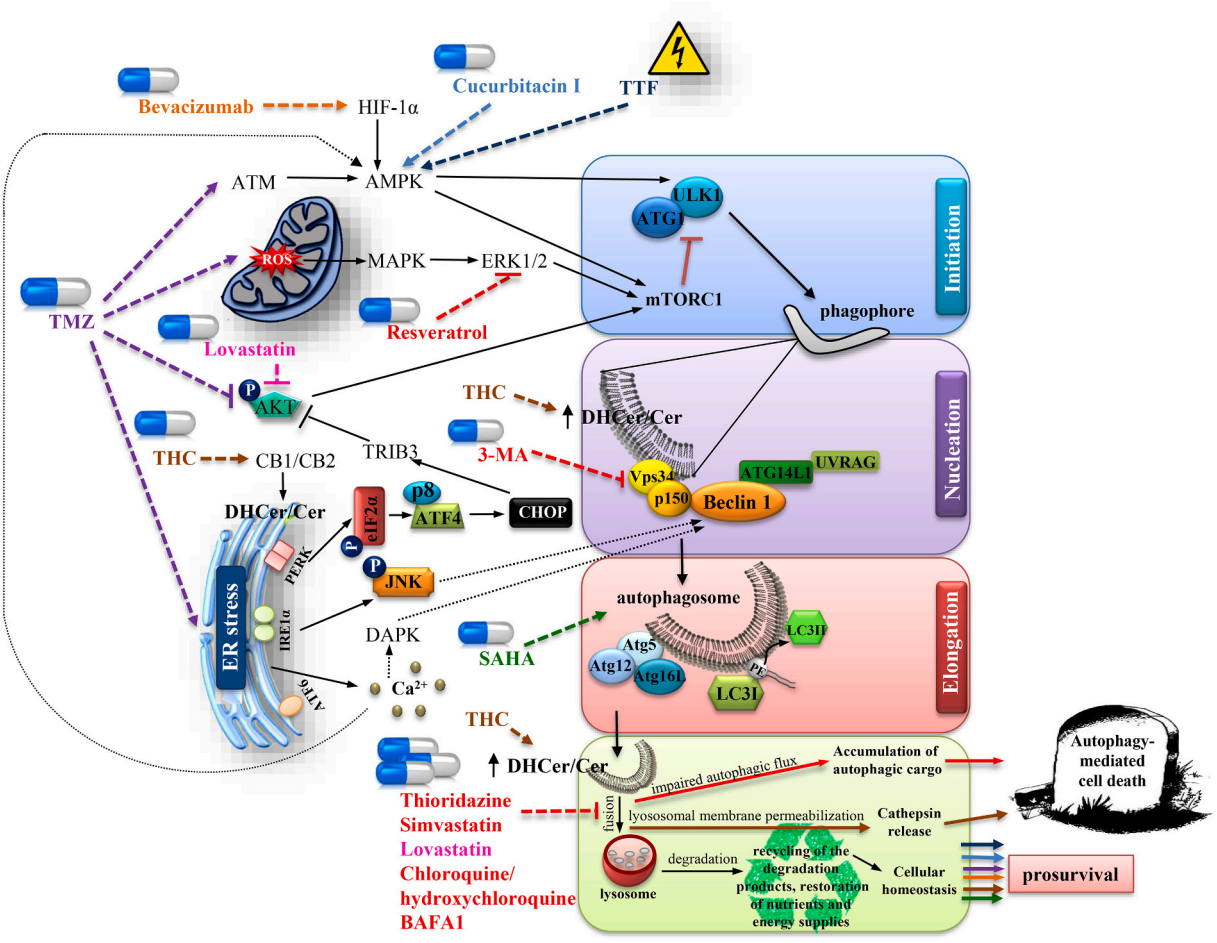

Fig. 5. Schematic representation of the principal signaling pathways connected with autophagic process, together with the modulatory effect of various drugs on autophagy-dependent cell survival and cell death. TMZ stimulates cytoprotective autophagy via several mechanisms including: ROS-mediated stimulation of MAPKs/ERK1/2 pathway; inhibition of the AKT/mTOR pathway; activation of the AMPK/ULK1 cascade; and ER stress-dependent activation of JNK and Ca^{2+} influx most probably followed by Ca^{2+}-mediated stimulation of AMPK and DAPK-mediated phosphorylation of Beclin 1. Resveratrol suppresses cytoprotective autophagy by suppressing ERK1/2-dependent signaling, and 3-MA by Vps34 inhibition. Thioridazine, simvastatin, chloroquine/hydroxychloroquine, and BAFA1 cause pro-death effect via impaired fusion between autophagosomes and lysosomes. Lovastatin activates cytoprotective autophagy via inhibition of the AKT/mTOR pathway, while having pro-death effect via inhibition of the late-stage autophagy. TTF causes pro-autophagic effect through activation of AMPK and subsequent up-regulation of ULK1. Cucurbitacin I stimulates cytoprotective autophagy via activation of AMPK. Likewise, bevacizumab activates HIF1α-mediated AMPK signaling. THC activates CB1/CB2 receptors, which stimulates the synthesis and accumulation of ceramides and dihydroceramides and a modification of the DHCer/Cer ratio, resulting in stimulation of the eIF2α-dependent axis of the UPR and induction of p8. This subsequently promotes the up-regulation of ATF4, CHOP, and TRIB3, followed by inhibition of the AKT-mTORC1 axis and the triggering of autophagy. At the same time the change in the DHcer/Cer ratio is transmitted to the autophagosomes and the autolysosomes where it induces lysosomal membrane permeabilization, cathepsin release and the apoptotic death of glioma cells. SAHA triggers prosurvival autophagy via enhanced autophagosome formation. Dashed colorful arrows indicate the autophagy-dependent targets of anti-GBM drugs; continuous colorful arrows indicate the effects of drug functioning. Color patterns of arrows match the drug-effect scheme. Dotted black lines indicate tentative mechanisms of action.

resulted in inhibition of autophagy, which was followed by improved sensitivity of T98 and U87 cells to TMZ and reduced glioma growth rate in *Atg4C* knockout mice [10]. Furthermore, it has been shown that resveratrol suppressed autophagy by suppressing ERK1/2-dependent signaling, thus sensitizing GBM cells to TMZ (Fig. 5) [125,126]. On the other hand, thioridazine inhibited late-stage autophagy by impairing fusion between autophagosomes and lysosomes, providing benefits to the TMZ treatment in vitro and in vivo (Fig. 5) [132]. Likewise, recent reports of the combination treatments of statins, simvastatin [133] or lovastatin [107] with TMZ have shown that both treatments synergistically sensitized GBM cells to TMZ and enhanced TMZ-mediated killing effect. Simvastatin inhibited the fusion of lysosome to the autophagosome and impaired the autophagic flux resulting in an accumulation of intracellular autophagic cargos and enhanced apoptotic death of GBM cells (Fig. 5) [133]. Interestingly, lovastatin showed the duality of action activating the process of autophagy via inhibition of the Akt/mTOR pathway on one hand and impairing the lysosome-autophagosome fusion via inhibition of two crucial mediators of this mechanism (lysosome-associated membrane proteins (LAMP1/2) and dynein) on the other (Fig. 5) [107]. Overall, lovastatin was demonstrated to increase apoptotic death of GBM cells due to suppression of the autophagic flux thereby showing potential as chemotherapeutic agonist increasing TMZ efficiency [107]. In contrast, several reports demonstrated that TMZ efficacy may be potentiated by the up-regulation of autophagy [59,134]. Josset et al. found that mTOR inhibitor everolimus increased cytotoxic effect of TMZ and radiation in U87 cells via augmented autophagic cell death [59], while Liu et al. showed that momelotinib inactivated JAK2/STAT3 signaling pathway resulting in enhanced autophagy and higher sensitivity of U251 cells to TMZ [134]. Moreover, a combined administration of TMZ and Δ^9-tetrahydrocannabinol (THC) strongly enhanced autophagy both in vitro and in tumor xenografts [135]. Of note, inhibition of autophagy with a non-selective Vps34 inhibitor 3-MA or by *Atg1* knockdown prevented TMZ/THC-induced cell death, supporting the notion that autophagy is a crucial process in the anti-tumoral action of this drug combination (Fig. 5) [135]. A similar effect was found when TMZ was combined with a mixture of a 1:1 ratio of THC and cannabidiol (CBD) [135–137].

In reference to these findings, autophagy was also found to hinder therapeutic effectiveness of tumor treating fields (TTF). Treatment with TTF resulted in enhanced autophagosome formation and increased LC3II expression not only in U87 and A172 cells, but also in Fisher rats inoculated intracranially with F98 glioma cells [83]. Pathway analysis revealed that enhanced autophagy was mediated by activation of AMPK, subsequent up-regulation of ULK1, and increased autophagic flux, which exerted the prosurvival effect under TTF treatment acting as a resistance mechanism (Fig. 5). Moreover, TTF-stimulated cells showed overexpression of GRP78 and depleted generation of intracellular ATP, suggesting that activation of autophagy might be ER stress-dependent. Importantly, inhibition of autophagy with chloroquine or by shRNA-mediated silencing of *Atg7* resulted in marked reduction in cell growth of TTF-treated cells [83].

Recently, autophagy has been found to have modulatory effect not only on the efficiency of TMZ or TTF, but also on the activity of novel potential therapeutics against GBM [138,139]. Lohitesh et al. demonstrated that U87 cells treated with histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) showed up-regulated autophagy as evidenced by enhanced autophagosome accumulation [138]. Moreover, co-stimulation with chloroquine a known inhibitor of the autophagosome-lysosome fusion, resulted in: hyperaccumulation of autophagic vacuoles, reduction in mitochondrial autophagy, accumulation of damaged mitochondria, enhanced generation of ROS, and finally increased cell death (Fig. 5) [138]. Of note, inhibition of autophagy at an early stage by 3-MA alleviated the cell death-inducing effect of the SAHA/chloroquine combo confirming that apoptosis induced by these agents was dependent on autophagosome accumulation [138]. Moreover, autophagy has been identified as a factor hindering responsiveness of gliomas to bevacizumab [140]. Hu et al. demonstrated that in glioma xenografts treatment with bevacizumab resulted in hypoxia-mediated autophagy occurring via HIF-1α/AMPK signaling (Fig. 5) [139]. This effect was accompanied by bevacizumab resistance and enhanced survival of tumor cells. However, inhibition of autophagy with chloroquine, 3-MA and BAFA1 (an autophagosome-lysosome fusion blocker) or by *Atg7* knockdown increased apoptosis of GBM cells in vitro and reduced tumor growth in vivo (Fig. 5) [139].

Lately, natural substances with anti-tumoral activity have been a focus of much research. Notably, autophagy was identified as crucial mechanism mediating cytotoxic effect of several phytochemicals including cannabinoids or cucurbitacin I [4,141,142]. Accordingly, cannabinoids such as THC and cannabidiol (CBD) have been investigated as potential anticancer agents for GBM. THC was found to trigger glioma cell death via stimulation of ER stress and autophagy. Thus, THC binding to CB1 and CB2 cannabinoid receptors in glioma cells stimulated the de novo synthesis of ceramides and the subsequent activation of the eIF2α-dependent axis of the UPR including the enhanced expression of (p8/NUPR1) and the transcription factors ATF4 and CHOP, which in turn led to the upregulation of Tribbles pseudokinase 3 (TRIB3) [143,144]. Hence, treatment with THC enhanced the inhibitory interaction of TRIB3 with AKT thereby leading to the inhibition of mTORC1 and the stimulation of autophagy (Fig. 5) [4]. Pharmacological suppression of autophagy or selective knockdown of *Atg1*, *Atg5* or *Ambra1* strongly reduced THC-induced autophagy and cell death, emphasizing the importance of this cellular process in the cytotoxic effect of cannabinoids [4]. Moreover, it was found that the activation of ERstress- and autophagy-dependent cell death mechanism of THC relies on a modification of the sphingolipid profile of the endoplasmic reticulum and of the autophagosomes. This change was associated with an increase in the levels of specific molecular species of di-hydroceramide (DHCer) which led to a change in the ceramide (Cer): DHCer ratio that is transmitted to the lysosomes and ultimately led to the permeabilization of the lysosomal membrane, cathepsin release, and subsequent activation of the apoptotic death of glioma cells (Fig. 5) [143]. Likewise, Huang et al. demonstrated that CBD induced a similar mechanism (mitochondrial dysfunction and lethal mitophagy arrest leading to autophagic cell death) [142]. This effect occurred via activation of transient receptor potential cation channel subfamily V member 4 (TRPV4) and downstream initiation of the above-mentioned ER stress-dependent ATF4/CHOP/TRIB3/AKT/mTOR signaling pathway (Fig. 5). The relevance of autophagy in CBD-induced cell death was confirmed by application of the early-stage autophagy inhibitors (LY294002, wortmannin) or by silencing of the *Atg*5, which reversed the pro-death effect of CBD. These results strongly suggested the occurrence of autophagy-mediated glioma cell death [142]. In oppose to these results, cucurbitacin I a plant-derived triterpene, was shown to induce cytoprotective autophagy in vitro as well as in animal model studies [141]. Exposure of GBM cells to cucurbitacin I resulted in up-regulation of Beclin 1 expression, conversion of LC3I to LC3II, and an overload of autophagosomes, occurring as downstream effects of the AMPK activation (Fig. 5) [141]. Interestingly, this effect was independent of the PI3K/AKT signaling. Silencing of *Becn1* or chemical inhibition of autophagy with 3-MA or chloroquine significantly increased cucurbitacin I-induced apoptosis of GBM cells, indicating that autophagy may promote cell survival and alleviate therapeutic efficiency of this phytochemical [141]. An overview of the influence of therapeutic compounds, alone or in combination with co-therapeutic agents, on stress responses together with main cellular effect triggered by the applied treatment is summarized in Table 1.

To date, therapeutic importance of autophagy modulation was tested in several clinical trials. The co-administration of radiotherapy and TMZ together with autophagy inhibitor – hydroxychloroquine in patients with newly diagnosed GBM was tested in phase I/II clinical study [145]. Administration of hydroxychloroquine was able to inhibit autophagy, however, dose-limiting toxicity prevented the escalation of dosage,

while tolerable concentration of 600 mg/day was not enough to improve overall survival of patients [145]. Similar treatment regimen but with chloroquine was applied in an open-label dose-finding phase I trial showing promising overall survival of patients [86]. These results indicate the alternate role of autophagy in mediating cell death-inducing effect of TMZ and support further clinical studies based on autophagy-modulating agents as potential adjuvant therapeutics for GBM. On the contrary, a few autophagy inducers have also been clinically tested as potential co-treatment drugs in therapeutic regimen of GBM. Thus, when combined with standard radio- and chemotherapy, mTOR inhibitors everolimus and temsirolimus, as well as AKT inhibitor perifosine, showed good safety profile, however, failed to significantly improve patients' outcome [87,91]. In contrast, a second line phase Ib clinical study carried out in recurrent GBM patients that were treated with nabiximols (a cannabinoid-based medicine containing equal amounts of THC and CBD) and TMZ showed promising results and significantly enhanced the survival of GBM patients [146]. This combination has been shown to strongly enhance autophagy in animal models of GBM, therefore reflecting a promising strategy to stimulate autophagy-mediated glioma cell death. Nonetheless the results of additional studies with a larger number of patients including those from currently ongoing clinical trials (NCT03529448; NCT05629702) will be necessary to cogently support this idea.

Altogether, pre-clinical and clinical data suggest that targeting autophagy may become a vital therapeutic strategy in GBM patients. However, only the combinational treatment of well-established chemotherapeutics with autophagy modulators in adjuvant settings seem to bring high hopes for the real improvement of patients' outcome. Thus, autophagy reprograming warrants further investigation before entering the standard therapeutic regimen in GBM.

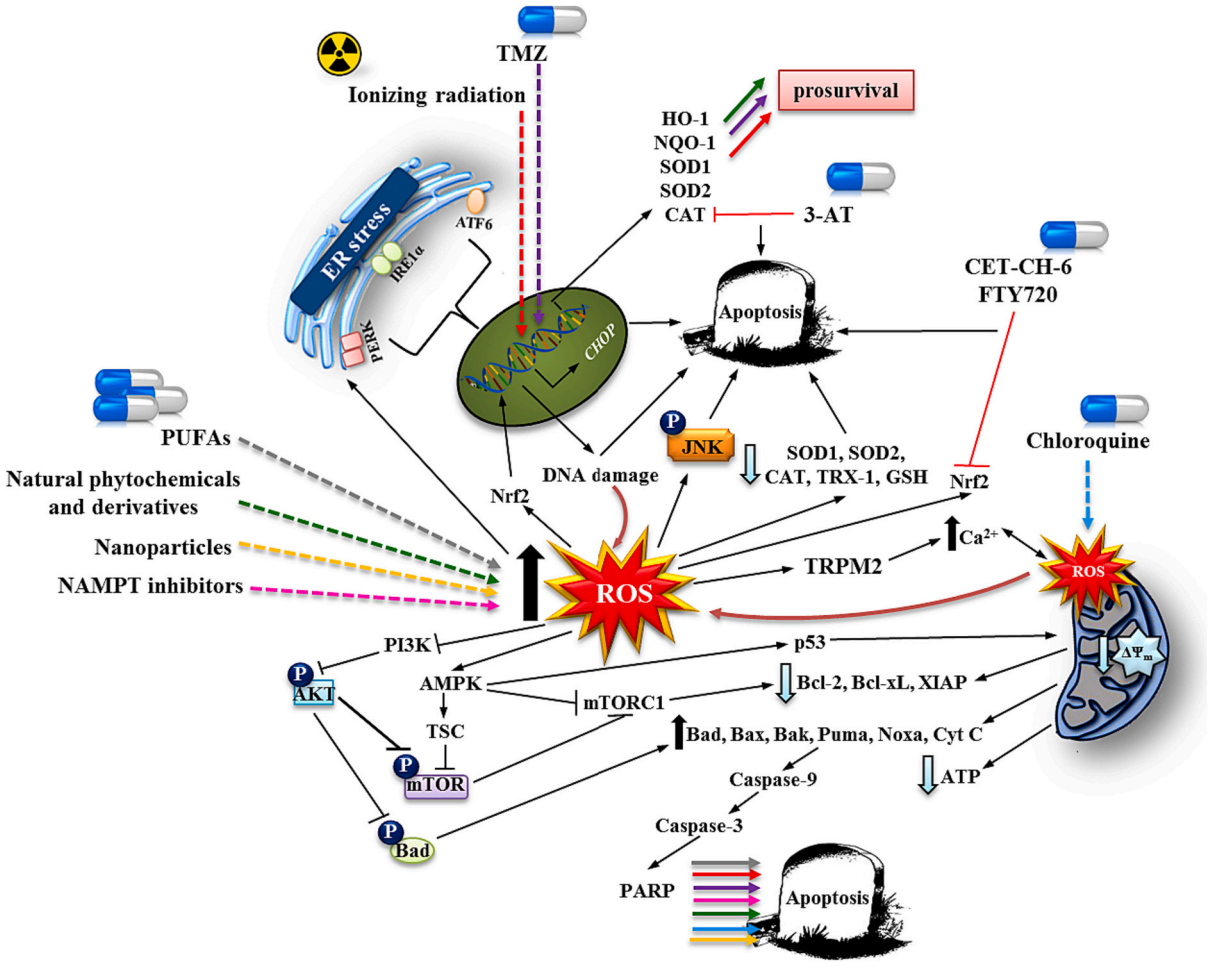

Fig. 6. Schematic representation of ROS-dependent pathways induced in GBM cells upon stimulation with various therapeutic agents. TMZ causes proapoptotic effect via DNA damage-associated overproduction of ROS, which leads to AMPK-dependent up-regulation of p53 and overexpression of proapoptotic Bax and Noxa, together with AMPK-dependent inhibition of mTORC1 complex resulting in down-regulation of antiapoptotic Bcl-2. In contrast, TMZ and ionizing radiation activate prosurvival response by causing ROS-mediated overexpression of Nrf2 followed by overexpression of HO-1, NQO-1 and CAT. A CAT inhibitor AT-3 and Nrf2 inhibitors CET-CH-6 and FTY720 counteract the prosurvival effect. The NAMPT inhibitors evoke proapoptotic effect via generation of ROS and reduced activity of SOD1/2 and activation of JNK signaling. Similarly, nanoparticles cause ROS accumulation, JNK phosphorylation and overexpression of proapoptotic proteins such as CHOP, Bax, Noxa, and Puma, while decreasing antiapoptotic ones. Natural phytochemicals cause proapoptotic effect by increasing ROS levels, which results in: activation of AMPK followed by TSC-dependent inhibition of mTOR and subsequent down-regulation of Bcl-2; inhibition of phosphorylation of AKT, mTOR, and Bad; and alleviation of the antioxidant response connected with the down-regulation of SOD1/2, TRX-1, CAT and GSH; while, synthetic derivatives of natural phytochemicals cause ROS-dependent activation of the ER stress followed by CHOP expression, with concomitant up-regulation of the antioxidant response hindering the proapoptotic effect. Chloroquine causes proapoptoic effect via generation of mitochondrial ROS and inhibition of mitochondrial autophagy. PUFAs cause ROS-dependent activation of TRPM2-induced Ca^{2+} influx and further cascade of proapototic events. Apoptotic processes are then accompanied by disruption of the mitochondrial membrane potential ($\Delta\Psi_m$), depletion of ATP levels, up-regulation of proapoptotic molecules, and promotion of caspase cascade. Dashed colorful arrows indicate ROS induction by various anti-GBM drugs; continuous colorful arrows indicate the final effect of drug activity. Color patterns of arrows match the drug-effect scheme.

3.3. Oxidative stress as modulator of drug functioning in glioblastoma

Recent molecular studies of novel as well as already well-established pharmacological treatments for GBM show the increasing role of oxidative stress in mediating therapeutic activity of radiotherapy and chemotherapeutic agents [64,76,108]. Consequently, TMZ in addition to the DNA alkylating properties, was also found to rely its cytotoxic effect on evoking oxidative stress [20,103]. Zhang et al. revealed that GBM cells treated with TMZ showed significant ROS overproduction associated with DNA damage, which resulted in strong up-regulation of AMPK (Fig. 6) [103]. Furthermore, TMZ caused overexpression of the proapoptotic proteins Bax and Noxa, via the AMPK-dependent up-regulation of p53 and reduced the expression of the anti-apoptotic protein Bcl-2 through the inhibition of the mTORC1 complex (Fig. 6) [103]. This effect was counteracted by NAC confirming the key role of oxidative stress in the mechanism of TMZ cytotoxicity [103]. On the other hand, co-treatment of TMZ with irradiation resulted in augmented activity of the cellular antioxidant systems including CAT and nuclear factor erythroid 2-related 2 (Nrf2), which aim at neutralizing the redox imbalance, therefore decreasing chemoradiosensitivity and promoting glioblastoma recurrence [95,147–149]. In fact, after TMZ and/or IR treatment the nuclear levels of Nrf2 were markedly increased in tissues of recurrent tumors in comparison to their primary counterparts [95,149,150]. Moreover, in experimental conditions TMZ- or IR-induced up-regulation of Nrf2 was followed by an overexpression of Nrf2-regulated antioxidant enzymes such as heme oxygenase-1 (HO-1) and NADPH:quinine oxidoreductase-1 (NQO-1), which contributed to alleviated cytotoxicity of TMZ and IR (Fig. 6) [149,150]. Likewise, Flor et al. have shown that the overexpression of CAT also promoted resistance to TMZ and irradiation [148]. Notably, chemical or siRNA-mediated inhibition of CAT or Nrf2 increased the TMZ- and IR-induced apoptosis of GBM cells [147–150]. As such, the application of an irreversible inhibitor of CAT – 3-amino-1,2,4-triazole (3-AT), or modulators of Nfr2 – chalcone derivative CET-CH-6 and fingolimod (FTY720) enhanced the cytotoxicity of irradiation and/or TMZ (Fig. 6) [147–149]. These findings suggest that inhibition of the antioxidant network might be a promising approach to potentiate the therapeutic effect and to overcome the resistance to TMZ and/or IR treatment [95,147,149,150]. The results of other in vitro studies showed that inhibitors of the nicotinamide phosphoribosyltransferase (NAMPT) such as FK866 and CHS-828 increased ROS production, reduced SOD activity and total antioxidative capacity, and activated c-Jun/JNK signaling pathway in TMZ-stimulated T98 and U251 cells (Fig. 6) [151]. Additionally, both agents enhanced TMZ-induced apoptosis by increasing the activity of caspase-3, caspase-9, and caspase-1, whereas scavenging of intracellular ROS with tocopherol resulted in a decreased sensitization effect of NAMPT inhibitors to TMZ, confirming the crucial role of oxidative stress in mediating the cytotoxic potential of the NAMPT inhibitors + TMZ combo (Fig. 6) [151]. Furthermore, chloroquine which is a well-known antimalarial drug repurposed for use as anticancer agent was also demonstrated to act through stimulation of oxidative stress [152]. Chloroquine, except for being a standard autophagy inhibitor, is also believed to be another TMZ-sensitizing agent in GBM [145,152,153]. It has been demonstrated that chloroquine increased the cytotoxicity of TMZ by augmenting mitochondrial levels of ROS and inhibiting mitochondrial autophagy. Accordingly, treatment with antioxidants caused marked inhibition of the chloroquine-potentiated death of cells exposed to TMZ, indicating that the apoptotic process in GBM cells is strongly dependent on the mitochondrial ROS (Fig. 6) [80,153].

Currently, high interest in adjunctive therapies stimulated a widespread quest for natural compounds with anticancer activity. As such, several phytochemicals, such as resveratrol, curcumin, steroidal saponin N45, polyphyllin VII and withaferin A have been tested in GBM cells in combination with TMZ [154–158]. In the human GBM cell line SHG44 resveratrol combined with TMZ demonstrated an additive effect in enhancing ROS generation and activating AMPK [154]. This activation of AMPK was then followed by tuberous sclerosis complex (TSC)-dependent inhibition of mTOR and subsequent down-regulation of Bcl-2 and increased apoptosis of GBM cells (Fig. 6) [154]. Importantly, NAC prevented the decrease in cell viability triggered by resveratrol/TMZ combination confirming the role of ROS in the cytostatic effect of the applied treatment [154]. On the other hand, curcumin enhanced the proapoptotic effect of TMZ through simultaneous generation of ROS and disruption of AKT/mTOR signaling in U87 cells and U87-grafted mice [155]. Curcumin and TMZ treatment markedly inhibited the phosphorylation of AKT, mTOR, and Bad. Remarkably, since AKT is known to promote cell survival through the phosphorylation of Bad (a proapoptotic member of Bcl-2 family of proteins), decreased level of p-AKT diminished phosphorylation of Bad, which resulted in stimulation of active unphosphorylated form of this protein and increased apoptosis (Fig. 6) [155]. In line with this, a steroidal saponin from *Paris vietnamensis* – N45 was found to significantly suppress the proliferation of glioblastoma U251 and U87 cells and TMZ-resistant glioblastoma U87R cells via ROS/PI3K/AKT signaling pathway, leading to induction of mitochondrial apoptosis upon TMZ treatment [156]. The N45 caused a ROS-dependent decrease in PI3K, and Bcl-2 expression as well as in AKT phosphorylation, followed by overexpression of cytochrome C (cyt C) and capase-3. Moreover, inactivation of the PI3K/AKT cascade down-regulated the expression of anti-apoptotic Bcl-2, thus shifting the Bcl-2 and Bax balance towards a higher and therefore proapoptotic Bax/Bcl-2 ratio (Fig. 6) [156]. Another steroidal saponin from *Paris polyphylla* – polyphyllin VII was found to evoke apoptosis and autophagy of U251 and U87 cells and to reduce the cells' resistance to TMZ [157]. Similarly, this effect was shown to occur via ROS-dependent inhibition of the AKT/mTORC1 pathway and increased Bax/Bcl-2 ratio (Fig. 6) [157]. Interestingly, cytotoxicity of polyphillin VII was partly rescued by 3-MA and BAFA1, however, both autophagy and cell viability-reducing effects were completely reversed by co-stimulation with NAC, suggesting a crucial role of ROS in the cytotoxicity of this saponin [157]. In line with this, the steroidal lactone withaferin A increased ROS levels and inhibited cell proliferation in several GBM cell lines mainly through decreased phosphorylation of the AKT/mTOR signaling pathway [158]. Additionally, withaferin A caused resensitization of GBM cells to TMZ via MGMT depletion [158].

Thus far, several in vitro studies investigated natural polyphenols also as single-agent treatments for GBM [64,96,97,100,106]. As such, Sharma et al. demonstrated that kaempferol (KMF) induced apoptosis in U87, T98, and LN229 cells through severe oxidative stress accompanied by overproduction of ROS and alleviation of antioxidant systems SOD1 and thioredoxin (TRX-1) [97]. Treatment with KMF resulted in decreased expression of Bcl-2, decreased $\Delta\Psi_m$ with concomitant elevation of membrane fluidity, as well as hyperactivity of caspase-3 and cleavage of PARP (Fig. 6) [97]. Of note, NAC pretreatment or further siRNA-mediated down-regulation of TRX-1 and SOD-1 resulted in enhanced ROS production and increased sensitivity of GBM cells to KMF-induced apoptosis [97]. Studies of resveratrol-treated GBM cells, showed that stimulation with this polyphenol caused enhanced generation of ROS and reduced expression of SOD2 and CAT, which resulted in activation of caspases-9 and -3 and apoptosis in U251 but not LN428 cells (Fig. 6) [96]. Likewise, a sesquiterpene lactone brevilin A induced oxidative stress as evidenced by ROS generation, GSH depletion, and increased phosphorylation of p38 MAPK and JNK (Fig. 6) [100]. Subsequently, brevilin A modulated the expression of Bcl-2 proteins towards increased Bak/Bcl-xL ratio, and decreased expression of Xiap. Moreover, it caused decrease in $\Delta\Psi_m$, induced cyt C release from mitochondria into the cytosol, and augmented the expression of the cleaved forms of caspase-9, −3 and PARP (Fig. 6) [100]. Of note, Massi et al. demonstrated that exposure to CBD also caused overproduction of ROS followed by cyt C release and activation of caspases-8, −9, and − 3 in U87 glioma cells (Fig. 6) [159]. Lately, it has also been demonstrated that newly designed derivatives of plant-origin small compounds such as kinetin riboside (KR) – the 8-azaKR, 7-deazaKR, and artemisin – the

dihydroartemisinin (DHA) - were able to interfere with the redox status of GBM cell lines causing ROS generation, ATP depletion, lipid peroxidation, DNA oxidation and deregulation of antioxidant systems [61,160]. Orlicka-Płocka et al. demonstrated that upon treatment with KR and 7-deazaKR T98 cells showed increased levels of intracellular and mitochondrial ROS, which was followed by lipid peroxidation and increased GSH levels [61]. Interestingly, mRNA analysis also demonstrated significant up-regulation of many genes of the antioxidant system, i.e. *NRF2*, *SOD1*, and *CAT*, suggesting that simultaneous overexpression of ROS scavengers might impair the cytotoxic efficiency of these agents (Fig. 6) [61]. Furthermore, Qu et al. revealed that DHA was able to induce ER stress, autophagy and apoptosis in U87 and U251 cells via increased expression of multiple proteins such as GRP78, CHOP, eIF2α, caspase-12, Beclin 1, LC3 II, caspase-9, caspase-3 as well as depolarization of mitochondrial membrane, and cyt C release [160]. However, all these effects were found to be preceded by increased ROS generation suggesting that cytotoxicity of DHA might be ROS-dependent. This observation was further confirmed by co-treatment with the cell-permeant SOD mimetic the manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), which reversed the cytotoxic, proautophagic, and proapoptotic effect of DHA [160]. In line with this, certain natural compounds such as berberine and luteolin seemed to evoke the proapoptotic response in GBM cells via the orchestrated response between oxidative and ER stress [64,106]. In this respect, ROS production and mitochondrial dysfunction were accompanied by ER stress, which finally led to an increased Bax/Bcl-2 ratio, the disruption of the $\Delta\Psi_m$, the activation of caspase-9 and -3, PARP cleavage and the overexpression of ER stress-associated proapoptotic protein CHOP (Fig. 6). Notably, the administration of NAC reversed the cytotoxic effects of both phytochemicals, confirming the principal role of oxidative stress in the proapoptotic effect of these polyphenols [64,106].

Interestingly, Hari et al. suggested that some PUFAs can be exploited for the therapy of GBM [161]. In general, the tumoricidal effect of various PUFAs is believed to occur mostly due to ROS generation, incorporation of PUFAs into membrane bilayers, and induction of changes in mitochondrial function. Indeed, studies revealed that arachidonic acid and eicosapentaenoic acid enhanced the accumulation of nitric oxide and lipid peroxides, which resulted in initiation of multiple molecular effects including increased Bax/Bcl-2 ratio, enhanced cyt C release and overexpression of caspase-9 and -3 (Fig. 6) [161]. In line with this, eicosapentaenoic acid was found to increase the anticancer activity of cisplatin via enhanced production of cytosolic and mitochondrial ROS, disruption of $\Delta\Psi_m$, overexpression of transient receptor potential melastatin 2 (TRPM2) channel and further TRPM2-induced Ca^{2+} influx and up-regulation of caspase-3, −8, and − 9 (Fig. 6) [162].

Additional evidence of the importance of oxidative stress in mediating cytostatic effect in GBM cells comes from nanoparticles (NPs)-based studies [94,101,104,105]. To date, treatment approaches based on nanomedicine have been increasingly tested in brain tumors, thus several research on various types of NPs, including disulfiram, silica, iron–platinum and graphene have shown that oxidative stress is generated upon treatment with these NPs [94,104,105]. Costa et al. demonstrated that graphene oxide enhanced oxidative stress and caused changes in lipids composition resulting in reduced viability of GBM cell lines but not of normal astrocytes [104]. Madala et al. reported that newly designed disulfiram nanoparticles evoked multiple effects including marked overproduction of ROS, loss of $\Delta\Psi_m$, increased JNK phosphorylation and decreased levels of p-AKT [105]. This effect was followed by an increased expression of proapoptotic proteins such as Bax, Noxa, CHOP and cleaved PARP, and decreased expression of anti-apoptotic Bcl-2, Xiap, and survivin in T98 and DAOY cells (Fig. 6) [105]. In line with this, Kusaczuk et al. demonstrated that silica NPs triggered apoptosis of LN229 cells via oxidative stress accompanied by enhanced ROS generation, $\Delta\Psi_m$ collapse, and deregulation of antioxidant defense systems, which led to the up-regulation of *BAX*, *PUMA*, and *NOXA* together with the down-regulation of *BCL-2* and *BCL-xL* and activation of caspase-9 and -3 (Fig. 6) [94]. An overview of the influence of therapeutic compounds, alone or in combination with co-therapeutic agents, on stress responses together with main cellular effect triggered by the applied treatment is summarized in Table 1.

Altogether, these findings show that oxidative stress can play a crucial role in modulating drug-mediated responses in GBM cells. Therefore, both prooxidant and antioxidant approaches might be attempted for glioma therapy especially when trying to alleviate oxidative stress in the tumor microenvironment as opposed to triggering excessive oxidative stress within the tumor cell. However, future studies are still required to precisely identify the most efficient co-therapeutic strategies for GBM and to develop pharmacological agents selectively targeting cytoprotective ROS-scavenging systems in the clinical practice.

3.4. Modulation of stress responses in glioblastoma stem-like cells

GBM is characterized by inter- and intratumoral heterogeneity, which has been suggested to contribute to treatment resistance. Numerous studies demonstrated the presence within the tumor mass of a subpopulation of self-renewing and pluripotent GBM stem-like cells (GSCs) responsible for GBM formation, maintenance, invasiveness, and recurrence [4,6,7,29,163]. Considering the outstanding role of GSCs in the development and chemoresistance of GBM it seems mandatory to comprehensively understand the biology of these cells. Given this, various studies have already explored the molecular mechanisms underlying self-renewal capacity and differentiation potential of GSCs. However, the impact of stress responses on their functioning and drug sensitivity remains elusive.

Existing data, although limited, indicate that stress-dependent signaling may play certain role in regulating the self-renewal capacity of GSCs. Hence, ER stress was shown to modulate stemness and tumor forming potential of these cells [29,163–165]. Chen et al. suggested that the cell surface fraction of GRP78 was responsible for maintaining stemness in human head and neck cancer cells [165]. Thus, GRP78-deficient cells or those with GRP78 bound to its interactome protein progranulin showed significantly down-regulated expression of five stemness-associated markers such as *NANOG*, *OCT4*, *SOX2*, *TERF-1* and *PRDM14* [165]. On the other hand, chemical induction of ER stress in GSCs resulted in reduced self-renewal potential [163,164]. As such, GSCs treated with tunicamycin (TM) – a known ER stress inducer, showed decreased self-renewal in vitro and reduced growth of tumor xenografts in vivo [164]. Interestingly, this tumor-suppressive potential was linked not only to the proapoptotic effect of TM, but also to the strong alleviation of SOX2 expression – an essential factor in maintaining cell stemness [164]. In reference to these results, TM and thapsigargin – another ER stress-inducing agent, also strongly reduced the neurosphere forming ability of GSCs via down-regulation of SOX2 [163]. Interestingly, further analyses demonstrated that SOX2 was down-regulated in a PERK-dependent although eIF2α/ATF4-independent manner, suggesting a noncanonical mechanism of PERK signaling [163]. Although preliminary, these data suggest that drugs evoking ER stress might have an additional advantage of suppressing the protumorigenic potential of GSCs by limiting their self-renewal capacity.

Moreover, several initial reports indicate that GSCs may display an impaired autophagy and oxidative stress-related networks [5,166–168]. Studies suggest that in comparison to differentiated cells, GSCs show diminished accumulation of ROS due to higher expression of ROS scavenging systems (e.g., prohibitin, GSS, GSTO1, hMTH1, SOD1, SOD2, APE1, and Nrf2), which results in maintenance of stemness and greater resistance to radio- and chemotherapy [5,166,167,169]. However, still little is known about the possible ROS-dependent control of self-renewal/differentiation mechanisms and the tumor-initiating capacity of GSCs. In this respect, Sato et al. demonstrated that in cells stimulated with hydrogen peroxide ROS played a fundamental role in controlling differentiation and tumor-initiating capacity of GSCs via induction of

p38MAPK [169]. As such, ROS caused a p38MAPK-mediated degradation of BMI1 – a stem cell marker, which was followed by decreased expression of other stemness-associated proteins such as SOX2, Musashi, and Nestin and further loss of self-renewal capacity. Furthermore, a p38MAPK-dependent activation of forkhead box protein O3 (FOXO3) resulted in differentiation of GSCs. Consequently, these results were confirmed in vivo evidencing that induction of oxidative stress may deprive GSCs of their tumor-initiating capacity via activation of ROS–p38MAPK cascade. Thus, although still little is known about the role of oxidative stress in regulating self-renewal and maintaining the stemness of GSCs, it is conceivable that interventions modulating of the intracellular ROS levels could bring potential therapeutic benefits derived of acting on this cell population. Interestingly, currently available reports suggest that modulation of autophagy influences the biology of GSCs as well. The shRNA-mediated targeting of ATG5 or chloroquine-mediated inhibition of this process resulted in an increased expression of stemness markers i.e. NANOG, SOX2, CD133 and BMI1, which was however counteracted by a decreased proliferation and clonogenicity of GSCs [168]. These findings show an ambiguous but potentially important role of stress responses in the modulation of GSCs biology, which encourages further investigation of these mechanisms in context of both regulation of self-renewal capacity *per se* and modulation of drug efficiency in therapy of GBM.

Considering the importance of GSCs in the treatment resistance and tumor recurrence, several reports have investigated the influence of standard therapeutics and novel drug candidates on GSCs biology. Recently, activation of stress responses has been reported to play a significant role in modulation of drug sensitivity in GSCs [74,170–173]. Shah et al. reported that IR-stimulated GSCs showed enhanced ER stress and increased autophagosome formation, the two events that correlated with increased cell survival [170]. Noteworthy, this cytoprotective effect switched to the proapoptotic response after potentiating ER stress by co-stimulation with 2-deoxy-D-glucose [170]. As such, Yang et al. analyzed the mechanism of GSCs resistance to IR-induced immune response [171]. They demonstrated that upon high radiation doses, GSCs showed reduced exposure and release of molecules of damage-associated molecular patterns (DAMPs) in comparison to their differentiated counterparts. Interestingly, siRNA-mediated silencing of GRP78 promoted IR-mediated ER stress and apoptosis, as well as elicited cell surface exposure and extracellular release of DAMPs molecules. Moreover, GRP78 knockdown increased activation of T lymphocytes and stimulated maturation of dendritic cells in IR-treated GSCs. In general, radiation accompanied by down-regulation of GRP78 alleviated post-radiotherapy tumor recurrence and efficiently prevented tumor generation [171]. In accordance with these results, Huynh et al. demonstrated that GRP78 silencing using siRNA technology or its inhibition via application of the resveratrol analog – pterostilbene (PSB) reduced the self-renewal ability and increased IR sensitivity of GSCs both in vitro and in vivo [174]. Interestingly, PSB was shown to decrease GRP78 expression via miR-205 pointing to the existence of a novel regulatory mechanism of expression for this chaperone [174]. Overall, these findings suggest that the overload of cytoprotective capacity of the UPR by chemical augmentation of ER stress or by inhibition of GRP78 might be a promising therapeutic approach in adjuvant GBM treatment to overcome GSCs radio- and chemoresistance.

To support the hypothesis of ER stress relevance in GSCs behavior upon pharmacological treatment, Yoo et al. demonstrated an intriguing role of the ER stress-associated proteins in the process of cell death mediated by proteasome inhibitors (PIs) [172]. PIs showed high selectivity in killing GSCs in comparison to their differentiated counterparts. The PIs such as MG132, epoxomicin, marizomib, or bortezomib induced phosphorylation of JNK and transcriptional activation of ER stress-associated mediators of apoptosis such as ATF3, CHOP, DDIT4, GADD34, Noxa and TRIB3. Of note, ATF3 was found to play pivotal role in GSCs selectivity to apoptosis as it showed 31-fold higher expression in GSCs compared to the non-stem cells [172]. Surprisingly, the proapoptotic effect of PIs in GSCs seemed UPR-independent, since the cells showed an increased expression of ATF3 and CHOP but not the upstream regulators of the ER stress such as GRP78, XBP1, and ATF4. Accordingly, the application of inhibitors of the UPR-mediated apoptosis such as salubrinal, STK047915 and TK064652 in MG132-treated GSCs resulted in potentiated killing effect. Moreover, a co-treatment of MG132 and STK047915 synergistically inhibited the capacity of GSCs to form tumors in vivo [172]. These results suggest that PIs and the UPR blockers might be a promising therapeutic approach in combinational treatment to increase GSCs apoptosis and reduce tumor recurrence in gliomas.

An interesting notion concerning the impact of autophagy on GSCs features comes from the study of Zhuang et al. [74]. They suggested that defective autophagy in GICs contributed to the blocked differentiation and radioresistance in GBM. Indeed, although they demonstrated that the basal level of autophagy in untreated GSCs was rather low, they confirmed the link between autophagy and radioresistance of GSCs both in vitro and in vivo. It has been shown that rapamycin – a well-known mTOR inhibitor stimulated differentiation and radiosensitivity of GSCs via increased autophagy. Rapamycin-induced augmentation of autophagy resulted in decreased tumorigenicity and enhanced killing effect of IR in GSCs, whereas inhibition of autophagy with 3-MA restored the clonogenic potential of these cells, decreased apoptosis and caused a more efficient repair of IR-induced DNA damage. Of note, in vivo combination of rapamycin and IR effectively suppressed tumor growth and associated mortality of mice subjected to intracerebral grafting of human GICs [74]. Likewise, Wang et al. demonstrated that resveratrol decreased proliferation and increased IR-sensitivity of radioresistant GSCs cell line SU-2 [175]. Resveratrol substantially attenuated the repair of IR-induced DNA damage, and the combined treatment resulted in increased autophagy and apoptosis of SU-2 cells. Moreover, resveratrol+IR decreased the ability of SU-2 to generate neurospheres and down-regulated the expression of neural stem cell marker CD133, suggesting loss of self-renewal capacity and activation of differentiation. Notably, these results were accompanied by enhanced autophagolysosome formation and marked overexpression of LC3II and Beclin 1, which inclined authors to suggest that radiosensitizing effect of resveratrol might occur through increased autophagy [175]. Nevertheless, this hypothesis requires further confirmation, since neither genetic nor chemical inhibition of autophagy was applied to support their notion. On the contrary, Wu et al. demonstrated that activation of autophagy in GSCs was associated with promotion of tumor development via formation of vasculogenic mimicry (VM) – a VEGF-independent microvascular circulation providing blood supplies and nutrients to the microenvironment of tumor [173]. Thus, it has been shown that bevacizumab-induced autophagy in GSCs correlated with the development of VM and resistance to the antiangiogenic therapy in NOD-SCID mice [173]. Consequently, administration of chloroquine blocked autophagy and significantly prolonged the survival of tumor-bearing animals. Furthermore, chloroquine application and *Atg5* knockdown in bevacizumab-treated GSCs prevented phosphorylation of kinase insert domain receptor (KDR)/VEGFR-2 and subsequent development of VM. Moreover, analysis of patient-derived specimens showed that high expression of p-KDR and ATG5 in GBM was correlated with the formation of VM and shorter progression-free survival and overall survival [173]. In this context, it might be worth mentioning that the neurotrophic factor Midkine (MDK) signaling through one of its receptors, the anaplastic lymphoma kinase (ALK) was found to maintain the self-renewal and tumorigenic capacity of GSCs by preventing the autophagic degradation of the transcription factor SOX9. Accordingly, pharmacological or genetic inhibition of the MDK/ALK axis prevented GSCs self-renewal activity in vitro and reduced the tumorigenic capacity of GSCs in vivo [176]. Moreover, the combined administration of the pharmacological inhibitors of ALK crizotinib and lorlatinib enhanced the anticancer activity of TMZ [176]. Of importance, these results led to the development of phase I clinical study in recently diagnosed GBM patients. The results

of this study suggest that the combination of radiotherapy, TMZ and crizotinib enhances the survival of GBM patients [177]. Although still preliminary these observations support the notion that enhancing selective autophagy may be a promising therapeutic strategy to target the GSCs` population and design more effective therapies against GBM. Altogether, the contradictory results of autophagy implications in GSCs-mediated chemoresistance of gliomas warrant further intensive investigations. Nevertheless, this also strongly suggests that autophagy ought to be considered as potential target in surpassing GSCs-dependent tumor recurrence and increasing therapeutic benefit of conventional therapies against GBM.

4. Possible clinical implications

Despite high recognition of stress-dependent signaling as modulator of drug efficiency in preclinical studies, stress-modulatory agents have not been sufficiently explored in a clinical setting. An effective application of such compounds might be hindered by the dual role of stress-mediated pathways in regulating cell fate, which may complicate the therapeutic use of stress-targeting drugs. Given this, controlled modulation of stress responses might become one of the emerging strategies in anticancer therapies.

One possible approach to utilize stress-dependent signaling in favor of oncopharmacology would be to determine the degree of ER stress activation characteristic of each cancer subtype/tumor. As such, tumors exhibiting high basal ER stress might be more prone to the use of drugs that selectively impair the adaptive branches of the UPR. Therefore, although still speculative, specific inhibition of ATF6 or IRE1 signaling could constitute an appealing therapeutic alternative in reinforcing anti-GBM potential of TMZ and irradiation as proven in several in vitro studies [98,108,178]. In line with this, a preliminary screening of publicly available transcriptome dataset indicated that a subset (up to 20%) of GBM tumors displayed high IRE1 activity signature, which was correlated with shorter survival of patients. Hence, categorization of tumors could be helpful in the optimization of the UPR-targeted strategies. For instance, generation of transcript signatures associated with high or low IRE1 activity might be used to predict sensitivity to IRE1 RNase inhibitors and select patients eligible for such therapy [179]. Nonetheless, to date, only a few compounds targeting stress responses have been introduced into clinical trials and even less with the aim of being tested specifically in GBM. Thus, the MKC-8866 (ORIN1001, an inhibitor of IRE1 RNase activity) has entered a recent early stage testing as a single agent or as a co-therapeutic drug with paclitaxel in patients with advanced solid tumors (NCT03950570) [29]. Despite promising anticancer effects of MKC-8866 in preclinical studies, no clinical data concerning the efficiency of this inhibitor in patients is yet available. Furthermore, although certain compounds known to potentiate ER stress such as salinomycin, tunicamycin or thapsigargin have been shown to exert anti-GBM actions as adjuvant therapeutics in a preclinical settings [79,158,159], the aggravators of ER stress have not been well explored in the clinics due to severe toxicity and adverse effects in patients [180–182]. This reflects the pitfalls of translating preclinical results into the clinical practice and warrants further research aimed at exploring the precise molecular events that determine the degree of activation of the UPR. This knowledge would facilitate the design/identification of substances capable of consistently inhibit its pro-survival activity, while enhancing the proapoptotic component. In this context some of the natural compounds or derivatives of lipid nature that have been shown to stimulate the cell death promoting brunch of ER stress could constitute a straighter forward strategy [4,183].

As opposed to ER stress, oxidative stress is better recognized as factor contributing to the cytostatic effect of many chemotherapeutic drugs currently used as standard of care treatment for various cancers. One such example of drugs that directly induce ROS, which further contributes to their anticancer activity is cisplatin, doxorubicin and arsenic trioxide [184,185]. Lamentably, due to their limited brain bioavailability these compounds cannot be successfully implemented into anti-GBM regimens in clinics. Nevertheless, a set of other potential ROS-inducing agents have been studied in clinical trials. As such, 2-methoxyoestradiol (2-Me, Panzem), an estradiol metabolite, and its nanoformulation, alone or in combination with TMZ have been investigated in phase I and II clinical trials for GBM (NCT00481455, NCT00306618) [186]. Although the main activity of 2-ME is attributed to its antiangiogenic potential, it was also found to act as ROS inducer via the mechanism most likely connected with nuclear localization of nitric oxide synthase, overproduction of nitric oxide and subsequent nitric oxide-dependent DNA damage [184]. Moreover, motexafin gadolinium, an electron acceptor that increases superoxide production, was investigated in phase I/II trials with TMZ and 60-Gy fractionated radiation for newly diagnosed supratentorial GBM [187]. Unfortunately, despite good tolerability of this treatment, median overall survival of patients was not improved. An interesting study of Tan et al. also showed that biosynthesis of ROS was significantly elevated in recurrent GBM and quantitative risk assessment system based on ROS might be used as predictor of GBM recurrence [188]. This suggests that identification of ROS-specific profile of recurrent GBM may provide early warning of tumor recurrence and thus assure guidance for therapy selection.

More data coming from the clinical testing in GBM patients was obtained for autophagy modulating agents. To date, the only FDA-approved modulators of autophagy are chloroquine and hydroxychloroquine [189]. As mentioned previously (section 3b), a phase I/II clinical trials of hydroxychloroquine with radiotherapy and TMZ showed efficient inhibition of autophagy but failed to improve the overall survival of patients [141]. On the other hand, a corresponding treatment but with chloroquine resulted in ameliorated survival of GBM patients [86]. As opposed to autophagy inhibitors, certain inducers of this process were also tested in clinics in GBM patients [87,91,142]. As such, everolimus, temsirolimus, and perifosine combined with standard radio- and chemotherapy showed favorable safety profile but did not manage to improve patients' outcome [87,91]. More optimistically, when nabiximols was combined with TMZ survival of patients with recurrent glioblastoma was significantly improved [142].

Altogether, despite the ability of many compounds to modulate stress responses and their promising efficacy in preclinical investigations, clinical trials for GBM continue showing unsatisfactory clinical benefits and insignificant prolongation of patients' survival. These failures are mostly attributed to sparse anti-glioma activity or severe side effects hindering further testing in patients. Thus, the last frontier to overcome in exploration of the therapeutic potential of stress-modulatory agents against GBM is the development of safe and target-specific drugs easily penetrating through the blood-brain barrier.

5. Conclusions and future perspectives

Stress-activated responses are an integral part of normal cellular physiology. Thus, in response to challenges that can put cells in danger these adaptive responses coordinate a cellular program that facilitates survival. Likewise, when the consequences of these challenges cannot be overcome by these stress responses, they also participate in the activation of programmed cell death. In this review we focused on three stress responses (ER stress/unfolded protein response, oxidative stress and autophagy) that are frequently deregulated in cancer cells. The activation of these stress responses in tumor cells has been intensively studied, however their implication in the therapeutic activity of anticancer treatments is still incompletely understood. In this manuscript we discussed how stress-dependent signaling may be activated primarily or secondarily upon exposure to different therapeutic agents that are frequently used in glioblastomas. Thus, the activation of some (or all) of these stress responses upon exposure to anticancer agents can determine the efficacy of the treatment by enhancing the cytotoxic activity of certain compounds or by promoting resistance to their action. Alas, despite the undeniable importance of these stress mechanisms, a

comprehensive evaluation of their impact on the efficiency of pharmacological treatments is very difficult considering that these pathways interplay, cross and merge with each other.

The future of drugs primarily targeting stress responses is therefore uncertain, however more and more efforts should be shifted towards development of such agents. Although there is still a lot of pitfalls to deal with while designing drugs efficiently and consistently targeting specific stress-related targets, newest advanced technology in oncopharmacology may provide significant progress on this field. Nonetheless, it remains to be established how to classify tumors according to their basal levels of cellular stress, and thus, how to identify patients susceptible to stress-targeted treatment. Moreover, to facilitate the design of more efficient therapies it is crucial to precisely understand stress responses at the molecular level, which should stimulate further extensive research on this topic. Finally, current treatments in oncology (including those used for the management of GBM) are based on combinational treatments. In this respect, conscious selection of drugs deliberately promoting the pro-death branch of stress responses together with those suppressing the prosurvival pathways may hold a promise in reducing tumor resistance, delaying tumor progression, and in consequence, prolonging life expectancy of GBM-patients.

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CRediT authorship contribution statement

Magdalena Kusaczuk: Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Elena Tovar Ambel:** Writing – review & editing, Visualization. **Monika Naumowicz:** Writing – review & editing, Visualization. **Guillermo Velasco:** Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Magdalena Kusaczuk reports financial support and article publishing charges were provided by Medical University of Bialystok. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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