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NOVEL APPROACHES TO REDUCE TEMOZOLOMIDE RESISTANCE IN GLIOBLASTOMA MULTIFORME: A REVIEW OF THE LITERATURE

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Abstract: *Temozolomide (TMZ), an oral alkylating agent, is currently used as a part of standard treatment for glioblastoma multiforme (GBM). GBM is considered one of the most lethal forms of human cancers, and despite recent improvement in cancer therapy, it remains an incurable disease, with a rare long-term survival of the patients due to the rapid emergence of cell clones resistant to treatment. Like other chemotherapeutic agents, resistance to TMZ is the major therapeutic obstacle to an effective therapy; thereby the development of new therapeutic strategies is required to overcome this problem. In the present review, an overview of the recent works on the reduction of TMZ resistance is presented.*

KEYWORDS: *Temozolomide, Glioblastoma multiforme, Drug resistance, Alkylating agents, O6 alkylguanine DNA alkyltransferase.*

BACKGROUND

Temozolomide (TMZ, $C_6H_6N_6O_2$) is an imidazotetrazine (a class of bicyclic aromatic heterocycles (derivative of dacarbazine[1]. It was first synthesized by Stevens et al[2] in the early 1980s. In the 1990s they identified a series of imidazotetrazine derivatives with different chemical, structural, and biological properties. Among these compounds, mitozolomide showed a strong anti-tumor activity against a broad spectrum of murine and human xenografts tumors[3], but in phase I clinical trials it showed a cruel and unpredictable life-threatening toxicity, including the human bone marrow suppression and deep platelet damage (thrombocytopenia) due to cross linking of DNA strands[4]. In 1987, Stevens described the activity of some analogues of mitozolomide, and reported the chemical and anti-tumor properties of a series of 3-substituted derivatives in which chloroethyl group of mitozolomide has been replaced by alkyl group, and in particular the methyl analogue of mitozolomide (TMZ) in mice[5]. Subsequent studies[5-7] demonstrated the anti-tumor activity of TMZ against a variety of cancers, including glioma, metastatic melanoma, and other cancers. Preclinical studies indicated that TMZ passed blood brain barrier and penetrated into the central nervous system. It had relatively low toxicity compared with mitozolomide, and good anti-tumor activity against a wide range of cancers[5-10]. Phase 1 and 2 clinical studies showed that after oral administration, TMZ was rapidly absorbed into the blood, had excellent penetration of the blood-brain barrier, and its bioavailability was approximately 100%. It showed an anti-cancer property against recurrent GBM, melanoma, and mycosis fungoides[11-14]. Finally, on March 15, 2005, the U.S. Food and Drug Administration (FDA) approved TMZ for the treatment of adult patients with newly diagnosed glioblastoma multiforme (GBM) showing progression after treatment with a nitrosurea drug (BCNU or CCNU) and procarbazine[15]. Recently the effect of temsirolimus (a m-TOR inhibitor that also showed anti-tumor effects in a wide range of different tumor in preclinical models) on GBM has been evaluated in trials in combination with other treatments. It was used in combination with standard radiation therapy (RT) with

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and without TMZ. No superiority of temsirolimus to TMZ in combination with standard RT was observed. Data suggested that TMZ could be safely substituted by temsirolimus in combination with standard RT in TMZ-resistant GBM patients. Infectious related toxicities are one of the side effects associated with standard RT/TMZ. It is also associated with temsirolimus as well. Therefore, Combination therapy with temsirolimus/TMZ/RT showed significant suppression of cellular, humoral, and innate immunity[16].

MECHANISM OF TMZ ACTION

As previously mentioned, TMZ is an oral alkylating agent administered as a chemotherapeutic drug for management of GBM and melanoma tumors growth. Alkylating agents are among the oldest kind of drugs used for cancer treatment[17]. TMZ is considered as a prodrug without the need for metabolic activation. Due to its lipophilic nature and small molecular weight, it can efficiently pass blood-brain-barrier and penetrate into the brain tissue. The property that makes TMZ suitable for oral administration is its stability at acidic pH values, so it does not change in the stomach. In systemic circulation and under physiological pH, TMZ rapidly hydrolyzes to its short-lived active form, 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) and CO_2[18]. This spontaneous reaction is started with the cleavage of the tetrazolium ring with the effect of water molecules[15].

pH in brain tumors is slightly more basic than normal tissue[19-21], and this condition is in favour of selective activation of prodrug in tumor environment. MTIC then hydrolyzes to 5-amino-imidazole-4-carboxamide (AIC) (which is excreted from kidneys) and methyldiazonium cation, which transfers alkyl group to DNA[22,23]. The methyldiazonium cation can also interact with cellular RNAs and proteins[24]. However, there is no evidence that RNAs alkylation and/or proteins alkylation or carbamylation has a significant role in TMZ cytotoxicity.

Methyl groups are added at N^7 and O^6 positions of guanine and the O^3 position of adenine in DNA[25]. The roles of N^7 methyl guanine and O^3 methyl adenine in TMZ cytotoxicity are controversial[26-28] and will be discussed later. O^6 methyl guanine plays the most important role in TMZ activity. TMZ cytotoxicity is due to the lack of ability of mismatch repair system to find a complementary base for methylated guanine. During subsequent DNA replication, O^6 methyl guanine is paired with a thymine instead of a cytosine and this mismatch pair bases activates DNA repair signaling pathway. Thymine in daughter strand is identified by DNA mismatch repair system and excised. However, the O^6 methyl guanine remains, and the lack of a suitable base for pairing with it cause the insertion of thymine in daughter strand again. This futile cycle of repair continues and finally results in activation of Checkpoint kinase 1, generation of double-strand breaks (DSBs), and incuction of apoptosis cell death[29].

MECHANISM OF TMZ RESISTANCE

Resistance to chemotherapy is one of the major challenges to the success of cancer treatment. Drug resistance is caused by various mechanisms including inactivation of the drug, inhibiting cell death (apoptosis suppression), changes in drug metabolism, epigenetic and drug targets, enhance DNA repair, and gene amplification. Chemotherapy resistance can be inherent in subpopulation of cancer cells or adopted over time and after exposing to the chemotherapeutic agents. Tumors often consist of a heterogeneous population of cells and respond differently to the chemotherapy. These cells usually show different sensitivity to chemotherapeutic drugs. As treatment continues, sensitive cells die, and resistance cells remain. These resistance cells will grow and in next step due to the resistance of all cells, chemotherapy will fail completely[30].

The most important factor associated to TMZ resistance in GBM is the expression of O6 methylguanine DNA methyltransferase (MGMT) gene in brain tumor cells[31], inefficient mismatch repair system[32], and activity of base excision repair (BER) system[33].

O^6 alkylguanine DNA alkyltransferase (AGT) (encoded by MGMT) eliminates the cytotoxic effect of TMZ by direct removal of methyl group from guanine base. The expression level of AGT varies in different tissues, for example the liver expressing the highest and haematopoietic tissues and brain the lowest levels[34,35]. In tumor cells also the expression level of AGT varies from high protein expression in breast, ovarian, and lung tumors to low protein expression in gliomas, pancreatic carcinomas, and malignant melanomas[36]. Expression of this protein in brain tumors predicts poor response to chemotherapy. *In vitro* studies showed that cell lines with high expression level of AGT were more resistance to TMZ than cell lines in which the expression level of this enzyme is low[37,38]. So, a good response to TMZ needs a low level of AGT.

Other reason contributing to TMZ resistance is the inefficiency of mismatch repair system in tumor cells. Studies showed that cells with deficient mismatch repair system were up to 100-fold less sensitive to alkylating agent's effect compared with normal cells[39,40]. Mutations in proteins that are responsible for identifying or repairing errors in mismatch repair system lead to lack of recognition O^6 methyl guanine mispairs, toleration of O^6 methyl guanine lesions, continuing of cell cycle and surviving[40].

The activity of BER system is the third reason for TMZ resistance. N^7 methyl guanine and N^3 methyl adenine are two main products of DNA methylation and overall make up 80% of TMZ activity products. After production, these methylated bases are identified and repaired by BER system rapidly. So TMZ can be cytotoxic when BER system is disrupted[41]. Studies about inhibition of BER system proteins and cell line with inefficient BER system, showed the role of this repair system in TMZ resistance discussed in this paper.

ISOCITRATE DEHYDROGENASE (IDH) MUTATIONS AND RESPONSE TO TMZ

Isocitrate dehydrogenase 1 and 2 (IDH 1 and 2) are key enzymes in cellular metabolism, epigenetic regulation, redox states, and DNA repair. These enzymes convert Isocitrate and $NADP^+$ into aKetoglutarate (aKG), CO_2, and NADPH. IDH1 and IDH2 genes are located on chromosome 2q33 and 15q26.1, respectively. IDH1 and IDH2 mutations have been identified in multiple tumor types, including gliomas. IDH mutations are found in >80% of low-grade gliomas and secondary GBMs, but <10% of primary GBMs[42-44]. Research showed that IDH1/2 mutations were associated with a relatively prolonged patient survival for glioma[45] and GBM[46]. Also, IDH1/2 mutations predicted for improved tumor responses to chemotherapy and/or RT in clinical trials[47,48] and retrospective analyses[49-52]. Furthermore, cancer cells are sensitized to RT and chemotherapy by the introduction of mutant IDH1/2 or silencing of wild-type IDH1/2. So, IDH1/2 mutations or the absence of IDH1/2 wild-type enzymes improve the treatment process[44]. A study about the impact of IDH 1/2 mutation on the prognosis and chemosensitivity of low-grade gliomas showed that it was associated with prolonged overall survival and a higher rate of response to TMZ. Response to TMZ was estimated by progression-free survival, as well as measuring the tumor size on successive MRI scans, then correlated with molecular alterations. Data showed that patients with both IDH 1/2 mutations had the best response rate to TMZ[50]. Therefore, it can be a significant marker of positive chemosensitivity in secondary GBM. Also, *in vivo* and *in vitro* studies showed that overexpression of IDH1 gene resulted in chemotherapy resistance to a high dose of TMZ. The IDH1 mutation caused cell cycle arrest in G1 stage and a reduction of proliferation and invasion ability, while raising sensitivity to chemotherapy[53]. The reaction of IDH1/2 leads to NADPH production and this product plays an important role in cellular protection from oxidative stress. This protective effect of NADPH may undo the oxidative stress induced by TMZ in cells. Therefore, IDH mutation sensitizes the cells to therapy.

OVERCOMING TMZ RESISTANCE

Down Regulation of MGMT

Nowadays, gene therapy is a promising approach to treat various diseases, including cancer. It is defined as a technology that aims to modify the genetic complement of cells to obtain therapeutic benefit[54]. As mentioned above, MGMT expression in brain tumor cells is the most important reason for chemotherapy resistance. So, a decrease in MGMT expression level can be a good strategy for enhancing the efficiency of TMZ in tumor cell death.

Interferons (IFNs) are signaling proteins produced by host cells in the presence of some pathogens. They activate immune cells and have important roles in the immune system[55]. They have been classified into three types based on their receptors: Interferon I binds to a specific cell surface receptor complex known as the IFN-α/β receptor[56]. The type I interferon present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω[57]. Human IFN-β enhances the cytotoxicity of TMZ[58,59], by down regulation of the MGMT expression. IFN-β sensitizes resistant glioma cells to TMZ and anti-tumor effect of TMZ is potentiated by a combination of IFN-β and TMZ[60,61]. Down regulation of MGMT by IFN-β, could be a good choice to overcome TMZ resistance. Detailed role of combined IFN-β and TMZ to increase tumor sensitivity to chemotherapy and the toxicity profile of combined IFN-β and TMZ is not clear.

p53 is a DNA-binding transcription factor that regulates the expression of genes involved in cell-cycle checkpoint and apoptosis in response to DNA damage[62]. Some studies[63-65] indicated the relationship between p53 and MGMT expression. Transient transfection of wild-type p53 protein into the IMR human fibroblasts cell line suppressed MGMT transcription and expression[66]. They proposed the improvement of chloroethylnitrosourea cancer treatment protocols by combining them with either a p53 inducing modality, such as ionizing radiation, or p53 adenoviral transduction. Also, expression of p53 in a tetracycline-regulated system, in a p53-null MGMT-proficient human lung tumor cells down-regulated the transcription of the MGMT gene and altered their sensitivity to alkylating agents[67]. Inhibition of MGMT expression by p53 is mediated by separation of the Sp1 transcription factor from cognate cis elements in the MGMT promoter. Overexpression of Sp1 can remove the inhibitory effect of p53 on the MGMT expression. So, a decrease of Sp1 enhances sensitivity of tumor cells to alkylating drugs like TMZ[68].

RNA interference (RNAi) is a process leading to post-transcriptional gene down-regulation or silencing by endogenous production or artificial introduction of small interfering double strand RNA (siRNA) with sequences complementary to the targeted

gene[69]. It was proposed as a potential therapeutic approach to treat cancer[70]. In a study, siRNA for MGMT was encapsulated in cationic liposomes and delivered to the U251SP, T98G, and U251 human glioma cell lines. Results showed that siRNA-based down-regulation of MGMT could enhance the sensitivity of glioma cells against TMZ[71].

In a study using lentiviral-based anti-MGMT small hairpin RNA (shRNA) technology, a specific inhibition of the MGMT expression in GBM cell lines and in subcutaneous tumors was observed. Bioluminescence imaging measurements indicated that luciferase and shRNA-expressing lentiviruses were able to efficiently transduce the GBM xenografts *in vivo*. Combination treatment with injection of a lentivirus expressing an anti-MGMT shRNA and TMZ induced a reduction of the size of the tumors more effective than TMZ alone[72].

Conditionally replicating adenoviruses (CRAds) represent a potential novel approach for cancer therapy[73]. CRAds specifically replicate in cancer cells and have no or negligible toxicity to normal cells[74,75]. Based on the fact that methylation and subsequent silencing of the MGMT promoter sensitize cells to TMZ, Alonso et al[76] hypothesized that the oncolytic adenovirus Delta-24-RGD in combination with TMZ can overcome the MGMT-mediated resistance. The results of delta-24-RGD and TMZ combination treatment showed an increase in cytotoxic effect of TMZ in glioma U87MG and T98G cells. Δ-24-RGD infection down-modulated the MGMT by preventing the recruitment of p300 to the MGMT promoter.

Recently, it has been shown that endoplasmic reticulum (ER) stress-inducing drugs such as Salinomycin sensitize glioma cells to TMZ through down-regulation of MGMT with unknown mechanism. It has been suggested that ER stress is involved in both development and treatment of cancers. ER stress causes a complex cellular response, including the up-regulation of aberrant protein degradation in the ER, with the goal of resolving that stress[77].

Valproic acid (VPA) is an approved drug for the treatment of epileptic seizures, bipolar disorders, and migraine. It acts via inhibition of the transamination of gamma-aminobutyric acid and shows the anti-cancer effect through inhibition of histone deacetylases. Combination of VPA and TMZ significantly enhanced the anti-tumor effect of TMZ in TMZ-resistant malignant glioma cells (U87, U138, T98, and U251) via down-regulation MGMT expression by unknown mechanism[78].

Bone morphogenetic protein 2 (BMP2) belongs to the TGF-β superfamily of proteins and plays an important role in the development of bone and cartilage[79-81]. A study indicated that BMP2 raised sensitivity to TMZ in GBM cells by down-regulation of MGMT expression. BMP2 decreased the hypoxia-inducible factor (HIF)-1α protein stability. MGMT expression is directly regulated by HIF-1α at the transcriptional level[82,83]. Overall, HIF-1α suppression promotes down-modulation of MGMT, and this is sufficient to override GBM resistance to TMZ.

DNA methylation [the addition of a methyl group (–CH3) covalently to the cytosine in the dinucleotide 5'-CpG-3'] directly prevents transcription factor binding to the gene promoter[84]. So, the lack of MGMT expression due to a methylated MGMT promoter is considered a good therapeutic strategy for TMZ-treated GBM patients. A retrospective study of MGMT promoter methylation in 10 pediatric GBM revealed that methylation of the MGMT promoter was correlated with survival. The average survival time for patients with methyltated MGMT was increased as compared to patients with unmethylated MGMT promoter. The patients with the methylated MGMT gene promoter responded better to treatment with TMZ[85].

Inhibition of BER

As mentioned above, the N^7-methylguanine and N^3-methyladenine are the majority products of the TMZ activity. However, these DNA adducts play little role in TMZ cytotoxicity due to the rapid repair by BER. Hence, the BER pathway is a major contributor to cellular resistance to TMZ and BER system inhibition could be an attractive strategy for enhancing TMZ toxicity independent of the O6-methylguanine DNA lesion[73].

Methoxyamine, an alkoxyamine derivative, is able to block the BER pathway through covalently binding to apurinic/apyrimidinic (AP) DNA damage sites. The formed adduct is a stable intermediate and refractory to the catalytic activity of AP endonuclease and polymerase b (pol b), which are downstream members of the BER pathway[86]. The results obtained from an *in vitro* study demonstrated the efficiency of methoxyamine to overcome GBM resistance to TMZ treatment regardless of MGMT or mismatch repair status[87].

Alkylpurine-DNA-N-glycosylase (APNG) is a BER enzyme that has an important role in TMZ resistance. It repairs the cytotoxic lesions N3-methyladenine and N7-methylguanine. Silencing of APNG in *in vitro* and *in vivo* test showed that expression of APNG attenuated the repair of TMZ-induced DNA damage and conferred resistance to TMZ[88]. So, APNG inhibition may be useful in the treatment of the disease.

Inhibition of Chitinase-3-Like Protein 1

Chitinase-3-like protein 1 (CHI3L1), also known as YKL-40, is a secreted glycoprotein expressed in several tissues and involved in activation of the innate

immune system. It plays some roles in cancer cell proliferation, survival, invasiveness, and cell-matrix interactions regulation. In a study, TMZ-resistant U87 cell line was established and novel targeting molecules, other than MGMT were investigated. Gene expression analysis indicated that YKL-40, MAGEC1, and MGMT mRNA expression were up-regulated in the TMZ resistant U87 cell line. Notably, short hairpin (sh) RNA-based inhibition against the YKL-40 resulted in moderate growth inhibition in the resistant cells. Also, inhibition of YKL-40 gene exhibited significant restored the sensitivity to TMZ. Therefore, YKL-40 is a key molecule in addition to MGMT, which is responsible for TMZ resistance in GBM cell lines and could be a new target to overcome TMZ resistance in recurrent GBM in the future[89].

MicroRNAs (MiRNAs)

MiRNAs regulate a wide spectrum of gene expression in a post-transcriptional manner. They play crucial roles in tumorigenesis, angiogenesis, invasion, and apoptosis in various types of tumors. Alteration of miRNAs expression in GBM cells compared with normal brain tissues, were reported. Taken together, 52 up-regulated miRNAs and 33 down-regulated miRNAs have been reported between 2005 and 2010. Recurrent aberrations of expression were detected in only four miRNAs (miR-21, miR-10b, miR-128-1, and miR128-2). So, these four miRNAs have the potential to contribute to the molecular pathogenesis of GBM. Also, some reports showed the relation between miRNA dysregulation and acquired TMZ resistance[90]. Hence, miRNA-targeted therapies could be another strategy for GBM.

MiR-21 overexpression was reported in all types of human cancers. To date, 16 miRNAs were identified that their expression was significantly altered in GBM compared with anaplastic astrocytoma and among them negative correlation of miR-196a/b overexpression with overall survival of GBM patients was reported[91,92]. In a study the expression profiles of 157 miRNAs in patients with glioma were investigated. The results showed that the expression levels of 12 miRNAs (miR-9, miR-15a, miR-16, miR-17, miR-19a, miR-20a, miR-21, miR-25, miR-28, miR-130b, miR-140, and miR-210) were increased, and expression levels of two miRNAs (miR-184 and miR-328) were reduced with progression of disease. Also, they suggested miR-17 and miR-184 as interesting candidates contributing to the glioma progression[93]. Also, clinical implications of miR-26 gene amplification in GBM patients were reported[94]. In a large-scale, genome-wide miRNA expression profiling of GBM, anaplastic astrocytoma and normal brain samples, several differentially regulated miRNAs between these groups were found. In malignant gliomas 55 up-regulated and 29 down-regulated miRNAs were reported. Also, a cluster of only 23 miRNAs was sufficient to distinguish GBM from anaplastic astrocytoma[95]. In another study 10 miRNA was identified that their expression signature could predict overall survival of GBM patients[96]. Expression profiles of 261 miRNA were analyzed and the results showed five clinically and genetically distinct subclasses of GBM. So, miRNAs are important determinants of GBM subclasses through their ability to regulate developmental growth and differentiation programs in several transformed neural precursor cell types[97].

Recent studies have shown that miRNAs play an important role in drug resistance. It was reported that some miRNAs like miR-21, miR-195, and miR-455-3p play some roles in the resistance of GBM cells to TMZ[98-104]. In a study TMZ-sensitive glioma cell lines (U-138MG, A172, LN382, AM-38, U-251MG, and KMG4) were used to generate TMZ resistant variants by continuous exposure to the drug. Then, comprehensive analysis of miRNA expression using miRNA microarray was performed to investigate the mechanisms of acquired resistance against TMZ. Data showed that miR-16 played a role in TMZ resistance. The selective miR-16 mimics and inhibitor were transfected into cells. Treatment with the mimics of miR-16 greatly decreased the sensitivity of cells to TMZ, while sensitivity to these drugs was increased by treatment with the miR-16 inhibitor. In addition, the down-regulation of miR-16 in TMZ-sensitive cells was concurrent with the up-regulation of Bcl-2 protein. Conversely, overexpression of miR-16 in TMZ-resistant cells inhibited Bcl-2 expression and decreased TMZ resistance[105]. Therefore, miR-16 modulates TMZ resistance by regulating Bcl-2 in human glioma cells. A similar study with an *in vitro* model of acquired TMZ resistance (D54MG cell line) showed that chronic TMZ exposure up-regulated the expression of miR-21, and inhibition of miR-21 resensitized chemoresistant GBM cells to TMZ[106]. So, miR-21 inhibitor can be a good chemotherapy adjunct in the treatment of TMZ-resistant GBM.

Combination Therapy With Other Anti-Cancer Agents

Currently, there is a growing interest in combination therapy using multiple anti-cancer agents, as a suitable solution to overcome the drug resistance. Different anti-cancer drugs affect different targets and cell subpopulations and therefore can enhance the therapeutic effects, reduce dose and side effects, and prevent or delay the induction of drug resistance[107].

TABLE 1. *In vitro* studies about the synergistic effect of TMZ and some anti-cancer agents.

Reference	*Cell line and tissue of origin*	*Agent*	*Mechanism*
Balça-Silva et al[108]	U87 (GBM), U118 (GBM)	Tamoxifen	Intensifying apoptotic cell death
Pazhouhi et al[109,110] and Khazaei et al[111]	U87MG (GBM)	Thymoquinone	Intensifying apoptotic cell death Inhibiting autophagy
Atif et al[112]	U87MG (GBM), U118MG (GBM)	Progesterone	Suppressing the EGFR/PI3K/Akt/mTOR signaling pathway and MGMT expression
Zandi et al[113]	A-172 (GBM)	Ciprofloxacin	Unknown
Ren et al[114]	U87MG (GBM), T98G (GBM), LNZ308 (Astrocytoma), RG (GBM), G44 (GBM), G112 (GBM), G130 (GBM), G168 (GBM)	Imatinib	Altering cell cycle control mechanisms
Bak et al[115]	C6 (Glioma)	Vitamin D	Enhancing autophagy cell death
Yu et al[116]	U87 (GBM) and C6 (Glioma)	Metformin	Increasing apoptotic rates
Zanotto-Filho et al[117]	C6 (Glioma), U251MG (GBM), U87MG (GBM)	Curcumin	Unknown
Marutani et al[118]	T98 (GBM), A172 (GBM)	Levetiracetam	Enhancing cellular senescence
Gupta et al[119]	A172 (GBM), U87 (GBM)	Hypericin	Intensifying apoptosis via the down-regulation of critical cell cycle-regulatory and prosurvival components
Chen et al[120]	U87MG (GBM), Hs683 (Glioma), DBTRG-05MG (GBM)	Valproic acid	Increasing apoptosis by p53 and Bax expression, mitochondrial trans-membrane potential loss, reactive oxygen species production, and glutathione depletion. Also decreasing in nuclear translocation of the Nfe-2 p45-related factor and heme oxygenase-1 and γ-glutamyl-cysteine synthetase expression
Brassesco et al[121]	T98G (GBM), U251 (GBM), U138MG (GBM), U87MG (GBM)	Dehydroxymethyle-poxyquinomicin	Unknown
Hanif et al[122]	U87 (GBM)	N-(2-hydroxy- phenyl) acetamide	Increasing apoptosis cell death with increased Bax/Bcl-2 ratio and Caspase-3 expression
Lee et al[123]	U87-MG (GBM), U373 (Astrocytoma)	Chloroquine	Enhancing autophagy, caspases activation and p53- dependent apoptosis
Torres et al[124]	U87MG (GBM), A172 (GBM), SW1783 (astrocytoma), U373MG (GBM), T98G (GBM), SW1088 (astrocytoma, LN405 (GBM)	Cannabinoids	Enhancing autophagy
Khazaei et al[125]	U87MG (GBM)	Trifolium Pratens L.	Enhancing apoptosis and autophagy
Khazaei et al[126]	U87MG (GBM)	Tranilast	Increasing apoptosis cell death with increased Bax/Bcl-2 ratio and p53 expression.

Some studies were carried out to determine the synergistic effect of TMZ and other anti-cancer agents in human glioma cell lines that are summarized in Table 1. However, the efficacy of these combination treatments has not yet been confirmed in clinical trials.

Some combinations were investigated in clinical trials. For example, a study with 30 patients with surgically confirmed GBM confined to one cerebral hemisphere, with a Karnofsky performance score greater than 70, no comorbid disease, and age younger than 60 years was performed. Along with conventional therapy, they used chloroquine (an autophagy inhibitor) orally for 12 months. Results showed that chloroquine improved mid-term survival for GBM that received conventional therapy. So, chloroquine as adjuvant therapy for GBM is warranted[127]. Phase I clinical study showed that the combination of TMZ and procarbazine was reasonably effective and well-tolerated in treating patients with relapsed gliomas. There is little difference in terms of side effects between TMZ alone and the combination with procarbazine[128]. Moreover, in clinical trials, the combination of carmustine and TMZ as neo-adjuvant therapy in GBM exhibited promising activity with a good safety profile[129].

CONCLUSIONS

Epidemiological studies showed a significant increase in incidence and death rate of brain cancers in the world[130]. Since GBM tumor cells are resistant to TMZ and the median survival of GBM patient is very short, TMZ is not a convincing therapeutic agent. Recently *in vitro* and *in vivo* studies proposed some strategies to enhance the efficacy of TMZ. But few clinical studies are performed in this field. It is worth re-examining the effect of TMZ with these agents on survival of patients with brain GBM tumor in clinical studies.

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Conflict of Interest:
The Authors declare that they have no conflict of interests.

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