Potential Strategies Overcoming the Temozolomide Resistance for Glioblastoma

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Abstract

Glioblastoma (GBM) is a highly malignant type of primary brain tumor with a high mortality rate. Although the current standard therapy consists of surgery followed by radiation and temozolomide (TMZ), chemotherapy can extend patient's post-operative survival but most cases eventually demonstrate resistance to TMZ. O^6-methylguanine-DNA methyltransferase (MGMT) repairs the main cytotoxic lesion, as O^6-methylguanine, generated by TMZ, can be the main mechanism of the drug resistance. In addition, mismatch repair and BER also contribute to TMZ resistance. TMZ treatment can induce self-protective autophagy, a mechanism by which tumor cells resist TMZ treatment. Emerging evidence also demonstrated that a small population of cells expressing stem cell markers, also identified as GBM stem cells (GSCs), contributes to drug resistance and tumor recurrence owing to their ability for self-renewal and invasion into neighboring tissue. Some molecules maintain stem cell properties. Other molecules or signaling pathways regulate stemness and influence MGMT activity, making these GCSs attractive therapeutic targets. Treatments targeting these molecules and pathways result in suppression of GSCs stemness and, in highly resistant cases, a decrease in MGMT activity. Recently, some novel therapeutic strategies, targeted molecules, immunotherapies, and microRNAs have provided new potential treatments for highly resistant GBM cases. In this review, we summarize the current knowledge of different resistance mechanisms, novel strategies for enhancing the effect of TMZ, and emerging therapeutic approaches to eliminate GSCs, all with the aim to produce a successful GBM treatment and discuss future directions for basic and clinical research to achieve this end.

Key words: glioma, temozolomide, chemosensitivity

Introduction

Glioblastoma (GBM) is one of the most common and aggressive primary malignant brain neoplasms in adults, with a low median survival period of only 12–15 months after the initial diagnosis. Although it's relatively uncommon, with a low incidence of about 5/100,000 when compared with other malignant tumors,[1] GBM still accounts for around 70% of all adult malignant brain tumors.[2,3] Owing to its rapid proliferation ability and highly infiltrative growth, complete surgical resection is still difficult to achieve.[1,3] The majority of diagnosed GBMs recur, even after an expanded resection of normal brain tissue was performed at the edge of the tumor mass. The benefits of nitrosoureas-based chemotherapy on GBM patient survival were established more than three decades ago.[2] Since then, surgical resection, followed by radiation therapy and chemotherapy, has been adopted as conventional therapies for patients with newly diagnosed GBM.

Temozolomide (TMZ) is an oral alkylating agent, for which the antitumor activity was first discovered in 1987,[4] and has been widely applied as an effective first-line chemotherapeutic agent for the treatment of GBM patients since FDA approved its efficacy in March, 2005.[5,6] When combined with radiation therapy, and employed as an adjuvant therapy, TMZ treatment contributed to a significant increase in median survival period, 2-year survival, progression-free survival, and improved quality of life when compared with radiotherapy alone.[6] Since then, hundreds of studies and papers have demonstrated the main cytotoxic mechanism by which TMZ may eliminate GBM tumors. TMZ elicits cytotoxicity by transporting a methyl group that attaches to guanine at the O^6 and N^7 positions, and adenine at

N^3 position during DNA replication to form cytotoxic O^6-methylguanine (O^6-MG), N^7-methylguanine (N^7-MG), and N^3-methyladenine (N^3-MA). These cytotoxic groups are composed of mismatched lethal base pairs that result in single and double-strand DNA breaks that induce cell cycle arrest at G2/M, eventually leading to cell apoptosis.[1,7–9]

Although subsequent radiation therapy and chemotherapy with TMZ contributed to lengthen survival and improve quality of life,[6,9,10] the survival advantages are still palliative.[11,12] The increase in TMZ resistance is one of the main reasons for GBM treatment failure.[9] O^6-methylguanine-DNA methyltransferase (MGMT) is the most important contributor to TMZ chemoresistance that could repair the main cytotoxic lethal base pairs, which are composed of the alkylating agent TMZ. Recently, a series of TMZ resistant cases have been reported, although these cases do not appear to have MGMT activity. So, there should be some other resistance mechanism independent of the MGMT repair system, such as that present in glioma stem cells (GSCs).

Following the first introduction of the concept of cancer stem cells, based on studies of blood formation in hematopoietic stem cells and cancer-initiating cells in leukemia,[13] reports gradually proposed that in various human solid tumors, including malignant brain tumors, there is a small population of undifferentiated highly tumorigenic cancer stem cells or cancer-initiating cells, that possess extensive self-renewal capacity and further differentiation ability. This self-renewal and differentiation induce cancer initiation, progression, metastasis, and resistant to conventional therapies.[14,15] Recent evidences also demonstrate that GSCs exhibit stronger resistance to conventional therapies than normal neural stem cells (NSCs).[16] This increased resistance is probably because of different proliferative ability, expression levels of key proteins and molecules related to cell behavior and survival, or various subsequent markers. In this paper, we will discuss the main mechanism of TMZ cytotoxicity. The TMZ resistance mechanism is caused by a conventional repair system and regulated by GSCs. Strategies to enhance TMZ chemosensitivity against glioma are the latest frontier in current treatments targeting GSCs.

Resistance Mechanism

The TMZ response varies in GBM, although TMZ is the standard chemotherapy for GBM treatment.[6] In GBM with TMZ resistance, it is difficult to prevent tumor progression or recurrence.[17,18] Clarifying the TMZ resistance mechanism is essential to increase drug efficacy, and could provide an important basis for individualized treatment according to different resistance mechanisms. Current researches indicated that TMZ resistance is the result of both the DNA repair system, such as MGMT, mismatch repair (MMR), and base excision repair (BER), as well as other mechanisms like autophagy and GSCs (Fig. 1).[1,17,19–28]

DNA repair systems
O^6-methylguanine-DNA methyltransferase   The antitumor effect of TMZ is reflected in the O^6-guanine methylation although TMZ-induced DNA methylation occurs at the N^7-guanine (>70%) and the N^3-adenine (>9%) to a greater extent than the O^6-guanine (5%).[17] MGMT is DNA repair enzyme that directly repairs the TMZ-generated cytotoxic lesion by removing the methyl group in the O^6-methylguanine, which leads to invalidation of TMZ-induced lethal DNA damage. MGMT expression levels directly correlate with the methylation status of the promoter site in the cysteine-phosphate-guanine (CpG) *MGMT* gene island. MGMT is diminished by methylation of MGMT promoter and stays active when MGMT promoter remains unmethylated. It was reported that GBM cases, with a methylated MGMT promoter, showed prolonged survival compared to cases with an unmethylated MGMT promoter in a phase II trial evaluating the combination effect of radiotherapy and TMZ for newly diagnosed GBMs.[19,21,23,26] Hence, MGMT is the main culprit contributing to TMZ resistance, providing a potentially sensitive target for TMZ therapy.
Mismatch repair   MMR is a system that corrects nucleotide base mismatches generated in the process of DNA synthesis. O^6-methylguanine (O^6-MeG), induced by TMZ treatment, mispairs with thymine during DNA replication. The MMR system recognizes mispaired O^6-MeG/T and excises the newly synthesized strand, leaving the parental strand with O^6-MeG intact. These futile cycles repeat, leading to cell cycle arrest and apoptosis. The loss of MMR function does not respond to TMZ-induced mispairing and can be associated with resistance to the cytotoxic effects of TMZ. MMR ability is impaired by mutation of MMR genes, such as melanocyte-stimulating hormone 2 (*MSH2*), MSH6, mutL homolog 1 (*MLH1*), and post-meiotic segregation-increased *Saccharomyces cerevisiae* 2 (*PMS2*). MSH6 somatic mutations were mainly identified in recurrent GBM, mediated by TMZ compared to primary GBM without TMZ therapy.[19,20,25,27] It indicates that mutations in MMR genes are rar in primary GBM, while these genes are vulnerable to TMZ-induced mutations, so that tumor cells will become resistant to TMZ because of a disrupted MMR pathway.[19,27]
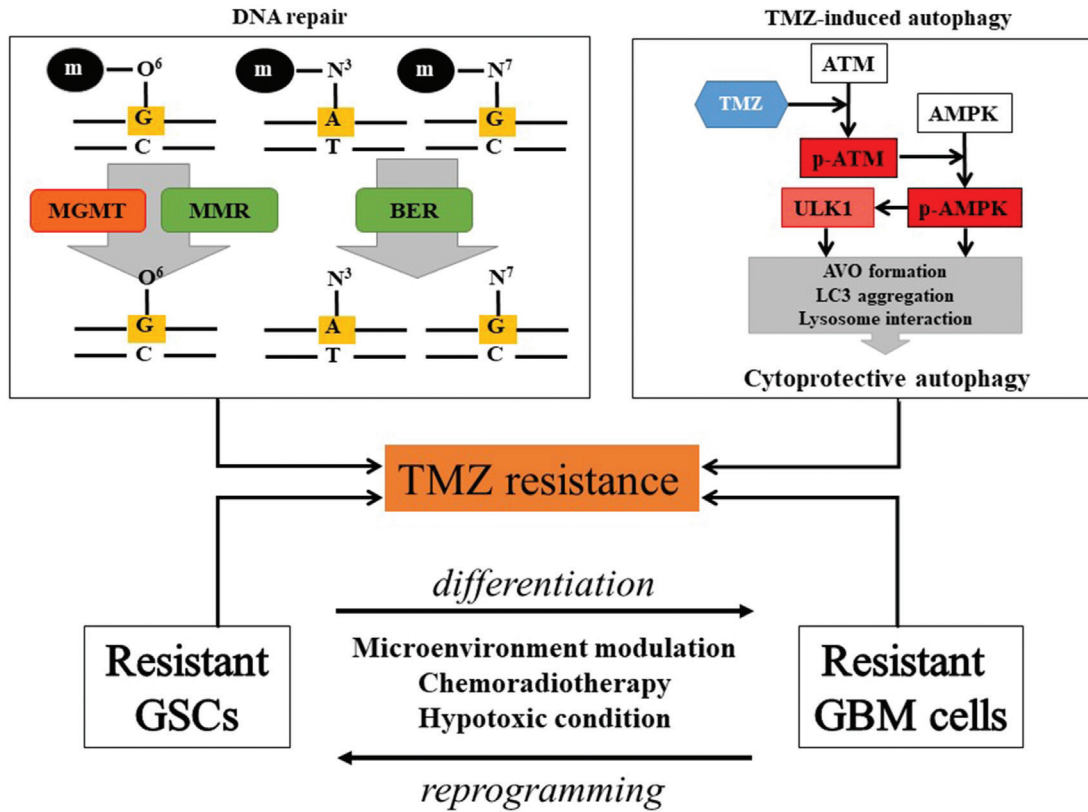
Fig. 1 TMZ resistant mechanism. The causes of TMZ resistance are mainly DNA repair system, autophagy, and GSC. The MGMT and MMR systems remove the O^6-guanine methylation, followed by usual DNA replication. Activated BER also contributes to DNA repair through removal of the methylation of N^7-guanine and the N^3-adenine. TMZ-induced autophagy via the ATM/AMPK pathway can induce AVO formation, LC3 aggregation, which are essential for autophagosome and lysosome interaction, facilitating cytoprotective autophagy and cell survival. GSCs may change their phenotype to TMZ resistant GSCs or differentiate into TMZ resistant GBM cells via tumor microenvironment modulation, chemoradiotherapy, or hypoxic condition. On the other hand, differentiated GBM cells can re-acquire stem cell capacity through reprogramming by the tumor microenvironment modulation. AMPK: AMP-activated protein kinase, ATM: ataxia telangiectasia mutated, AVO: acidic vesicular organelles, BER: base excision repair, GSC: glioblastoma stem cell, LC3: microtubule associated protein light chain 3, MGMT: methyl-guanine methyltransferase, MMR: mismatch repair, TMZ: Temozolomide, ULK: unc51-like kinase.

Base excision repair The BER system is involved in the repair of DNA damage induced by an oxidizing agent, ionizing radiation or alkylating agents. This system consists of multicatalysis reactions by DNA glycosylase, endonuclease, polymerase, and DNA ligase. The methylation of N^7-guanine and the N^3-adenine represent more than 90% of the methylation caused by TMZ and are rapidly repaired by BER, so that would promote GBM survival. When one or more components of BER are mutated, it results in the deficiency in the ability of BER to repair DNA damage, furthermore, contributing to TMZ cytotoxicity to GBM. Notably, N^3 lesions are lethal if not repaired, unlike N^7 lesions. Among the components of BER, poly (ADP-ribose) polymerase-1 (PARP-1) is known as an important enzyme with dual effect. Inhibition of PARP-1 leads to the accumulation of broken

DNA in the cells, resulting in cell death. PARP-1 hyper-activation, generated by DNA damage, leads to a depletion of nicotinamide adenine dinucleotide (NAD+) and adenosine triphosphate (ATP) followed by cell death. Thus, the regulation of BER activity will contribute to the treatment of TMZ resistant GBM.[17,19–21,27] However, reports indicated that the role of BER in TMZ resistance is less important than MGMT and MMR, although the rate of N^7-guanine and the N^3-adenine methylation is higher than that of O^6-guanine.

Autophagy

Autophagy is the mechanism to maintain homeostasis and survival through the degradation and recycling of cellular proteins, organelles, and other cellular components. Autophagy is commonly regulated by

various stimuli including conditions, such as starvation, hypoxia, pathogens, radiation toxic agent, and DNA damage. Autophagy involved in cancer cell behaviors and its implications for cancer therapy have been an appealing direction for cancer research for decades. However, the role of autophagy is still controversial.[29,30] It is possible that the effect on autophagy depends on the cellular context and treatment conditions.[28] Studies that investigated the relation of autophagy to TMZ treated glioma cells have demonstrated that autophagy, but not apoptosis, is induced in GBM cells treated with TMZ.[31,32] Autophagy activity is reduced in cancer cells than in their normal counterparts to maintain the potential to activate self-defensive autophagy.[33] Once cancer cells are exposed to radiation or chemotherapeutic agents, a high rate of autophagy is triggered[34,35] and cancer cells degrade unnecessary components or molecules for their survival as an adaptation to detrimental conditions caused by cancer therapies, such as TMZ chemotherapy.[31] Another study also has reported that very few of GBM cells treated with TMZ underwent apoptosis.[36] The role of autophagy is preliminary protective and autophagy induction is considered as a mechanism of chemoresistance.[30,37] Based on the findings described above, combination treatments of TMZ with autophagy inhibitors or regulators would be a promising strategy to treat highly resistant GBM cases. However, Investigations showed that additional treatment of TMZ-treated GBM cells with Bafilomycin A1, which inhibits autophagosome and lysosome fusion, resulted in more effective apoptotic cell death as compared with TMZ and/or Bafilomycin A1 treatment alone because of the mechanism by which Bafilomycin A1 blocked TMZ-induced cytoprotective autophagy at the late stage and activated caspase-3 as well as mitochondrial and lysosomal membrane permeabilization.[31] In the same study, another autophagy inhibitor, 3-methyladenine (3-MA), which is known to inhibit autophagy at the early stage, has decreased TMZ cytotoxicity when employed in combination therapy, in contrast with Bafilomycin A1.[31] Furthermore, it has been reported that inhibition of adenosine monophosphateactivated protein kinase (AMPK), an initiator of autophagosome formation interacting with mammalian autophagyinitiating kinase unc51-like kinase 1 (ULK1),[38] augments the cytotoxicity of TMZ in GBM cells in combination treatment,[32] These discoveries suggest that autophagy is the main result of TMZ cytotoxicity and inhibition of autophagy significantly influences the TMZ anti-glioma effect. Meanwhile, there should be a focus on how different autophagy inhibitors or regulators play different roles in different steps of the process of TMZ induced autophagy to potentially induce different results.

Glioma stem cells Since the first description of the cancer stem cell hypothesis was proposed for hematopoietic cancers, GSC was isolated from the bulk of the GBMs. Molecules that have been identified as GSC markers include CD133, CD15, stage-specific embryonic antigen-1 (SSEA1), and nestin. GSCs are capable of self-renewal, tumorigenesis, differentiation, chemo-resistance and radio-resistance.[18,24,39,40] GSC chemo-resistance was related to drug efflux transporter (ATP-binding cassette, ABCG2) and GSC diversity.[41] GSCs exhibit diversity because the tumor bulk consists of numerous heterogenetic GSC phenotypes based on distinct genomic profiles. The tumor microenvironment can also modulate GSC phenotypic change and may produce different type of GBM cell lines. In contrast, differentiated GBM cells can be reprogrammed by the tumor microenvironment and re-acquire stem cell capacity. Hypoxic condition promotes stemness and enhances MGMT upregulation through hypoxia-inducible factor 1-alpha (HIF-1α) in GSCs.[23,42] Chemo-resistance based on GSC theory could be important as a hallmark of recurrent GBM.

Here, it is explained that TMZ resistance is associated with MGMT function, MMR function, BER function, autophagy, and GSC. GBM cell with MGMT promoter unmethylated status and GSC are primarily tolerant of the effect of TMZ. GBM treated with TMZ can acquire TMZ resistant via changes in the MGMT promoter status, mutation of MMR related genes, TMZ induced-autophagy activation, and heterogeneity of GBM cells. It is important that TMZ therapy can be a trigger of resistance.

Strategy for Enhancing TMZ Effect

Strengthen TMZ effect

Modulation of TMZ dosing schedule MGMT is one of the mechanisms involved in TMZ resistance. MGMT repairs damaged DNA by transferring a methyl group from the O^6 position of guanine, methylated by TMZ, to its cysteine in an active enzymatic domain (Fig. 2). Methylated MGMT loses its enzymatic activity leading to subsequent degradation. Continuous exposure to TMZ, dose-dense TMZ (ddTMZ) or metronomic TMZ (mTMZ) causes accumulation of methylated MGMT and sensitization to TMZ. Biologically, a low dose (20–50 mg/m²) mTMZ regimen might deplete CD4+CD25+Foxp3+ regulatory T cells (Tregs), which play a significant role in hampering antitumor immunity.[43]

Among several regimens of alternate TMZ dosing, 7 d on/7 d off (7/14 d), 21 d on/7 d off (21/28 d), and
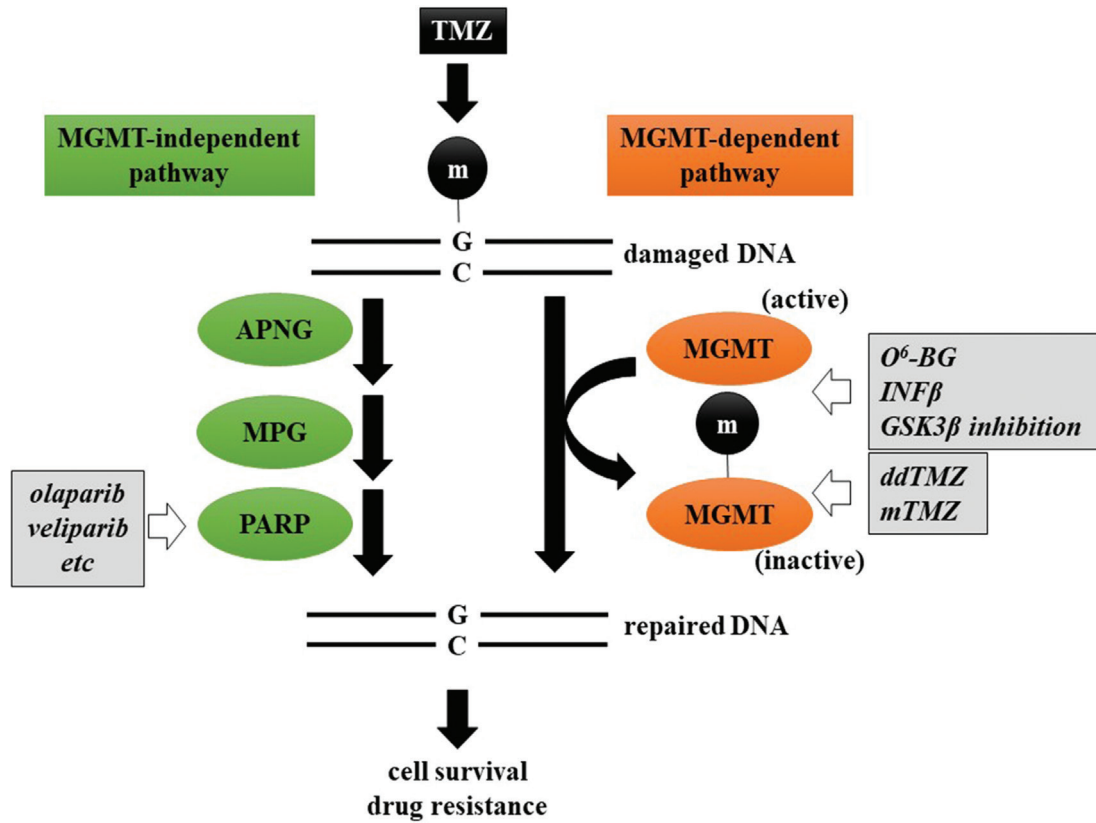
Fig. 2 Potential therapeutic approach enhancing the effect of TMZ. TMZ resistance by DNA repair is divided into MGMT independent and dependent mechanisms. Therapeutic approaches to overcome the former include methylation of the MGMT promoter with O⁶-BG, IFN*β*, and GSK3*β* inhibition and depletion of MGMT by ddTMZ. Currently, PARP inhibition is the only available intervention for the latter mechanism. *Circled m* represents a methyl group. APNG: alkylpurine-DNA-*N*-glycosylase, GSK3*β*: glycogen synthase kinase 3*β*, INF*β* interferon-*β*, MPG: *N*-methylpurine DNA glycosylase, O⁶-BG: O⁶-benzylguanine, PARP: poly (ADP-ribose) polymerase.

continuous or metronomic administration (28/28 d) have been vigorously investigated.[44)] For newly-diagnosed GBM, a large randomized phase III trial (RTOG0525) that compared the ddTMZ regimen (75–100 mg/m^2, 21/28 d) with the standard 5/28 d TMZ regimen showed no survival benefit with ddTMZ.[45)] It is suggested that newly-diagnosed GBM, which has no history of exposure to TMZ (TMZ-naïve) is susceptible to TMZ. In contrast, a 7/14 d regimen for treatment of recurrent or progressive GBM was superior to the standard regimen with respect to both progression-free survival (PFS) and overall survival (OS), without significantly increasing adverse events.[46)] Based on these data, ddTMZ should be recommended for the patients with refractory disease formerly exposed to TMZ. However, a phase II trial comparing two ddTMZ regimens (7/14 d vs. 21/28 d) for patients with their first recurrent GBM after standard treatment showed no difference between the two regimens for median overall survival (mOS) 298 d vs. 322 d.[47)] This study also demonstrated that TMZ rechallenge

was suitable for MGMT promoter-methylated GBM after standard therapy.[47)]

Most ddTMZ regimens also increase the amount of TMZ accumulation over one course (28 d) related to potential toxicity. Based on the available data, no significant increase in toxicity of myelosuppression, which is hallmark of TMZ toxicity, was found. CTCAE grade 3/4 lymphopenia was found 24–53% and 12–68% in 21/28 d and 7/14 d regimen, respectively.[44,48)] A prophylaxis against opportunistic infection and careful MRI follow-up should be required for all ddTMZ regimens in addition to standard treatment. Dose-intense TMZ could promote invadopodia formation, one of the infiltrating phenotype of tumor cells, via MMP-2 activation.[49)] Thus, it is also necessary to carefully observe neurological deterioration and tumor progression MRI.

Combination treatment with TMZ enhancer Concomitant therapy is another approach for enhancing the activity of TMZ. The majority of TMZ-induced methylation site is N^7 portion of guanine (>70%), whereas N^3 portion of adenine and O^6 portion of

guanine are methylated at 9% and 5%, respectively. N^7-MG and N^3-MA are substrates for the BER system consisting of a multistep reaction by several DNA glycosylases (alkylpurine-DNA-N-glycosylase; APNG, etc.), endonucleases (N-methylpurine DNA glycosylase; MPG, etc.), polymerases (poly (ADP-ribose) polymerase; PARP, etc.), and DNA ligases (Fig. 2).[17] PARP is one of the most intensively investigated BER enzymes and several PARP inhibitors applied to ovarian, prostate, and breast cancers in the United States, Europe, and Japan.[50] Some preclinical studies showed that the combination of TMZ with PARP inhibitor was effective for IDH-mutant glioma.[51,52] Currently, several clinical trials have investigated the efficacy of PARP inhibitors (olaparib, veliparib, etc.), with or without TMZ, against WHO grades II–IV gliomas (http://www.clinicaltrials.gov).

It is clear that methylation of the MGMT promoter is a favorable prognostic and a predictive factor for GBM; thus, the agents depleting MGMT and methylation of the MGMT promoter could potentiate TMZ cytotoxicity. O^6-benzylguanine (O^6-BG), a false substrate of MGMT, could irreversibly inactivate MGMT by antagonizing O^6-MeG leading to a reversal of TMZ resistance.[17] In phase II clinical trial, O^6-BG was shown to restore sensitivity of recurrent high-grade glioma (HGG) to TMZ, but no significant survival benefit was observed.[53] Interferon-β (INFβ) enhances chemosensitivity by downregulating MGMT transcription via p53 induction.[54] INTEGRA study (JCOG0911), a phase II trial examining the effect of IFNβ on RT + TMZ standard therapy for newly-diagnosed HGG, was conducted in Japan. However, it was disappointing that median PFS (mPFS) and mOS were not significantly different between RT + TMZ and RT + TMZ + IFNβ (mPFS, 10.1 vs. 8.5 months; mOS, 20.3 vs. 24.0 months).[55] In addition, we identified several drug candidates enhancing the activity of TMZ (Kitabayashi et al., unpublished data). Among them, glycogen synthase kinase 3β (GSK3β) inhibition has been revealed to enhance the activity of TMZ via conversion of unmethylated guanine to methylguanine at the MGMT promoter.[56] A phase I/II clinical study that investigated the efficacy and safety of concomitant GSK3β inhibitors with TMZ against recurrent GBM demonstrated an anti-tumor effect, survival benefit, and enhancement of the TMZ effect without adverse side effects.[57]

Treat the GSCs based on TMZ

As mentioned, GSCs play an indispensable role in developing resistance to chemoradiotherapy and are the main culprit behind GBM recurrence after initial therapy, owing to their stem-cell-like properties such as self-renewal, capacity of differentiation

and tumor initiation. Therefore, novel therapeutic approaches that are effective and successful in eliminating both GSCs and entire tumor bulk are urgently required. Hypothesizes and studies support the idea that molecules involved in maintaining the stem-cell-like properties of GSCs could be novel therapeutic targets to overcome chemoresistance. Recent reports demonstrated that new agents may be effective as single treatments or to synergistically enhance TMZ cytotoxicity against GSCs and eliminate GBM tumor bulks via MGMT promoter methylation, or other MGMT independent pathways (Fig. 3).

MGMT dependent manner

1. JNK inhibition sensitizes TMZ via regulation of MGMT expression.
   The c-Jun NH2-terminal kinases (JNKs), also known as stress-activated MAP kinase (SAPK), is a member of the mitogen-activated protein (MAP) kinase family.[58] JNK interacts with signals from numerous extracellular stimuli, and is involved in important cellular processes such as proliferation, apoptosis, and differentiation.[58,59] JNK is commonly upregulated in a number of human cancers, including GBM. The activation level of JNK in self-renewing cells is obviously superior to that of differentiated cells.[59,60] Studies targeting JNK showed that, regardless of whether JNK was inhibited by the JNK specific inhibitor SP600125 or silenced by JNK-shRNA, GSCs after JNK inhibition exhibits weakened stem cell properties with reduced ability to form tumor spheres, lowered expression of stem cell markers, such as Nestin and Sox-2, and elevated expressions of differentiated cell markers such as GFAP and βIII-tubulin.[60] Further studies showed that when, GSCs were treated with SP600125, it suppressed the expression of MGMT in both a dose- and time-dependent manner. In combination treatment with TMZ, SP600125 synergistically sensitize TMZ cytotoxicity in GSCs. Knockdown of JNK by shRNA resulted in the same outcome with SP600125. Another novel JNK inhibitor, AS602801, also inhibited GSCs both *in vitro* and *in vivo*.[59] However, JNK inhibition is ineffective to GSCs without MGMT expression.[61] The JNK pathway could be a therapeutic target to overcome GSC related chemo-resistance, and a novel approach to treat GSCs expressing MGMT.

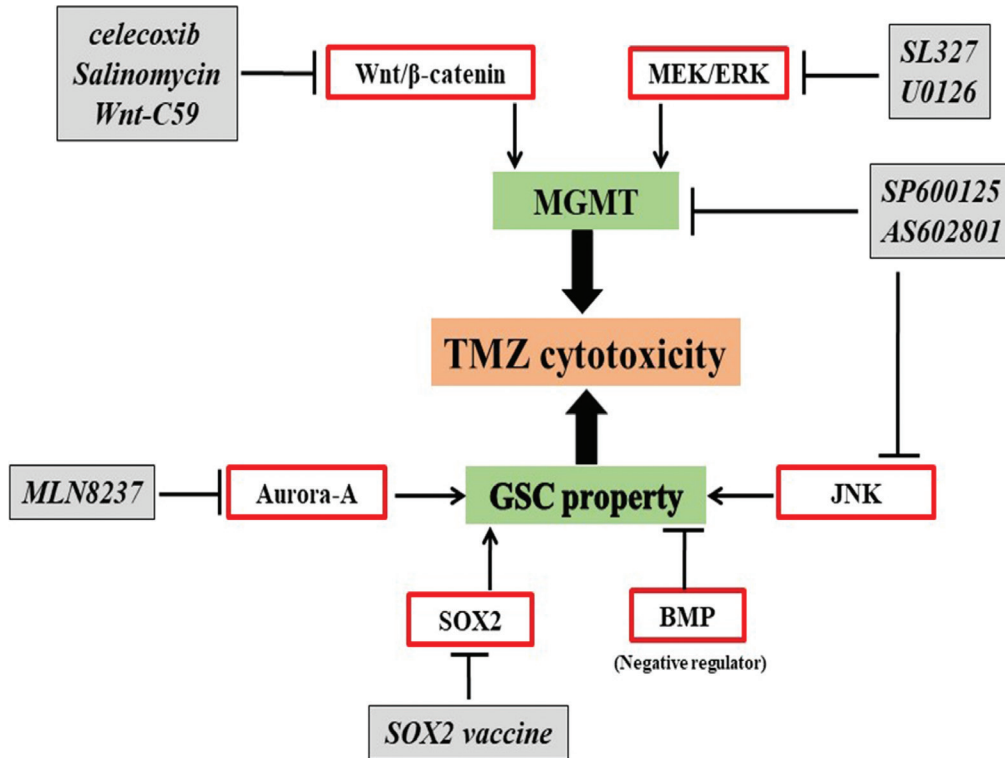2. MEK inhibitors potentiate TMZ efficacy to GSCs.

Fig. 3 Treat the GSCs based on TMZ. JNK, MEK/ERK pathways, and Wnt signaling maintain extensive proliferation and self-renewal ability of GSCs in a MGMT dependent manner. Aurora-A kinase, SOX2, and BMP regulate GSC stemness and correlate with tumor aggressiveness and poor prognosis. Targeting these molecules is a promising therapeutic strategy to enhance TMZ. BMP: bone morphogenetic proteins, ERK: extracellular signal regulated kinase, JNK: c-Jun *N*-terminal kinase, MEK: mitogen-activated protein kinase, SOX2: sex determining region Y-box 2.

MEK-ERK signaling is an important signaling pathway involved in many biological processes like proliferation, differentiation, and apoptosis. Studies demonstrated that MEK/ERK signaling is continuously activated as the consequence of upregulation or abnormalities of upstream molecules of receptor tyrosine kinases, such as EGFR, PDGFR.[62,63] Other studies also revealed that ERK phosphorylation is active in glioma cells.[64] These findings indicate that therapies targeting MEK/ERK signaling may be potential approaches for treating GSCs. A study discussing the relationship between MEK/ERK signaling and MGMT expression level showed that MEK inhibitors, SL327 and U0126, suppressed MGMT expression in GSCs via activation of p53. MGMT suppression is capable of inhibiting MGMT expression,[65] suggesting that MEK activity is required for the maintenance of MGMT expression, regulating TMZ resistance of GSCs. Combination of the MEK inhibitor SL327 with TMZ treatment synergistically sensitized resistant GSCs to TMZ and suppressed the tumorigenic properties of GSCs.[66] *In vivo* experiments also exhibited a significant increase in survival when a combination treatment of SL327 with TMZ was employed, as compared with either SL327 or TMZ treatment alone. These data suggested that the MEK inhibitor plays an effective role as enhancer for TMZ chemotherapy targeting GSCs.

3. Inhibition of Wnt/β-catenin signaling prevents chemo-resistance by down-regulation of MGMT.

Wnt/β-catenin signaling is an ancient and highly conserved system that controls embryonic development and gene homeostasis. According to previous studies, activated Wnt/β-catenin signaling is a key feature of epithelial cancers that regulates self-renewal and differentiation in several adult stem cell niches and interacts with the surrounding microenvironment to influence stemness, cell proliferation and invasive behavior of tumor cells.[67,68] Activation of Wnt/β-catenin signaling enhances the motility of GBM

cells and contributes to the mesenchymal transition.[69] Gene ontology analysis, and pathway-specific gene-expression profiling, also showed that high MGMT expression is significantly related to aberrant Wnt signaling.[70] Therefore, Wnt signaling may provide a novel approach to GSC treatment. Wnt signaling inhibitors celecoxib, salinomycin, and porcupine inhibitor Wnt-C59, and genetic inhibition of Wnt signaling by specific shRNA, suppressed MGMT expression.[70] The combination of Wnt signaling inhibitors with TMZ restores TMZ sensitivity *in vivo*.[70] The above data illustrates that inhibition of Wnt/β-catenin signaling may offer a potential strategy to treat chemoresistant GSCs expressing MGMT.

MGMT independent manner

1. Aurora-A kinase inhibitor suppresses GSC and potentiates TMZ efficacy.

    Aurora-A is a serine-threonine kinase essential to centrosome maturation and mitotic entry and exit.[71,72] Overexpression of Aurora-A has been reported in several human tumors, including gliomas.[73–75] Moreover, Aurora-A kinase activity influences multiple signaling pathways related to cell growth and differentiation and is associated with GBM patient survival.[76] Inhibition of Aurora-A by MLN8237, a selective Aurora-A kinase inhibitor, suppressed the ability to form GSC tumor spheres and synergistically potentiated TMZ chemosensitivity in combination treatment.[76,77] When treating glioma cell lines with MLN8237 combined with TMZ, it strongly suppressed cell proliferation and significantly inhibited colony formation of GSCs compared to either MLN8237 or TMZ alone.[77] Interestingly, MLN8237 at a low concentration renders GSCs sensitivity to radiation therapy.[76,77] In summary, Aurora-A kinase may be a new molecular target for GSC treatment.

2. Inhibition of SOX2 decreased GSC activity and TMZ resistance.

    Sex-determining region X (SOX) and sex-determining region Y (SRY)—box is a family of transcriptional factors characterized by conserved high mobility group DNA-binding domains that control several important functions and processes involved in the maintenance of stem cell properties in lots of tissues during embryonic development and adulthood, while its genetic inactivation

induces stem cell differentiation.[78–80] SOX2, a member of the SOX family, is overexpressed in clinical GBM samples and higher SOX2 levels are correlated with tumor aggressiveness and poor prognosis.[10,81] Except for promoting the maintenance of GSC stemness, SOX2 also contributes to TMZ resistance.[10] These results indicate the potential to target SOX2 as a strategy to eliminate GSCs and potentiate TMZ sensitivity. Several strategies have been proposed to target SOX2, directly or indirectly, to overcome GSCs. Indirect inhibition of SOX2, by inhibition of its upstream molecules such as PDGFR, SHH, and mTOR, suppressed tumor growth significantly through SOX2 downregulation and GSC sensitization. Meanwhile, increased cytotoxicity was observed in GSCs when SOX2 inhibition was combined with TMZ, except for the SHH inhibitor.[10,82,83] Immunotherapy, using a peptide vaccination against SOX2, prolonged the survival of GSCs transplanted mice by monotherapy and the vaccination, in combination with TMZ, doubled the survival time.[84] Another study showed that direct inhibition of SOX2, using miRNA delivery in GSCs, strongly suppressed tumorigenicity in a mouse xenograft model and increased GSC chemosensitivity to TMZ.[85] These data prove that inhibiting SOX2 is a promising and effective strategy targeting GSCs.

3. BMP as new agent to eliminate stemness and chemoresistance.

    Bone morphogenetic proteins (BMP) are a member of the TGF superfamily demonstrated to be involved in cell growth, differentiation, and defining stem cell properties. BMP, especially BMP4, acts as a negative regulator of GSC behaviors. Administration of BMP4 prevents tumor growth and motility through BMP receptor-mediated Smad activation, along with induction of anti-proliferative differentiation.[14,86] A high-density microarray analysis using high-dose TMZ resistant GSCs demonstrated that *BMP7* was the most down-regulated gene, which indicated that internal *BMP7* expression correlated with TMZ resistant.[87] Administration of BMP7 made highly TMZ resistant GSCs sensitive and suppressed cell proliferation and migration. Furthermore, in a mouse xenograft model, BMP7 treatment synergized to improve TMZ efficacy and extended survival significantly as compared to TMZ alone.[87] The main reason for BMP7 potentiation of the TMZ effect may

be because BMP activation suppresses the stem cell properties and protective factors involved in chemoresistance, thereby sensitizing GSCs to low-dose TMZ treatment. Overall, these findings indicate that identification of BMPs in GSCs have advantages in individualized treatment, providing an effective strategy for GBM treatment targeting GSCs based on TMZ.

Other therapies that might potentiate the TMZ effect

Existing treatments (for GBM or under clinical trial)

1. Anti-angiogenic therapy
    An anti-vascular endothelial growth factor (VEGF) antibody bevacizumab with TMZ therapy unfortunately showed no survival benefit for GBM patients in two randomized clinical trials (AVAglio, RTOG0825).[88,89] Interestingly, subanalysis of the AVAglio study revealed that the anti-angiogenic therapy could improve clinical symptoms by reducing cerebral edema, resulting in better quality of life. Recently, bevacizumab combined with ddTMZ regimen is expected to have efficacy on progressive or recurrent GBM previously exposed to TMZ.[90,91]
2. Tumor treating fields (TTF)
    TTF are electric fields of low intensity and intermediate frequency. They demonstrated that cell death is induced by anti-mitotic properties in several cancers. For GBM, its therapeutic potential was first reported in 2007.[92] Thereafter, clinical trials were performed for recurrent patients (EF-11 study)[93] and then for primary GBM patients (EF-14 study).[94] In those studies, TTF with TMZ therapy prolonged both PFS and OS compared with TMZ alone. TTF might delay repair of DNA damage by TMZ,[95] suggesting that TTF could potentiate the effect of TMZ. However, cost-effectiveness should be considered.[96]
3. Immunotherapy
    Cancer immunotherapy is the process by which the body activates its own immune system to fight against existing cancer cells. For several cancers, such as malignant melanoma, prostate cancer, and lung cancer, a few kinds of immunotherapies are incorporated into the standard therapies.[97] A variety of immunotherapies, dendritic cell (DC) vaccines, peptide vaccines, such as the EGFRvIII and WT-1, tumor antigen vaccines, adoptive immunotherapy, therapy with NK cells derived from umbilical cord blood, oncolytic virus therapy, and gene

transfer therapy, are reported.[97–99] For cancer immunotherapy, effective antigens exposed to T cells would be an essential step to generate and maintain immune responses to cancer cells.[100] Several GBM antigens have been identified as potential immunotherapeutic targets.[101] Some trails assessing the combination of TMZ chemotherapy with immunotherapies also showed enhanced anti-glioma effects in highly TMZ resistant cases.[102] Therefore, immunotherapy is also a promising new potential therapeutic strategy for GBM.

Recently, immunotherapy with fusions of DCs and glioma cells (FC therapy) appears promising in GBM patients. In phase I/II trail, treating GBM patients with a combination of standard TMZ chemotherapy and DC-based vaccination significantly prolonged mOS and PFS,[102–105] as compared with the standard radio-chemotherapy treatments.[6] FC therapy helped the immune system recognize and eliminate the TMZ-induced chemo-resistant peptides, such as WT-1, gp100, and MAGE-A3.[102] Aglatimagene besadenovec (AdV-tk) plus valacyclovir (gene-mediated cytotoxic immunotherapy), autologous formalin-fixed tumor vaccine, and dendritic cell vaccination are also promising therapies.[106–108] However, tumor-mediated immunosuppression masked the effect when the residual tumor burden is large.[107,109] The EGFRvIII vaccine did not demonstrate a benefit for survival.[110,111]

4. Immune checkpoint inhibitors
    The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) immune checkpoint pathways are negative regulators of T-cell immune function.[112] Flow cytometric analysis on tumor infiltrating lymphocytes showed that, in patients with GBM, the expression of immune checkpoint molecules like PD-1 and CTLA-4 were significantly higher than that in T-cells from peripheral blood mononuclear cells (PBMCs).[113] Glioma cells evade immune surveillance by expressing PD-1, CTLA-4, and other immune checkpoint molecules that negatively regulate cytotoxic T-lymphocytes (CTL)[114,115] (109, 110) and activate regulatory T-cells (Treg), which suppresses effector T-cells.[112] Collectively, PD-1/PD-L1 inhibitors and CTLA-4 inhibitors, called immune checkpoint inhibitors, have been developed for several cancers, including GBMs.[114,116,117] Therefore, immune checkpoint inhibitors (nivolumab, pembrolizumab, and

ipilimumab) are potential novel therapeutics for GBM.[118)] Nivolumab has been assessed in a large scaled trial for recurrent GBM treatment (CheckMate-143 trial), but it did not demonstrate a remarkable effect when compared with consistent chemotherapy,[119)] though the currently randomized trials for newly diagnosed GBM (CheckMate-498, 548) are ongoing. The clinical trials for other inhibitors (NCT 02337686, RTOG 1125, etc.) are currently underway; therefore, the effect of these treatments is yet to be established. The results of these studies, and the analyses of interactions between these drugs and TMZ in GBM, are anticipated.

Potential treatment

1. Existed drugs or techniques
   Metformin (MET), the first-line drug for treating diabetes, has been proven to suppresses cell proliferation and selectively kill cancer stem cells.[120,121)] This common medicine modulated apoptosis by increasing the Bax/Bcl-2 ratio, reduced reactive oxygen species (ROS) production, and inhibited the TGF-β1-induced epithelial-mesenchymal transition-like process and stem-like properties in GBM cells via the AKT/mTOR/ZEB1 pathway.[122,123)] It is also been reported that MET sensitized TMZ cytotoxicity to GBMs when employed in combination.[124,125)] AKT inhibitors or PI3K/mTOR inhibitors also potentiate TMZ.[126,127)] MET is a potential novel therapeutic directing drug repositioning for GBM. For other drugs, glycogen synthase kinase 3β (GSK3β) inhibitors, cyclin dependent kinase (CDK) inhibitors, and other drugs used for clinical treatment of various diseases have been identified as TMZ enhancers via apoptosis or inhibition of DNA repair[56,57,128–134)] (Table 1).

2. Targeted molecules
   Mutations in ATRX are well known as the most prevalent genetic abnormalities in diffuse astrocytoma with IDH mutation. The presence of ATRX mutations in glioma implies an increased sensitivity to radiotherapy and DNA-damaging agents that primarily induce double-stranded breaks.[135,136)] Knockdown of ATRX indicates suppression of ATM (ataxia telangiectasia, mutated) dependent DNA damage repair by modulating histone H3 lysine 9 trimethylation (H3K9me3) to enhance TMZ.[137)] Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive

transcription factor reported to regulate the expression of various cytoprotective genes.[138,139)] Constitutive Nrf2 activation in many cancers enhances cell survival and resistance to anti-cancer drugs,[140,141)] including TMZ,[142)] through glutathione (GSH) synthesis, which plays a critical role in neutralizing reactive oxygen species (ROS) induced by chemotherapy.[142)] Inhibition or genetic silencing of Nrf2 sensitizes highly resistant GBM to TMZ.[142,143)]

With the identification of oncogenic microRNAs (miRs), and their critical function in tumorigenesis, sequence-specific targeting of growth promoting miRs has emerged a novel and promising therapeutic avenue.[144)] Many reports have identified miRs are an important key to regulating many cellular behavior, such as cell growth, stemness, apoptosis, and drug resistance of GBM.[145,146)] As miR-125b has been proven necessary for GSCs fission and for making stem cells insensitive to chemotherapy, inhibition of miR-125b demonstrates increased apoptosis targeting Bak1.[147)] MiR-128 is upregulated in TMZ-treated glioma cells and considered to be one of the reasons for TMZ-induced apoptotic cytotoxicity via JNK2/c-Jun signaling-mediated mTOR-inhibition.[148)] MiR-141-3p promotes glioma cell growth and TMZ resistance by directly targeting p53,[149)] indicating that inhibition of miR-141-3p would provide new therapeutic methods for GBM treatment. Moreover, miR-16 mediates TMZ resistance in glioma cells by modulation of apoptosis via targeting Bcl-2, suggesting that miR-16 and Bcl-2 could be potential therapeutic targets for glioma therapy.[150)] Introduction of miR-17, miR-21, miR-30a, and miR-101 into chemo-resistant glioma also resulted in an increase in chemosensitivity to TMZ treatment by various regulating pathways and mechanisms.[151–154)] There are several miRs reported to be overexpressed in GBM compared to normal brain tissues;[145)] therefore, there is always a potential to provide a broader view in the exploration for new anti-glioma agents. In the near future, more novel breakthroughs in the area of miRs are expected.

Future Prospective

TMZ has been used as standard chemotherapy for malignant glioma since 2005. To date, various

Table 1 Existing TMZ enhancing drugs

Classification	Common indications	Administration	Mechanism of combination with TMZ	References
GSK3β inhibitors				
Cimetidine (Tagamet)	Gastroduodenal ulcer	IV injection or oral	GSK3β inhibition	57)
Lithium (LIMAS)	Mood disorder	Oral		
Olanzapine (Zyprexa)	Mood disorder	Oral		
Valproate (Depaken)	Seizure	IV injection or oral		
PI3K inhibitors				
Dactolisib	Breast cancer (trial)	Oral	PI3K/mTOR inhibition	128, 129)
CDK inhibitors				
Flavopiridol (Aivocidib)	Acute myeloid leukemia	IV injection	Suppresses DNA repair activity in the G2/M transition	130)
Palbociclib (Ibrance)	ER(+)/HER(−) breast cancer	Oral	Inhibit CDK4/6 to influence cell cycle by inducing G1 arrest and apoptosis	131)
Abemaciclib (Verzenio)	Advanced or metastatic breast cancer			
Others				
Glucophage (metformin)	Type-2 diabetes	Oral	Akt/mTOR inhibition	120, 121, 124, 125)
Levetiracetam (Keppra)	Epilepsy	IV injection or oral	Demethylate the methylated MGMT promoter via suppressing wt-p53 MGMT suppressive function	133)
Bevacizumab (Avastin)	Colorectal cancer, lung cancer, breast cancer, brain cancer eye disease	IV injection	Blocks angiogenetic VEGF-A	134)

CDK: cyclin dependent kinase, ER: estrogen receptor, GSK3β: glycogen synthase kinase 3β, HER: human epidermal growth factor, IV: intravenous injection, MGMT: methylguanine methyltransferase, mTOR: mammalian target of rapamycin, PI3K: phosphoinositide 3-kinase, TMZ: temozolomide, VEGF: vascular endothelial growth factor.

kinds of clinical trial have been performed for GBM with novel drugs. However, no drugs exceeded the effect of TMZ. Therefore, understanding the mechanisms of TMZ resistance and identifying the novel modalities of therapy overcoming chemoresistance remains an important focus in the management of GBM patients. While the understanding of these mechanisms underlying intrinsic and acquired chemoresistance in GBM is expanding rapidly, promising therapeutic options will hopefully be discovered in the near future.

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Conflicts of Interest Disclosure

The authors declare that they have no conflict of interest. All authors who are the members of The Japan Neurosurgical Society (JNS) have registered online self-reported COI Disclosure Statement Forms through the website for JNS members.

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