Acta Poloniae Pharmaceutica – Drug Research, Vol. 79 No. 6 pp. 763-775, 2022
DOI: 10.32383/appdr/159449
Received 30 November 2022, Received in revised form 3 January 2022, Accepted 19 January 2023
ISSN 2353-5288
Polish Pharmaceutical Society

REVIEW

TEMOZOLOMIDE: A CYTOSTATIC DRUG THAT IS STILL IMPORTANT TODAY

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Abstract: TMZ has an advantage over other traditional alkylating agents (carmustine, lomustine, procarbazine), which are highly toxic and have poor patient survival. TMZ circumvents these problems because cytochrome P450 enzymes and the kidneys are not involved in its metabolism, it has predictable side effects (nausea, vomiting, thrombocytopenia, neutropenia), which are usually reversible and only mild to moderate, have been widely described. About half of the patients treated with TMZ have high drug resistance induced by the activity of O^6-methylguanine methyltransferase. Cancer stem cells (CSCs), which are found among the neoplastic cell population, have also been shown to be responsible for resistance to TMZ. Additionally, acquired immunity, induced by TMZ's epigenetic and genetic alterations, may develop. Currently, there are new therapeutic strategies for glioblastoma multiforme (GBM) based on nanotechnology, which are aimed at improving TMZ treatment (e.g. the use of apolipoprotein), or other techniques (siRNA, which increases the oxygen level in the tumor). Thus, although TMZ was discovered more than three decades ago, this drug will be used to treat not only GBM but also a large number of neoplastic pathologies. Further research focused on understanding the mechanisms of action and resistance to TMZ is required to improve its clinical application today and in the future.

Keywords: alkylating agents, drug resistance, chemotherapy, nanoparticles, cancer, glioblastoma multiforme.

Temozolomide (TMZ) (a DNA alkylating agent, nitrogen mustard derivative, dacarbazine analog, $C_6H_6N_6O_2$) was first introduced in 1999 (the first license was obtained) in the treatment for GBM, the most common malignant brain tumor. TMZ was first used in oncology as an alkylating agent (1-3).

TMZ is a small alkylating molecule, with a low molecular weight of 194.154 g/mol, which is responsible for introducing methyl groups into DNA. It is an anti-cancer dacarbazine analog that was developed by Professor Malcolm Stevens. A medical chemist, he founded a drug discovery laboratory in Great Britain less than 40 years ago in the pharmacy department at Aston University in central Birmingham. In the 1970s, a new era in the discovery of new drugs in cancer therapy began. Robert Stone joined Stevens' team in 1978 as part of his doctoral scholarship. Stevens mobilized him with one sentence: 'Make some interesting molecules.' Stone was particularly interested in a reactive heterocyclic ring (rich in nitrogen atoms). Two strands of nitrogen chemistry led to the final discovery of

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temozolomide's nucleus (imidazotetrazine ring system) (4).

By the late 1970s, anti-cancer drugs known as triazenes had been discovered (chemical formula N_3H_3; triazene H_2NNNH – molecules containing a linear chain of three nitrogen atoms) (4-5). Dacarbazine (DTIC), a triazene, was discovered and is still used in the treatment of malignant melanoma. DTIC is a prodrug that forms the *in vivo* alkylating agent MTIC (5-(3-methyl-1-triazen-1-yl) imidazole-4-carboxamide) (4). Currently, TMZ is the best cytostatic alkylating drug compared to carmustine (BCNU) and lomustine (CCNU) (6). TMZ is a cytostatic drug that is marketed as Temodar in the United States and Temodal in Europe.

The aim of this article is to review pharmacokinetics, application, contraindications, and adverse reactions of the use of a TMZ. We also summarize new alternative forms of GBM therapy.

DISCUSSION

Pharmacokinetics

The chemical structure of TMZ (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0] nona-2,7,9-triene-9-carboxamide) is shown in Figure 1 (2-3, 6-8, 11-14). TMZ (an alkylating cytostatic drug) is a pharmacologically inactive prodrug (chemical name: 3,4-dihydro-3-methyl-4-oxoimidazole), which is converted into an active metabolite in the vascular system at physiological pH and is rapidly converted to an active metabolite with anti-tumor activity (11). Its anti-tumor effect leads to the disruption of DNA replication (DNA alkalization) in the neoplastic cell. The TMZ metabolite shows an alkylating activity towards biological macromolecules, in particular towards DNA nucleic acid. Alkylation is a chemical modification that causes structural changes and fragmentation of DNA chains and, as a consequence, disturbs DNA and RNA synthesis disturbs protein synthesis, and prevents cell division. These changes ultimately lead to cell death. The action of TMZ is particularly applicable to rapidly dividing cells (where the DNA repair mechanisms are insufficient), such as neoplastic cells (12).

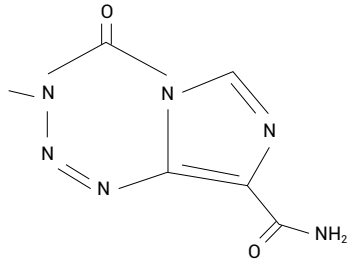

Figure 1. Temozolomide.

As a prodrug, TMZ is spontaneously cleaved hydrolytically at physiological pH to form the unstable, active metabolite MTIC. MTIC is very unstable and quickly decomposes into two products: a side metabolite – AIC (5-aminoimidazole-4-carboxamide) – and methylhydrazine (a methyldiazonium cation). Methylhydrazine as a methyldiazonium cation then methylates the bases in DNA, that is, the methyl group is added at the N^7 (70%) and O^6-guanine positions (5%) and also at the N^3-adenine position (25%) (11). Although the percentage of O^6-methyl guanine (O^6-meG) formed is small, it exerts the greatest influence on the induction of apoptosis. This is because O^6-meG is impaired with thymine in the cell cycle, instead of cytosine as is normally the case. Cells that produce an O^6-meG/T pair must go through a second cell cycle, which leads to a DNA double-strand break. If it is not repaired, the genome becomes unstable, which in turn introduces neoplastic cells to the apoptosis or autophagy pathways (12-14).

The advantage of TMZ over the older active DTIC is that activation of methylhydrazine levels is completely spontaneous. On the other hand, DTIC must be enzymatically activated. Absorption is rapid and the maximum concentration of the active ingredient is reached after about 20 minutes. The plasma half-life is 1.8 hours (15-16). TMZ can penetrate the cerebrospinal fluid and cross the blood-brain barrier (BBB), making it suitable for treating brain tumors. This drug penetrates through passive diffusion and does not require hepatic metabolism for activation. Moreover, cytochrome P450 enzymes and the kidneys are not involved in its metabolism (12, 15-17).

TMZ resistance

The BBB is nothing but a preliminary filter in the resistance mechanism of brain tumors (18). In brain tumors, a new blood-brain tumor barrier (BBTB) is additionally produced and abnormal neovascularisation occurs, leading to hypoxia and an increase in some angiogenic mediators in tumors (19-20). However, some cancer cells have a repair mechanism that makes them resistant to this active ingredient. Approximately half of the patients treated with TMZ have high drug resistance (21). Among the reasons for this is the activity of O^6-methylguanine-DNA methyltransferase (MGMT), an enzyme that removes the methyl group from O^6-methylguanine and consequently neutralizes the anti-cancer activity of TMZ. After acquiring the methyl group, methylated MGMT is

degraded by ubiquitin. Inhibition of MGMT activity increases the cytotoxicity of TMZ, and epigenetic methylation of the MGMT gene promoter blocks the repair activity of this enzyme (22-23). It was shown that primary glioblastoma-derived cells harboring a methylated promoter were more sensitive to the induction of programmed death after TMZ use than those in which no gene modification was observed. Therefore, low MGMT expression and enzyme promoter methylation are currently important diagnostic and strategic factors in the application of TMZ therapy in patients with anaplastic astrocytoma or GBM (12, 21, 23-27). Summing up, it has been shown that cancer cells showing higher MGMT activity are more resistant to the cytotoxic effect of TMZ in contrast to cells lacking MGMT activity (7, 28-30). Therefore, the methylation state of MGMT 'classifies' whether the patient should receive standard treatment (chemo- and radiotherapy) or, alternatively, radiotherapy or TMZ monotherapy (31-33). In conclusion, methylation in patients receiving combination therapy (radiotherapy and TMZ) followed by adjuvant TMZ administration is an independent prognostic factor – it increases survival time and also disease-free time (31-32, 34-37).

The mismatch repair system

Other DNA repair mechanisms include DNA mismatch repair (MMR) and base excision repair (BER). These mechanisms partially explain why TMZ-induced alkylation fails (27). MMR has three steps (38): 1. recognition and association of the mismatch through the subunits MSH2-MSH6 or MSH2-MSH3; 2. resection by EXO1 exonuclease; 3. LIG1 ligase – repair and ligation (39-40). Tolerance to TMZ increases in GBM when the MMR complex is inactivated or mutated (mismatched O^6-meG/thymine pairs are not recognized) (13, 41-42). It should be noted that the MSH6 gene is very sensitive to inactivating somatic mutations, and even its expression is inhibited after TMZ chemotherapy (43). Research by Higuchi et al. has shown that the administration of a PLK1 inhibitor (Volasertib) in patients with MMR deficiency inhibits the proliferation of glioblastoma cells (44). In contrast, Maxwell et al. (45) have found no relationship between inactivated MMR and resistance to TMZ.

Base excision repair

BER is the main measure in the repair of nucleotides damaged by alkylating agents, ionizing radiation, or reactive oxygen species (46-47). Over 90% of methylations caused by TMZ (N3-MeA and N7-MeG methylations) are quickly and efficiently repaired by the BER pathway. However, it has been proven that MGMT and MMR are more important pathways in TMZ resistance than BER (21, 42). In the BER pathway, poly (ADP-ribose) polymerase (PARP) is a very important DNA-damage signaling protein (48-49). It has been proven that the inhibition of PARP increases the frequency of DNA strand breaks, making PARP-deficient cells hypersensitive to carcinogens (49). Kinsella et al. (50) have shown that inhibition of PARP increases cytotoxicity (BER-repaired lesions), thus improving the cytotoxicity of TMZ *in vitro* and *in vivo*. Therefore, disruption of the BER pathway by PARP inhibition is a way of overcoming resistance to TMZ (51-52).

Cancer stem cells

Cancer stem cells (CSCs), which are found among the neoplastic cell population, have also been shown to be responsible for resistance to TMZ treatment. Glioblastoma stem cells (GSCs), as neoplastic cells, have the highest proliferation among GBM cells (53) and are located in a special microenvironment – the 'stem cell niche' (54). Thanks to this niche, GSCs are constantly maintained and survive, which additionally leads to the interaction with non-cancerous cells and the extracellular matrix, without recognizing the immune system of a GBM patient (55-58).

Acquired immunity

Acquired immunity may develop under the influence of chemotherapy, such as TMZ chemotherapy. This type of acquired immunity is caused by epigenetic and genetic alternations due to the increased action of the drug. This acquired immunity, which leads to numerous modulations (induction and selection of genes, DNA damage, alteration of genes related to apoptosis) leads to the selection of resistant cells, where cancer may relapse (59-60). These numerous modulations may select resistant cells, which in response will contribute to the relapse (61-62). Additionally, certain miRNAs are involved in acquired TMZ resistance, including miR-195, miR-455-3p, and miR-10a* (63), miR-1268a (64), miR-30a (65), miR-181b and miR-181c (66). These authors confirmed that miRNA can be considered a predictive marker of the effect of TMZ treatment in glioblastoma patients.

TMZ: application

1. TMZ is used in the treatment of malignant gliomas (such as GBM and anaplastic astrocytoma) showing recurrence or

progression after standard treatment. This type of therapy is used for children from three years of age, adolescents, and adult patients;

2. TMZ is used in the treatment of newly diagnosed GBM, in combination with radiotherapy, and then as monotherapy after radiotherapy is completed (in adults) (1);
3. TMZ in combination with capecitabine (CAPTEM) and the targeted radiopeptide ^{177}Lu-octreotate is applicable as multimodality therapy for advanced neuroendocrine neoplasms (NENs) – a particular option for patients with metastatic cancer (67-68). A better effect is observed in the case of gastric and pancreatic neuroendocrine tumors (NETs) compared to primary intestinal NETs (68-69);
4. This drug has been proven effective in the treatment of lung cancer, metastatic melanoma, large intestine (colon), and ovarian cancers (70);
5. TMZ is used in malignant melanoma when previous therapies have failed or have not been eligible for immunotherapy. The combination of an alkylating agent, fotemustine, and TMZ, acting as a chemomodulator, has been proposed as an alternative treatment (71).

A GBM (astroglial tumor) is one of the most aggressive tumors with a poor prognosis. The group of infiltrating brain gliomas includes astrocytomas with a diffuse growth pattern (filamentous, gemistocytic, and protoplasmic types), oligodendrogliomas, and mixed brain gliomas. The distinguishing feature of this group is the ability to extensively infiltrate the brain structures and spinal cord, and the tendency to the gradual progression of malignancy in subsequent tumor relapses. Until recently, the World Health Organization (WHO) categorized gliomas according to their histological appearance; now, molecular parameters are also taken into account (72).

An important genetic feature distinguishing most oligodendrogliomas is the loss of heterozygosity within chromosomes 1 and 19, which is associated with chemosensitivity (1, 17). Gliomas have different grades of severity that represent their potential for malignant transformation, although low-grade gliomas can develop into high-grade tumors. Grades I and II are low-grade gliomas, while grades III and IV are high-grade gliomas with increased aggressiveness (73). A grade IV GBM (grade IV glioma) is the most malignant primary brain tumor (74), which leads to extremely low median survival (14.5-16.6 months) during classical treatment (surgery, radiotherapy, and TMZ chemotherapy) (75).

Contraindications for the use of TMZ

Absolute contraindications

TMZ, as a cytostatic antineoplastic drug, is wholly contraindicated in patients who are hypersensitive to the substances contained in the drug or to dacarbazine (a methylated imidazoltriazene derivative). It is also contraindicated in galactose intolerance and the malabsorption of both galactose and glucose, and in congenital Lapp lactase deficiency. It should be noted that this drug is completely contraindicated in severe myelosuppression. It should be emphasized that cytochrome P450 enzymes and the kidneys are not involved in its metabolism and so its toxicity is moderate and usually reversible (13).

Relative contraindications

Older age and impaired renal and hepatic function in certain situations are not absolute contraindications (17). The doctor must be informed if the patient is allergic to TMZ, dacarbazine (DTIC-Dome), or any of the ingredients in TMZ capsules. They should also be informed about the consumption of dietary supplements, herbal remedies, or vitamins. It is important to let the doctor know about the use of drugs, such as steroids (dexamethasone, methylprednisolone, prednisone), antiepileptic drugs (carbamazepine, valproic acid), or sulphonamides. The doctor must be informed whether the patient had or has any kidney or liver disease. Pregnancy and breastfeeding are contraindications (6, 76).

TMZ treatment

TMZ treatment is used as monotherapy (chemotherapy) and in combination therapy (radiotherapy and chemotherapy) (77-78).

Before using TMZ, the following parameters should be considered: absolute neutrophil count (ANC) $\geq 1.5 \times 10^9$/L and platelet count $\geq 100 \times 109^9$/L. On the 22nd day (21 days after the first dose) or within 48 hours thereafter, total blood counts should be assessed weekly until the ANC is $> 1.5 \times 10^9$/L and the platelet count is $> 100 \times 10^9$/L. If the ANC drops to $< 1.0 \times 10^9$/L or the platelet count is $< 50 \times 10^9$/L during chemotherapy, the dose of TMZ must be reduced in the next cycle. Therapeutic doses of TMZ are 100 mg/m^2, 150 mg/, and 200 mg/m^2 of body surface area. The lowest dose is 100 mg/m^2 of body surface area (8, 79).

Combination therapy

Combination therapy, that is, radiotherapy and chemotherapy, is given to adults with newly diagnosed GBM. The TMZ is administered orally, usually at a dose of 75 mg/m^2 of body surface area per day for 42 days (up to 49 days) during targeted radiotherapy (60 Gy given in 30 doses). If there are abnormal blood test results and the patient is intolerant to the drug during combination therapy, the TMZ therapy may be delayed or discontinued. If no complications have occurred after the completion of radio- and chemotherapy, the therapy is interrupted for four weeks, followed by monotherapy (7, 80). During combination therapy and monotherapy, prophylactic treatment is used – antiemetics (metoclopramide or 5-hydroxytryptamine) (6).

TMZ combination therapy is performed under hematological (neutrophilic granulocytes, platelets) and non-hematological (except for alopecia, nausea, and vomiting) control. TMZ therapy is continued when the total neutrophil count is $\geq 1.5 \times 10^9$/L and the platelet count is $\geq 100 \times 10^9$/L. If the neutrophil count ≥ 0.5 and $< 1.5 \times 10^9$/L and the platelet count ≥ 10 and $< 100 \times 10^9$/L, the therapy is temporarily interrupted and the body is potentiated by, for example, leukomax (granulocyte-macrophage colony-stimulating factor). However, it should be discontinued when the total neutrophil count is $< 0.5 \times 10^9$/L and the platelet count is $< 10 \times 10^9$/l (Common Toxicity Criteria) (7-8, 79-81).

Monotherapy

Four weeks (28 days) after the end of combination therapy with TMZ and radiotherapy, TMZ monotherapy begins with up to six cycles (in exceptional cases, up to 12 cycles). Each cycle is 28 days long, and a new dose of TMZ is taken once daily for the first five days of each cycle. The initial dose is 150 mg/m² of body surface area. Thereafter, the patient will not receive chemotherapy and so the next (second) treatment cycle will follow after the 28th day. When the patient has not received chemotherapy before, the initial dose of TMZ is 200 mg/m² of body surface area once daily for the first five days. Before starting each new cycle, the doctor will perform a blood test to see if the TMZ dose needs to be adjusted. Depending on the results of the blood test, the attending physician may adjust the dose before starting each new cycle (82).

Depending on the blood test results and on how well the patient tolerates the drug in each treatment cycle, the doctor may adjust the dose, delay or discontinue the drug application. TMZ is available in doses of 5 mg, 20 mg, 100 mg, 140 mg, 180 mg, or 250 mg in a bottle or in sachets (17, 79, 82-83).

Modification of TMZ therapy

Recently, the effectiveness and benefits of combining TMZ with trans sodium crocetinate (TSC) (84) or tumor-treating fields (TTFields) (7) have been proven. The addition of TSC (a drug increasing oxygen supply) to classic treatment (TMZ with radiotherapy) showed an increased survival after two years (84). Research by Stupp et al. (8) also showed a life extension of about three months in patients treated with TMZ plus TTFields compared to treatment with TMZ alone. Similar benefits have been demonstrated when using bevacizumab with chemotherapy (TMZ) and radiotherapy in GBM patients (85-86). The hypermethylated MGMT promoter is currently being investigated as a prognostic biomarker (2-3, 87-88).

In recent years, the effectiveness and safety of Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) have been proven in both classical therapy and in the case of GBM recurrence. Gupta et al. (89) showed that THC/CBD therapy contributed to a significant increase in survival (83% – the experimental group and 56% – the placebo group/year).

Biomarkers as a response to the effects of TMZ

While the methylation status of MGMT has long been considered a biomarker that helps predict the response to TMZ in gliomas, other new biomarkers have recently been discovered, such as a variant of the epidermal growth factor receptor (EGFR) (C11), MSH2, and MSH6 (29-30, 90-93). EGFR amplification and overexpression have been implicated as prognostic and predictive biomarkers (94). RAS or BRAF mutations have also been recognized as potential prognostic biomarkers for response to TMZ treatment (95-96), while Calegari et al. did not observe any significant mutations (96).

In fact, some microRNAs (98) are correlated with MGMT expression, such as the enhancer gene located between the proliferation marker Ki-67 (MKI67) and the MGMT promoters (99). In addition, other DNA repairs systems such as MMR, BRCA2, and XRCC2 may play an important role in repairing damage caused by alkylation (100). Moreover, resistance to apoptosis may reduce the efficacy of TMZ as TMZ-related apoptosis inducers have synergistic effects in melanoma (101).

Some studies showed the important role of cystatin A (CSTA) overexpression. It was associated with ensuing resistance to any treatment including

TMZ, and also the combination of TMZ with nitrosoureas. CSTA plays a main role in the growth and expansion of brain tumor cells. CSTA is a regulator of proteolytic enzymes and cysteine cathepsins and its overexpression may signal an inflammatory tumor environment and promote leukotrienes synthesis and metabolism. CSTA expression displayed an important correlation with markers of invasive/migratory GBMs, CD68 and CXCR4. Excessive expression of CSTA has been previously identified as a significant prognostic factor of shorter survival in gliomas. It suggests the role of inflammation in therapy resistance, because leukotrienes are crucial immune modulators of leukocyte migrations, and are involved in numerous inflammatory disturbances, together with cancer (102-104). Another side, increased Fc Fragment of IgG Receptor IIb (FCGR2B) expression as a section of a local immune signature has been already associated with high-risk GBMs (105).

One of the research indicated that 1H magnetic resonance spectroscopy (MRS)-detectable glutamate/glutamine/ GLX can be used as early biomarkers of TMZ response in mutant isocitrate dehydrogenase 1 (IDH1) models, and 13C MRS-detectable production of hyperpolarized [1-13C] glutamate and [5-13C]glutamate from hyperpolarized [1-13C]α-KG and [2-13C]pyruvate, respectively, have potential as supportive approaches to monitor response. Overall, these metabolic imaging biomarkers could help improve presently available imaging methods and supply an early indication of response to TMZ treatment in lower-grade mutant IDH1 glioma (106).

Pasqualetti et al. described a potential new biomarker to predict the outcomes of up-front therapy of elderly glioblastoma patients. All of these patients were above 65 y.o. They had the examination of thickness temporal muscle (TMT) measurement. Patients in the lower quartile of TMT, with TMT thinner than 7 mm, exist longer (107).

During the therapy of glioblastoma, periodic assessment of the stage of the disease plays an extremely important role. Imaging follow-up allows not only assessing response to the treatment but also modifying the therapy strategy. One research showed the place of the first magnetic resonance (MRI) following radio-chemotherapy (RT-CT) in patients diagnosed with high-grade glioma (HGG). Near 10% of asymptomatic patients were given the diagnosis of disease recurrence at the time of three consecutive MRIs. It is worth noting that half of the symptomatic patients changed their treatment regimen after MRI (108).

Fractionated stereotactic radiotherapy (FSRT) plus concomitant TMZ is a safe and effective treatment option associated with survival benefits and low risk of complications in selected patients with recurrent GBM. Positron emission tomography (PET) / single photon emission computed tomography (SPECT) / computed tomography (CT) / MRI imaging compared with treatment planning using only CT/MRI may indicate on better quality treatment base. This is evidenced by higher median survival time (11 months for patients receiving FSRT based on biologic imaging plus TMZ vs. 6 months for patients who got FSRT without biologic imaging, without TMZ, or both) (109-110).

One of the most hopeful developments in translational cancer medicine has been the emergence of circulating tumor cells (CTC) as a minimally invasive universal biomarker. CTCs are involved in the process of hematogenous metastatic spread to distant places. Krol et al. suggest circulating glioblastoma clusters can overcome the blood-brain barrier and can be detected in the peripheral circulation. Exome sequencing of GBM CTC clusters accents variants in 58 cancer-associated genes including ATM, PMS2, POLE, APC, XPO1, TFRC, JAK2, ERBB4, and ALK. This may be one of the new preferences for monitoring glioblastoma treatment in the future, including with the use of TMZ (111).

Nanotechnology, other techniques, TMZ analogs, and future prospects

Nanomedicine

Currently, there are new therapeutic strategies for GBM based on nanomedicine (2-3). The aim is to improve the TZM therapy, for example, by using apolipoprotein E (to functionalize nanoparticles {NPs} and facilitate their passage through the BBB) (112). Other agents have been used to promote the passage through the BBB, such as BBB glucose transporters, which promote NP transcytosis, and the transferrin receptor, which is overexpressed in brain tumors due to the high metabolic requirements of cancer cells (78, 113-115).

Folic acid is another ligand used to functionalize NPs loaded with TMZ. An example is magnetite NPs used in combined hyperthermia and chemotherapy with TMZ, stimulating an increase in temperature to release TMZ (116-117).

TMZ treatment can be difficult to implement, even in the case of nanomedicine. This is particularly true of poly(lactic-co-glycolic acid) (PLGA) NPs, which are suitable for transporting TMZ since GBM cells require high levels of this drug to obtain

an adequate cytotoxic effect, but PLGA NPs cannot transport large amounts of the drug (and therefore may not achieve the desired therapeutic effect) (118). Ramaho et al. demonstrated good performance of PLGA NPs in TMZ transport, possibly due to the addition of a functionalizing agent (119).

Other techniques (siRNA, hypoxia)

Among other techniques is silencing MGMT expression (120). For this purpose, NPs of iron oxide functionalized with the chlorotoxin peptide were loaded with siRNA (121). In addition, the p53 protein was used, which acts by regulating MGMT (122-123).

GBM has multiple zones of hypoxia (124) and, therefore, increasing the oxygen level in the tumor may enhance the anti-tumor effects of TMZ, as is the case with hyperbaric oxygen (HBO) therapy (125). Moreover, hypoxic tumors are resistant to radiotherapy because the production of reactive oxygen species is the main cause of deaths induced by this treatment (126).

TMZ analogs

Rai et al. (127) generated the 8-(N,N-dimethylcarboxamide) synthesizing TMZ analogs (γ-carbolines and β-carbolines series). These analogs showed better DNA alkylation activity and an improvement in the brain/plasma ratio (up to 30-fold) compared to TMZ. However, this anti-tumor activity was not observed in *in vivo* studies. TMZ analogs produced by N3-methyl substitution with propargyl or sulfoxide showed high anti-tumor activity in MGMT-positive GBM cells (39, 128). TMZ analogs: C8-imidazolyl and C8-methylimidazole tetrazine also showed better anti-tumor activity in MGMT-positive GBM cells and MMR-deficient colon cancer cells (129). Nanoformulation – N3-propargylimidazotetrazine analog bound to the targeted liposomal nanocarrier – overcame TMZ resistance in GBM cells (130), and the TMZ analog NEO212, a covalent conjugation of TMZ and perillyl alcohol (POH), increased anti-tumor activity in nasopharyngeal carcinoma (NPC) cells compared to TMZ. Moreover, this analog made cells more sensitive to the second cycle of drug treatment by inactivating MGMT (131).

Future prospects

Despite a large number of preclinical studies (*in vitro* and *in vivo*), nanopreparations loaded with TMZ have not yet been introduced into clinical trials. Consequently, there is much more to be discovered in this field of the use of nanocarriers. Clinical trials are ongoing in which TMZ is administered together with other nanoformulas, usually in combination with other drugs. For example, patients with relapsed GBM are treated with a combination therapy of oral TMZ and SGT-53, a cationic liposome carrying a plasmid with a p53 DNA sequence that can induce apoptosis in a cancer cell. TMZ has also been studied in combination with Doxorubicin-loaded PEGylated liposomes in patients with relapsing GBM. The results of these studies are no better than chemotherapy (TMZ) with radiotherapy (132).

Adverse effects of TMZ

TMZ is an embryotoxic, teratogenic, and genotoxic alkylating agent. It has been shown that radiotherapy and chemotherapy (42 days of therapy) led to the occurrence of opportunistic infections associated with lymphocyte depletion, including lung infections caused by Pneumocystis jirovecii pneumonia, formerly known as *Pneumocystis carinii*, and caused reactivation of infections (including cytomegalovirus). It should be noted that TMZ therapy is a high risk, for example, in patients with immunological diseases such as HIV (6, 79). During TMZ treatment, cases of the reactivation of hepatitis caused by the hepatitis B virus, including fatal cases, have been reported (133). It should be remembered that during TMZ treatment, liver dysfunction (increased bilirubin, increased liver enzymes, hepatitis) may occur. The hepatotoxic effects of TMZ may even occur several weeks or months after the end of therapy (79).

The most undesirable effects are thrombocytopenia and neutropenia (6). There is a risk of lymphocytopenia/neutropenia, sometimes severe, and other hematological disorders (such as thrombocytopenia, and anemia) (134). Thrombocytopenia may increase the risk of bleeding, and neutropenia or leukopenia may increase the risk of infections (135-136). The affected cells are mainly CD4 T lymphocytes, followed by B lymphocytes, and then there is an increased production of cytokines, such as interleukin 7 or 15 (137). To counteract lymphocytopenia, one study performed adoptive lymphocyte transfer after treatment, but the results were poor because the lymphocyte counts did not increase, indicating a long-term effect of TMZ on the lymphocyte population (138). Skin pruritus, dryness, irritation, reddening, rash, and alopecia are common during TMZ treatment, and toxic epidermal necrolysis or Stevens-Johnson necrosis may very rarely occur (139-141). The use of TMZ is associated with a high risk of side effects for the nervous

system: aphasia/dysphasia, convulsions, headaches and dizziness, consciousness disorders, speech disorders, memory disorders, somnolence, confusion, ataxia, coordination disorders, hemiparesis and peripheral neuropathy (79). During chemotherapy, partial loss of vision or even, in extreme cases, hearing loss, may occur (79). An additional endocrine symptom may be diabetes insipidus (polyuria, a feeling of thirst), which may lead to dehydration and dyselectrolytemia, and symptoms resembling Cushing's syndrome (79). The detailed adverse effects of TMZ are detailed in Table 1 below (6, 79, 133-134).

New alternative methods of glioblastoma therapy

A brain glioblastoma is a tumor of the central nervous system (CNS) (brain and spinal cord). It is the most invasive primary tumor of the brain and it is the most common primary malignant brain tumor with an incidence of more than 3 out of 100,000 (32). Conventional treatment options for brain tumors

Table 1. Adverse effects of TMZ

Target organ	Side effects
Skin and hair	– itching, dry, irritated, or reddened skin, increased sensitivity to sunlight, hair loss, increased sweating, skin eruption (on the body or in the mouth); – severe rash with swelling of the skin (including on hands and feet), peeling skin, rash, allergic reactions, hives, and shingles; – severe skin reactions: Stevens-Johnson syndrome, toxic epidermal necrolysis.
Digestive tract	– inflammation of the mouth (fungal infections), mouth pain, discoloration of the tongue, change in taste, tooth infections, dyspeptic symptoms (abdominal pain, nausea, vomiting, heartburn, loose stools/diarrhea or constipation), hemorrhoids, loss of appetite, anorexia, weight loss or increased thirst, weight gain and increased blood glucose levels, hyperglycemia; – hepatotoxicity including increased bilirubin (cholestasis), increased liver enzymes, hepatitis, and liver damage including liver failure; – viral diseases: 1. hepatitis B virus (reactivation of the infection), 2. cytomegalovirus (CMV) from the Herpesviridae family.
Respiratory system	– shortness of breath, cough, stuffy nose, cold, flu, sinusitis, bronchitis; – Pneumocystis jirovecii pneumonia and, in severe cases, pulmonary fibrosis and respiratory failure.
Hematological system	– leukopenia, neutropenia, thrombocytopenia, thrombocytopenia, anemia; – myelodysplastic syndrome, myeloid leukemia, sepsis.
Peripheral and central nervous systems	– convulsions, seizure, stroke, headache and dizziness, consciousness disorders, speech disorders, memory disturbances (forgetfulness), disorientation, confusion, impaired coordination, paresis; – peripheral neuropathy, visual disturbances, hearing disturbances, a false sense of spinning, difficulty with balance; – herpetic meningitis, anxiety, depression, emotional changes, difficulty sleeping (sleepiness or insomnia), difficulty concentrating.
Eyes	– partial loss of vision, dryness, nystagmus, or eye pain.
Auditory system	– hearing impairment, ringing in the ears, ear pain or discomfort (caused by loud noises), hearing loss, middle ear infection, false sense of spinning.
Cardiovascular system	– palpitations (arrhythmia), hypertension, peripheral edema.
Osteoarticular system	– muscle and joint damage (back pain, joint pain).
Endocrine system	– Cushing's like syndrome; – diabetes insipidus (frequent urination, feeling thirsty).
Urinary system	– pain when urinating, impaired urinary concentrating ability, and the formation of diabetes insipidus (polyuria).
Sexual system	– teratogenic (women) and genotoxic (women and men) effects; in men – fertility disorders, even irreversible infertility; – vaginal bleeding, vaginitis, amenorrhoea, oligomenorrhoea or menorrhagia or dysmenorrhoea, breast pain, sexual impotence.

include surgical resection, radiotherapy, and/or chemotherapy – the choice of treatment options depends on the size of the tumor, its location, and the pathological diagnosis (1). TMZ is the most frequently used cytostatic agent in anaplastic astrocytoma and glioblastoma (142).

As a DNA alkylating agent, TMZ is distinguished by the fact that it is an FDA-approved anti-cancer drug for the first-line treatment of GBM. However, treating GBM remains a challenge. This is attributed to the toxic nature of TMZ, its severe side effects, and its rapid degradation *in vivo* (143-145).

Anaplastic astrocytoma (AA, III°) and GBM (GBM, IV°) are the most malignant tumors of the CNS. The prognosis is very bad in the case of untreated neoplasms; survival ranges from nine months to five years depending on the patient's age and the degree of malignancy. The malignancy and high mortality of these tumors and their resistance to standard treatment are attributed to overexpression of the intracellular survival signaling pathways PI3K-Akt/PKB-mTOR, Ras-Raf-MEK-ERK, and PLCγ1-PKC, which are regulated by the TrkB receptor (145-146).

A recent study has shown that the combined application of PI3K, Raf, PLCγ1, and TrkB inhibitors in combination with TMZ may be of practical importance and constitute the basis for further research on their use in sensitizing cells of AA and GBM to the induction of apoptosis. Research using AA (MOGGCCM) and GBM (T98G) cells has shown that LY294002, sorafenib, TMZ, U-73122, and LOXO-101 successfully eliminated cancer cells by programmed death (147).

Research on cytostatic drugs, including TMZ, is usually conducted on commercial human glioma cell lines: AA – MOGGCCM (grade 3 malignancy according to the WHO) (The European Collection of Authenticated Cell Cultures {ECACC}) (148) and GBM – T98G (grade 4 malignancy according to the WHO) (American Type Culture Collection {ATCC}) (149).

Apart from TMZ, triple combination therapy is used in chemotherapy for gliomas including lomustine, procarbazine, and vincristine. Currently, there are also new therapy strategies, such as the use of monoclonal antibodies (for example, bevacizumab and cetuximab), protein kinase inhibitors (for example, enzastaurin), tyrosine kinase inhibitors (vatalanib and vandetanib), integrin inhibitors (for example, cilengitide) or mTOR inhibitors (temsirolim and everolim) (150).

Gliomas are also very resistant to pharmacological treatment. Therefore, new, more effective therapies are being sought that will eliminate neoplastic cells by programmed death (apoptosis, autophagy) and limit their migration (147).

Currently, nanotemozolomide is being tested and a new branch of medicine, nanomedicine, is being developed. The use of nanocarriers in glioblastoma therapy is promising. Polymer nanoparticles have a polymer coating to protect the drug from early degradation. As a result, nanotemozolomide is released continuously. This drug may also act in a targeted manner through surface modification (the attachment of specific ligands, peptides, antibodies, and so on) (143). In order to improve the resistance of glioma to TMZ, an angiopep-2 (A2) modified nanoprodrug of polytemozolomide (P(TMZ)n) that combines with MGMT siRNA (siMGMT) targeting MGMT was developed (A2/T/D/siMGMT). It escalated the quantity of TMZ inside tumor lesion, and also reduced MGMT expression in glioma. The in vitro investigations indicated that the A2/T/D/siMGMT successfully amplify the cellular uptake of TMZ and siMGMT, and resulted in considerable cell apoptosis and cytotoxicity in the glioma cells (151).

During the TMZ treatment of malignant tumors of the CNS, a new alternative form of therapy with oncolytic viruses has recently been used. The main advantage of oncolytic viruses is that they show a synergistic effect, have much fewer side effects, and increase the effectiveness of treatment. Therapy with oncolytic viruses is an effective and very safe method. They only act on cancer cells, and when they spread too much, they are naturally removed by the cells of the immune system. Observing the development of medicine, there is a good chance that treatment with oncolytic viruses can replace standard methods of treatment (152–155). Montemurro et al. found that extent of resection (EOR), female sex, progression-free survival (PFS), and adjuvant chemotherapy after the second surgery were associated with longer survival. These outcomes hold up the role of maximal EOR in patients with recurrent GBM (156).

CONCLUSIONS

TMZ is an oral alkylating agent and it is a first-line drug in combination therapy (plus radiotherapy) of GBM. TMZ crosses the BBB and therefore acts directly on the activity of the CNS. It is well tolerated, making it a suitable candidate for combination chemotherapy. However, approximately

half of GBM patients are resistant to temozolomide because of their MGMT DNA repair system. A lot of clinical data show that reducing the expression of MGMT may enhance the chemotherapeutic efficacy of TMZ. Currently, TMZ has an advantage over other traditional alkylating agents (carmustine, lomustine, procarbazine, or vincristine) which are highly toxic and have low patient survival. In addition, these agents are highly toxic (mainly causing myelosuppression and respiratory changes), limiting their use and even stopping treatment. TMZ circumvents these problems because cytochrome P450 enzymes and the kidney are not involved in its metabolism, has predictable side effects, and its toxic effects (fatigue, nausea, vomiting, thrombocytopenia, and neutropenia), which are usually reversible and only mild to moderate, have been widely described in our publication. Nowadays there are new nanotechnology-based GBM therapeutic strategies that aim to improve the treatment of TMZ (e.g., the use of apolipoprotein) or other techniques (siRNA increasing the oxygen level in the tumor). So, while TMZ was discovered more than three decades ago, the drug is still gaining attention.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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