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► **To cite this version:**

Khaled Messaoudi, Anne Clavreul, Frédéric Lagarce. Toward an effective strategy in glioblastoma treatment. Part I: resistance mechanisms and strategies to overcome resistance of glioblastoma to temozolomide.. Drug Discovery Today, 2015, 20 (7), pp.899-905. 10.1016/j.drudis.2015.02.011 . hal-01392441

HAL Id: hal-01392441

<https://hal.science/hal-01392441v1>

Submitted on 7 Sep 2021

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Toward an effective strategy in glioblastoma treatment. Part I: resistance mechanisms and strategies to overcome resistance of glioblastoma to temozolomide

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Glioblastoma multiforme (GBM) is a devastating disease and the most lethal of adult brain tumors. Treatment is based on surgery, radiotherapy and chemotherapy by oral temozolomide (TMZ), which is the most potent chemotherapy agent for the treatment of GBM. Despite TMZ efficiency, the prognosis of these tumors remains poor. This is because of inherent or acquired resistance of glioma tumor cells to TMZ. This resistance is caused by DNA repair enzyme activity, overexpression of epidermal growth factor receptor (EGFR), galectin-1, murine double minute 2 (Mdm2), p53 and phosphatase and tensin homolog (PTEN) mutations. Many strategies to overcome this resistance have been developed. In this review, we will describe the main mechanisms of GBM resistance to TMZ and different strategies developed to reverse the phenotype of these tumor cells. Finally, we will discuss the drawbacks and limitations of these strategies.

Introduction

Glioblastoma multiforme (GBM) is the most common and the most aggressive primary brain tumor in adults [1]. Median survival is 14.6 months [2] and the percentage of patients living for five years or more is less than 10% [3]. Despite the advances made during the past few years in brain tumor therapy, the prognosis of this disease has not improved as a result of tumor resistance. Current therapy includes surgical intervention, radiotherapy and chemotherapy with temozolomide (TMZ) [4]. TMZ is used at the beginning of GBM treatment at the daily dose of 75 mg/m² body surface area in association with radiotherapy for six weeks followed by six cycles of TMZ alone at the dose of 150–200 mg/m² for five days every 28 days. With this combination (TMZ plus radiotherapy), the median survival reached 14.6 months compared with 12 months for patients treated by radiotherapy alone [4]. The efficacy of TMZ for treating GBM is thus limited; this is partly because of the high levels of activity of O⁶-methylguanine DNA methyltransferase (MGMT) DNA repair in tumor cells, which reduces the effect of this alkylating agent and leads to a resistant

phenotype. Unfortunately, other mechanisms contribute significantly to the resistance to TMZ such as overexpression of epidermal growth factor receptor (EGFR), galectin-1, murine double minute 2 (Mdm2) and p53 gene mutation. To reduce acquired resistance, different therapeutic molecules have been developed, for example, O⁶-benzyl-guanine which inhibits MGMT, tyrosine kinase inhibitors that act on EGFR, nutlin-3 which inhibits Mdm2, which contributes to the restoration of p53 activity. Despite their effectiveness, these molecules do not seem to be able to cure GBMs. However, the considerable development of RNA interference (RNAi)-based therapy seems to give hope to patients with GBMs. In this review, the main mechanisms of resistance of GBMs to TMZ and strategies that have been developed to overcome this resistance are described. Finally, we provide a critical evaluation of future prospects and challenges for treatment of GBMs.

Structure and mechanism of action of temozolomide

TMZ is an alkylating agent from the imidazotetrazine family which is stable only at acidic pH [5]. This prodrug undergoes rapid chemical conversion in the systemic circulation at physiological pH to the active compound 3-methyl-(triazene-1-yl)

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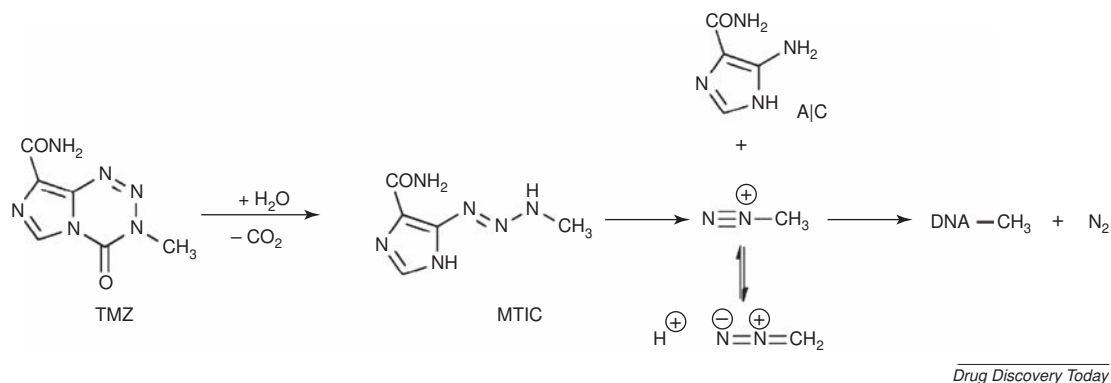


FIGURE 1
Structure and activation of temozolomide (TMZ) and production of active compound imidazole-4-carboxamide (MTIC) [5].

imidazole-4-carboxamide (MTIC), which will react with water to produce 5-aminoimidazole-4-carboxamide (AIC) and the highly reactive methyl diazonium cation (half life = 0.4 s). This unstable cation transfers a methyl group to DNA causing the cytotoxic effect of TMZ [6] (Fig. 1). The methylation occurs at purine bases of DNA at the O^6 and N^7 positions of guanine and the N^3 position of adenine. The O^6 methylation accounts for 5% of the total lesions caused by TMZ in DNA but it is the main cause of the TMZ cytotoxicity [7]. The N^7 and N^3 methylations are very frequent and represent 80–85% and 8–18% of total alkylations, respectively [8].

Mechanisms of GBM resistance to TMZ

Through time, GBM cells become resistant to damages caused by TMZ. This resistance is related to the implementation of several mechanisms such as DNA repair mechanisms, the overexpression of EGFR, galectin-1 and Mdm2 and the mutation of p53 and phosphatase and tensin homolog (PTEN). In addition, certain microRNA (miRNA) expression profiles are altered in GBM with overexpression of some and a significant reduction in the production of others.

DNA repair mechanisms

MGMT

The main mechanism of GBM resistance involves MGMT DNA enzyme repair. MGMT is a 22 kDa protein, capable of removing not only the methyl groups attached to the O^6 position in guanines but also other alkyl groups such as ethyl, isopropyl and butyl [9]. However, MGMT removes methyl groups much faster than other alkyls [9]. It allows a direct repair of the lesion caused by TMZ by removing the methyl group in position O^6 of guanine [10] (Fig. 2). It acts as a suicide enzyme because the fixing of the methyl group on cysteine residue 145 located in its catalytic pocket causes its inactivation [11] (Fig. 2). MGMT is not recycled and undergoes degradation by the proteasome [12]. The levels of MGMT vary widely according to the type of tumor and also within the same type of tumor [13]. The MGMT gene is not commonly mutated or deleted [13]. MGMT expression is correlated to the methylation profile of the MGMT promoter. Some studies have shown that the level of MGMT is inversely related to the density of the cysteine-phosphate-guanine (CpG) methylation in CpG islands [14].

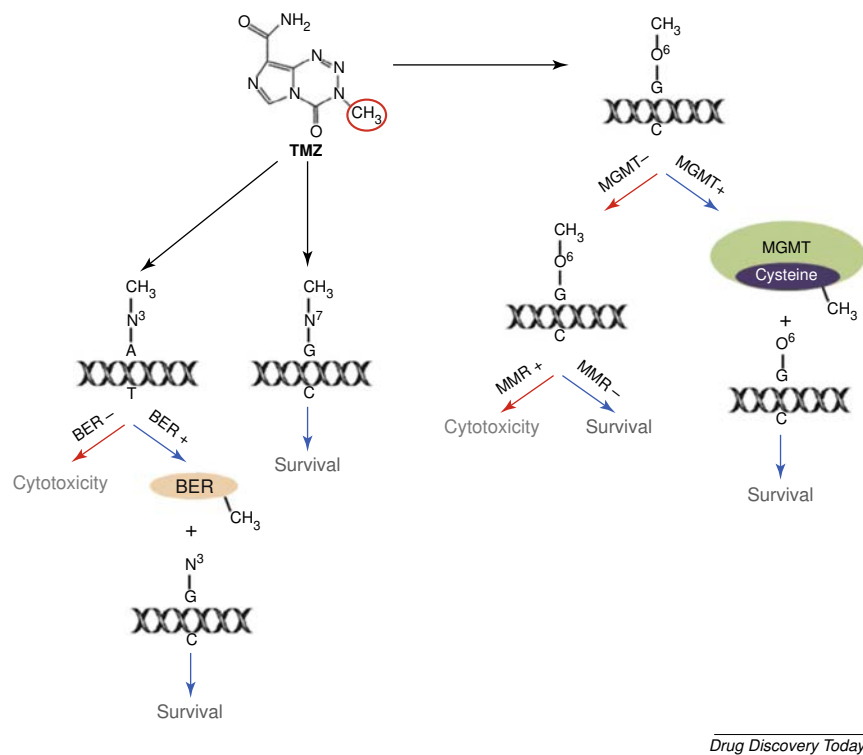
MGMT gene silencing is ensured by hypermethylation of promoter CpG islands [15]. Approximately, 45% of patients with newly diagnosed GBM present a methylation of the MGMT promoter and, thus, respond better to TMZ [16]. Therapeutic molecules that inhibit MGMT such as O^6 -benzyl guanine (O^6 -BG) and O^6 -(4-bromothienyl) guanine have been used in clinical trials preceding treatment with TMZ [8,17,18] (Table 1). These pseudosubstrates gave promising results by enhancing TMZ activity *in vitro* and *in vivo* in tumor cells possessing high levels of MGMT. They react with MGMT by covalent transfer of the benzyl group or bromothienyl group to the active cysteine residue of MGMT and cause an irreversible inactivation of this enzyme. Despite the effectiveness of the association of these pseudosubstrates with TMZ, their high toxicity against normal cells especially bone marrow cells is an obstacle to the use of these molecules [19].

MMR alteration

DNA mismatch repair (MMR) is a system that corrects errors of nucleotide base mismatches generated during DNA synthesis [8]. In the absence of MGMT, the O^6 -MG persists and it can pair with thymine. The resulting O^6 -MG/T is recognized by MMR and only newly synthesized strands are excised, keeping O^6 -MG intact. This repair cycle is repeated when another strand is generated. These futile cycles of insertion and excision of thymine occur and lead to cell cycle arrest and apoptosis [8,20]. An impaired MMR pathway caused by mutations in MMR protein complexes causes a failure to recognize and repair O^6 -MG adducts produced by TMZ. This causes DNA replication and allows the cell cycle to continue and thereby makes TMZ less effective [21]. These mutations can be present naturally or acquired by TMZ regimens [22]. Because of its importance, strategies to restore the effect of the MMR system should be developed to improve the effect of TMZ.

BER

This system is involved in the repair of DNA damage caused by oxidizing agents, ionizing radiation or alkylating agents [23,24]. The base excision repair (BER) system comprises proteins and enzymes. Among them, poly(ADP-ribose) polymerase-1 (PARP-1) is an enzyme activated in response to DNA damage and plays an important part in the BER system. This enzyme binds to DNA and begins the synthesis of a poly(ADP-ribose) (PAR) from NAD⁺, which enables the recruitment of BER complex proteins (XRCC1, DNA polymerase, ligase, flap endonuclease 1) and the DNA repair [25].



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FIGURE 2

Temozolomide (TMZ) interactions with DNA repair mechanisms. Abbreviations: MGMT, O^6 -methylguanine DNA methyltransferase; MMR, DNA mismatch repair; BER, base excision repair.

The BER pathway is involved in repair of N^3 and N^7 methylations caused by TMZ [8,20,26]. The N^3 lesions are lethal if not repaired contrary to N^7 lesions [8,27]. These methylations that represent more than 90% methylation caused by TMZ are rapidly repaired by BER. However, TMZ becomes toxic when one or more components of BER have mutations. Thus, the development of PARP inhibitors, limiting BER activity, seems to be a promising strategy for enhancing the effect of TMZ [28]. However, despite the high rate of methylation at N^7 and N^3 compared with O^6 methylation, the role of BER in TMZ resistance is less important than MMR or MGMT.

Overexpression of EGFR and galectin-1

EGFR

EGFR (170 kDa) is one of the receptors that plays an important part in tumor development by stimulating cell proliferation, migration, angiogenesis and cell resistance to chemotherapy [29]. Ligand binding to wild-type EGFR will result in the activation of one of the following signaling pathways: Ras/Raf/MAPK (mitogen-activated kinases) [30] or PI3K/AKT/mTOR [31]. These signaling pathways are activated strongly in GBMs, causing inhibition of cell autophagy and apoptosis responsible for the decrease of TMZ efficiency [32] (Fig. 3).

EGFR gene amplification is the most frequent genetic alteration in primary GBMs with frequency of 40% [33] and approximately half of these tumors carry the rearranged EGFR gene resulting in tumors expressing wild-type EGFR as well as a mutated EGFR [34]. EGFR variant III (EGFRvIII) is the most common variant of EGFR in GBMs, it is characterized by a deletion of 267 amino acids in the

extracellular domain, which leads to the formation of a receptor unable to bind a ligand and which has a constitutive tyrosine kinase activity.

Therapeutic molecules have been developed to inhibit the EGFR signaling pathway. For example, Cetuximab[®], a monoclonal antibody binding specifically to EGFR, inhibits downstream signal transduction pathways [35] (Table 1). Cetuximab[®] is able to recognize the wild-type EGFR and EGFRvIII leading to an inhibition of cell proliferation *in vitro*. However, its use in the clinic gave disappointing results with a median survival of five months [36]. Currently, a Phase I/II clinical trial is ongoing to study the efficacy of combining radiotherapy, TMZ and Cetuximab[®] for treatment of patients with primary GBM [37]. Gefitinib[®] and Erlotinib[®] which are tyrosine kinase inhibitors (TKIs) are other therapeutic molecules used to inhibit EGFR signaling pathways (Table 1). They act by binding to the cytoplasmic ATP pocket domain blocking EGFR phosphorylations and, thereby, EGFR-mediated activation of downstream pathways [38]. These molecules have been tested in clinical trials (Phase I and II) in patients with GBM [39,40]. Unfortunately, disappointing results were obtained. Indeed, the use of Gefitinib[®] or Erlotinib[®] in association to radiotherapy has shown a modest improvement in patient survival [41,42]. The associations of TKIs and TMZ have been evaluated and showed an improvement of median survival compared with TMZ associated to radiotherapy but only a small subpopulation experienced sustainable disease control. Indeed, the response of GBM patients was correlated with the presence of intact PTEN [36]. In addition, patients with MGMT promoter methylation and intact PTEN had a significant survival advantage [40].

TABLE 1

Different strategies tested *in vitro* and in preclinical and clinical trials for inhibition of molecules involved in TMZ resistance in GBM

Molecule	Target	Mechanism of action	Effect	Limitations to use in clinic	Refs
O⁶-Benzyl guanine (O⁶-BG) and O⁶-(4-bromothienyl) guanine	MGMT	Irreversible inactivation of MGMT by covalent link of benzyl or bromothienyl groups to the active cysteine residue of MGMT	Enhancing TMZ activity <i>in vitro</i> and in animal models on tumor cells possessing high levels of MGMT	High toxicity against normal cells especially bone marrow cells	[8,17–19]
Cetuximab[®]	EGFR	Monoclonal antibody binding specifically to EGFR	Specific recognition of the wild-type EGFR and EGFRvIII <i>in vitro</i> which causes an inhibition of cell proliferation	Disappointing results in the clinic	[36]
Gefitinib[®] and Erlotinib[®]		TKIs	Association of TKIs and TMZ showed an improvement of survival median compared with the combination TMZ + radiotherapy	In clinical trials: effective only in subpopulation of patients with intact PTEN and MGMT promoter methylation	[36,40]
Nutlin-3	Mdm2	Binding to Mdm2 and activation of the p53 pathway	Activation of the p53 pathway in cancer cells, leading to cell cycle arrest, apoptosis and growth inhibition of human tumor xenografts in nude mice	Effective only in cancer cells expressing wild-type p53 Potential toxic effect by p53 accumulation	[61,64,65]
Synthetic lactulose amines	Galectin-1	β-Galactoside which has an affinity for the CRD of Galectin-1	Tumor cell apoptosis and suppression of tumor growth and metastasis	Potential gastrointestinal side-effects	[53,54,80]
Galactomannan Davanat[®]		Binds Galectin-1 at a site distinct from the CRD	Davanat [®] increased the longevity of patients with colorectal cancer by 46% compared with the best standard of care, and reduced the serious adverse effects by 41% compared with the best standard of care	Not yet tested in GBM in clinical trials	

Abbreviations: CRD, carbohydrate recognition domain; EGFR, epidermal growth factor receptor; GBM, glioblastoma multiforme; Mdm2, murine double minute 2; MGMT, O⁶-methylguanine DNA methyltransferase; PTEN, phosphatase and tensin homolog; TKIs, tyrosine kinase inhibitors; TMZ, temozolomide.

Galectin-1

This protein belongs to the lectin family containing a carbohydrate recognition domain (CRD) with a high affinity for β-galactosides [2,43]. Galectin-1 is present inside and outside cells, and has intracellular and extracellular functions. The intracellular activity of galectin-1 is dependent on protein–protein interaction, whereas its extracellular activity is dependent on its lectin activity [44]. In astrocytomas there is a relationship between the expression level of galectin-1 and the grade of malignancy [45].

Galectin-1 is a hypoxia-regulated protein; its production and secretion are stimulated by hypoxia [46]. Galectin-1 has been shown to have important functions in several aspects of cancer biology implicating cell migration by interaction with integrins and components of the extracellular matrix [47], metastasis formation [48], stimulation of angiogenesis by regulating the ORP150 that controls the maturation and secretion of vascular endothelial growth factor (VEGF) [49], chemo- and radio-resistance by interaction with Ras protein [50,51] and modulation of p53 migration in the nucleus [50] (Fig. 3). In addition, galectin-1 inhibits T cell effector function by promoting apoptosis, allowing tumors to evade the immune system [46,52]. In view of its important role

in the chemo- and radio-resistance, targeting galectin-1 by different strategies was considered. Among them, the use of synthetic lactulose as β-galactoside, which has an affinity for the CRD of galectin-1 [53] (Table 1), and the use of galactomannan (Davanat[®]), which binds to galectin-1 at a site distinct from the CRD [54] (Table 1). Davanat[®] was approved by the FDA for treatment of colorectal cancers and gave promising results. However, more investigations must be done for evaluation of its effect on GBM.

p53, Mdm2 and PTEN**p53 and Mdm2**

In humans, p53 is a tumor suppressor protein encoded by the TP53 gene that can induce a temporary or permanent cessation of cell growth in response to cellular stress such as DNA damage [55,56]. Certain frequent TP53 mutations lead to the loss of function of tumor suppressor p53 and a p53 mutant protein accumulation. Unlike wild-type p53, which under normal conditions has a short half-life owing to the inhibitory action of Mdm2 [56], the mutated p53 does not undergo this inhibition and accumulates to high levels in cancer cells [57]. Mdm2 is a frequently amplified gene in GBM [58]. Its overexpression causes a loss of function of p53, in

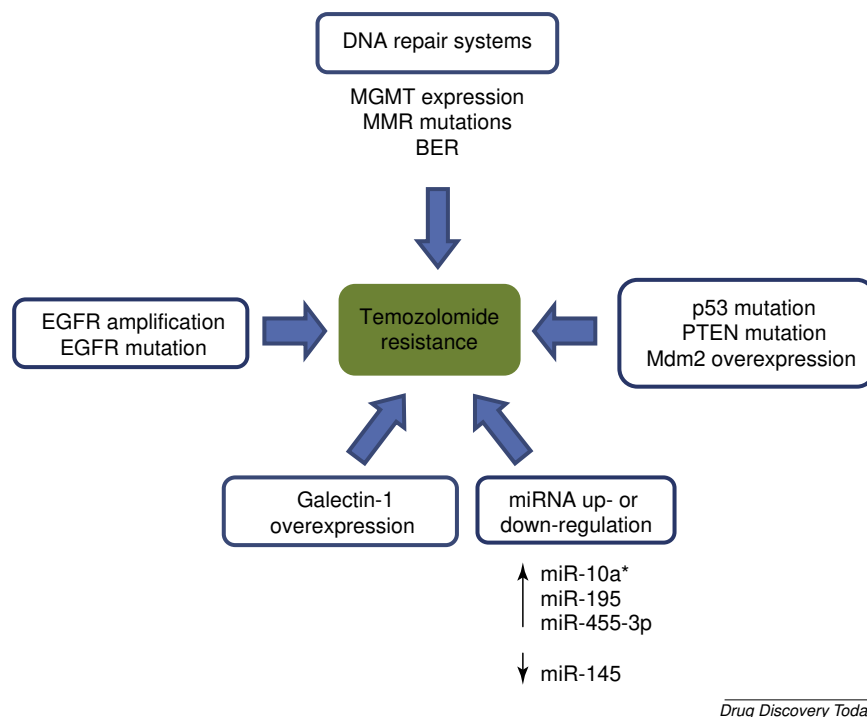


FIGURE 3
Different actors involved in the resistance of glioma tumor cells to TMZ.

addition to mutation of the TP53 gene [59] leading to the promotion of tumor cell growth and a resistance to chemoradiotherapy [60] (Fig. 3).

Because of the crucial role of p53 deficiency in glioma development and resistance to anticancer drugs, many strategies were developed to restore the effect of p53. Gene therapy to restore p53 function and inhibitors of p53–Mdm2 interaction are the most important [61]. Indeed, gene therapy provides the direct delivery of wild-type p53 in cancer cells. The adenoviral delivery of p53 was tested and has shown that it can improve the quality of life and the prognosis in patients with recurrent malignant gliomas [62]. Targeting the interaction of Mdm2 with p53 is also a potential cancer therapeutic strategy because of Mdm2 overexpression in gliomas [63]. The treatment of cells with an inhibitor of p53–Mdm2 binding should result in stabilization and accumulation of the wild-type p53 protein [64]. Nutlin-3 is one of the molecules that has been developed for this purpose and has yielded interesting results in preclinical studies. Its action is related to its binding to Mdm2 in the p53 binding pocket, activating the p53 pathway [61,64] (Table 1). The main limitation of inhibitors of p53–Mdm2 interaction is that they are effective only in cancer cells expressing wild-type p53, and have no effect in cells expressing mutant p53. Moreover, it is important to consider the toxic effect that can cause overexpression of p53 on normal cells expressing wild-type p53 [65].

PTEN

PTEN is a tumor suppressor enzyme, frequently mutated in GBM leading to an enhancement of tumor cell proliferation [66,67]. PTEN protects wild-type p53 from inactivation and degradation through the inhibition of PI3K and inhibition of Mdm2 transcription [68]. By contrast, p53 is able to increase the PTEN activity by

enhancing PTEN gene transcription by binding to the PTEN promoter [69], p53 can be stabilized by binding with PTEN which can also enhance the transcriptional activity of the TP53 gene [70]. Restoration of wild-type PTEN into tumors in which it is absent or mutated suppresses tumorigenicity, promotes apoptosis and can induce chemosensitivity [68,71]. This indicates that the association of wild-type PTEN and wild-type p53 can enhance the sensitivity of tumor cells to anticancer drugs especially to TMZ (Fig. 3).

miRNAs

miRNAs have a crucial role as a novel class of oncogenes or tumor suppressor genes [72]. Some studies have shown that aberrant miRNA expression could affect GBM sensitivity to TMZ [73]. For example, miR-21, miR-195, miR-455-3p and miR-10a* are up-regulated in a TMZ-resistant cell line [74,75]. By contrast, miR-145 is downregulated in GBM tumor cells, this enhances TMZ resistance [76] (Fig. 3). miRNAs can be exploited for the development of a new therapeutic strategy. Indeed, the oncogenic miRNAs can be downregulated using antisense oligonucleotides or miRNA sponges [77,78], whereas tumor-suppressive miRNAs can be replaced with miRNA mimetics [79].

Concluding remarks

The development of molecular biology has identified the targets involved in the resistance of GBMs to TMZ and helped in understanding their mode of cross involvement. This accelerated the development of new strategies to inhibit the effect of these targets and potentially increase the effectiveness of TMZ. However, despite promising results *in vitro* and *in vivo*, these strategies have shown their limits in terms of efficacy and side-effects in the clinic. Indeed, the systemically administered treatments lack selectivity

because they will be distributed in the target tissue and healthy tissue causing side-effects of varying severity. It also appears that inhibiting a target is not sufficient to reverse resistance to TMZ; in fact there are so many pathways in tumor cell biology that compensation mechanisms often occur. Moreover, the targets are located in the brain and thus the drugs must cross the blood-brain barrier, which is difficult to achieve. For these reasons these

tumors remain incurable at present and new effective strategies to deliver innovative drugs at their targets are strongly needed. This issue will be addressed in the second review in this series.

Acknowledgment

The authors are very grateful to the Ligue contre le Cancer, Comité du Maine et Loire, France that funded this work.

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