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# Temozolomide does not influence the transcription or activity of matrix metalloproteinases 9 and 2 in glioma cell lines



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#### ABSTRACT

Glioblastoma multiforme (GBM) is a treatment-resistant malignancy with poor prognosis. Temozolomide (TMZ) is widely used as a first-line drug for GBM. Although this improves patient prognosis, it does not completely eradicate the tumour. Even after total surgical resection, GBM can exhibit uncontrollable invasiveness at the tumour margins owing to activation of matrix metalloproteinases (MMPs) such as MMP-2 and -9; these degrade collagen IV in the basement membrane, which normally prevents cancer invasion. TMZ induces DNA damage and activates transcription factors including c-jun, c-fos, nuclear factor- $\kappa\beta$ , and early growth response protein-1, which have putative binding sites on the MMP-9 promoter. TMZ may therefore enhance tumour invasion by stimulating MMP-9 transcription and enzymatic activity. To test this hypothesis, we investigated MMP-2 and -9 mRNA transcription and activity in GBM cell lines treated with TMZ. Human A172 GBM cells were exposed to TMZ (25% and 50% inhibitory concentrations) for 24 or 48 h; cell cycle distribution and mRNA levels of MMP-2 and -9 were evaluated using flow cytometry and semi-quantitative reverse transcription PCR, respectively. MMP-2 and -9 enzymatic activities were assessed using gelatin zymography in human A172 and U373 MG GBM cells exposed to TMZ under the same conditions. TMZ altered A172 cell cycle distribution, but not MMP-2 or -9 mRNA levels. TMZ did not affect MMP-2 or -9 enzymatic activities in A172 or U373 MG cells. These findings indicated that TMZ is therefore unlikely to promote GBM invasiveness.

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#### 1. Introduction

Gliomas are common malignant brain tumours, among which glioblastoma multiforme (GBM) has the worst prognosis [1]. GBM is treated by surgical resection, along with radiotherapy and some types of chemotherapy, but this does not greatly prolong median survival [2]. Temozolomide (TMZ), an orally bioavailable alkylator [3], increases the median survival to 14.6 months [4] and is widely used as a first-line drug for GBM treatment.

TMZ is a small molecule (194 kDa) that has lipophilic properties and shows good biodistribution, including high penetration through the blood–brain barrier into the central nervous system [5,6]. The cytotoxicity of TMZ is attributed to methylation of the O6 position of guanine [7,8], induction of DNA double strand breakage, and tumour cell death by apoptosis [9]. This process does not depend on cell cycling—that is, it affects cells in mitosis and also those in G0/G1 phase (about 50–90% of any proliferative cell population) [10]. However, since the introduction of TMZ as a standard chemotherapy in the U.S., the median survival has remained well below 1 year [11]. One reason for this poor prognosis is the development of therapeutic resistance mediated by the DNA repair protein, O6-methylguanine-DNA methyltransferase, which suppresses the effects of TMZ [12]. Another reason for the poor prognosis is a failure to control the invasiveness of GBM tumour cells, which can infiltrate into the contralateral brain hemisphere or resection plane, even after gross total resection [13,14].

Tumour invasion involves three steps, namely tumour attachment via cell surface receptors that specifically bind to matrix components such as lamin and fibronectin; tumour cell secretion of hydrolytic enzymes that degrade the local extracellular matrix (ECM); and tumour cell locomotion into the proteolytically modified ECM [15]. Malignant brain tumour invasion is accompanied by remodelling of the vasculature and destruction of the ECM in the adjacent normal tissue [16]. Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade ECM components and thus promote tumour invasiveness [17,18]. In particular, the MMP-2 and -9 gelatinases enable cancer cells to degrade collage IV in



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**Fig. 1.** Cytotoxicity of TMZ in A172 ( $\blacksquare$ ) and U373 MG ( $\bullet$ ) cells. Cells were exposed to the indicated concentrations of TMZ for 48 h and then fixed and stained with Methylene Blue; the absorbance at 595 nm was measured and expressed as a percentage of the level observed in cells cultured without TMZ, which was set as 1. Results represent the mean ± SD of eight experiments.

the basement membrane, a process that is essential for ECM invasion [19,20]. MMP-2 and -9 thus play important roles in glioma invasion and are associated with glioma malignancy [16,21–25]. In the present study, we hypothesised that MMP-2 and -9 could serve as markers of tumour invasiveness.

The DNA damage caused by TMZ induces cellular stress, which can activate transcription factors such as c-jun, c-fos, nuclear factor (NF)- $\kappa\beta$  and early growth response protein (egr)-1 [9,26–30]. These transcription factors can bind to the *MMP*-9 promoter and induce gene transcription [31,32]; the resultant increase in the MMP-9 protein level is directly associated with its enzymatic activity [33,34]. However, the relationship between TMZ exposure and the transcription and activities of MMP-9 and -2 is unclear. We speculated that TMZ would enhance *MMP*-9 transcription and thus modify glioma invasiveness. To test this hypothesis, we examined the transcription and enzymatic activities of MMP-2 and -9 in GBM cells exposed to TMZ.

#### 2. Materials and methods

#### 2.1. Cell lines and TMZ

A172 and U373 MG human glioblastoma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Cat. No. 12800-082) supplemented with 10% foetal bovine serum in a culture flask until the start of the experiments. TMZ was obtained from Merck (Kenilworth, NJ, USA).

#### 2.2. TMZ cytotoxicity assay

A total of 10<sup>4</sup> dispersed A172 or U373 MG glioma cells were allowed to attach to the bottom of the culture flask for 24 h prior to treatment. Cells were seeded in 96-well plates and exposed to a range of TMZ concentrations (16  $\mu$ M–4 mM); TMZ was dissolved in distilled water and diluted 1:1 in 2 × DMEM to achieve these final concentrations. After 72 h in culture, the cells were fixed with 5% glutaraldehyde and stained with 0.01% Methylene Blue. The plates were washed prior to eluting the dye using 0.3 N hydrochloric acid. The absorbance of the eluate was measured at 595 nm on a

microplate reader (Bio-Rad, Hercules, CA, USA) and used to calculate the 25% and 50% inhibitory concentrations (IC25 and IC50, respectively).

#### 2.3. Cell treatment by TMZ

Cells were exposed to no TMZ or the IC25 or IC50 for TMZ for 0, 24 and 48 h before the assays were carried out. Twofold the required TMZ concentrations were prepared in distilled water and then diluted 1:1 in  $2 \times$  DMEM. Cells were cultured in 2 ml of this liquid per 25 cm<sup>2</sup> flask. For semi-quantitative reverse transcription (RT)-PCR, cells were exposed to the TMZ IC50 for 48 h.

#### 2.4. Cell cycle analysis

Cells were dispersed with trypsin, resuspended in phosphatebuffered saline, fixed with 100% ethanol, and stained with 0.05  $\mu$ M propidium iodide with 180 U of RNase for 30 min. Cell cycle distribution was evaluated using flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA). The cell populations in each phase of the cell cycle were analysed manually.

#### 2.5. Semi-quantitative RT-PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Tokyo, Japan), according to the manufacturer's instructions. After treatment with RNase inhibitor and RNase-free recombinant DNase I (Takara Bio, Otsu, Japan) for 30 min, total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara Bio). The resultant cDNA was used as a template for semi-quantitative RT-PCR with the following primers: MMP-2, 5' TTCTTGTCGCGGTCGTAGTC and 3' ACAGCCCTGCAAGTTTCCAT; MMP-9. 5' CTTCTACGGCCACTACTGTG and 3' GCCAGTACTTCC-CATCCTTG; and glyceraldehyde 3-phosphate dehydrogenase CCCAGCAAGAGCACAAGAG (GAPDH). 5 and 31 CCTCTTCAAGGGGTCTACATG. The reactions were carried out on an ABI 7300 system (Applied Biosystems, Foster City, CA, USA) at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s (CYBR Premix Ex Tag II; Takara Bio). The specificity of the amplification was confirmed by melting curve analysis, and MMP-2 and -9 transcript levels were compared to that of the internal control (*GAPDH*) using the relative quantification ( $\Delta\Delta$ Ct) method [35].

#### 2.6. Gelatin zymography

Cell culture medium conditioned by treated A172 and U373 MG cells was collected. Sodium dodecyl sulphate (SDS) polyacrylamide (7.5%) was co-polymerised with 2.5 mg/ml gelatin (Nitta Gelatin, Osaka, Japan) and the gels were loaded with 10  $\mu$ l gel-loading buffer [62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 10% SDS, and 0.1% Bromophenol Blue] and the same volume of sample. These were separated by SDS polyacrylamide gel electrophoresis. The gel was washed with denaturation buffer consisting of Tris-buffered saline (50 mM Tris-HCl. pH 7.5) supplemented with 1% Triton X-100, and then subjected to zymography for 24 h at 37 °C in developing buffer composed of Tris-buffered saline (50 mM Tris-HCl, pH 7.5) supplemented with 5 mM CaCl<sub>2</sub> and 1 µM ZnCl<sub>2</sub>. The gel was then stained with 0.1% Coomassie Brilliant Blue. Gelatinolytic regions were visible as white bands against a blue background. The bands were monochromed and highlighted using WinRoof image analysis software (Mitani Corporation, Fukui, Japan).

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