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Mechanism of temozolomide-induced antitumour effects on glioma cells

Wei Shen¹, Jun-An Hu¹ and Jie-Sheng Zheng²

Abstract

Objective: To investigate the mechanisms of action of the tumoricidal effects of temozolomide against the human glioma cell line U251 in vitro, and to provide preclinical proof-of-concept studies of the effects of temozolomide-containing regimens.

Methods: U251 cells were exposed to 100 μmol/l temozolomide. Morphological alterations were monitored by light microscopy. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle analysis and the rate of apoptosis were determined using flow cytometry and the number of acidic vesicular organelles stained with acridine orange were analysed by fluorescence microscopy. The scratch recovery test was used to measure cell migration.

Results: U251 cells that were treated with temozolomide displayed morphological alterations indicative of a rounder shape and impaired cellular adhesion to the cell culture plate compared with control U251 cells. Temozolomide reduced cell viability as measured by the MTT assay, caused cell cycle arrest in the gap 2/mitosis phase, inhibited cell migration and promoted autophagy in U251 cells.

Conclusion: Temozolomide induced autophagic, but not apoptotic processes, in U251 cells and thus reduced their viability and migration.

Keywords
Glioma, temozolomide, apoptosis, autophagy, flow cytometry, cell culture, in vitro study

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Introduction
Temozolomide is an imidazotetrazine derivative and a novel oral alkylating agent with enhanced cytotoxicity. Temozolomide is also able to penetrate the blood–brain barrier and does not require hepatic metabolism for activation. Temozolomide (TMZ) has been shown efficacious in the treatment of some malignant tumours. However, clinical trials show not high response rates and recent studies have examined the possibility of improving the chemotherapeutic efficacy of TMZ by combining it with other agents. The specific mechanisms of action of temozolomide remain elusive. The aim of the present study was to investigate the cytotoxicity of temozolomide on the human glioma cell line U251 in vitro and the possible pharmacological mechanisms of action.

Materials and methods
Cell line
The human glioma cell line U251 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and was continuously cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO® Cell Culture, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 mmol/L L-glutamine (all from Thermo Fisher Scientific, Rockford, IL, USA) in a humidified incubator at 37°C with 5% CO₂.

Experimental design
The IC₅₀ value of temozolomide, defined as the concentration that reduces the global growth of cells by 50%, was previously determined to be approximately 200 mmol/L. Therefore, the concentration of temozolomide that was used in this study was set at 100 µmol/l, which was considered to be approximately similar to the plasma concentration found in human subjects. Temozolomide (Sigma-Aldrich, St Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich), which was the vehicle. The U251 cells were treated with either 100 µmol/L temozolomide (TMZ group) or 100 µmol/L DMSO (vehicle control group) for the stated periods (see below).

Morphological assessment
The U251 cells were seeded at a density of 1.0 × 10⁶ cells/ml in six-well cell culture dishes, followed by treatment with 100 µmol/l temozolomide or DMSO during the logarithmic phase of growth. Following treatment for 72 h, morphological alterations were analysed by observation under an inverted phase contrast microscope (Leica DMIL; Leica, Solms, Germany) at ×20 magnification.

Cell viability assay
Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, U251 cells were seeded into 96-well plates at a density of 2000 cells/0.10 ml and incubated overnight at 37°C. Temozolomide (100 µmol/l) was then added for 24 h, 48 h, 72 h and 96 h to the respective wells. After incubation, cells were incubated with 300 µl MTT reagent (Sigma-Aldrich) at a concentration of 0.50 mg/ml for 4 h. For solubilization of the resultant formazan product, 10% v/v sodium dodecyl sulphate and 0.10 mol/l HCl (both reagents from Thermo Fisher Scientific) were added to each well and the supernatant fractions were removed at 1000 g for 5 min at room temperature using a table-top low-speed centrifuge (Model 80-2; Shanghai Medical Equipment Company, Shanghai, China). Finally, the intracellular formazan crystals were solubilized with 150 µl DMSO.
The absorbance of each well was quantified using a microplate spectrophotometer 1500 (Thermo Fisher Scientific) at a wavelength of 490 nm. The results were expressed as the percentage of the absorbance measured for the control group. All treatment groups were measured in triplicate.

**Scratch recovery assay**

The U251 cells at the logarithmic phase of growth were plated into six-well culture dishes at a density of $1.0 \times 10^6$ cells/ml. After incubation for 24 h, cells were synchronized with 2% FBS. A straight line was then introduced to each well by scratching with a sterile 200 µl pipette tip when cells had reached 80% confluence. Detached cells were removed by gently washing the well three times with 100 mM phosphate-buffered saline (PBS; pH 7.4) at 37°C. Cells were then allowed to grow for an additional 24 h in culture medium that was supplemented with 2% FBS and either 100 µmol/l temozolomide or DMSO. Migration of the cells was photographed at ×40 magnification under an Olympus IX71® inverted microscope (Olympus, Tokyo, Japan).

**Cell cycle analysis**

For cell cycle analysis, U251 cells were treated with 100 µmol/l temozolomide or DMSO for 72 h and then harvested by trypsinization. Harvested cells were fixed with 70% v/v ethanol for 24 h followed by digestion with 100 g/ml RNase A (Sigma-Aldrich) for 15 min at room temperature. After 30 min of staining with 50 µg/ml propidium iodide (Sigma-Aldrich), $1 \times 10^6$ cells were assayed using an AccuriTM C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with an excitation wavelength of 488 nm. The proportion of cells in the following cell cycle phases was determined: gap 0/gap 1 (G0/G1); gap 2/mitosis (G2/M, mitosis); and synthesis (S).

**Fluorescence staining of AVOs with acridine orange**

To detect the presence of acidic vesicular organelles (AVOs), a marker of autophagic cell death, in temozolomide- and DMSO-treated U251 cells, vital staining with acridine orange was undertaken as described previously. After 72 h of exposure to 100 µmol/l temozolomide or DMSO, the U251 cells were stained with acridine orange at a final concentration of 1 µg/ml for a period of 15 min, followed by washing three times with 100 mM PBS (pH 7.4), and then cells were observed under a Nikon confocal microscope (DIGITAL ECLIPSE C1 Plus C1; Nikon, Sendai, Japan) after 30 min in the dark. For each sample of cells, five images were examined and the mean fluorescence intensity within 15–20 regions-of-interest per image was determined using EZ-C1 Freeviewer software version 3.0 (Nikon).

**Apoptosis detection**

After 72 h incubation with 100 µmol/l temozolomide or DMSO, U251 cells were resuspended by trypsinization and their density adjusted to $5 \times 10^5$ cells/ml. Then, 2 ml of cell suspension from each group was centrifuged at 1000 g for 5 min at room temperature using a table-top low-speed centrifuge (Model 80-2; Shanghai Medical Equipment Company), washed three times in 100 mM PBS (pH 7.4) at 4°C, and then resuspended in 200 µl binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2). The cell suspension was then double-labelled with 5 µl of 20 µg/ml Annexin V-fluorescein isothiocyanate (Sigma-Aldrich) and 10 µl of 50 µg/ml propidium iodide (Sigma-Aldrich) at room temperature in the dark for 15 min. The cells were harvested using a table-top low-speed centrifuge (Model 80-2; Shanghai Medical Equipment Company) at 1000 g for
5 min at room temperature, then suspended in 300 µl binding buffer, and excited with an argon laser emitting at a wavelength of 488 nm using an Accuri™ C6 flow cytometer (BD Biosciences).

**Statistical analysis**

All statistical analyses were performed using the SPSS® statistical package, version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data are presented as mean ± SD. One-way analysis of variance (ANOVA) and two-way factorial ANOVA with multiple factors were used to compare groups of data, followed by Q-test on the differences between paired groups. The percentage of cells in different phases of the cell cycle was compared using χ²-test. A P-value < 0.05 was considered to be statistically significant.

**Results**

The U251 cells treated with 100 µmol/l temozolomide were morphologically distinct from the control DMSO-treated cells. Cells in the vehicle control group were larger in number, attached to the bottom of the well and had a fusiform shape. Control-treated cells also appeared glossy under fine light refraction and were characterized by rapid proliferation. The U251 cells treated with 100 µmol/l temozolomide were rounder in shape and smaller in size with poor light transmittance. The addition of temozolomide resulted in impaired cellular adhesion. The cell culture medium above the temozolomide-treated U251 cells was cloudy in appearance.

After treatment with 100 µmol/l temozolomide for 24 h, cell viability as assessed using the MTT assay in the TMZ group was significantly decreased compared with the control group (P = 0.0001) and the degree of inhibition increased over time (Table 1; P = 0.0001 for each time-point).

The scratch recovery assay created wounds in the adherent U251 cell layer by producing a uniform linear denuded region using a sterile pipette tip. The control cells exhibited a higher migratory potential than the temozolomide-treated cells as demonstrated by the migration of the control cells across the denuded region at 24 h after the wound was created (Figure 1). In contrast, the temozolomide-treated cells showed limited migration at 24 h after the wound was created.

Flow cytometric cell cycle analysis was undertaken on control- and temozolomide-treated U251 cells. The exposure of cells to temozolomide for 72 h significantly decreased the percentage of cells in the G0/

**Table 1.** Cell viability (%) of the human glioma cell line U251 cultured in vitro with either dimethyl sulfoxide vehicle control (control group) or 100 µmol/l temozolomide (TMZ group) as measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Control group</th>
<th>TMZ groupa</th>
<th>Statistical significanceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>100.00 ± 0.00</td>
<td>86.29 ± 1.58</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>48 h</td>
<td>100.00 ± 0.00</td>
<td>75.34 ± 1.02</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>72 h</td>
<td>100.00 ± 0.00</td>
<td>72.42 ± 1.45</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>96 h</td>
<td>100.00 ± 0.00</td>
<td>69.82 ± 3.04</td>
<td>P = 0.0001</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD.

aCell viability compared with the control group, which was the reference group.

bOne-way analysis of variance (ANOVA) and two-way factorial ANOVA with multiple factors were used to compare groups of data, followed by Q-test on the differences between paired groups.
G₁ and S phases of the cell cycle and significantly increased the percentage of cells in the G₂/M phase compared with the control cells (P < 0.001 for each comparison) (Table 2).

When the presence of AVOs was analysed, the control group demonstrated a low mean fluorescence intensity (1.19 x 10⁶ units), indicating a lack of AVOs under normal culture conditions. After 72 h of 100 μmol/l temozolomide treatment, the mean fluorescence intensity increased to 1.79 x 10⁶ units, which was significantly higher than that of the control group (P < 0.01) (Figure 2).

There was no significant difference in the rate of apoptosis as detected by flow cytometry 72 h after treatment with either DMSO control or 100 μmol/l temozolomide (5.12 ± 0.41% versus 5.20 ± 0.33%, respectively).

**Discussion**

This present study investigated the effect of temozolomide on the morphology, viability, migration, apoptosis and autophage of U251 cells. We found that U251 cells that were treated with temozolomide displayed
morphological alterations indicative of a rounder shape and impaired cellular adhesion to the cell culture plate compared with control U251 cells. Temozolomide reduced cell viability as measured by the MTT assay, caused cell cycle arrest in the gap 2/mitosis phase, inhibited cell migration and promoted autophagy in U251 cells. These data suggest that temozolomide induced autophagic, but not apoptotic processes, in U251 cells and thus reduced their viability and migration.

Malignant gliomas of neuroepithelial origin occur more frequently than other types of central nervous system tumours, accounting for 35.3–61.0% (mean 44.7%) of observed intracranial tumours. Surgery is the first-line treatment for glioma at the current time, and this can be combined with radiotherapy, chemotherapy and

Table 2. Cell cycle analysis of the human glioma cell line U251 cultured in vitro with either dimethyl sulfoxide vehicle control (control group) or 100 μmol/l temozolomide (TMZ group) for 72 h as measured using flow cytometry.

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Control group</th>
<th>TMZ group</th>
<th>Statistical significancea</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>63.42 ± 1.57</td>
<td>8.17 ± 1.25</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>G2/M</td>
<td>4.16 ± 0.11</td>
<td>79.90 ± 3.03</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>S</td>
<td>32.43 ± 1.60</td>
<td>11.93 ± 3.78</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Data presented as the mean ± SD percentage of cells.

G0, gap 0; G1, gap 1; G2, gap 2; M, mitosis; S, synthesis.

Figure 2. Representative fluorescent photomicrographs showing the detection of acidic vesicular organelles (red fluorescence) in the human glioma U251 cell line. U251 cells were cultured in vitro for 72 h with either dimethyl sulfoxide vehicle control (A) or 100 μmol/l temozolomide (B). The colour version of this figure is available at: http://imr.sagepub.com.
immune therapy. Adjuvant chemotherapy following initial surgery is effective in destroying the cancerous cells that remain, and thus reduces the risk of cancer recurrence. Newer agents designed to be directed antiglioma therapy are currently being explored with exciting preliminary results.

Temozolomide is an imidazotetrazine derivative and a novel oral alkylating agent. Its availability as an oral agent, favourable safety profile and efficacy support its potential in the treatment of glioma. It is also able to penetrate the blood–brain barrier and it does not require hepatic metabolism for activation. The methylation of DNA is the principal mechanism responsible for the cytotoxicity of temozolomide against malignant cells, resulting in the fragmentation of DNA and disrupted DNA replication, and thus growth suppression and apoptotic cell death. However, the specific mechanisms of action of temozolomide remain elusive. The aim of this present study was to evaluate the effect of temozolomide on U251 cells in vitro and explore the possible pharmacological mechanisms of action.

There are three classical forms of cell death; apoptosis, autophagy and necrosis. Many chemotherapeutic drugs exhibit their cytotoxic effect by inducing apoptosis in tumour cells. Apoptosis is not the only form of programmed cell death. Research has shown that there is another mechanism of programmed cell death associated with the appearance of autophagosomes and autophagic proteins. Although the same signalling pathways regulate both processes, apoptosis and autophagy differ considerably in terms of their mechanisms, processes and morphological changes. The regulation of protein degradation and organelle turnover by autophagy involves the participation of lysosomes, which are cellular organelles rich in acid hydrolase, an enzyme that is responsible for cellular and molecular degradation. Therefore, autophagy is characterized by the formation and promotion of AVOs.

These present observations revealed that temozolomide induced autophagy in U251 cells, suggesting autophagy as an underlying mechanism of action of temozolomide. Autophagy is a cellular pathway for the degradation and turnover of proteins and cellular organelles. Under normal conditions, autophagy plays an important role in maintaining intracellular homeostasis. In this role, autophagy removes damaged or dysfunctional organelles and promotes cell survival under conditions of nutrient deprivation by degrading disposable intracellular content, thereby generating energy and the building blocks for protein synthesis. Moreover, studies report that once cancer cells are exposed to radiation or chemotherapeutic agents, a high rate of autophagy is observed in those cancer cells.

Inhibited cell growth and G2/M cell cycle arrest was indicated by a decrease in the proportion of U251 cells in the G0/G1 and S phases following exposure to temozolomide in the present study. These observations suggested that temozolomide interferes with DNA replication. Temozolomide did not trigger the apoptosis of U251 cells, although cytotoxicity was observed as indicated by the MTT assay. These present findings were consistent with previous reports that autophagy, and not apoptosis, was induced in malignant glioma cells following treatment by temozolomide. Furthermore, it was shown that the inhibition of autophagy increased the therapeutic efficacy of temozolomide against malignant glioma cells.

These present observations demonstrated that temozolomide potently induced autophagy in U251 cells, and thus inhibited their proliferation and migration. Moreover, these observations provide novel insights that might assist or inform new treatment strategies for the clinical management of malignant gliomas.
Declaration of conflicting interest
The authors declare that there are no conflicts of interest.

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