Effect of temozolomide on cell viability in gonadotroph adenoma cell lines

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Abstract. Invasive pituitary adenomas are usually refractory to routine neurosurgery, radiosurgery or medications, and alternative therapies are needed. The effects of temozolomide (TMZ) on the inhibition of gonadotroph adenoma cell viability and hormone secretion were evaluated. Cell viability and IC_{50} values were evaluated after αT3-1 cells were treated with TMZ (31.25-1000 μM) or vehicle for 0-72 h. Cell cycle changes and the extent of apoptosis were detected using flow cytometry, TUNEL and TEM. The molecular mechanism of TMZ action was investigated by the Caspase-Glo® assay and immunoblotting. Gonadotropin secretion was assessed using an immunoassay system. TMZ dose- and time-dependently suppressed cell proliferation (P<0.01 vs. control, 250 μM, 24 h) and induced S-phase accumulation and G2/M-phase arrest (P<0.05 vs. control, 250 μM, 24 h). Early apoptotic cells increased following a 24-h TMZ incubation (P<0.001 vs. control, 250 μM), consistent with TEM and TUNEL detection that exhibited morphological features of apoptosis. TMZ (250 μM) increased the level of caspase-3/7 by 6-fold, caspase-9 by 7-fold and caspase-8 by 3-fold after a 24-h incubation, while it attenuated Bcl-2 expression (P<0.001 vs. control) and raised the proteolysis of PARP. Both FSH and LH levels were significantly decreased by TMZ (P<0.01 vs. control, 250 μM, 24 h). TMZ inhibited cell proliferation and hormone secretion, and induced cell cycle arrest and apoptotic cell death in gonadotroph adenoma cells via both death receptor and mitochondrial pathways, suggesting that it may represent a useful medical management strategy of invasive gonadotroph adenomas.

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Abbreviations: CCK-8, cell counting kit-8; DSBs, double-strand breaks; IC_{50}, median inhibitory concentration; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MGMT, O^6-methylguanine-DNA methyltransferase; NFPAs, non-functioning pituitary adenomas; O^6MeG, O^6-methylguanine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; RLU, relative light units; TEM, transmission electron microscope; TUNEL, terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end labeling

Key words: gonadotroph adenoma, αT3-1, temozolomide, apoptosis

Introduction

Pituitary adenomas account for nearly 15% of clinical intracranial neoplasms (1), with autopsy studies demonstrating an incidence of 25% (2). Gonadotroph tumors comprise 15-35% of all pituitary adenomas, and most of the clinically non-functioning adenomas, which constitute approximately one-third of all tumors of the anterior pituitary, are actually gonadotropin-secreting adenomas or gonadotroph adenomas (3-5). Although most gonadotroph adenomas are benign, slow-growing tumors, some are characterized by fast invasive growth, increased mitotic activity, a Ki-67 labeling index greater than 3%, and positive p53 immunoreactivity (6). These patients generally consult for headache and/or a visual field disorder, and baseline concentrations of gonadotropins or their free subunits are elevated in 30-50% of these patients (7). These tumors are refractory to neurosurgery, radiosurgery or alternative medical therapies (such as dopaminergic agonists, somatostatin analogs, and GnRH agonists/antagonists) (7). There is an urgent need to identify new therapeutic targets and develop new agents for the treatment of these refractory adenomas.

TMZ, one of the alkylating chemotherapeutic agents, has been approved for the treatment of patients with metastatic melanoma and glioblastoma multiforme (8). In addition, TMZ clinical trials have been performed to test its activity against brain metastatic solid tumors, leukemia (8), pancreatic neuroendocrine tumors (9) and refractory pituitary adenomas (10-14). Two case studies related to gonadotroph adenoma patients report that their lesions decreased after TMZ therapy (15,16), and studies of TMZ on MMQ, GH3, and AtT20 cell lines suggested that this drug may have efficacy in pituitary adenoma cells (17). However, in another study, TMZ failed

to attain biochemical control in a severe case of acromegaly (18). These conflicting data prompted us to further study the effectiveness and mechanism of TMZ on pituitary adenoma cell death in detail. In this study, the effects of TMZ on cell viability, apoptosis, and gonadotropin secretion are assessed in the mouse gonadotroph adenoma cell line, αT3-1, which is derived from a transgenic mouse and maintains differentiated functions unique to gonadotropes (19).

Materials and methods

Chemicals and reagents. TMZ was obtained from Sigma-Aldrich (Shanghai, China). The cell counting kit (CCK)-8 was obtained from Dojindo Laboratories (Kumamoto, Japan). The DeadEnd™ Fluorometric TUNEL System and the Caspase-Glo® 3/7, 8 and 9 assay systems were supplied by Promega (Beijing, China). Annexin V-FITC apoptosis assay kit was provided by Biosea Biotechnology (Beijing, China).

Cell culture. The αT3-1 cells used in this study were kindly provided by Dr P.L. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA) and were maintained in monolayer cultures in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin (Invitrogen, China) in humidified 5% CO_2, 95% air, at 37˚C. The cells were passaged at ~90% confluence using a trypsin-EDTA solution (0.05% trypsin, 0.5 mM EDTA). Cells were kept in phenol red-free medium containing charcoal-treated fetal bovine serum for 4 days before the experiments.

Drug treatment and assessment of cell viability. The αT3-1 cells were trypsinized, centrifuged and resuspended in 1 ml of medium, then plated in 96-well plates at $1x10^4$ cells/100 μl per well. After incubation for 24 h, TMZ (31.25, 62.5, 125, 250, 500 or 1000 μM) was added to the respective wells, with DMSO serving as solvent control. Cell viability was evaluated every 24 h by the CCK-8 assay. Briefly, 10 μl of CCK-8 was added to each well followed by incubation at 37˚C for 3 h. Absorbance was then measured at a wavelength of 450 nm using a Victor-2 plate reader (Perkin Elmer). Each CCK-8 assay was performed in triplicate. Cell viability was calculated as a percentage of the control, and the median inhibitory concentration (IC_{50}) of TMZ were calculated from growth inhibition curves fitted to the data using the OriginPro 7.5 software (OriginLabs, Northampton, MA, USA).

Cell cycle analysis. Cell cycle distribution was determined by staining DNA with propidium iodide (PI). Briefly, αT3-1 cells were treated with TMZ or DMSO for 24-48 h and then harvested. Cells were then washed and fixed in 70% ethanol on ice for 30 min. After centrifugation, the cell pellets were washed and resuspended in phosphate-buffered saline (PBS). Cells were then treated with RNase and stained with PI. DNA content was analyzed using a FACS flow cytometer (Becton-Dickinson).

Annexin V staining. αT3-1 cells were seeded at $1x10^5$ cells/well in 6-well plates and treated with 125, 250 or 500 μM TMZ, or DMSO after cells were grown to 50% confluence. After drug or vehicle incubation for 24 h, Annexin V assays were performed according to the manufacturer's protocol (Biosea, China). Briefly, the cultured cells were trypsinized and collected, washed twice with PBS and resuspended with 200 μl binding buffer before addition of 10 μl of Annexin-V-FITC. After a 15-min incubation at room temperature in the dark, nuclei were counterstained with 5 μl PI (Sigma). The percentage of apoptotic cells was determined using a FACS flow cytometer.

TUNEL staining. αT3-1 cells were cultured on glass-bottomed dishes (Nest Biotechnology Corporation) at a density of $2x10^4$ cells/well. After cells were grown to 50% confluence, the cells were treated with 125, 250, 500, 1000 μM TMZ or DMSO. Treatment was terminated at 24 h, and the cells were fixed in 4% methanol-free formaldehyde in PBS for 25 min. TUNEL staining was performed for detection of apoptotic cells using the fluorometric TUNEL staining kit (Promega Corporation, Madison, WI). In brief, the fixed cells were washed twice in PBS and permeabilized with 0.2% Triton X-100 for 5 min. The cells were again washed twice in PBS, equilibrated with the equilibration buffer at room temperature for 5 min and then incubated with 50 μl of TdT fluorescein-12-dUTP for 1 h at 37˚C in a humidified chamber. The reaction was terminated by 2X SSC for 15 min at room temperature and slides were washed three times in PBS and mounted with anti-fading mounting medium (Golden Bridge International, Inc., Mukilteo, WA, USA). The cells were observed and photographed under a confocal microscope (Zeiss, Heidelberg, Germany).

Electron microscopy. Following a 24-h treatment with TMZ (250 μM), cell samples were collected and fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. After dehydration in graded ethanol, all cultures were embedded in Epon epoxy resin. Semi-thin and ultra-thin sections were then cut with an ultramicrotome. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (TEM) (JEM-1010, JEOL, Japan).

Caspase-3/7, -8, and -9 activity assays. Caspase activity was measured using Caspase-Glo 3/7, 8 and 9 assay kits (Promega, Madison, WI). Cells were seeded at $1x10^4$ cells/well in 96-well, white-walled plates and treated with 125, 250 or 500 μM TMZ, or DMSO after cells were grown to 50% confluence. After incubation for 0, 2, 4, 8, 12 or 24 h, an equal volume of Caspase-Glo reagent was added. The plates were shaken at 500 rpm for 30 sec, incubated for 1 h, and measured for luminescent output (relative light units, RLU) using a Veritas Microplate Luminometer (Turner BioSystem, Sunnyvale, CA). Data are presented as the mean ± SD from three replicates.

Western blotting. Analysis of the expression of Bcl-2 and poly(ADP-ribose) polymerase (PARP) (intact and cleaved fragments) was performed after TMZ (250 μM) treatment for 0, 4, 8, 12, 24 h, 48 or 72 h. Briefly, cell lysates were separated on a 10% acrylamide gel, electrophoretically transferred onto a PVDF membrane (PALL Corp., East Hills, NY, USA) and probed by standard techniques with primary antibodies for Bcl-2 and PARP. HRP-conjugated goat anti-rabbit IgG (1:1000

dilution, Cell Signaling Technology) was used as a secondary antibody for rabbit anti-PARP (1:1000 dilution, Cell Signaling Technology) and rabbit anti-Bcl-2 (1:1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) primary antibodies, whereas horse anti-mouse IgG (1:1000 dilution, Cell Signaling Technology) was used as a secondary antibody for the mouse anti-β-actin primary antibody (1:1000 dilution, Santa Cruz Biotechnology, Inc).

Gonadotropin secretion assay. The effects of TMZ on gonadotropin secretion were analyzed by measuring follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in the culture medium from αT3-1 cells with a Centaur® XP Immunoassay System (ADVIA) (Siemens, USA). In brief, the αT3-1 cells were cultured in 24-well plates and treated with 125, 250 or 500 μM TMZ, or DMSO after cells were grown to 50% confluence. After incubation for 24, 48, 72 or 144 h, the supernatant was collected and diluted 5-fold to bring the typical hormone concentration within the middle of the standard range for the hormone assay. Results are expressed as the mean ± SD from three independent experiments.

Statistical analysis. The normal distribution and homogeneity of variance of the data sets were analyzed by the Kolmogorov-Smirnov test and Levene's test, respectively, in advance. Comparisons of the means for cell proliferation, Annexin V Staining, Caspase-Glo assay, immunoblotting, and gona-dotropin assay for αT3-1 cells at the examined time points for vehicle and TMZ concentrations were analyzed using one-way ANOVA. The cell cycle data for vehicle and TMZ was compared by independent-samples t-test. A P-value <0.05 was considered to be statistically significant.

Results

Inhibition of cell proliferation and dose response to TMZ. TMZ significantly reduced the viability of αT3-1 cells in a concentration- and time-dependent manner following a 24-72-h treatment, as assessed by the CCK-8 assay (Fig. 1A). Cell viability decreased linearly with the increasing TMZ concentrations and the IC_{50} value at 72 h was 260.44 μM. The lowest examined concentration of TMZ (31.25 μM) did not demonstrate a statistically significant inhibition of cell proliferation (P>0.05). The lowest concentration of TMZ to demonstrate an effect (62.5 μM) reduced αT3-1 cellular viability to 88.4±3.66% (P<0.001 vs. vehicle) following a 72-h treatment. The maximal effect was observed following a 72-h treatment with 1000 μM TMZ, which remarkably dropped cellular viability to 2.4±0.67% (P<0.001 vs. vehicle) in the αT3-1 cell line. TMZ treatment was also associated with obvious morphological changes in αT3-1 cells, including cellular floating and cell debris accumulating following a 24-72-h treatment with 250 μM TMZ (Fig. 1B).

TMZ induces growth arrest and apoptosis. To address whether the anti-proliferative effect of TMZ in pituitary adenoma cells is associated with cell cycle regulation, DNA cell cycle analysis was performed in αT3-1 cells treated with TMZ or vehicle for 24 or 48 h. Incubation with TMZ (250 μM, a dose close to the IC_{50} value) for 24 or 48 h increased the

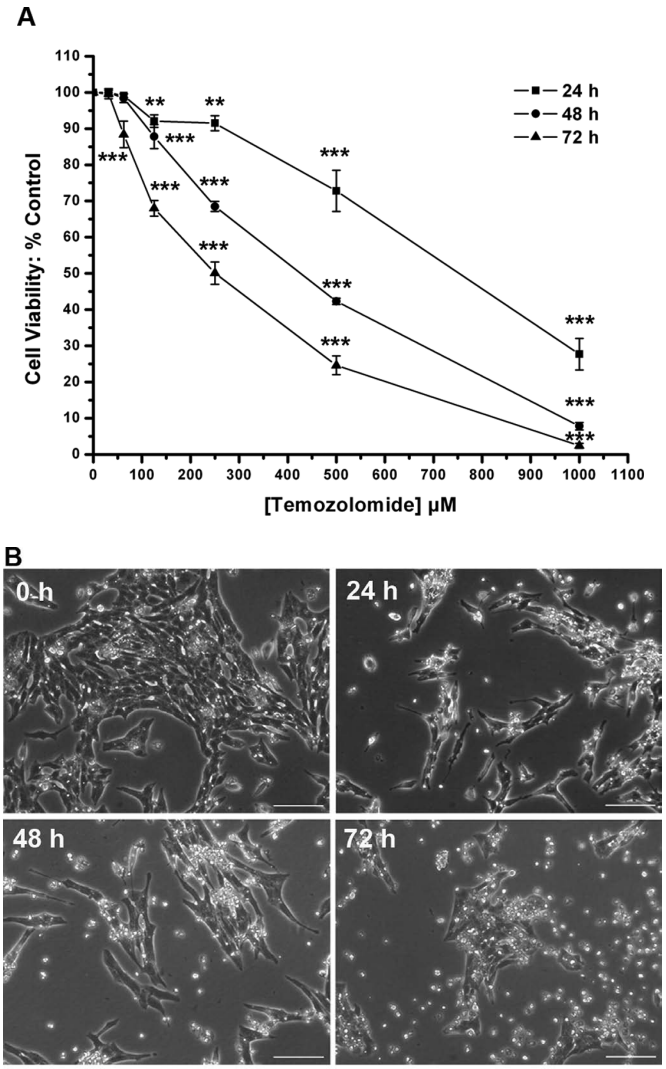

Figure 1. Effect of temozolomide (TMZ) on pituitary adenoma cellular viability. (A) TMZ (31.25-1000 μM) concentration- and time-dependently reduced the viability of αT3-1 cells following 24-72-h treatment, as assessed by CCK-8 assay. Data are expressed as the mean ± SD from three independent experiments and analyzed using one-way ANOVA. **P<0.01, ***P<0.001 vs. vehicle-treated cultures. (B) Cellular morphology of αT3-1 cells following 0-72-h treatment with 250 μM TMZ. Bars, 100 μm.

number of S-phase (37.60±8.63 vs. 21.00±2.83% in control, P<0.05 and 33.03±6.65 vs. 15.00±4.76% in control, P<0.05, respectively) and G2/M-phase (30.75±8.70 vs. 11.70±3.82% in control, P<0.05 and 29.90±9.82 vs. 7.97±1.68% in control, P<0.05, respectively) cells, accompanied by a corresponding decrease in the proportion of cells in G0/G1 (31.65±10.07 vs. 67.30±6.65% in control, P<0.01 and 37.07±10.16 vs. 77.03±5.67% in the control, P<0.01, respectively) (Fig. 2).

To determine whether TMZ increased apoptotic cell death, flow cytometry analysis was performed. The percentage of early apoptotic cells (Annexin V^+/PI^-) increased from 0.27±0.10% in vehicle-treated cultures to 5.67±1.12% (P<0.01 vs. vehicle), 9.03±0.70% (P<0.001 vs. vehicle), and 18.20±2.49% (P<0.001 vs. vehicle) following a 24-h treatment with 125, 250, or 500 μM TMZ, respectively (Fig. 3A and B). These data suggest that both cell cycle arrest and cell apoptosis could be the reasons for the anti-proliferative effects of TMZ and may result in the inhibition of αT3-1 cell viability.

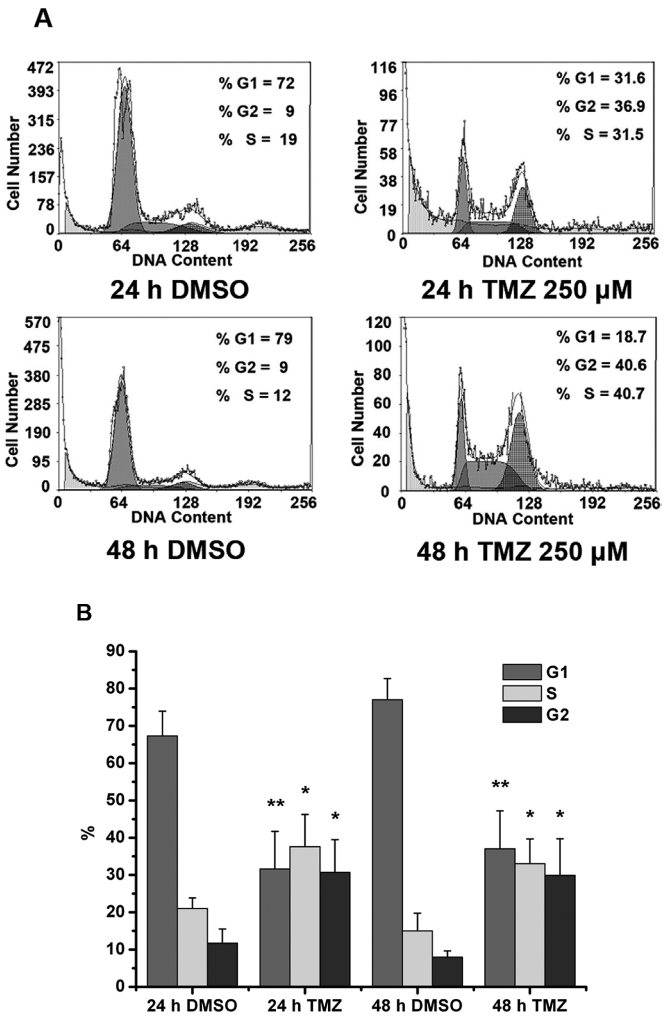

Figure 2. Cell cycle analysis of αT3-1 cells following temozolomide (TMZ) treatment. Cells were incubated in either medium plus DMSO or medium containing TMZ (250 μM) plus DMSO for 24-48 h before harvesting. The harvested cells were stained with propidium iodide and analyzed by fluorescence-activated cell sorting to determine the proportion of cells in each phase of the cell cycle. (A) Representative flow cytometry histograms obtained from treated cells. (B) Quantitation of cell population distribution over the various stages of the cell cycle. TMZ increased the percentage of cells in the S and G2/M phases and decreased the percentage of cells in G0/G1 phase of the cell cycle. Data are expressed as the mean ± SD from the three independent experiments and analyzed using independent-samples t-test. *P<0.05, **P<0.01 vs. vehicle-treated cultures.

The above hypotheses were further proven by visualization of apoptotic cells using TUNEL staining (Fig. 3C) and electron microscopy scaning (Fig. 3D). After TMZ (250 μM) treatment for 24 h, αT3-1 cells exhibited certain morphological features of apoptosis, including nuclear shrinkage, chromatin condensation and margination.

Caspase activation and Bcl-2, PARP attenuation induced by TMZ. To investigate the mechanism of the proapoptotic effect of TMZ on pituitary adenoma cells, we detected the effect of TMZ on cleavage of caspases by Caspase-Glo 3/7, 8 and 9 assay. Compared with vehicle, both 250 and 500 μM TMZ dramatically augmented caspase-3/7 (Fig. 4A), -8 (Fig. 4B) and -9 (Fig. 4C) activation after treatment for 2-24 h, while 125 μM induced a partial increase. Basal cellular caspase-3/7, -8 and -9 levels were 4.95±0.16x10^3 RLU, 1.03±0.07x10^3 RLU and 3.67±0.29x10^3 RLU, respectively. The lowest tested dose of TMZ (125 μM) increased caspase-3/7, -8 and -9 to 10.59±0.44x10^3 RLU (P<0.001 vs. vehicle), 1.40±0.13x10^3 RLU (P>0.05 vs. vehicle) and 5.60±0.32x10^3 RLU (P<0.05 vs. vehicle), respectively, following a 2-h treatment. TMZ (250 μM) increased the level of caspase-3/-7 6-fold, caspase-9 7-fold, and caspase-8 3-fold after a 24-h incubation. A maximal effect was found following a 24-h incubation with 500 μM TMZ, which significantly raised caspase-3/7, -8 and -9 to 155.65±1.56x10^3 RLU (P<0.001 vs. vehicle), 23.73±1.13x10^3 RLU (P<0.001 vs. vehicle) and 143.08±8.12x10^3 RLU (P<0.001 vs. vehicle), respectively. These results suggest that the pro-apoptotic effect of TMZ treatment in αT3-1 cells could be mediated by both the death receptor and the mitochondrial pathways.

To further confirm the above postulations we performed immunostaining for antiapoptotic proteins, including Bcl-2, an inner mitochondrial membrane protein that blocks apoptosis, and PARP, a nuclear polymerase involved in DNA repair, predominantly in response to environmental stress. The cleavage of the 116-89 kDa PARP by caspase-3/7 is considered to be a marker for cells undergoing apoptosis. We performed Western blot analysis to determine changes in Bcl-2 and the cleaved form of PARP after treatment of cells with TMZ. Consistent with caspase-9 and -3/7 activation and the increase in apoptotic cell death, TMZ (250 μM) down-regulated the expression of Bcl-2 in a time-dependent manner (Fig. 5A). Densitometric analysis revealed a maximal reduction following a 72-h treatment, which decreased Bcl-2 expression to 46.04±12.51% (P<0.001 vs. control) (Fig. 5B). TMZ also raised the proteolysis of PARP following 4-72-h exposure, coinciding with Bcl-2 attenuation, caspase activation and apoptotic cell death (Fig. 5A). These results support our belief that the mitochondrial apoptotic pathway contributes to the pro-apoptotic effect of TMZ treatment in αT3-1 cells.

Effects of TMZ on gonadotropin secretion. To detect the functional inhibition of TMZ on the hormone secretion of αT3-1 cells, the supernatant was examined by a Centaur® XP Immunoassay System after TMZ (125, 250 or 500 μM) or DMSO treatment for 24, 48, 72 and 144 h. In agreement with the anti-proliferative effect above, both FSH (Fig. 6A) and LH (Fig. 6B) were significantly decreased by TMZ in a concentration- and time-dependent manner. Basal FSH and LH levels in the αT3-1 cell culture medium were 10.87±1.48 mIU/ml and 1.95±0.17 mIU/ml, respectively. The lowest examined dose of TMZ (125 μM) reduced FSH and LH to 9.17±0.65 mIU/ml (P<0.05 vs. vehicle) and 1.52±0.05 mIU/ml (P<0.05 vs. vehicle), respectively, following a 24-h incubation. A maximal effect was found following a 144-h incubation with 500 μM TMZ, which dramatically dropped FSH to 2.79±0.80 mIU/ml (P<0.001 vs. vehicle) and LH to 0.41±0.05 mIU/ml (P<0.001 vs. vehicle), respectively.

Discussion

As the effect and mechanism of TMZ on gonadotroph adenoma cells is largely unknown, this study was performed to elucidate the mode of death of αT3-1 cells upon treatment with TMZ. Here, we demonstrate that TMZ time- and dose-

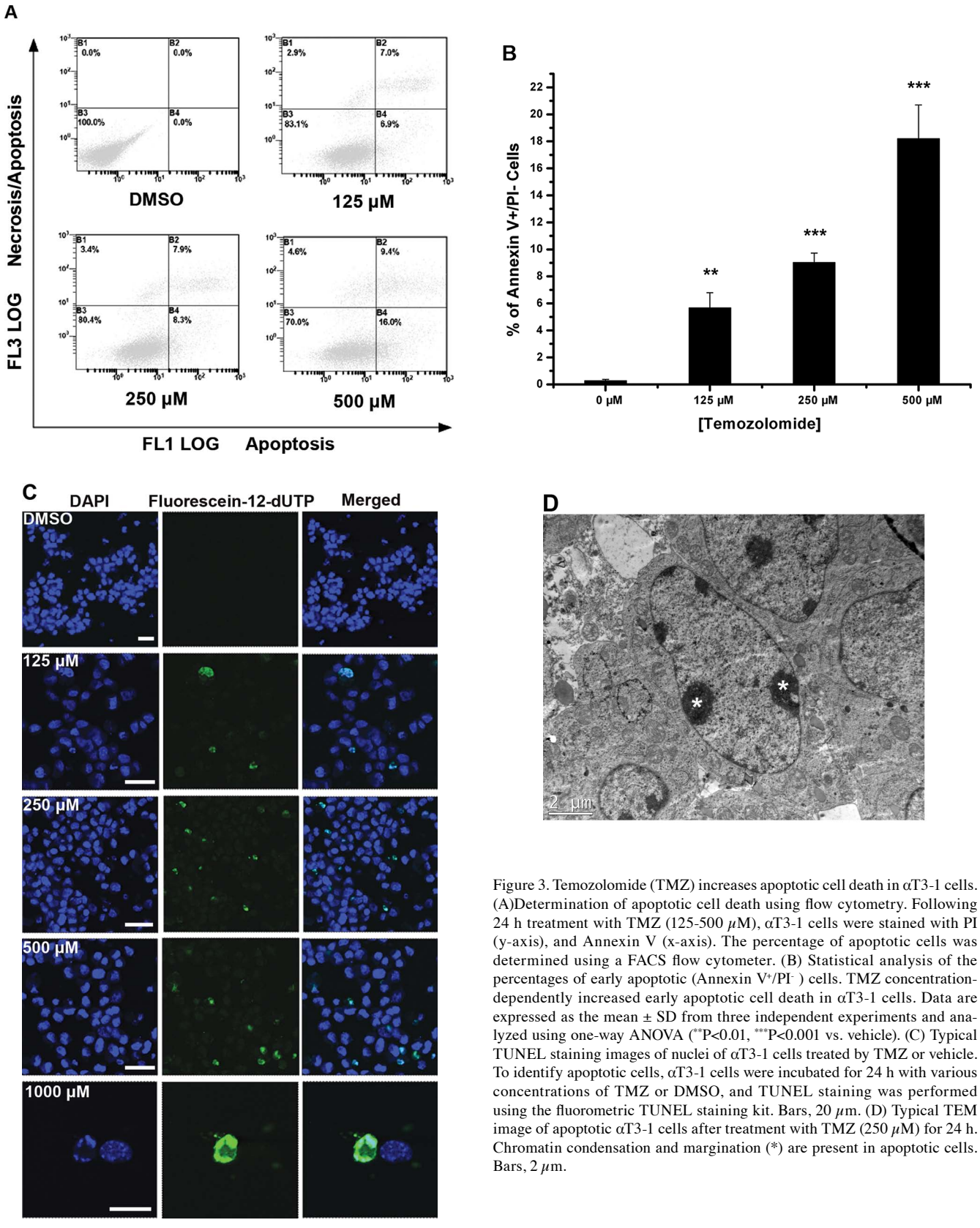

Figure 3. Temozolomide (TMZ) increases apoptotic cell death in αT3-1 cells. (A)Determination of apoptotic cell death using flow cytometry. Following 24 h treatment with TMZ (125-500 μM), αT3-1 cells were stained with PI (y-axis), and Annexin V (x-axis). The percentage of apoptotic cells was determined using a FACS flow cytometer. (B) Statistical analysis of the percentages of early apoptotic (Annexin V^+/PI^-) cells. TMZ concentration-dependently increased early apoptotic cell death in αT3-1 cells. Data are expressed as the mean ± SD from three independent experiments and analyzed using one-way ANOVA ($^{**}P<0.01$, $^{***}P<0.001$ vs. vehicle). (C) Typical TUNEL staining images of nuclei of αT3-1 cells treated by TMZ or vehicle. To identify apoptotic cells, αT3-1 cells were incubated for 24 h with various concentrations of TMZ or DMSO, and TUNEL staining was performed using the fluorometric TUNEL staining kit. Bars, 20 μm. (D) Typical TEM image of apoptotic αT3-1 cells after treatment with TMZ (250 μM) for 24 h. Chromatin condensation and margination (*) are present in apoptotic cells. Bars, 2 μm.

dependently reduces cell viability of gonadotroph adenoma cells by inducing cell cycle arrest and apoptosis, with a parallel decrease of Bcl-2 and full-length PARP expression and increase in caspase-3/7, -8 and -9 activities. Functionally, TMZ reduced gonadotropin levels, including FSH and LH, in the cell culture supernatant.

Among pituitary adenoma-specific chemotherapy studies, our results are consistent with previous data. Previous studies have shown that TMZ significantly inhibited cell proliferation of the somatotropin/prolactin-secreting cell line, GH3, and the prolactin-secreting cell line, MMQ, at a concentration of 250 μM, and similarly affected the corticotrophin-secreting

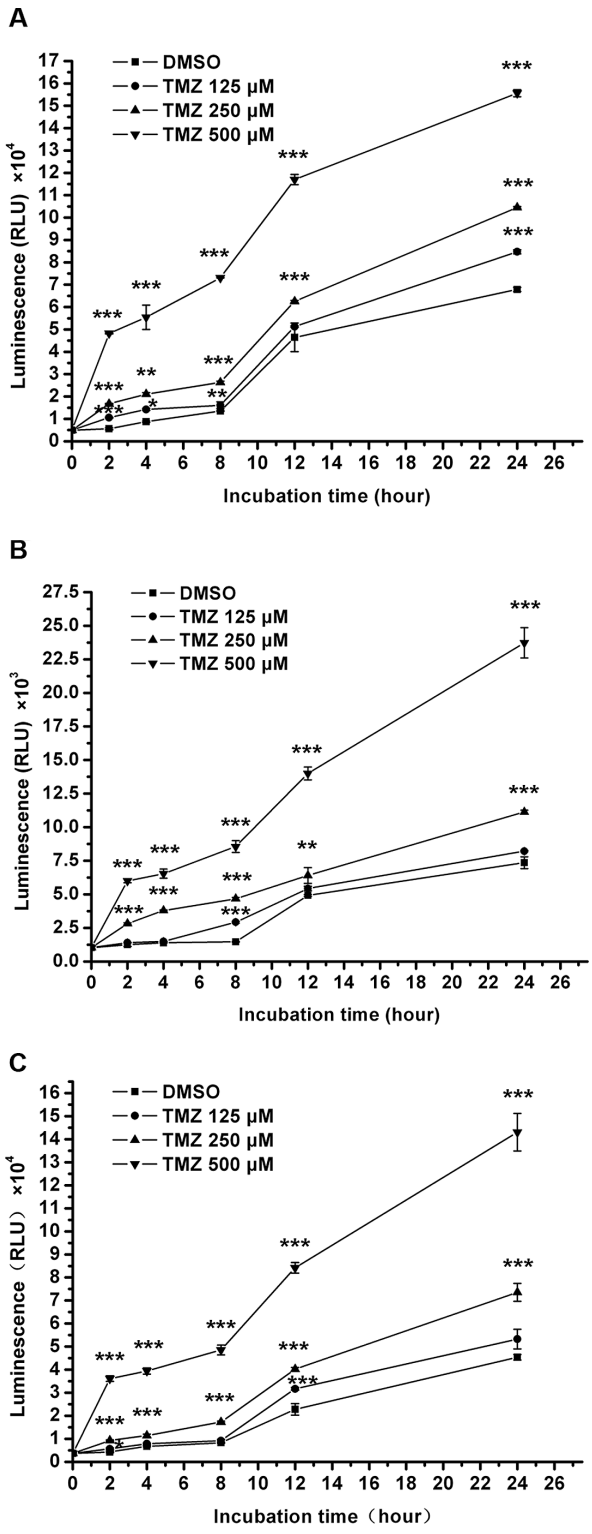

Figure 4. Temozolomide (TMZ) time- and dose-dependently enhances caspase activity in pituitary adenoma cells. To investigate the mechanism of the pro-apoptotic effect of TMZ on αT3-1 cells, the activities of cellular caspase-3/7 (A), -8 (B), and -9 (C) were evaluated after treatment with TMZ (125, 250 or 500 μM) or DMSO for 0, 2, 4, 8, 12 or 24 h with Caspase-Glo reagent and Veritas Microplate Luminometer. Results, recorded as RLU, are expressed as the mean ± SD from three independent experiments and were analyzed using one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 vs. vehicle treated cultures).

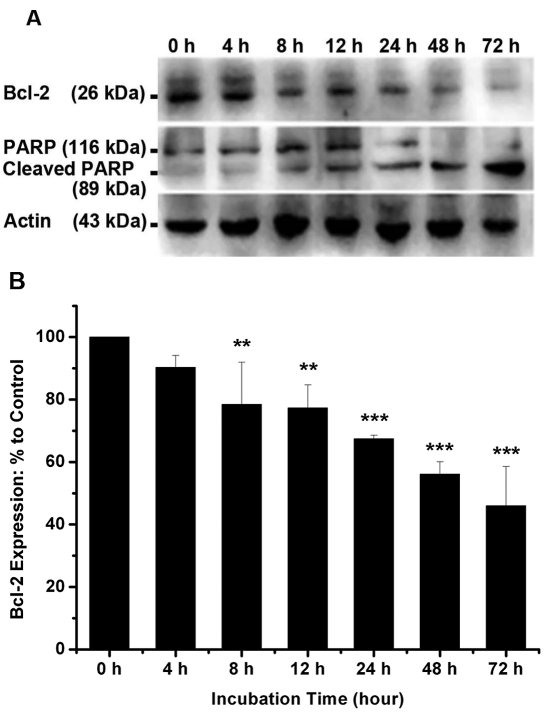

Figure 5. Temozolomide (TMZ) time-dependently attenuates the expression of anti-apoptotic proteins in pituitary adenoma cells. To evaluate the effect of TMZ on anti-apoptotic proteins, Western blot analysis of the expression of Bcl-2 and PARP (intact and cleaved fragments) was performed after TMZ (250 μM) treatment of αT3-1 cells for 0, 4, 8, 12, 24, 48 or 72 h. (A) Representative blots of three independent experiments. β-actin was used as loading control. TMZ reduced Bcl-2 expression and increased PARP cleavage, in agreement with capase-8, -9 and -3/7 activation (Fig. 4). (B) Densitometric analysis of Western blots. Data are expressed as the mean ± SD from three independent experiments and analyzed using one-way ANOVA (**P<0.01, ***P<0.001 vs. control).

cell line, AtT20, at a concentration of 50 μM (17). In our study, the IC_{50} value of TMZ for the αT3-1 cells was 260.44 μM, close to the value of 258 μM reported by Sheehan *et al* (17). This suggests that in the present experiment TMZ had comparable cytotoxicity, and that the αT3-1 cells had acceptable TMZ sensitivity. These findings further confirm the notion that TMZ is potentially fitting for the chemotherapy of relatively slow-growing pituitary adenomas, because it is not cell cycle-specific and restrains all phases of tumor cell growth (14).

Reports exist describing gonadotroph adenoma patients whose lesions decreased after TMZ therapy. Fadul *et al* (15) reported two cases of a persistent response to TMZ, one in a patient with a recurrent luteinizing hormone-secreting pituitary carcinoma, the other of a patient with hyperprolactinemia from a prolactinoma. After TMZ treatment, eradication of neck and shoulder pain, improvements in visual field deficits and tumor shrinkage were seen in the first patient, while serum prolactin concentration decreased in the second patient. The responses lasted for more than 1 year in both patients after TMZ chemotherapy. In addition, Syro *et al* (16) reported a case of a recurrent oncocytic gonadotropic pituitary adenoma that was partly immunopositive for LH and O^6-methylguanine-DNA methyltransferase (MGMT), a major determinant of resistance to some types of cytostatic treatments (10). After TMZ administration, MRI demonstrated significant tumor necrosis, indicating responsiveness to TMZ therapy. These examples demonstrate that gonadotropic pituitary adenomas may be sensitive to TMZ even when MGMT is partially immunopositive.

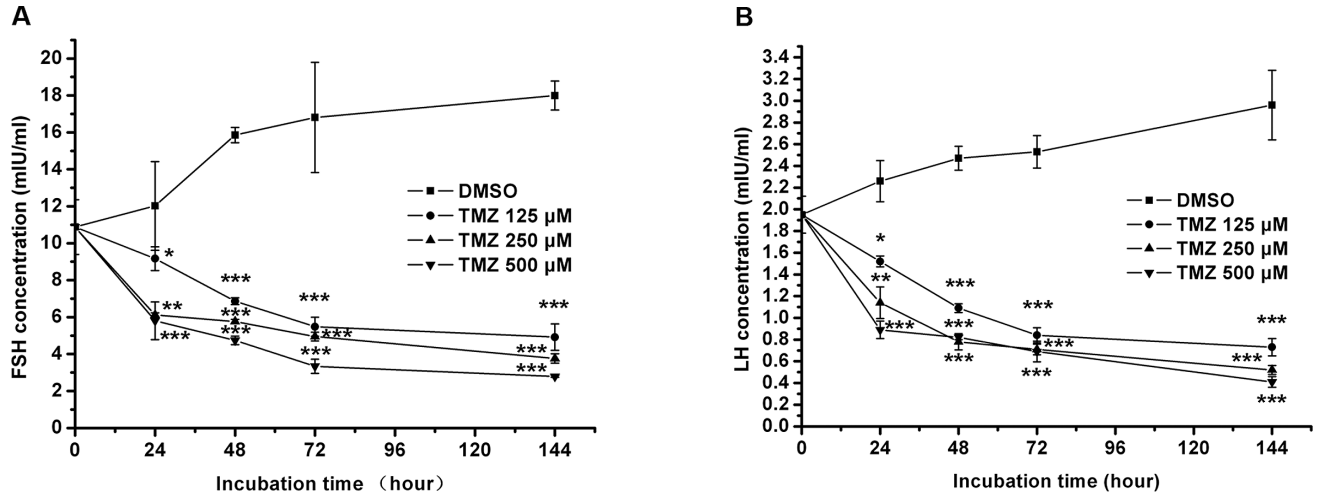

Figure 6. Effects of temozolomide (TMZ) on gonadotropin secretion. To detect the effect of TMZ on hormone secretion of αT3-1cells, the supernatant was examined by the Centaur® XP Immunoassay System after TMZ (125, 250 or 500 μM) or DMSO treatment for 24, 48, 72 or 144 h. Both FSH (A) and LH (B) were decreased by TMZ in a time- and dose-dependent mode. Data are expressed as the mean ± SD from three independent experiments and analyzed using one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001 vs. control).

Our data are also consistent with previous investigations characterizing the mechanism of action of TMZ. We have shown that TMZ induced statistically significant S-phase accumulation and G2/M-phase arrest, which suggest that a large number of cells in gonadotroph pituitary adenomas failed to complete DNA synthesis smoothly, followed by increased apoptosis. What is the mechanism by which TMZ initiates these anti-proliferative effects on pituitary adenoma cells? Kaina *et al* (20) proved that O^6-methylguanine (O^6MeG) is the major proapoptotic DNA lesion in malignant glioma cells upon O^6-methylating agent treatment. At a pH >7 TMZ is transformed into 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide (MTIC), which methylates DNA at the N^3 position of adenine and the O^6MeG. During the next cycle of DNA replication, O^6MeG mismatches with thymine (21), which is recognized and degraded by a mismatch repair system (MMR) (22). Owing to the template strand location of the adduct, another thymidine residue will replace the mismatched one during repair synthesis. Double-strand breaks (DSBs) in the DNA are generated due to repeated and invalid attempts to repair the damage, which then activates cell cycle checkpoints, G2/M cell cycle arrest and apoptosis through a signaling cascade (8,23). In addition to O^6MeG, the N^3-methyladenine adduct exerts its cytotoxic effect by hindering the binding of DNA polymerase to the adenine in the DNA template strand and is repaired by the base excision repair system (BER) (21,24). PARP plays an important role in this repair system and cleavage of full-length PARP to the cleaved form by executioner caspase-3/7 facilitates cellular disassembly, namely cell apoptosis (25), which is in agreement with the results of our study with TMZ treatment.

How does O^6MeG-triggered apoptosis get carried out in pituitary adenoma cells? After TMZ treatment, αT3-1 cells presented the decline in Bcl-2, a hallmark of O^6MeG-triggered apoptosis (26), accompanied by caspase-9 activation and the activation of the executioner caspase-3/7. Meanwhile, caspase-8, the initiator caspase of death receptor apoptotic pathway, was also evoked. These data indicate that both death receptor and mitochondrial apoptotic pathways likely participate in TMZ-induced αT3-1 cell apoptosis. Moreover, statistical analysis indicates that the death receptor pathway is less efficiently evoked than the mitochondrial apoptotic pathway in this process. Similarly, it has been shown that O^6MeG-triggered malignant glioma cell apoptosis was followed by Fas/CD95/Apo-1 receptor and caspase-8 activation in non-p53-mutated glioma cells, and intrinsic mitochondrial apoptotic pathway activation in p53 mutated glioma cells via Bcl-2 degradation, activation of caspase-9, -7 and -3 and formation of DSBs (27).

Baseline concentrations of gonadotropin are elevated in 30-50% of gonadotroph adenoma patients, and may result in ovarian hyperstimulation and dysgenesia (28-31). In previous clinical treatment of gonadotroph adenoma patients with TMZ, changes in gonadotropin levels before and after drug administration were not evaluated (15,16). In our *in vitro* study using gonadotroph adenoma αT3-1 cells, TMZ resulted in a dramatic decrease in gonadotropin production, which could have clinical significance for those patients suffering from hormone-hypersecreting and invasive gonadotroph adenomas. Similarly, for hyperprolactinemia, a previous *in vitro* study showed that prolactin secretion in the prolactin-secreting MMQ and GH3 cell lines was inhibited by TMZ (17) and a clinical trial demonstrated that a PRL carcinoma patient presented with reduced hormone secretion and obvious tumor shrinkage after TMZ treatment (12).

It is necessary to note that the αT3-1 cell line is not representative of human pituitary adenomas, and its human counterpart is needed to further evaluate the therapeutic effects of TMZ. In addition, the minimum stably effective concentration of TMZ (250 μM) in this study is above that reasonably achievable clinically (100 μM) (32). This may help explain why some patients with invasive pituitary adenoma are resistant to TMZ alone (18) and rapidly respond to the combination chemotherapy regimen of TMZ and capecitabine (33). Meanwhile, some caution must be exercised in extrapolating these results to the *in vivo* situation.

In summary, we report for the first time the inhibitory effects of TMZ on gonadotroph adenoma cell viability and

hormone secretion *in vitro* and provide further insight into the possible mechanisms triggered by both mitochondrial and death receptor apoptosis pathways. Our results suggest that TMZ may be useful in the treatment of aggressive gonadotroph adenomas, although its *in vivo* efficacy has yet to be fully examined.

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