Temozolomide-induced inhibition of pituitary adenoma cells

Laboratory investigation

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Object. Aggressive pituitary adenomas frequently require multimodality treatment including pituitary-suppressive medications, microsurgery, and radiation therapy or radiosurgery. The effectiveness of temozolomide in terms of growth suppression and decreased hormonal production is evaluated.

Methods. Three pituitary adenoma cell lines—MMQ, GH3, and AtT20—were used. A dose escalation of temozolomide was performed for each cell line, and inhibition of cell proliferation was assessed using an MTT assay. Concentrations of temozolomide that produced statistically significant inhibition of cell proliferation for each cell type were identified. Extent of apoptosis for each selected temozolomide concentration was studied using TUNEL staining. The effect of temozolomide on prolactin secretion in MMQ and GH3 cells was also measured via ELISA.

Results. Significant inhibition of cell proliferation was noted for MMQ and GH3 cells at a concentration of 250 μM temozolomide. The AtT20 cells demonstrated statistically significant cell inhibition at a concentration of only 50 μM temozolomide (p < 0.05). Apoptosis significantly increased in all cell lines in as little as 24 hours of incubation at the respective temozolomide concentrations (p < 0.05). Prolactin secretion in the prolactin secreting MMQ and GH3 cell lines was inhibited by 250 μM temozolomide.

Conclusions. Temozolomide inhibits cell proliferation and induces apoptotic cell death in aggressive pituitary adenoma cells. A reduction in hormonal secretion in prolactinoma cells was also afforded by temozolomide. Temozolomide may prove useful in the multimodality management of aggressive pituitary adenomas.

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Key words • pituitary adenoma • temozolomide • apoptosis • prolactin

Pituitary adenomas are one of the most common types of intracranial tumors, with autopsy studies demonstrating an incidence of 25%.1 The biology of pituitary adenomas can vary substantially. Some pituitary adenomas are slow growing, incidentally found, and require no treatment whatsoever. On the other extreme, some pituitary adenomas prove refractory to surgery, radiation, and pituitary-suppressive medications (for example, dopamine receptor agonists and somatostatin analogs) and continue to progress. Pituitary carcinomas occur in 0.2% of patients and may metastasize within the CNS.4

Temozolomide has proven effective in the treatment of patients with high-grade gliomas.2 Anecdotal case reports have suggested that temozolomide may have efficacy for some patients with aggressive pituitary adenomas, including pituitary carcinomas.3,4,10,15,16 Low MGMT expression has been linked to a favorable clinical response in patients with aggressive pituitary adenomas undergoing treatment with temozolomide.13,21 This study evaluates the response of 3 aggressive pituitary adenoma cell lines to temozolomide. In particular, the effects of temozolomide on cell proliferation, apoptosis, and prolactin production are assessed for the MMQ, GH3, and AtT20 pituitary adenoma cell lines.

Methods

Chemicals and Reagents

All GH3, MMQ, and AtT20 cells used in this study were purchased from the American Type Culture Collec-
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tion. The GH3 cells are a growth hormone- and prolactin-secreting line from the rat a secretes prolactin (ATCC number CCL-82.1). The MMQ cells are a prolactinoma cell line derived from a rat that is known to secrete prolactin and have functional dopamine receptors (ATCC number CRL-10609). The AtT20 cells are corticotrophs derived from a mouse anterior pituitary cell line; they do not produce prolactin (ATCC number CRL-1795). Temozolomide was obtained from Schering-Plough. The prolactin assay was obtained from MD Biosciences. The TUNEL reagents were supplied by BD Biosciences.

**Cell Culture**

The MMQ, GH3, and AtT20 cells were grown in F12 K medium with 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin and incubated at 37°C. The medium was changed every 3–4 days depending on the confluence, and cells were subcultured as needed for the experiments. For initial plating of cells in experiments, manual cell counts were performed using a trypan blue dye and a hemacytometer.

**Assessment of Cell Proliferation**

The MMQ, GH3, and AtT20 cells were spun down and resuspended in 1 ml of medium then plated in a 96-well plate at 10,000 cells/200 μl per well. The GH3 cells were trypsinized before being spun down and resuspended, as these are adherent cells. Temozolomide was then added at the various concentrations to the respective wells. After the incubation period of 1–4 days, 100 μl of MTT was added to each well. Between 2 and 3 hours later, 100 μl of developer solution (50% v/v dimethylformamide (DMF); 20% w/v sodium dodecyl sulfate (SDS); 0.24% v/v glacial acetic acid; 60 mM sodium acetate) was added to each well. The cells were then incubated overnight at 37°C. Using a plate reader, the absorbance was then calculated at a wavelength of 570 nm, and the OD values were determined. These values are represented as OD values or percentage change compared with a control (no temozolomide present).

A dose escalation of temozolomide for each cell line was performed. The effects of temozolomide concentrations from 50 to 500 μM were evaluated. Data are analyzed and expressed as an mean OD ± SEM and as an average percentage of control cells not exposed to temozolomide. Each MTT assay was performed in triplicate (that is, 3 times per well). Statistical differences in OD values were determined using a 2-tailed, equal-variance t-test. A p value ≤ 0.05 was deemed statistically significant.

**The TUNEL Staining Assays**

After review of the results from the MTT assay wherein dose escalation of temozolomide was performed, the temozolomide dose that resulted in consistently significant (p < 0.05 in the MTT data as compared with controls) inhibition of cell proliferation was selected for each of the cell lines. The temozolomide concentrations chosen for each cell line were as follows: MMQ, 250 μM; GH3, 250 μM; and AtT20, 50 μM.

In a similar fashion to the cell proliferation assay, cells were placed in 96-well plates as noted above. The respective cell lines were then incubated with temozolomide for 1, 2, 3, and 4 days. At each time point, cells were labeled with TUNEL staining to determine the percentage of apoptotic cells. Cell counts were performed in triplicate and at least 100 cells were counted in 5 high-powered fields per well. The percentage of apoptosis was expressed as total number of TUNEL-positive cells divided by the total number of cells counted.

**Prolactin Secretion Assay**

A commercially available prolactin ELISA was obtained from MD Biosciences, Inc. The prolactin assay was performed as per the recommended protocol. In brief, pituitary adenoma cells were cultured in 96-well plates using either normal culture medium or medium with temozolomide. After periods of incubation ranging from 1 to 4 days, the plates were centrifuged and the supernatant was removed to be used as the assay sample. A 100-fold dilution of the supernatant was performed to place the typical prolactin concentration within the middle of the standard range for the prolactin assay.

Each sample was then placed on the microtiter strips of the ELISA plate; 50 μl of sample, 50 μl of tracer, and 50 μl of antiserum were placed into each well and then allowed to incubate for 16–20 hours at room temperature. The plate was then emptied of the samples and each well was washed 5 times with buffer (300 μl/well). Next, 200 μl of Ellman’s reagent was then added to each well. Plates were incubated in the dark by covering them with aluminum foil and placing them on an orbital shaker for 1 hour. Then absorbance per well was measured on a plate reader at 405 nm (yellow color). Prolactin concentration was plotted against a standard prolactin curve (0.39 ng/ml–50 ng/ml). All samples were run in duplicate, and the prolactin level reported for each time point was the result of 3 independent experiments. Thus, the prolactin level reported was the mean of 6 samples.

**Statistical Analysis**

Cell proliferation (MTT), apoptosis (TUNEL), and prolactin assay results of cell lines at time points for control (no temozolomide) and temozolomide concentrations were compared via t-test using StatView (SAS Institute Inc.). A p value ≤ 0.05 was considered to denote statistical significance.

**Results**

**Inhibition of Cell Proliferation and Dose Response to Temozolomide**

Inhibition of cell proliferation was assessed in MMQ, GH3, and AtT20 cells relative to the equivalent number and type of cells grown in a temozolomide-free medium. For the MMQ cells, 50 μM temozolomide did not demonstrate a statistically significant inhibition of cell proliferation. However, statistically significant inhibition of MMQ cell proliferation was demonstrated on Days 1–4 at 250 μM and 500 μM concentrations of temozolomide (p < 0.05, Fig. 1A). Similarly, at Days 1–3, significant in-
Inhibition of GH3 cells was noted at 100, 200, 250, and 500 μM temozolomide, but the 50 μM temozolomide had no significant effect on cell proliferation (Fig. 1B, p < 0.05). The AtT20 cells exhibited significant inhibition of cell proliferation when exposed to 50 μM temozolomide at 2, 3, and 4 days (p < 0.05). Dose escalation of temozolomide to concentrations of 100–500 μM resulted in minimal escalation of cell inhibition of the AtT20 cells beyond that observed at the 50 μM concentration (Fig. 1C).

Extent of Apoptotic Cells Identified by TUNEL Staining

The minimum concentration of temozolomide sufficient to induce statistically significant inhibition of proliferation for each cell line was used from the preceding results. The concentrations of temozolomide used for this section were 50 μM for the AtT20, 250 μM for the MMQ, and 250 μM for GH3 cells.

Under normal in vitro culture conditions, the percentage of TUNEL-positive cells for the MMQ and GH3 cells was 1% or less (Figs. 2A and B). With 4 days of exposure of MMQ cells to 250 μM temozolomide, apoptosis as indicated by TUNEL-positive staining increased 4.2% (p = 0.018, Fig. 2A). At 4 days of exposure to 250 μM temozolomide, 5.3% of GH3 cells exhibited positive TUNEL staining consistent with apoptosis (p = 0.036, Fig. 2B).

The AtT20 cells demonstrated a higher rate of apoptosis in normal growth conditions. However, the TUNEL staining increased with temozolomide exposure. The AtT20 cells demonstrated a 15% rate of TUNEL staining after 4 days of exposure to 50 μM temozolomide, compared with a 4.5% rate in the control (temozolomide-free medium, p = 0.048, Fig. 2C).

Prolactin Secretion

The AtT20 cells do not produce prolactin. However, MMQ and GH3 cells are known to secrete prolactin into the culture medium. Thus, to explore the functional inhibition of prolactin by temozolomide, prolactin levels were measured in MMQ and GH3 cells under normal culture conditions and in the presence of 250 μM temozolomide.

After 2 days of exposure of MMQ and GH3 cells to 250 μM temozolomide, there was a statistically significant reduction in prolactin secretion. The MMQ cells exposed to temozolomide had a prolactin production of 8.04 × 100 ng/ml as compared with 10.6 × 100 ng/ml for controls (p = 0.02, Fig. 3 left). The GH3 cells exposed to 250 μM temozolomide for 2 days had a prolactin production of 11.7 × 100 ng/ml as compared with 21.8 × 100 ng/ml in the control cells (p = 0.04, Fig. 3 right). These trends of decreased prolactin secretion for MMQ and GH3 cells

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**Fig. 1.** A: Cell proliferation results for MMQ cells expressed as a function of control cells (no temozolomide in culture medium). Cells were exposed to varying concentrations of temozolomide for 1, 2, 3, and 4 days. Error bars denote SEM. The 250 μM temozolomide resulted in a statistically significant (p < 0.05) difference from control cells at Days 1–4. B: Cell proliferation results for GH3 cells expressed as a function of control cells (no temozolomide in culture medium). Cells were exposed to varying concentrations of temozolomide for 1, 2, 3, and 4 days. Error bars denote SEM. The 250 μM temozolomide resulted in a statistically significant (p < 0.05) difference from control cells at Days 1–3. C: Cell proliferation results for AtT20 cells expressed as a function of control cells (no temozolomide in culture medium). Cells were exposed to varying concentrations of temozolomide for 1, 2, 3, and 4 days. Error bars denote SEM. The 50 μM temozolomide resulted in a statistically significant (p < 0.05) difference from control cells at Days 2–4.

**Fig. 2.** A: Percentage of apoptotic (TUNEL-positive) MMQ cells as a function of days of incubation in control (no temozolomide [Temodar]) and 250 μM temozolomide–containing medium. Error bars denote the SEM. Temozolomide increased apoptosis in a statistically significant fashion at all 4 days evaluated (p < 0.05). B: Percent apoptotic (TUNEL-positive) GH3 cells as a function of days of incubation in control (no temozolomide) and 250 μM temozolomide–containing medium. Error bars denote the SEM. Temozolomide increased apoptosis in a statistically significant fashion at all 4 days evaluated (p < 0.05). C: Percentage of apoptotic (TUNEL-positive) AtT20 cells as a function of days of incubation in control (no temozolomide) and 50 μM temozolomide–containing medium. Error bars denote the SEM. Temozolomide increased apoptosis in a statistically significant fashion at all 4 days evaluated (p < 0.05).
50% of patients, suggesting that they might be candidates for pituitary adenomas, low MGMT expression was seen in the assessment of patients with progressive nonfunctioning pituitary adenomas. In a recent study a systematic assessment of temozolomide’s in vitro effects on pituitary cells had not been performed. The concentrations required for growth inhibition were higher than for human malignant glioma cells, which exhibited 50% inhibition of cell proliferation after exposure to 258 μM temozolomide. Thus, the growth inhibitory effects demonstrated in the 3 pituitary adenoma cell lines should be realizable in vivo. There is, of course, the possibility for development of some degree of drug resistance to temozolomide over time in aggressive pituitary adenoma cells much like the resistance that is observed in gliomas. This was suggested by the lack of significant growth inhibition at Day 4 of treatment in the GH3 cells (Fig. 1B).

Growth inhibition of the pituitary adenoma cells was accompanied by increasing apoptosis. Apoptosis was increased over controls in as little as 24 hours of temozolomide exposure. The rate of apoptosis remained elevated during the 4-day period of temozolomide exposure used during this research (Fig. 2A–C).

Moreover, in those cell lines secreting prolactin (that is, the MMQ and GH3 cell lines), temozolomide resulted in a demonstrable decrease in hormone production. Such a functional consequence in terms of improvement in hormone overproduction could have profound clinical benefits for patients experiencing the sequelae of an aggressive, functioning adenoma. In patients with aggressive prolactinomas that have a temozolomide-induced decrease in adenoma volume (number of cells), the prolactin assay data suggest that prolactin output should also decrease appreciably, thereby reducing the functional consequences of a secreting adenoma.

Temozolomide may afford beneficial results for aggressive pituitary adenoma cells on its own. However, as earlier noted, the mainstay of treatment for aggressive pituitary adenomas is a multimodality approach that frequently involves radiation therapy or radiosurgery. Fortunately, temozolomide is known to be a radiosensitizing agent. To date, this radiosensitization has largely been explored in the setting of high-grade gliomas. However, given the response to temozolomide of aggressive pituitary adenomas as reported in this paper and small case series, the sensitizing effect of temozolomide in the setting of planned stereotactic radiosurgery or radiation therapy may offer added benefit over the effects of either one alone. Further characterization of the benefit-to-risk pro-
file of temozolomide in patients with pituitary adenomas refractory to conventional treatments seems justified.

Limitations of this study include the fact that the cell lines were not derived from human pituitary adenomas. Also, this was not an in vivo study, and, as such, the in vitro results need to be translated into clinical applications with caution. Human-derived pituitary cell lines and useful in vivo models of pituitary adenoma should be developed to better evaluate the therapeutic effects of temozolomide and other potentially useful therapeutic agents.

Conclusions

Temozolomide can induce inhibition of cell proliferation and eventual apoptosis in aggressive pituitary adenoma cells. It may also lead to improvement in hormonal hypersecretion in functioning adenoma cells. Further clinical evaluation of temozolomide in the setting of patients with recurrent pituitary adenomas seems warranted.

Disclosure

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