Tumor-inhibition Effect of Levetiracetam in Combination with Temozolomide in Glioblastoma Cells

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Abstract—Glioblastoma (GBM) is a malignant brain tumor with a poor prognosis. The standard postoperative chemotherapy is temozolomide (TMZ), which does not greatly improve survival. The DNA repair gene *O*-6-methylguanine-DNA methyltransferase (*MGMT*) contributes to the response of TMZ-induced DNA damage. The commonly prescribed antiepileptic drug levetiracetam (LEV) has been shown to enhance TMZ's antitumor effect via inhibition of histone deacetylases (HDACs), but the therapeutic advantages of the LEV and TMZ combination remain poorly understood. Mechanisms of response to chemotherapy include apoptosis and mitotic catastrophe, and recent studies have suggested that premature senescence may also be invoked when cancer cells are exposed to therapeutic agents. In our study, we evaluated cell proliferation and premature senescence after single and combined treatments of TMZ and LEV in two GBM cell lines that differ in TMZ sensitivity caused by the absence (A172) or presence (T98) of the MGMT protein. Both LEV and TMZ reduced cell proliferation in a dose-dependent manner in A172 cells. A senescent-like phenotype, as determined by β-galactosidase activity, was induced by both TMZ and LEV. Overall, there was a greater effect following combined treatment compared to the monotherapy groups. Thus, LEV appears to have a tumor-suppressive effect and induces cellular senescence, and combined treatment of LEV and TMZ enhanced these effects. Because LEV treatment results in few adverse effects, its use in GBM treatment may allow for reduction of the TMZ dose to enhance the clinical efficacy of TMZ chemotherapy and improve quality of life.

Keywords: temozolomide, levetiracetam, premature senescence, glioblastoma

INTRODUCTION

Glioblastoma (GBM) is a highly malignant brain tumor, representing more than half of all cases of gliomas. Since 2005, the most standard treatment for GBM is a combination of chemotherapy with temozolomide (TMZ) and radiation therapy; however, the survival rate of GBM remains poor, with a 5-year survival rate of less than 10% [1,2]; therefore, there is an urgent need to identify novel therapeutic drugs, modifications to the TMZ treatment protocol, or a combination of agents that will increase the anticancer potency of TMZ.

Epigenetic silencing of the DNA repair mechanism gene *O*-6-methylguanine-DNA methyltransferase (*MGMT*) by promoter methylation has been associated with longer overall survival in patients with GBM [1,2]. Some studies have identified certain factors that make tumor cells more sensitive to TMZ therapy through MGMT-dependent or MGMT-independent mechanisms, including interferon-β, resveratrol, carnitine, levetiracetam (LEV), and valproic acid (VAP) [2-6]. Thus, confirming that enhanced MGMT expression promotes clinical resistance to TMZ would help to improve the predictive diagnosis and establish effective treatment strategies for GBM patients.

Although the effects of chemotherapeutic agents on specific molecular pathways have been studied to a great degree, the effects that other drugs may have on such pathways are less recognized. The anticancer effects of antiepileptic drugs (AEDs) can be significant, because many kinds of AEDs are administered to patients with brain tumors that can cause epileptic seizures. The intrinsic anticancer potency of AEDs and their synergistic effects with chemotherapy or radiotherapy have also been suggested [7,8]; however, AED therapy is associated with several adverse events, including dementia, myelosuppression, liver dysfunction, and Steven-Johnson syndrome [9,10].

LEV is also an approved drug with anti-epileptic effects, with 91% of glioma patients treated with LEV remaining seizure-free in a clinical study [11-13]. Bobustuc et al. [14] reported that LEV is the most potent MGMT inhibitor compared to other AEDs tested. LEV decreased MGMT protein and mRNA expression levels in GBM cells, and single treatment of LEV showed a tumor suppression effect against several GBM cell lines. Thus, LEV appears to inhibit GBM cell proliferation and increase the sensitivity of GBM cells to TMZ.

Recently, substantial evidence has identified cellular senescence as an alternative tumor suppressor mechanism to apoptosis [6,15]. Cell senescence represents an arrested state in which the cells remain viable, and is thought to be a tumor-suppressive mechanism. Thus, it has been proposed that premature senescence could contribute to the antitumor efficacy of currently used chemotherapeutics [16,17]. Nevertheless, there is no direct evidence that the clinical use of TMZ induces premature senescence in GBM cells *in vivo*.

To further investigate whether LEV could control tumor progression, the effects of single and combined treatments of TMZ and LEV were evaluated using two GBM cell lines (A172 and T98). We used cell proliferation and senescence assays to evaluate the anti-tumor ability of the combined treatment of TMZ and LEV against the GBM cell lines.

MATERIALS AND METHODS

Cell culture. Human glioma cell lines T98 and A172 were obtained from the American Type Culture Collection (ATCC). T98 and A172 cells (2×10^5 cells/mL) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

Cell proliferation assay. T98 and A172 cells (2×10^4) were plated in a 96-well plate for 24 h, treated daily with 4 concentrations of LEV (0, 80, 160, or 320 μg/mL), and harvested at 72 h for cell proliferation assays. In another experiment, both cells were plated for 24 h, treated daily with different concentrations of TMZ (0, 18.75, 37.5, 75, or 150 μg/mL) for 72 h, and then harvested for cell proliferation assays. In the third experiment, cells were treated daily with LEV (0, 80, 160, 320 μg/mL) for 72 h and then treated with TMZ (0, 18.75, 37.5, 75, or 150 μg/mL) for another 72 h before assays were performed. The control group was GBM cells that did not receive any LEV or TMZ treatment. These wells were washed carefully with phosphate buffered saline (PBS). The cells were placed in the microplate and stored at −80°C for more than 3 h until samples were assayed. Cell proliferation assays were performed using CyQUANT® following the manufacturer's instructions. Briefly, the plates were thawed at room temperature, and then 200 μL of the CyQUANT GR dye/cell-lysis buffer was added to each well. The plate was incubated for 2–5 min at room temperature, and protected from light. The next day, fluorescence was measured using a fluorescence reader with filters appropriate for ~480 nm excitation and ~520 nm emission maxima.

Senescence assay. A cell senescence kit (Senescence Detection Kit, BioVision Research Products; Milpitas, CA, USA), designed to histochemically detect β-galactosidase (SA-β-Gal) activity in a cultured cell, was used to detect the number of senescent cells in each group. The aging process, or senescence, is considered to be a defense mechanism to control the growth of tumors; cell division slows down in the aging cell to maintain metabolism. This causes enlargement of the cell, which results in enhanced SA-β-Gal activity. Since this

activity is not found in a standard cell, SA-β-Gal activity can be used as a marker of cell senescence, which is indicated by the blue color resulting from its substrate X-Gal. Cells were plated in a 72-well plate containing 3 mL of 1× PBS per well. The X-gal solution (25 μL of 20 mg/mL) was dissolved in 1 mL dimethyl sulfoxide to prepare a 20× stock solution. Staining Solution (470 μL) and Staining Supplement (5 μL) (provided in the kit) were heated to solubilize the precipitate at 24°C. The culture medium was removed and the cells were washed once in 1 mL of 1× PBS. The cells were mixed with 0.5 mL of fixative solution for 10–15 min at room temperature. The cells were washed twice with 1 mL of 1× PBS. Staining Solution Mix (0.5 mL) was added to each well. The plate was covered and incubated overnight at 37°C. Cells were observed under the microscope for development of blue color.

Statistical analysis. Each experiment was repeated three times. All results are expressed as the mean ± SD. Statistical analysis was performed with SPSS version 20 software. For the parametric data, one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was used to statistically examine the differences between multiple groups. The results were considered significant when a value of $P < 0.05$ was obtained.

RESULTS

Combined LEV + TMZ treatment inhibited glioma cell proliferation more than each drug alone. At 72 h, the T98 cell numbers were reduced to a certain degree relative to the number of control cells after daily treatment with 40, 80, 160, and 360 μg/mL LEV, respectively (data not shown). However, the reduction in cell proliferation was not statistically significant. Similarly, T98 cell

numbers were slightly reduced in a dose-independent manner relative to the control cell numbers after daily treatment with 18.75, 37.5, 75, and 150 μg/mL TMZ, respectively, at 72 h, but again there was no significant difference (data not shown). In contrast, after daily treatment with both LEV (40 or 80 μg/mL) and TMZ (18.75 or 75 μg/mL), T98 cell growth inhibition was enhanced compared to treatment with either drug alone at these concentrations (Fig. 1). In particular, a significantly greater inhibitory effect on GMB cell proliferation was observed with the combined LEV (80 μg/mL) + TMZ (75 μg/mL) treatment compared to TMZ-only treatment at either dose (18.75 or 75 μg/mL). Interestingly, the combined high dose of LEV (80 μg/mL) + low dose of TMZ (18.75 μg/mL) treatment showed a significantly greater inhibitory effect than the high dose of TMZ (75 μg/mL) treatment alone (Fig. 1).

With respect to the A172 cell line, the cell numbers were reduced by 20.2%, 23.5%, and 24.8% relative to the number of control cells after daily treatment with 80, 160, and 360 μg/mL LEV, respectively (data not shown). The reduction in cell proliferation was not statistically significant between each dose, although that at 80 μg/mL LEV was significantly reduced compared to the control level and that observed with treatment of 40 μg/mL LEV. In contrast to LEV induction, after daily treatment with 18.75, 37.5, 75, and 150 μg/mL TMZ, A172 cell numbers were reduced in a dose-dependent manner, by 33.3%, 35.1%, 39%, and 53% relative to the control cell numbers, respectively, at 72 h (data not shown), although the reduction in cell proliferation was not statistically significant for any TMZ concentration. After daily treatment with both LEV (40 or 80 μg/mL) and TMZ (18.75 or 75 μg/mL), a significantly greater inhibitory effect on A172 cell proliferation was observed, especially with the combined treatment of LEV at a higher (80 μg/mL) + TMZ (75 μg/mL) concentration

compared to the TMZ-only treatment.

These results demonstrate that combined treatment of LEV and TMZ has a greater effect on inhibiting GMB cell proliferation than either treatment alone.

Combined LEV + TMZ treatment induced cell senescence more than each drug alone. We assessed the TMZ sensitivity of the two GBM cell lines by using a cell senescence assay. Senescence was detected histochemically by staining for SA-β-Gal activity, a known characteristic of senescent cells. T98 or A172 cells started to undergo senescence at about 24 h after treatment with TMZ, LEV, or both, and the number of cells showing positive SA-β-Gal staining was determined (Fig. 2). At 72 h, the number of senescent T98 and A172 cells increased in a dose-dependent manner after daily treatment with 0, 80, and 800 μg/mL TMZ, with significant differences from untreated cells noted at both concentrations ($P < 0.001$). Similarly, dose-dependent increases in senescent cells were observed with LEV treatment alone at 0, 5, 10, 25, 50, and 100 μg/mL at 72 h (Fig. 3). Significant increases in senescence from the control were found in the treatments of 0 and 80 μg/mL TMZ ($P < 0.001$), 80 and 800 μg/mL TMZ ($P = 0.011$), 0 and 10 μg/mL LEV ($P = 0.02$), 25 and 50 μg/mL LEV ($P = 0.04$), and 25 and 100 μg/mL LEV ($P < 0.01$) in the T98 GBM cell line. Similarly, the increases in senescence from the control were statistically significant at 0 and 80 μg/mL TMZ ($P < 0.001$), 10 and 50 μg/mL LEV ($P = 0.012$), and at 50 and 100 μg/mL LEV ($P = 0.02$) in A172 GBM cells. Significant increases in senescence from the control were also observed in the cells treated daily with both TMZ (80 μg/mL) and LEV (0, 10, 50, 100 μg/mL) in both cell lines. GBM cells treated with 80 μg/mL TMZ + 10 μg/mL LEV and 80 μg/mL TMZ + 50 μg/mL LEV (T98 cells: $P = 0.04$; A172 cells, $P = 0.01$),

and with 80 μg/mL TMZ + 50 μg/mL LEV and 80 μg/mL TMZ + 100 μg/mL LEV (T98 cells: $P = 0.013$; A172 cells, $P = 0.03$) had significantly higher rates of senescence than the control cells. Significantly higher rates of senescence were observed in the combined treatment groups as compared to treatment with LEV or TMZ alone for concentrations of 50 and 100 μg/mL LEV (Fig. 3).

DISCUSSION

The present study revealed, for the first time, that LEV induces senescence in GBM cells. Sole LEV treatment could inhibit cell growth in only A172 cells, as determined by a cell proliferation assay, whereas treatment with LEV increased the number of SA-β-gal-positive senescent cells in both cell lines compared to controls, suggesting that LEV promotes cellular senescence in both GBM cell lines. Thus, our results showed that combinatorial treatment of LEV and TMZ showed an increased anticancer effect in a dose-dependent manner.

Several preclinical and clinical studies have proven that hypomethylating drugs such as 5-aza-2'-deoxycytidine (5-aza-CdR; decitabine), and mSin3A/histone deacetylases (HDACs) such as valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA; vorinostat) have potent anticancer activity and promising therapeutic potential [18,19]. Previous experimental results indicated that the anticancer effects of VPA can be increased when administered in combination with other drugs [20-22]. Determining potential interactions among drugs prescribed to patients with brain tumors and epilepsy is an important clinical consideration.

LEV appears to exert a neuroprotective effect via free radical activity, which can reduce inflammation and neuronal death [23,24]. Therefore, LEV may help

to prevent the nerve damage caused by radiochemotherapy [12]. Synaptic vesicle glycoprotein 2A (SV2A) is the protein-binding region of LEV, and *Sv2a* knockout mice have epilepsy characterized by severe tonic spasm attacks [25]. GBM and oligodendroglioma showed stronger SV2A expression compared to the other tumor types, indicating that the SV2A protein level may be a suitable marker to predict a patient's response to LEV [26]. Therefore, brain tumor patients, in particular GBM patients with high expression of SVA2, may be more likely to benefit from LEV treatment.

HDACs are attractive targets in cancer therapy because their inhibition can induce cell differentiation, growth arrest, and apoptosis [27]. LEV does not directly inhibit HDAC activity *in vitro* when applied at the therapeutic concentration [28]. A limited number of studies have reported that LEV increased the transcription of *HDAC1* and could recruit the HDAC1/mSin3A corepressor complex to the p53-binding site, which ultimately silenced MGMT protein and mRNA expression levels *in vitro* [8,14,29]. Thus, LEV may increase TMZ-induced cytotoxicity in patients with GBM who highly express the MGMT protein, while also inducing fewer adverse side effects [24]. Indeed, a high dose of LEV inhibited the proliferation of malignant glioma cells and increased the effects of inhibiting the sensitivity of glioma cells to TMZ [30]. Our proliferation assay also showed that a high dose of LEV inhibited A172 (MGMT-negative) cell proliferation and increased the effects of inducing the sensitivity of A172 cells to the monofunctional alkylating agent TMZ. In contrast, we found no antagonistic or synergistic effect of LEV combined with TMZ in T98 (MGMT-positive) cells. Collectively, these results suggest that if a GBM patient is administered an increased dose of LEV, the TMZ dose can be

reduced, which would minimize the incidence of adverse effects related to TMZ and improve quality of life. However, despite the reports of the few side effects of LEV, caution should be taken to not overmedicate with respect to a patient's age, underlying disease, and other factors.

A hallmark of cancers is unlimited cellular proliferation; furthermore, cellular senescence is a potent tumor suppression mechanism [31]. Evidence has also accumulated that induction of senescence contributes to the treatment efficacy of chemotherapeutic agents and other anticancer strategies [16]. Hence, suitable evaluation of chemotherapeutic effectiveness should take both forms of cellular response into account [32]. Resistance to cell death as well as escape from senescence could be crucial for malignant transformation, which may contribute to the failure of chemotherapy [33]. Evidence has also accumulated that the chemotherapy-mediated senescence entry is p53-independent [34].

Although initially identified in tumors following radiation or genotoxic chemotherapy [35,36], treatment-induced senescence might also be induced at lower drug doses, in contrast to the higher doses at which DNA damage and apoptotic responses are elicited. TMZ has been reported to induce senescence without apoptosis in melanoma cells [37]. Yamada et al. [7] showed that carnitine induced senescence in glioma cells, and suggested that carnitine in combination with chemotherapy could represent an attractive alternative therapy for glioblastoma, which currently lacks an effective treatment. However, to our knowledge, few studies have documented the TMZ- and/or LEV-induced senescence changes to GBM cells. Our study demonstrated that treatment of a relatively low dose of LEV (10 μg/mL) increased the number of senescent cells compared to controls in the T98 GBM cell assay. This suggests that even small amounts of LEV may have an effect of inducing senescence on neoplastic cells.

In addition, combination treatment of TMZ and a low dose of LEV (10 μg/mL) did not show a significantly increased effect on senescence, whereas the number of senescent cells did increase at higher LEV doses in both GBM cell lines. Collectively, our senescence assay suggested that LEV treatment in combination with TMZ might induce higher rates of senescence as compared to treatment with LEV or TMZ alone. Considering its beneficial effects on the side effects of anticancer drugs, the use of LEV in combination with TMZ could represent an attractive alternative therapy for GBM. However, further work needs to be performed in relation to the safety and toxicity of LEV and TMZ at optimal therapeutic concentrations.

CONCLUSIONS

LEV has a tumor-inhibiting effect in GBM cell lines, which was enhanced when treated in combination with TMZ. LEV could therefore be used in conjunction with TMZ, which would enable reduction of the TMZ dose to achieve an improved tumor response and reduced side effects. Although further studies are needed to determine the exact mechanism underlying regulation of TMZ sensitivity, the use of drugs with tumor-suppression effects is considered to lead to further improvement in the vital prognosis of glioma patients.

REFERENCES

1. Stupp, R., Hegi, M.E., Mason, W.P., et al. *Lancet Oncol.,* 2009, vol. 10, pp. 459–466.
2. Stupp, R., Mason, W.P., van den Bent, M.J., et al. *N. Engl. J. Med.,* 2005, 352, pp. 987–996.
3. Milligan, T.A., Hurwitz, S., and Bromfield, E.B. *Neurology*, 2008, vol. 71, pp. 665–669.
4. Motomura, K., Natsume, A., Kishida, Y., et al. *Cancer*, 2011, vol. 117, pp. 1721–1730.
5. Kim, Y.H., Kim, T., Joo, J.D., et al. *Cancer*, 2015, vol. 121, pp. 2926–2932.
6. Lin, C.J., Lee, C.C., Shih, Y.L., et al. *Free Radic. Biol. Med.* 2012, vol. 52, pp. 377–391.
7. Yamada, S., Matsuda, R., Nishimura, F., et al. *Exp. Ther. Med.* 2012, vol. 4, pp. 21–25.
8. Rossetti, A.O., and Stupp, R. *Curr. Opin. Neurol.* 2010, vol. 23, pp. 603–609.
9. Kargiotis, O., Markoula, S., and Kyritsis, A.P. *Cancer Chemother. Pharmacol.* 2011, vol. 67, pp. 489–501.
10. Jaeckle, K.A., Ballman, K., Furth, A., and Buckner, J.C. *Neurology*, 2009, vol. 73, pp. 1207–1213.
11. Perucca, E., and Tomson, T. *Lancet Neurol.,* 2011, vol. 10, pp. 446–456.
12. Rosati, A., Buttolo, L., Stefini, R., et al. *Arch. Neurol.,* 2010, vol. 67, pp. 343–346.
13. Fonkem, E., Bricker, P., Mungall, D., et al. *Front. Neurol.,* 2013, vol. 4, pp. 153.

14. Lee, Y.J., Kim, T., Bae, S.H., et al. *CNS Drugs,* 2013, vol. 27, pp. 753–759.

15. Gewirtz, D.A. *Autophagy,* 2009, vol. 5, pp. 1232–1234.

16. Bobustuc, G.C., Baker, C.H., Limaye, A., et al. *Neuro-oncology* 2010, vol. 12, pp. 917–927.

17. Courtois-Cox, S., Jones, S.L., and Cichowski, K. *Oncogene,* 2008, vol. 27, 2801–2809.

18. Xue, W., Zender, L., Miething, C., et al. *Nature,* 2007, vol. 445, pp. 656–660.

19. Campisi, J., and d'Adda di Fagagna, F. *Nat. Rev. Mol. Cell Biol.* 2007, vol. 8, pp. 729–740.

20. Kern, K., Schebesch, K.M., Schlaier, J., et al. *J. Clin. Neurosci.,* 2012, vol. 19, pp. 99–100.

21. Collado, M., Gil, J., Efeyan, A., et al. *Nature,* 2005, vol. 436, p. 642.

22. Ryu, C.H., Yoon, W.S., Park, K.Y., et al. *J. Biomed. Biotechnol.* 2012, vol. 2012, 987495.

23. Garcia-Manero, G., Yang, H., Bueso-Ramos, C., et al. *Blood,* 2008, vol. 111, 1060–1066.

24. Osuka, S., Takano, S., Watanabe, S., et al. *Neurol. Med. Chir. (Tokyo),* 2012, vol. 52, pp. 186–193.

25. Ryu, C.H., Park, K.Y., Kim, S.M., et al. *Biochem. Biophys. Res. Commun.* 2012, vol. 421, pp. 585–590.

26. Zhou, Y., Xu, Y., Wang, H., et al. *Oncol. Lett.* 2014, vol. 7, pp. 203–208.

27. Li, X.N., Shu, Q., Su, J.M., et al. *Mol. Cancer Ther*., 2005, vol. 4. no.12, pp. 1912–1922.

28. Eyal, S., Yagen, B., Sobol, E., et al. *Epilepsia,* 2004, vol. 45, no. 7, pp.

737–744.

29. de Groot, M., Douw, L., Sizoo, E.M., et al. *Neuro-oncology,* 2013, vol. 15, pp. 216–223.

30. Cardile, V., Pavone, A., Gulino, R., et al. *Brain Res.,* 2003, vol. 976, no. 2, pp. 227–233.

31. Ueda, Y., Doi, T., Takaki, M., et al. *Brain Res.,* 2009, vol. 1266, pp. 1–7.

32. Custer, K.L., Austin, N.S., Sullivan, J.M., and Bajjalieh, S.M. *J. Neurosci.,* 2006, vol. 26, pp. 1303–1313.

33. de Groot, M., Aronica, E., Heimans, J.J., and Reijneveld, J.C. *Neurology,* 2011, vol. 77, 532–539.

34. Nakada, M., Furuta, T., Hayashi, Y., et al. *Front. Oncol.,* 2012, vol. 2, p. 98.

35. Shay, J.W., and Roninson, I.B. *Oncogene,* 2004, vol. 23, pp. 2919–2933.

36. Litwiniec, A., Grzanka, A., Helmin-Basa, A., et al. *J. Cancer Res. Clin. Oncol.,* 2010, vol. 136, pp. 717–736.

37. Rebbaa, A. *Cancer Lett.* 2005, vol. 219, pp. 1–13.

FIGURE LEGENDS

Fig. 1. The effect of levetiracetam (LEV), temozolomide (TMZ), and combination treatment of LEV and TMZ at different doses in a glioblastoma cell line (A: T98, B: A172). Both single and combined treatment resulted in dose-dependent decreases in the numbers of cells compared to untreated control cells. Asterisks indicate significance of one-way analysis of variance (ANOVA) with Bonferroni correction testing. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Fig. 2. Cytochemical staining for SA-β-gal activity of T98 and A172 cells. Microphotograph shows positive (blue) cells. T98 or A172 cells started to undergo senescence at about 24 h after treatment with TMZ, LEV, or both, as indicated by an increased number of cells showing positive SA-β-Gal staining. At 72 h, the number of senescent T98 and A172 cells increased in a dose-dependent manner after daily treatment with 0 and 80 μg/mL TMZ, with significant differences from TMZ untreated cells noted at both concentrations. Original magnification, 200×.

Fig. 3. The effect of levetiracetam (LEV), temozolomide (TMZ), and combined treatment of LEV and TMZ on senescence in T98 and A172 cells. LEV and TMZ combined treatment induced higher rates of senescence as compared to LEV or TMZ treatments alone. Asterisks indicate significance of ANOVA testing. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.