

Original Research Article

Effects of mTOR inhibitor, everolimus, on proliferation, autophagy and temozolomide sensitivity of glioma cells

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Abstract

Purpose: To study the effect of the mTOR inhibitor, everolimus, on glioma cell proliferation, autophagy, and drug sensitivity to temozolomide (TMZ).

Methods: Human glioma cell lines were cultured *in vitro*, and the effects of different concentrations of everolimus on the proliferation of brain glial cells were determined using CCK-8 method. The effect of different concentrations of everolimus on brain glial cell levels of autophagy protein were assayed by western blot method.

Results: The results of CCK-8 analysis showed that everolimus inhibited the proliferation of glial cells in a time- and concentration-dependent manner. Western blot results showed that the expression levels of autophagy proteins, LC3-II and LC3-II/I, were gradually and concentration-dependently up-regulated, while p62 protein level was gradually decreased concentration-dependently, when compared with blank control ($p < 0.05$). Treatment with different concentrations of TMZ alone, and in combination with everolimus for 48 h inhibited the proliferation of brain glial cells in a concentration-dependent manner, but the inhibition due to TMZ-everolimus combination was significantly higher than that of TMZ single treatment ($p < 0.05$). After 48 h, the expression level of Beclin-1 increased with the ratio of LC3-II/LC-I in TMZ-everolimus group, while the expression level of p62 decreased, when compared with TMZ alone, or control ($p < 0.05$).

Conclusion: Everolimus significantly inhibits the proliferation of glioma cells and promotes the occurrence of autophagy. Combined use of TMZ and everolimus significantly enhances the sensitivity of TMZ to glioma cells, inhibits cell proliferation, and promotes autophagy better than TMZ alone.

Keywords: mTOR inhibitor, Everolimus, Glioma cells, Proliferation, Autophagy

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INTRODUCTION

Glioma is a high-incidence malignant tumor of the central nervous system due to carcinogenesis in the brain and spinal cord. The incidence of glioma accounts for 1- 3 % of all malignant tumors. Most glioma occur in the

cerebral hemisphere. Glioma is caused by the interaction between susceptible congenital genetic factors and carcinogenic environmental factors [1]. The symptoms and signs of glioma are related to the location and pathological grade of the tumor. Usually, patients present with headache, nausea, vomiting and blurring, among

other symptoms. At present, the commonly used clinical treatments are radiotherapy and chemotherapy. However, these treatments are not effective because high-grade gliomas grow rapidly, infiltrate strongly, and easily recur after operation. The median survival time of glioma patients is only 13 months [2].

Autophagy is a cellular protective mechanism which is associated with tumors, degenerative diseases and other human diseases. Autophagy induces programmed cell death through cell membrane degradation and specific gene activation [3]. It is known that mTOR is a serine/threonine protein kinase which regulates the occurrence and development of tumors through P13K/Akt signaling pathway. In addition, the transcription of gene information involved in mTOR signaling pathway regulates autophagy [4].

Everolimus is a specific blocker of the mTOR signaling pathway which is often used to avoid rejection after kidney and heart transplantation. It has been reported that everolimus significantly reduced the proliferation of cancer cell lines *in vitro* in breast cancer and pancreatic neuroendocrine tumors, and patients treated with TMZ chemotherapy showed good tolerance [5]. Therefore, the purpose of this study was to determine the effects of the mTOR inhibitor everolimus on proliferation, autophagy and TMZ drug sensitivity of glioma cells.

EXPERIMENTAL

Reagents and instruments

Glial cell lines were provided by Shanghai Academy of Life Sciences, Chinese Academy of Sciences. Everolimus powder was purchased from Dalian Meilun Biotechnology Co. Ltd, while TMZ powder was purchased from Simga USA. Fetal bovine serum (FBS) was obtained from Hycfone; Cck-8 kit was purchased from Dojindo, Japan, while RPMI1640 medium was purchased from Shanghai Jingke Chemical Technology Co. Ltd. Super clean table was product of Jiabao Purification Engineering Equipment Company. Cell culture kit was purchased from Syme Fisher Technology Co. Ltd, while -80 °C-refrigerator was bought from Qingdao Haier Co. Ltd. Desk-top high-speed centrifuge was purchased from Simga Warner Precision Instruments. Electron microscope was obtained from Olympus Inc.

Cell culture

Human brain glial cells were added to RPMI1640 medium containing 10% fetal bovine serum, and

cultured in an atmosphere of 5 % CO₂ at 37 °C and saturated humidity. The cell culture medium was changed every 2 - 3 days, and cells in good state of logarithmic growth were selected for the experiments.

CCK-8 method

The CCK-8 method was used to determine the effect of different concentrations of everolimus on brain glial cell proliferation. The brain glioma cells at logarithmic phase were subjected to trypsin digestion, and a broth containing serum preparation was added to the cell suspension in 96-well plates, each well containing about 2×10^5 cells, with 100 μL cell suspension per well. The plates were incubated at 5 % CO₂ in a constant temperature incubator for 24 h. Then, into the culture plate was put 10 μL culture solution containing everolimus at concentrations of 5, 10, 20 and 40 μM. The blank control group contained only 0.1 % DMSO. Five replicate-wells were set for each group, and the experiment was repeated 3 times. After incubation for 12, 24 and 48 h, CCK solution was added for further incubation for 3 h, and the absorbance (OD) value at 450 nm was determined in a microplate reader. The degree of inhibition of proliferation was calculated as in Eq 1.

$$CPI (\%) = \frac{(1 - OD_e)}{OD_c} \times 100 \dots\dots\dots (1)$$

where *CPI* is % inhibition of cell proliferation, *OD_e* is OD of experimental group, and *OD_c* is OD of control group.

Western blotting

Western blotting method was used to determine the effect of different concentrations of everolimus on autophagy protein levels in glial cells. Human glioma cells in logarithmic growth phase were digested with trypsin and placed in a constant temperature incubator at 5% CO₂ incubator overnight, with everolimus working solution added to the culture medium at concentrations of 5, 10, 20 and 40 μM. Thereafter, the cells were washed with PBS solution, and 100 μL of cell lysing solution was added to the cell precipitate obtained after centrifugation. The mixture was incubated on ice at 4°C for 10min, followed by centrifugation. The protein content of the supernatant (10 μL) was determined using bicinchoninic acid (BCA) test kit. The protein was subjected to SDS-polyacrylamide gel electrophoresis at a voltage of 20 V for 30 min, and the protein bands were transferred to PVDF membrane. Non-specific binding was blocked by incubating the membrane with non-fat milk solution for 1 h.

Then, the membrane was incubated overnight at 4°C with primary antibodies i.e. rabbit anti-human LC3B, mouse anti-human Beclin-1, and rabbit anti-human P62, each at a dilution of 1:500. Thereafter, the membrane was washed thrice with TBST solution. The hybridized PVDF membrane was placed in a color-developing box for 4 min. Then, the protein bands were scanned and quantified using Odyssey infra-red scanner. The CCK-8 method was also used to determine the inhibitory effect of combination of everolimus and TMZ on human glioma cell proliferation. The TMZ working concentrations were 25, 50, 100 and 200 μM . The blank control group contained only 0.1% DMSO. The CCK-8 procedure followed the same steps as described above. Western blotting was also used to determine the effect of everolimus/TMZ combination treatment on human brain glioma cell autophagy protein levels, using the same procedure described above.

Statistical analysis

Measurement data in this study are expressed as mean \pm SD. Differences among groups were analyzed using analysis of variance (ANOVA), while differences between two groups were analyzed with *t*-test. All statistical analyses were done with SPSS 20.0 software package. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Effect of everolimus concentration on glial cell proliferation

CCK-8 method was used to determine the effects of different concentrations of everolimus on the proliferation of glial cells. The results showed that

everolimus significantly inhibited the proliferation of glial cells in time- and concentration-dependent manner (Table 1).

Effect of everolimus concentration on autophagy protein levels in glial cells

Western blot results showed that after treatment of glial cells with different concentrations of everolimus, the expression levels of the autophagy proteins LC3-II, LC3-II / I ratio and Beclin-1 were gradually increased in a concentration-dependent manner, when compared with the blank control group, and the protein level of P62 was gradually decreased in a concentration-dependent manner ($p < 0.05$; Table 2, Figure 2).

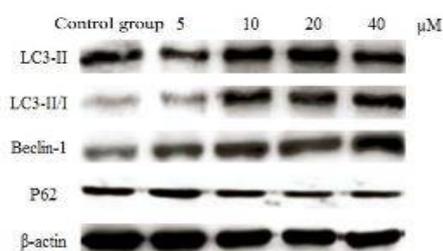


Figure 2: Effect of different concentrations of evimox on autophagy protein levels in glial cells

Effect of TMZ alone, and in combination with everolimus on glial cell proliferation

The CCK-8 test results showed that treatment of glial cells with different concentrations of TMZ alone or in combination with everolimus for 48h inhibited the proliferation of glial cells in a concentration-dependent manner, and the inhibition due to TMZ/ everolimus combination was significantly higher than that of TMZ alone ($p < 0.05$; Figure 3).

Table 1: Inhibitory effect of everolimus on the proliferation of glial cells (%)

Time (h)	Control group	Concentration of everolimus (μM)			
		5	10	20	40
12	2.78 \pm 1.15	5.21 \pm 1.48*	8.67 \pm 1.55*	10.54 \pm 1.89*	12.33 \pm 2.10*
24	4.68 \pm 2.11	9.45 \pm 2.22*	12.46 \pm 2.57*	20.88 \pm 2.67*	31.87 \pm 3.12*
48	7.49 \pm 2.25	13.66 \pm 2.31*	21.48 \pm 2.77*	32.65 \pm 2.87*	52.74 \pm 3.28*

* $P < 0.05$, compared with blank control group at the same time

Table 2: Effect of different concentrations of everolimus on autophagy protein levels in glial cells

Autophagy protein	Control group	Concentration of everolimus (μM)			
		5	10	20	40
LC3-II	0.11 \pm 0.02	0.15 \pm 0.01	0.36 \pm 0.11*	0.42 \pm 0.09*	0.58 \pm 0.12*
LC3-II/I Ratio	0.28 \pm 0.05	0.38 \pm 0.06	0.55 \pm 0.07	0.61 \pm 0.11*	0.76 \pm 0.13*
Beclin-1	0.11 \pm 0.03	0.19 \pm 0.02*	0.23 \pm 0.05*	0.48 \pm 0.08*	0.77 \pm 0.17*
P62	0.59 \pm 0.12	0.52 \pm 0.10	0.38 \pm 0.12*	0.22 \pm 0.04*	0.18 \pm 0.05*

* $P < 0.05$, compared with blank control group

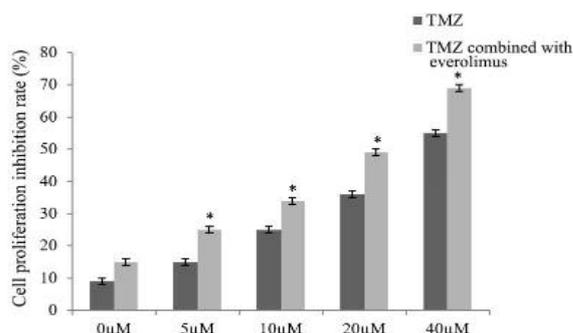


Figure 3: Effect of different concentrations of TMZ alone, and in combination with everolimus on glial cell proliferation. * $p < 0.05$, compared with TMZ alone

Effect of TMZ alone, and in combination with everolimus on autophagy protein levels in glial cells

Western blot results showed that after treatment of glial cells with TMZ alone or in combination with everolimus for 48 h, LC3-II / I ratio and Beclin-1 expression level in TMZ/everolimus combination group showed increasing trends, while P62 expression level showed a decreasing trend, when compared with TMZ alone, and control group (Table 3 and Figure 4).

Table 3: Effect of TMZ alone and in combination with everolimus on autophagy protein levels in glial cells

Autophagy protein	Control group	TMZ alone	TMZ combined with everolimus
LC3-II/I Ratio	0.62±0.15	1.21±0.23*	1.93±0.25*#
Beclin-1	0.32±0.09	0.81±0.10*	1.68±0.24*#
P62	1.72±0.28	1.02±0.21*	0.62±0.22*#

* $P < 0.05$, compared with blank control group; # $p < 0.05$, compared with TMZ alone group

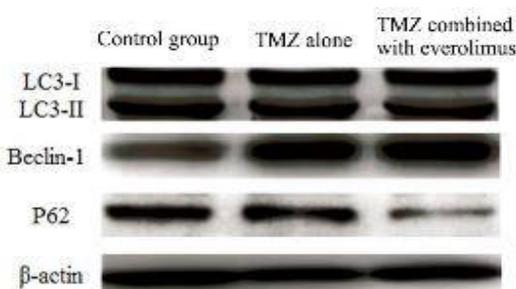


Figure 4: Effect of TMZ alone, and in combination with everolimus on autophagy protein levels in glial cells

DISCUSSION

Brain glioma is a highly heterogeneous tumor with invasive growth and no obvious boundary

with the surrounding brain tissue. The pathogenesis of glioma is not yet clear. However, it is generally believed to be related to genetic, environmental, immune dysfunction and other factors [6]. At present, effective chemotherapy after surgical resection and radiotherapy is still the main method for clinical treatment of brain glioma. However, due to difficulty in achieving complete surgical resection, the patients are prone to recurrence after surgery, with poor prognosis and five-year survival of only 2 % [7]. Therefore, there is need for new treatment methods to improve the survival and prognosis of patients.

mTOR is a downstream effector of mammalian P13K pathway, and it belongs to P13K-related kinases. It regulates tumors through the P13K /Akt signaling pathway. The P13k/Akt/mTOR signaling pathway is activated by multiple mechanisms in glioma, and is closely related to cancer [8]. Everolimus is an mTOR inhibitor which induces apoptosis by inhibiting mTOR kinase activation and blocking the regulation of downstream effectors, and by blocking G1-S transformation of cell cycle. Previous studies have demonstrated that everolimus induces autophagy and cell death, and affects the proliferation of glioma cell lines through cytotoxic effects [9,10]. Scholars elsewhere have found that everolimus improves the progression-free survival of patients with pancreatic neuroendocrine tumors, and speculated that it may prevent the occurrence of tumors by blocking the P13k/Akt/mTOR pathway [11].

Currently, the US Food and Drug Administration (FDA) has approved everolimus for clinical use in the treatment of renal cell carcinoma and other diseases [12]. Therefore, everolimus plays a significant role in inhibiting tumor proliferation and promoting autophagy. The results of this study showed that treatment of glioma cells with different concentrations of everolimus led to significant inhibition of the proliferation of glial cells, and the inhibitory effect was gradually enhanced with increase in drug concentration and exposure duration.

Autophagy is an effective way to induce excessive self-degradation and death of cells. Previous studies have proved that autophagy is closely related to precancerous lesions and proliferation of tumor cells, and may become a new method of anticancer therapy [13]. The results of this study showed that treatment of glial cells with different concentrations of everolimus led to gradual and concentration-dependent increases in autophagy protein LC3-II, LC3-II /LC-I ratio and Beclin-1 expression

levels, when compared with the blank control group, and the protein level of P62 was gradually decreased in a concentration-dependent manner. These results suggest that everolimus significantly inhibits cell proliferation and promotes autophagy after treatment of glioma cells.

Temozolomide (TMZ) is an oral second-generation alkylating agent and imidazotetrazine derivative which is absorbed quickly after oral administration. It directly crosses the blood-brain barrier, enters the cerebrospinal fluid, and acts on brain tumor tissues. It produces reasonable efficacy and acceptable toxicity, and has obvious efficacy on primary and recurrent malignant glioma [14]. Temozolomide (TMZ) does not exert a direct effect. Rather, it mainly plays a cytotoxic role through DNA guanine methylation, causing autophagic cell death. Relevant data show that TMZ adjuvant chemoradiotherapy significantly improved long-term survival of patients with brain glioma, with obvious synergistic effects [15].

The results of this study show that treatment of glial cells with TMZ alone or in combination with everolimus for 48h led to inhibition of the proliferation of glioma cells, with higher inhibition by TMZ/ everolimus combination than TMZ alone. Compared with TMZ alone group and control group, LC3-II /LC-I ratio and Beclin-1 expression level in the TMZ/ everolimus group showed upward trends, while P62 expression level showed a downward trend. These results suggest that everolimus enhances the sensitivity of glioma cells to TMZ. Its inhibitory effects on cell proliferation and autophagy are better than that of TMZ alone, but the specific underlying mechanism needs to be elucidated in further studies.

CONCLUSION

The results obtained in this study indicate that the mTOR inhibitor everolimus significantly inhibits the proliferation of glioma cells and promotes autophagy. However, combined use of TMZ and everolimus has significantly better enhancing effect on the sensitivity of TMZ to glioma cells, better inhibitory effect on cell proliferation, and better autophagy-enhancing effect than TMZ alone.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Jialiang Wang conceived and designed the study, Wei Dong, Jialiang Wang, Haipeng Liu, Shuo Sun, Yanbin Wang collected and analysed the data, while Wei Dong wrote the manuscript.

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REFERENCES

1. Lenting K, Verhaak R, Laan MT, Wesseling P, Leenders W. Glioma: experimental models and reality. *Acta Neuropathol* 2017; 133: 263-282.
2. Buckner JC, Shaw EG, Pugh SL, Chakravarti A, Gilbert MR, Barger GR, Coons S, Ricci P, Bullard D, Brown PD, et al. Radiation plus Procarbazine, CCNU, and Vincristine in Low-Grade Glioma. *N Engl J Med* 2016; 374: 1344-1355.
3. Ho TT, Warr MR, Adelman ER, Lansinger OM, Flach J, Verovskaya EV, Figueroa ME, Passequé E. Autophagy maintains the metabolism and function of young and old stem cells. *Nature* 2017; 543: 205-210.
4. Mathews ES, Appel B. Cholesterol Biosynthesis Supports Myelin Gene Expression and Axon Ensheathment through Modulation of P13K/Akt/mTor Signaling. *J Neurosci* 2016; 36: 7628-7639.
5. Nuñez JE, Donadio M, Filho DR, Rego JF, Barros M, Formiga MN, Lopez R, Riechelmann R. The efficacy of everolimus and sunitinib in patients with sporadic or germline mutated metastatic pancreatic neuroendocrine tumors. *J Gastrointest Oncol* 2019; 10: 645-651.
6. Yang P, Qiu Z, Jiang Y, Dong L, Yang W, Gu C, Li G, Zhu Y. Silencing of cZNF292 circular RNA suppresses human glioma tube formation via the Wnt/ β -catenin signaling pathway. *Oncotarget* 2016; 7: 63449-63455.
7. Ceccarelli M, Barthel FP, Malta TM, Sabedot TS, Salama SR, Murray BA, Morozova O, Newton Y, Radenbaugh A, Pagnotta SM, et al. Molecular Profiling Reveals Biologically Discrete Subsets and Pathways of Progression in Diffuse Glioma. *Cell* 2016; 164: 550-563.

8. Wu J, Hu G, Dong Y, Ma R, Yu Z, Jiang S, Han Y, Yu K, Zhang S. *Matrine induces Akt/mTOR signalling inhibition-mediated autophagy and apoptosis in acute myeloid leukaemia cells.* *J Cell Mol Med* 2017; 21: 1171-1181.
9. Meng J, Lister J, Vataire AL, Casciano R, Dinot J. *Cost-effectiveness comparison of cabozantinib with everolimus, axitinib, and nivolumab in the treatment of advanced renal cell carcinoma following the failure of prior therapy in England.* *Clinicoecon Outcomes Res* 2018; 10: 243-250.
10. Gottlieb J, Neurohr C, Müller-Quernheim J, Wirtz H, Sill B, Wilkens H, Bessa V, Knosalla C, Porstner M, Capusan C, et al. *A randomized trial of everolimus-based quadruple therapy vs standard triple therapy early after lung transplantation.* *Am J Transplant* 2019; 19: 1759-1769.
11. Franz DN, Lawson JA, Yapici Z, Brandt C, Kohnman MH, Wong M, Milh M, Wiemer-Kruel A, Voi M, Coello N, et al. *Everolimus dosing recommendations for tuberous sclerosis complex-associated refractory seizures.* *Epilepsia* 2018; 59: 1188-1197.
12. Mohamed A, Romano D, Saveanu A, Roche C, Albertelli M, Barbieri F, Brue T, Niccoli P, Delpero JR, Garcia S, et al. *Anti-proliferative and anti-secretory effects of everolimus on human pancreatic neuroendocrine tumors primary cultures: is there any benefit from combination with somatostatin analogs?* *Oncotarget* 2017; 8: 41044-41063.
13. Liu L, Liao JZ, He XX, Li PY. *The role of autophagy in hepatocellular carcinoma: friend or foe.* *Oncotarget* 2017; 8: 57707-57722.
14. Ma MF, Kong LD, Du ZY, Xie ZY, Chen L, Chen RJ, Li ZQ, Liu J, Li ZL, Hao AY. *A novel stimulus-responsive temozolomide supramolecular vesicle based on host-guest recognition.* *Colloid Polym Sci* 2019; 297: 261-269.
15. Huang J, Chaudhary R, Cohen AL, Fink K, Goldlust S, Boockvar J, Chinnaiyan P, Wan L, Marcus S, Campian JL. *A multicenter phase II study of temozolomide plus disulfiram and copper for recurrent temozolomide-resistant glioblastoma.* *J Neurooncol* 2019; 142: 537-544.