

Original article

Mechanism of thalidomide to enhance cytotoxicity of temozolomide in U251-MG glioma cells *in vitro*

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Keywords: glioma; thalidomide; temozolomide; autophagy

Background Glioma is the most common primary brain tumor with poor prognosis. Temozolomide has been used with thalidomide to treat gliomas. We investigated the synergistic mechanism of these two drugs *in vitro*.

Methods Human malignant glioma cells U251-MG were cultured and assigned to four groups with different treatments for 3 days: temozolomide group (100 $\mu\text{mol/L}$), thalidomide group (100 $\mu\text{g/L}$), temozolomide (100 $\mu\text{mol/L}$) plus thalidomide group (100 $\mu\text{g/L}$) and control group. MTT assay was applied to evaluate the cell viability. Cell cycle was analyzed by flow cytometry. The ultra-structural features of autophagosomes were observed with electron microscope. Acridine orange and monodansylcadaverine were adopted to label autophagosomes and flow cytometry was applied for quantification of autophagosomes. The expression of autophagy-associated protein was detected by Western blotting.

Results Proliferation of tumor cell was obviously suppressed by temozolomide with thalidomide treatment than by either drug used alone ($P=0.000$ for each day). The combination treatment induced cell cycle arrest at G0/G1 phase. Typical autophagic ultra-structural character was found after the combined treatment. Thalidomide promoted the autophagy induced by temozolomide. The autophagy-associated proteins — microtubule associated protein 1 light chain 3 (MAP1LC3) and Beclin1 were more significantly up-regulated by the combined treatment than temozolomide used alone (MAP1LC3, $P=0.000$; Beclin1, $P=0.004$). The expression level of phosphatase and tensin homolog deleted on chromosome ten (PTEN), which promoted autophagy by suppressing PI3K/Akt/mTOR signaling pathway, was elevated by thalidomide (thalidomide group: $P=0.000$; combined group: $P=0.002$).

Conclusions Thalidomide enhances the cytotoxicity of temozolomide by promoting the autophagy induced by temozolomide. Contributing to the up-regulation of PTEN by thalidomide, the expression of autophagy associated protein-MAP1LC3 and Beclin1 was enhanced, which leads to a reinforced autophagy in the combined treatment of temozolomide and thalidomide *in vitro*.

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Glioma is the most common primary brain tumor associated with a high degree of morbidity and mortality.¹ As the molecular mechanisms of gliomas have been clarified gradually, improving chemotherapeutic effect turns out to be the key part in combination treatment.² The therapeutic goal of chemotherapy triggers tumor-selective cell death.³ Compared with necrosis, programmed cell death (PCD) is more feasible to be induced by chemoagents. Until now, two types of PCD have been known: apoptosis and autophagic cell death. Glioma cells are more likely to respond to therapy through autophagy than through apoptosis.⁴

Temozolomide (TMZ), is a novel oral chemotherapeutic drug with broad-spectrum antitumor activity which penetrates into the brain and does not require hepatic metabolism for activation. Kanzawa et al⁵ reported TMZ inhibited the viability of malignant glioma cells by induction of autophagic cell death. However, Using TMZ alone would induce drug resistance in tumor and decrease the ratio of efficacy to price. Fortunately, the favorable safety profile of temozolomide allows it to be co-administered with various agents. Thalidomide (THD) has anti-angiogenic properties.⁶ A phase II study demonstrated that the combination of TMZ and THD was

more efficient than either agent alone.⁷ The two drugs act in different ways to exert the treatment effects: TMZ is related to cytotoxicity while THD inhibits tumor growth by anti-angiogenesis. It is poorly documented whether a synergistic mechanism exists or not.

In our preliminary related study *in vitro*, TMZ combined with THD more significantly inhibited tumor cell growth compared with TMZ used alone. The result implies us that besides anti-angiogenesis in the treatment of glioma, the synergistic effect of THD and TMZ may exist.

METHODS

Tumor cell line

Human GBM cell line U251-MG was obtained from the

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Institute of Neurology of Tianjin Medical University. U251-MG is a human brain tumor cell line that was established from a patient with glioblastoma multiforme by Ponten, with the characteristics such as low terminal cell density, strong density dependent inhibition of proliferation, diploidy and contact inhibition of membrane mobility.⁸ The cells were cultured as adherent cells in DMEM (GIBCO BRL Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 4 mmol/L glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Reagents

TMZ was kindly supplied by Tasly Pharmaceutical Co., Ltd. (Tianjin, China). THD was purchased from Changzhou Pharmaceutical Factory (Jiangsu, China). TMZ and THD were dissolved in 10% dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) to make a stock solution (TMZ: 100 mg/ml; THD: 100 mg/ml). DMSO concentration was kept below 0.1% in all the cell cultures and did not exert any detectable effect on cell growth or cell death. Acridine orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and monodansylcadaverine (MDC) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Goat anti-human polyclonal antibodies against Beclin1, MAP1LC3, PTEN and β -actin; horseradish peroxidase-conjugated secondary antibody (rabbit anti goat) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Treatments

The IC₅₀ (concentration resulting in cell viability of 50% of control group) of TMZ for U251-MG was less than 200 mmol/L. So we fixed the concentration of TMZ at a clinically achievable dose at 100 μ mol/L.⁹ Cultures of U251-MG were assigned to four groups based on different treatments: TMZ (treated with TMZ 100 μ mol/L), THD (treated with THD 100 μ g/L), TMZ plus THD (treated with TMZ 100 μ mol/L plus THD 100 μ g/L) and solvent control (DMSO) group.

Cell viability assay

The cytotoxicity of TMZ and THD on U251-MG was determined by MTT assay. Tumor cells were seeded at 2×10^3 cells/well (0.1 ml) in 96-well flat bottom plates and incubated overnight at 37.1°C. After exposure to the above described treatment for 24, 48, 72, and 96 hours. MTT (0.5 mg) was added to each well every 4 hours before the ending of incubation, when the MTT reaction was stopped by addition of 10% SDS-0.1 mol/L HCL. The formazan crystals of the cell were dissolved in DMSO and then its absorbance at 490 nm was measured by a microplate reader (Bio-Tek ELX800uv, Bio-Tek Instrument Inc. Winooski, VT., USA). All experiments were repeated in triplicate.

Cell cycle analysis

For cell cycle analysis, after different treatments for 3

days, U251-MG cells were trypsinized and then fixed in chilled 70% ethanol for at least 24 hours before being stained with 50 μ g/ml propidium iodide and 100 μ g/ml RNase A (Sigma, USA). After being stained for thirty minutes, the samples were analyzed using flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed by Modifit system (Becton Dickinson Company, USA). All experiments were repeated in triplicate.

Ultra-structural features of treated cells with electron microscopic observation

To morphologically demonstrate the induction of autophagy in treated tumor cells, we performed the ultra-structural observation as described previously.¹⁰ After different treatments for 3 days, U251-MG were harvested by trypsinization, washed twice with PBS and fixed with ice-cold glutaraldehyde (3% in 0.1 mmol/L cacodylate buffer, pH 7.4) for 30 minutes. After being washed in PBS, the cells were fixed in OsO₄ and embedded in Epon; 0.1 mm thin sections were stained with uranyl acetate/lead citrate (Fluka) and viewed in a Philips EM 400 electron microscope.

Detection and quantification of acidic vesicular organelles (AVOs) stained with acridine orange

Autophagy is characterized by formation and promotion of AVOs. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green, whereas acidic compartments fluoresce bright red. The volume of the cellular acidic compartment, which reflects the number of autophagic vacuoles, can be quantified. After different treatments for 3 days, cells were stained with acridine orange at a final concentration of 1 μ g/ml for 15 minutes, removed from the plate and collected in phenol red-free growth medium. Green (510–530 nm) and red (>650 nm) fluorescence emission from 10^4 cells illuminated with blue (488 nm) excitation light were measured with flow cytometry and the data were analyzed with Cell Quest software (Becton Dickinson company).

Visualization of MDC-labeled autophagic vacuoles

A fluorescent compound, MDC, has been proposed as a special tracer for autophagic vacuoles.¹¹ The U251-MG cells were treated as previously described, respectively. The autophagic vacuoles were labeled with MDC by incubating cell growth on cover-slips with 0.05 mmol/L MDC in PBS at 37°C for 1 hour. After incubation, cells were washed twice with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Olympus IX-71, Japan) equipped with a filter system. Images were obtained with a CCD camera (Olympus DP30BW, Japan) and processed using the program Meta View, version 4.5 (Universal Images corporation, USA).

Quantification of MDC labeled autophagic vacuoles

The cells were stained with the method mentioned above. Then the samples were analyzed by flow cytometry to detect the ratio of MDC staining positive cells. Excitation

wave length was 530 nm, emission filter was 590 nm. All experiments were repeated in triplicate.

Western blotting

For Western blotting, protein was abstracted from the treated cells. After the protein concentration was determined, equivalent amounts of proteins was boiled in sample buffers with DL-Dithiothreitol (DTT) and then centrifuged the protein at 12000 r/min for 15 minutes at 4°C. The protein was separated by SDS-PAGE (PAGE: 15% gel for MAP1LC3; 10% gel for Beclin1). Separated proteins in gel were transferred onto PVDF membranes by an electroblot apparatus (Bio-Rad, USA). Filters were blocked for 2 hours in 5% low fat milk and were incubated with goat anti-human antibody for MAP1LC3/Beclin1/PTEN and β -actin at 4°C for 12 hours. Membranes were then washed with PBS-T and incubated with HRP-conjugated anti-goat antibody for 1 hour. Specific signals were detected from quantitative gel and Western blotting imaging system (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Multiple groups were compared using analysis of variance (ANOVA) followed by post LSD testing where appropriate. Data were considered statistically significant at $P < 0.05$. All data are expressed as mean \pm standard deviation (SD).

RESULTS

Combination of TMZ with THD exhibited an obvious inhibition on cell viability

As shown in Figure 1: after different treatments for 3 days, the inhibition of cell proliferation in TMZ with THD group was more remarkable than that in TMZ group ($P < 0.05$). Compared with the control group ($P = 0.63$), THD used alone showed no significant inhibition on cell viability of U251-MG.

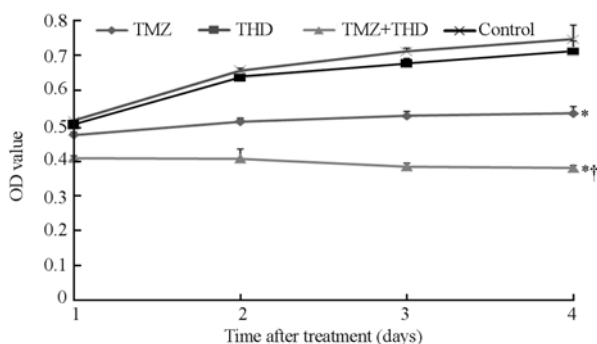


Figure 1. The OD value measured from MTT assay represented cell viability of U251-MG after different treatment at 1, 2, 3, and 4 days respectively. Results shown are the means of three independent experiments. * $P < 0.05$ as compared with the control group, † $P < 0.05$ as compared with the TMZ group.

Combination of TMZ with THD induced cell cycle arrest of G0/G1 phase on U251-MG

Results of cell cycle analysis shown in Table indicated that after different treatments for 3 days, TMZ alone increased the population of the G2/M phase ($P < 0.01$), while TMZ with THD significantly increased the population of G0/G1 phase ($P < 0.01$). The results indicated that TMZ alone induced G2/M phase arrest in tumor cells while TMZ combined with THD advanced the cycle arrest to G0/G1 phase, which demonstrated a promoted inhibition of cell proliferation.

Table. Cell cycle distribution of U251 cells treated with TMZ and THD for 3 days

Groups	n	Cell cycle (%)		
		G ₀ /G ₁	S	G ₂ /M
Control	3	47.30 \pm 3.05	35.40 \pm 4.45	17.30 \pm 2.65
TMZ	3	22.53 \pm 4.94*	26.07 \pm 1.80*	51.40 \pm 5.16*
TMZ+THD	3	65.33 \pm 4.16*	15.23 \pm 1.78*	15.43 \pm 4.42
THD	3	52.86 \pm 6.55	32.87 \pm 3.58	15.97 \pm 6.46

The population of cells at each phase after different treatments for 3 days. Results shown are the means of percentage from three independent experiments. * $P < 0.05$ as compared with the control group.

Ultrastructural features of autophagy were observed after TMZ and THD treatment

As shown in Figure 2, while untreated tumor cells exhibited few such features (Figure 2A), autophagosomes and secondary lysosomes were observed in TMZ treated and combined treatment group (Figure 2B–2E). The sum of autophagosomes in TMZ+THD group was definitely larger than that of the TMZ group. Most of the autophagosomes contained lamellar structures (Figure 2D) or residual digested materials (Figure 2E). These results indicated that U251-MG cells treated with TMZ for 3 days were in the stage of autophagic process. No apoptotic body was found in either group, which indicated that only autophagy was induced by TMZ rather than apoptosis.

Development of AVOs in TMZ with THD treated U251-MG cells

In the results of AVOs detection with flow cytometry, the percentage of cells in Q1 and Q2 quadrants stands for positive-staining rate. As shown in Figure 3, after 3 days treatment, compared with TMZ used alone (10.41%), the combination of TMZ with THD significantly increased the number of AVOs (36.05%), which represents the degree of autophagy in cells. THD used alone did not increase AVOs obviously. The result indicated that THD may enhance the TMZ induced autophagy, but THD itself did not do.

MDC accumulation increases after treatment of TMZ with THD

The fluorescent compound, MDC, could be specifically accumulated in autophagic vacuoles. As shown in Figure 4A, by using fluorescence microscope, autophagic vacuoles labeled by MDC were observed concentrated in spherical structures distributed in the cytoplasm and also localized in the perinuclear region, probably corresponding

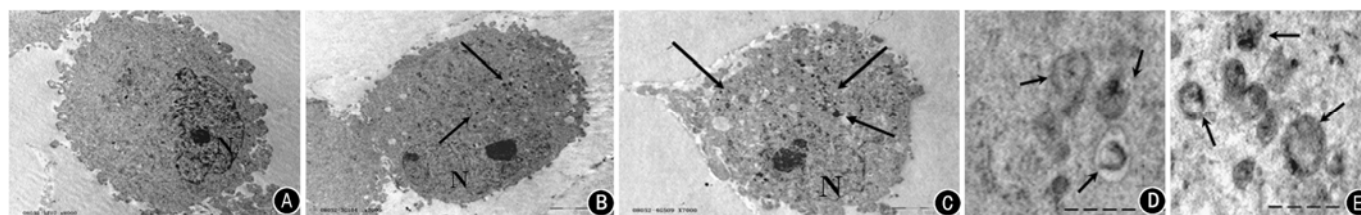


Figure 2. Ultrastructure features of U251-MG after different treatments for 3 days with electron microscopic observation, N indicated nucleus. **A:** Untreated cells. **B:** TMZ (100 μmol/L) for 3 days. **C:** TMZ (100 μmol/L)+THD (100 μg/L) for 3 days. **D:** Autophagosomes including lamellar structure. **E:** Autophagosomes including residual digested material. The arrows in the pictures indicate autophagosomes in cytoplasm.

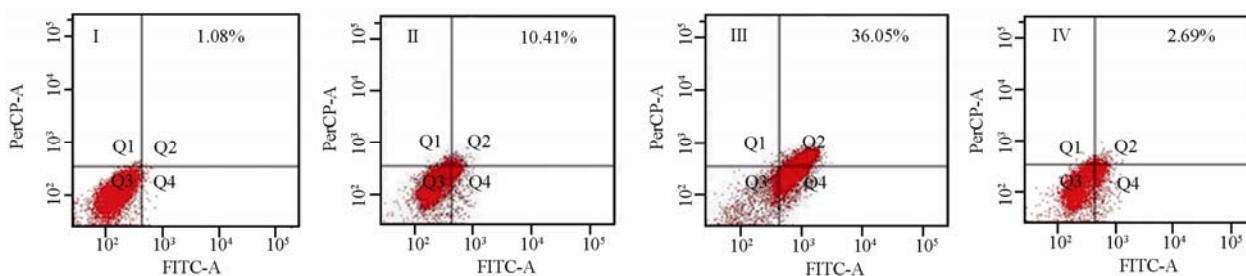


Figure 3. Quantification of AVOs with flow cytometry in U251-MG after different treatments for 3 days. **I:** control; **II:** TMZ (100 μmol/L) treatment. **III:** TMZ (100 μmol/L) with THD (100 μg/L) treatment. **IV:** THD (100 μg/L) treatment. The data shown are the addition percentage from Q1 and Q2 quadrants.

to the microtubule organizing center. In the control and THD group, MDC stained U251-MG was hardly found, nevertheless, there were many MDC stained U251-MG in TMZ and TMZ with THD group. Quantification of MDC staining positive cells were determined by flow cytometry. Results were shown in Figure 4B. The ratio of MDC positive staining cell in TMZ with THD group was higher than that in the TMZ group, which indicated that more autophagic vacuoles were formed in the combined treatment group than TMZ alone group. The result further demonstrated that THD may reinforce the TMZ induced autophagy.

Combination of TMZ with THD increased the expression of MAP1LC3, Beclin1 and PTEN protein

Micro-tubule associated protein 1 light chain 3 (MAP1LC3 or LC3) is an attractive biomarker for autophagy because it functions at least in part as a structural component during autophagosome formation.¹² Beclin1 is a tumor suppressor gene and induces autophagy when over-expressed in mammalian cells.¹³ It may activate and bind with Type-III PI3K to form the complex attending in the formation of autophagosome. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor which inhibits the class I phosphatidyl-inositol 3-kinase (PI3K)/Akt/mTOR signaling pathway,¹⁴ while the activation of this signaling pathway may suppress autophagy. We detected LC3, Beclin1 and PTEN protein expression after different treatments with Western blotting. An illustration should be made before showing the result. Two forms of LC3, the cytosolic LC3-I and the membrane-bound LC3-II, are produced post-translationally. When autophagy is induced, some LC3-I is converted into LC3-II. The amount of LC3 II or the LC3 II /LC3 I ratio correlates with the number of autophagosomes. In this study, the

amount of LC3 II was used to evaluate autophagy. Beta-actin was used as the internal control to calculate the relative expression level.

The results were shown in Figure 5, after 3 days treatment, compared with the control group, the protein expression of LC3II and Beclin1 was up-regulated in TMZ group and TMZ with THD group but almost stable in THD group (LC3II: $P=0.227$; Beclin1: $P=0.229$). The up-regulation of LC3II and Beclin1 expression were more significant in TMZ with THD group than that in TMZ group ($P < 0.05$). While compared with the control group, the level of PTEN was more obviously elevated in THD and TMZ with THD group ($P < 0.05$). No difference was shown between these two groups ($P=0.716$). No significant alteration of PTEN expression was shown in TMZ group compared with the control group ($P=0.635$). The result indicated: compared with TMZ used alone, the combination of THD with TMZ increased the expression level of LC3II and Beclin1. THD rather than TMZ promoted the expression of PTEN.

DISCUSSION

This study focused on the synergistic mechanism of THD to enhance the cytotoxicity of TMZ. In the present study, we confirmed TMZ may induce autophagy in glioma cells, which resulted in proliferative inhibition of the tumor cells and cell cycle arrest at G2/M phase. Meanwhile we found that although THD used alone was not sufficient to inhibit tumor cell growth, its combination with TMZ suppressed the cell viability of U251-MG more significantly than TMZ used alone and advanced the cell cycle arrest to G0/G1 phase. We also demonstrated the enhancement of tumor growth

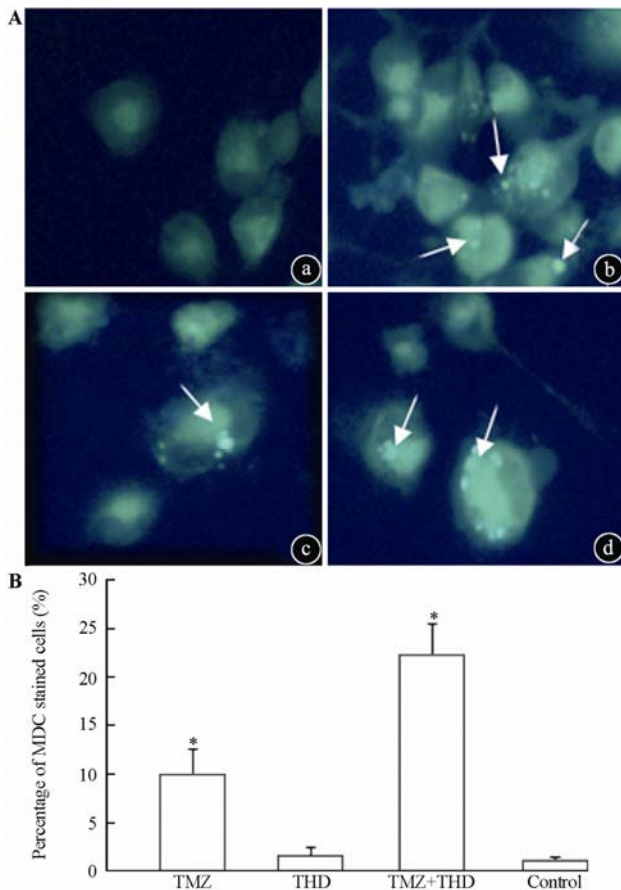


Figure 4. A: (a) MDC staining negative U251-MG; (b-d) MDC staining positive U251-MG, the arrow heads show MDC labeled autophagic vacuoles. **B:** Different ratios of MDC positive staining cells were shown between treatment groups (%): TMZ group: 10.04±2.48; THD group: 1.65±0.80; TMZ with THD group: 22.32±3.06; control group: 1.18±0.24. Results shown are the means of three independent experiments, **P* <0.05 as compared with the control group.

inhibition may attribute to the promotion of TMZ induced autophagy by THD.

Autophagy is a dynamic process of cytoplasmic protein and organelles degradation, which is observed in hepatic cells cultured *in vitro* during nutrient deprivation.¹⁵ The process of autophagy begins with sequestering cytoplasmic protein or organelles in a membrane vacuole to form autophagosome. Autophagosomes then fuse with lysosomes, where the materials inside are degraded and recycled. Normally autophagy is reversible and acts as a self-protection mechanism. When the process of autophagy turns irreversible, it would lead to autophagic cell death which is also referred as Type II programmed cell death. Oncologists announced that different types of cancer cells undergo autophagy after various anticancer therapies. Autophagy plays a key role in the elimination of cancer cells by triggering a non-apoptotic cell death program.¹⁶ When autophagic cell death is induced, autophagy functions as a self-destructive mechanism in cancer cells. Besides the signaling pathway that would be discussed as follows, many other factors also attend the

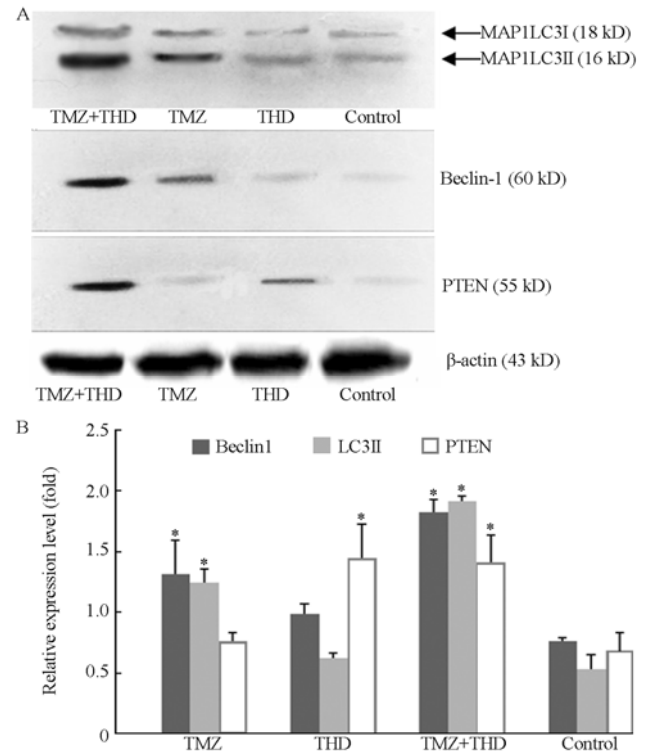


Figure 5. LC3II, Beclin1 and PTEN protein expression after different treatments for 3 days in U251-MG. A: Western blotting analysis. **B:** The densitometry was applied to qualify the protein expression of the Western blotting. *n*=3, **P* <0.05 compared with the control.

regulation of autophagy. For example, the Gαi3 protein, as combined with GTP, the GTP-Gαi3 is an inhibitor of autophagy; while GDP-Gαi3 turns to be an autophagy activator. As a member of RGS (regulators of G-protein signaling) family, GAIP (G alpha interacting protein) promotes autophagy by accelerating the hydrolysis of GTP combined with Gαi3.¹⁷ AGS3 (activator of G-protein signaling 3) is another factor that would enhance autophagy. Amino acid, the product of autophagy, could provide a negative feedback regulation of autophagy. The removal of exogenous amino acid would block the mTOR signaling pathway, which inhibits autophagy, while the endogenous amino acid produced by autophagy may complement the deficiency of amino acid and that would resume the mTOR signaling pathway. What's more, hormone, insulin and calcium all play important roles in regulation of autophagy, but the mechanisms are not poorly known.

PI3K/Akt/mTOR signaling pathway plays an important role in cellular proliferation, development and death.¹⁸ It has been demonstrated the activation of PI3K/Akt/mTOR signaling pathway inhibits the process of autophagy.¹⁹ PTEN, a tumor suppressor, is a phosphatase with a variety of substrate specificities. It functions as a negative regulator of the PI3K/Akt/mTOR signaling pathway.²⁰ Leslie et al²¹ reported PTEN is often absent or mutated in many malignant tumors, which leads to an attenuation of autophagy. Beclin1, another tumor suppressor gene, is

essential for autophagy. It forms complex with class III PI3K and localizes at the trans-Golgi network, suggesting that the Beclin 1-class III PI3K complex plays a role in sorting putative autophagosomal components and lysosomal protein.²² Moreover, some small molecular proteins are also involved in autophagy. LC3 functions as a structural component during autophagosome formation. Detection of LC3 by immunoblotting or immunofluorescence is a reliable method for monitoring autophagy and autophagy-related processes, including autophagic cell death.

Vital cell staining is another dependable method to investigate autophagy. As described previously, MDC and acridine orange staining are reliable but simple methods to detect autophagy. They may be used as qualitative methods as well as quantitative methods.

In our study, after exposure to TMZ (100 μ mol/L) with THD (100 μ g/L) for 3 days, the acridine orange and MDC staining positive tumor cells increased significantly which indicated a promotion of autophagy. Considering the enhanced inhibition of cell proliferation in TMZ with THD group, we inferred that autophagic cell death was induced. To find out the mechanism of THD to reinforce the effect of TMZ, we detect the expression of MAP1LC3 and Beclin1, both of which play crucial roles in the process of autophagy, at translational level. The result of immunoblotting showed the expression of MAP1LC3 and Beclin1 was up-regulated in both TMZ group and TMZ with THD group, while the result of TMZ with THD group was more significant. Interestingly, no significant difference was shown between THD and negative control group. So it seems THD may up-regulate the expression of autophagy-associated protein indirectly. Knobloch et al²³ reported THD-induced signaling protects active PTEN from proteasomal degradation, resulting in suppression of Akt signaling. Enlightened by this view, we detected the protein expression level of PTEN in different treatment groups. The results of THD and THD with TMZ group were obviously higher than those of TMZ group. So we concluded the up-regulation of PTEN may facilitate the process of autophagy rather than induce autophagy. In other words, PTEN is a facilitative but not crucial factor to promote autophagy. The evidence is that despite THD up-regulated the expression of PTEN, essential autophagy-associated protein MAP1LC3 and Beclin1 stayed still in THD alone group; while in TMZ with THD group, both of the expression level of PTEN and autophagy-associated protein were up-regulated. It seems that more factors would participate in and regulate the process of autophagy and further experiments are necessary to clarify them.

It should be noted that the study of synergistic mechanism only focused on the translational level. More small molecules involved in autophagy and PI3K/Akt/mTOR signaling pathway should be detected.

Despite its preliminary character, this study freshly indicated: besides anti-angiogenesis effect, THD may also reinforce the effect of TMZ by promoting autophagy. Furthermore, our study provided the experimental basis for more reasonable and effective clinical use of combination of TMZ with THD. Kanzawa et al⁵ once performed the similar experiment in U87-MG and A172 cell lines. The result was close to ours, while they announced inhibition of autophagy at a late stage could increase the therapeutic efficacy of TMZ for malignant glioma cells. It seems that the combination of TMZ with THD, which promote autophagy, may not benefit the patient with gliomas at a late stage of treatment. Moreover, we applied a series of methods to study autophagy and put forward the idea to combine the research of effectors in PI3K/Akt/mTOR signaling pathway with autophagy regulation. The methods and idea provided a fresh development in the research of autophagy.

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