Tanshinone I induces apoptosis and protective autophagy in human glioblastoma cells via a reactive oxygen species-dependent pathway

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Abstract. Glioma is the most common primary malignancy of the central nervous system and is associated with high mortality rates. Despite the available treatment options including surgery, radiotherapy and chemotherapy, the median patient survival rate is low. Therefore, the development of novel anticancer agents for the treatment of glioma is urgently required. Tanshinone I (TS I) is a tanshinone compound that is isolated from Danshen. Accumulating evidence indicates that TS I exhibits antiproliferative activity in a variety of cancer types. However, the role of TS I and its mechanism of action in human glioma remain to be elucidated. In the present study, the anticancer potential of TS I against human glioma U87 MG cells was investigated. The results indicated that TS I exerted a potential cytotoxic effect on human glioma U87 MG cells. TS I was found to induce cell proliferation, inhibition, cell cycle arrest, apoptosis and autophagy in U87 MG cells. Mechanistic experiments indicated that TS I activated endoplasmic reticulum (ER) stress and inhibited AKT signaling and apoptosis in human glioma U87 MG cells. Furthermore, the present study demonstrated that TS I induced protective autophagy in U87 MG cells. Additionally, ER stress and AKT signal-mediated apoptosis and protective autophagy were found to be induced by TS I via intracellular reactive oxygen species accumulation. The results of the present study demonstrated that TS I may be a potential anticancer drug candidate that may be of value in the treatment of human glioma.

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Introduction

It has been well established that malignant glioma is one of the most common types of brain neoplasms, and is associated with high mortality and poor 5-year survival rates worldwide (1). The currently available treatment for glioma involves surgical removal of the tumor, chemotherapy and radiotherapy, which can significantly improve the survival rates of the patients (2). However, for the majority of patients with glioma, prognosis is poor, with a median survival of ~14 months (3). Temozolomide (TMZ) is the most effective chemotherapeutic agent for glioma treatment (3). However, the response rate to TMZ treatment is accompanied by resistance to chemotherapy and high rates of toxicity, which may lead to patients experiencing severe nausea, vomiting and fetotoxic effects (4). Therefore, the development of novel anticancer agents that may be used to decrease glioma progression requires further investigation.

Danshen (*Salvia miltiorrhiza* Bunge) is a traditional Chinese herb that has been successfully used for the treatment of cardiovascular disease in Asian countries (5,6). TS I has been demonstrated to be one of the bioactive components of Danshen, and has been reported to possess antioxidant, anti-inflammatory and anticancer properties (7). Recent studies on TS I have focused on its anticancer activity (8-10). These results have demonstrated that TS I may induce the apoptosis of cancer cells in gastric (10), human breast (11,12) and human colon cancer (13,14). However, to the best of our knowledge, the exact mechanisms underlying the effects of TS I on human glioma have not yet been determined.

To determine the mechanisms underlying the anticancer activity exhibited by TS I in human glioma, the present study was performed to elucidate the biological mechanisms through which TS I may induce the inhibition of human glioma U87 MG cell growth.

Materials and methods

Reagents and antibodies. TS I was purchased from Sigma-Aldrich; Merck KGaA. The anti-p-AKT (cat. no. 4058), anti-AKT (cat. no. 9272), anti-cleaved poly(ADP-ribose)

polymerase (PARP) (cat. no. 5625), anti-GADPH (cat. no. 2118), anti-cyclin B1 (cat. no. 4138), anti-B-cell lymphoma (Bcl)-2 (cat. no. 15071), anti-beclin-1 (cat. no. 3738), anti-C/EBP homologous protein (CHOP) (cat. no. 2895), anti-p-eukaryotic initiation factor (eIF)2α (Ser51) (cat. no. 9721), anti-eIF2α (cat. no. 9722), anti-LC3B (cat. no. 2775) and anti-Bcl-2-associated X protein (Bax) (cat. no. 2774) antibodies were purchased from Cell Signaling Technology, Inc. The anti-p21 antibody (cat. no. MAB1047) was purchased from R&D Systems, Inc. LY294002 was purchased from Merck KGaA. The Annexin V-FITC and propidium iodide (PI) kit was purchased from BD Biosciences; Becton, Dickinson and Company. N-acetyl-L-cysteine (NAC), a reactive oxygen species (ROS) scavenger and 3-methyladenine (3-MA; an inhibitor of autophagy) were purchased from MedChem Express LLC.

Cell culture. The U87 MG glioma cell line was purchased from Procell Life Science & Technology Co., Ltd. (cat no. CL-0238). The cell line was established in the University of Uppsala and was authenticated using STR profiling. Cells were maintained in DMEM supplemented with 10% FBS (Procell) and 1X penicillin-streptomycin solution.

Cell viability assay. U87 MG glioma cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay. U87 MG cells were then seeded into a 96-well plate ($6x10^3$ cells/well) for 24 h. Cells were then treated with TS I (0, 0.625, 1.25, 2.5, 5 or 10 μM), and incubated for an additional 24 h. A total of 10 μl CCK-8 solution was subsequently added to each well and cells were then incubated for 4 h. The absorbance value of each well was measured using an ELISA reader at a wavelength of 450 nm. All experiments were performed three times, and results are expressed as the mean ± standard deviation.

Cell cycle assay. The cell cycle distribution of human glioma U87 MG cells was analyzed using flow cytometry and PI staining. Cells were seeded ($1x10^6$ cells/well) into 6-well plates and treated with different TS I concentrations (0, 0.625, 1.25 and 2.5 μM) for 24 h. The treated cell groups were then fixed in cold 70% ethanol for 2 h. RNase A (60 μg/ml) and PI (50 μg/ml) in PBS were added, and samples were incubated for 30 min in the dark at room temperature. The cell cycle distribution was analyzed using flow cytometry (FACSCalibur; BD Biosciences; Becton, Dickinson and Company). A total of 10,000 cells were collected for each cell group. The percentage of cell populations at subG0/G1, G2/M and S phases were examined using Modfit LT version 2.0 software (Verity Software House). Three independent experiments were performed.

Cell apoptosis assay. Cell apoptosis was assessed using an Annexin V-FITC/PI kit (BD Pharmingen; BD Biosciences) and the apoptotic rate was analyzed using a flow cytometer (FACSCalibur; BD Biosciences; Becton, Dickinson and Company). U87 MG cells were seeded ($1x10^6$ cells/well) into 6-well plates and treated with different TS I concentrations (0, 0.625, 1.25 and 2.5 μM) for 24 h. Cells were then suspended in a 5-ml culture tube with 1X binding buffer (provided with the Annexin V-FITC/PI kit) at a density of $1x10^6$ cells/ml, stained using Annexin V-FITC and counterstained using PI in binding

buffer at room temperature for 15 min. The number of apoptotic cells was then determined using a flow cytometer (FACSCalibur; BD Biosciences; Becton, Dickinson and Company).

Western blot analysis. Cells were seeded onto 35-mm plates ($2x10^6$ cells), with 2 ml complete DMEM for 24 h. Following TS I treatment, cells were washed with PBS, and lysed with RIPA lysis buffer (150 mmol/l; NaCl 50 mmol/l Tris-HCl; pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS) with 1 mM sodium orthovanadate, 1 mM MPMSF and 1% cocktail of protease inhibitors (Sigma-Aldrich; Merck KGaA). Protein concentration was determined using the BCA method. Equal quantities of protein (40 μg/lane) were separated using 6-12% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk for 2 h at 25°C. The membranes were incubated with a variety of primary antibodies as follows: AKT (1:1,000), p-AKT (1:1,000), cyclin B1 (1:1,000), cleaved PARP (1:1,000), Bcl-2 (1:1,000), Bax (1:1,000) or p21 (1:1,000), beclin-1 (1:2,000), CHOP (1:1,000), p-eIF2α (Ser51) (1:3,000), eIF2α (1:4,000) and LC3B (1:2,000), followed by incubation with anti-rabbit HRP secondary antibody (1:20,000, cat. no. 7074, Cell Signaling Technology, Inc.) or anti-mouse HRP-conjugated secondary antibody (1:20,000, cat. no. 7076, Cell Signaling Technology, Inc.) for 2 h at 25°C. GAPDH (1:3,000) served as a loading control. Visualization was achieved using SuperSignal West Pico chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.).

Measurement of ROS generation. Intracellular ROS levels were determined using a ROS assay kit (Keygen Biotech Co., Ltd.). Briefly, the cells were plated in 6-well plates at a density of $2.0x10^5$ cells/well for 24 h. Following treatment with TS I (0, 0.625, 1.25 and 2.5 μM) for 12 h, cells were then incubated with 10 μM DCFH-DA for 15 min at 37°C in the dark. Next, the cells were examined by flow cytometry using a FACSCalibur Flow Cytometer (BD Biosciences) and the data were analyzed by FlowJo 7.6 software (FlowJo LLC).

Statistical analysis. All data are presented as the mean ± standard deviation. A Student's t-test and one-way ANOVA followed by Dunnett's post hoc test were used to determine significance for differences among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Result

TS I exerts potent cytotoxic effects on human glioma U87 MG cells. To investigate the potent anticancer effects of TS I on U87 MG cells, cell viability was analyzed using a CCK-8 assay. In the present study, U87 MG cells were treated with TS I at different concentrations (0, 0.625, 1.25, 2.5, 5 and 10 μM) for 24 h. The results demonstrated that TS I significantly inhibited U87 MG cell proliferation in a dose-dependent manner. Cell viability was reduced by 26.92±1.06, 42.37±4.38, 51.17±2.25 and 66.39±2.24% at 1.25, 2.5, 5 and 10 μM TS I, respectively, after 24 h (Fig. 1). The IC_{50} value was found to be 3.35±0.44 μM at 24 h. The results demonstrated that TS I exerted marked cytotoxic effect on U87 MG cells by inhibiting cell proliferation.

TS I induces cell cycle arrest at the G2/M phase in human glioma U87 MG cells. To investigate the mechanisms through which TS I inhibited U87 MG cell growth, flow cytometry was used to analyze the distribution of cells within the cell cycle following treatment with 0, 0.625, 1.25 and 2.5 μM TS I for 24 h. Flow cytometry indicated that TS I significantly increased the percentage of cells in the G2/M phase in a dose-dependent manner (6.61±0.40, 11.84±1.08 and 15.79±1.79% in the 0.625, 1.25 and 2.5 μM treatment groups, respectively) compared with the control group (5.82±0.67%) (Fig. 2A). Additionally, TS I treatment significantly decreased the expression of cyclin B1 and increased the expression of p21 (Fig. 2B). These results indicated that the downregulation of cyclin B1 expression and the upregulation of p21 expression contributed to TS I-induced G2/M phase arrest and antiproliferation effect in U87 MG cells.

TS I induces apoptosis in human glioma U87 MG cells. The present study assessed whether the inhibition of TS I-induced U87 MG cell growth was associated with apoptosis. Flow cytometry, which was performed using Annexin V-PI staining, demonstrated that, following treatment with TS I for 24 h, the proportion of apoptotic U87 MG cells was markedly increased in a concentration-dependent manner. The percentage of apoptotic cells was 6.34±1.54, 10.57±2.28, 21.26±2.48 and 30.33±3.13% in the 0, 0.625, 1.25 and 2.5 μM TS I groups, respectively (Fig. 3A). The levels of apoptosis-associated proteins were also determined using western blot analysis. As shown in Fig. 3B, TS I treatment decreased Bcl-2 and increased the cleaved PARP and Bax expression in a dose-dependent manner. These results suggested that TS I induces U87 MG cell apoptosis via the downregulation of Bcl-2 expression and the upregulation of Bax and cleaved PARP expression.

TS I induces apoptosis via ROS production in human glioma U87 MG cells. TS I has been previously shown to induce apoptosis by increasing ROS levels in colon cancer cells (9). Therefore, whether ROS production was associated with TS I-induced apoptosis of U87 MG cells was assessed in the present study. Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μM) for 12 h and analyzed using flow cytometry. As shown in Fig. 4A, ROS generation was significantly increased in a dose-dependent manner. Furthermore, cells were pre-treated with 4 mM ROS scavenger NAC for 1 h, and exposed to TS I (1.25 μM) for 24 h. Cell viability was analyzed using a CCK-8 assay. The results indicated that 4 mM NAC significantly reduced TS I-induced inhibition of cell growth (Fig. 4B). Furthermore, pretreatment with NAC significantly restored the TS I-induced upregulation of Bax and cleaved PARP expression, and the downregulation of Bcl-2 expression (Fig. 4C). These data suggest that TS I promotes ROS generation and this effect is associated with TS I-induced cell apoptosis.

TS I induces apoptosis by suppressing the AKT signaling pathway in human glioma U87 MG cells. AKT signaling is a key downstream effector of phosphoinositide 3-kinase (PI3K) that regulates a variety of biological processes, including cell cycle progression, proliferation and apoptosis in cancer cells (15,16). Previous studies have focused on the role of the AKT signaling pathway in human gliomas (17,18). To determine the molecular
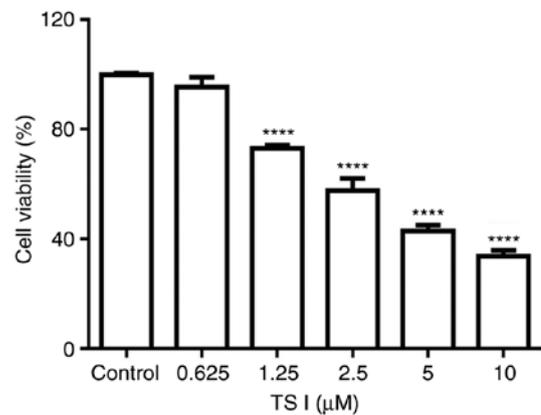
Figure 1. TS I inhibits the proliferation of U87 MG cells. The growth inhibitory effect of TS I was measured using a Cell Counting Kit-8 assay. Cell viability was examined after treatment with TS I (0, 0.625, 1.25, 2.5, 5 and 10 μm) for 24 h. Data are presented as the mean ± standard deviation of three separate experiments. ****P<0.0001. TS-I, tanshinone I.

mechanisms underlying the TS I-induced apoptosis of U87 MG cells, the phosphorylation/activation of AKT in cells treated with TS I (0, 0.625, 1.25 and 2.5 μM) was assessed for 12 h. The phosphorylation of AKT was found to decrease following TS I treatment in a dose-dependent manner. However, TS I exerted no effect on total AKT expression in U87 MG cells (Fig. 5A). To further elucidate whether TS I-induced apoptosis was associated with AKT signaling, cells were treated with 5 μM PI3K inhibitor (LY294002) for 1 h prior to treatment with TS I (1.25 μM) for 24 h. Cell viability was measured in U87 MG cells treated with TS I alone or in combination with LY294002. As shown in Fig. 5B, TS I (1.25 μM) in combination with 5 μM LY294002 significantly inhibited U87 MG cell proliferation compared with TS I treatment alone. Additionally, apoptosis-associated protein expression was also determined using western blot analysis. As shown in Fig. 5C, LY294002 with TS I significantly decreased Bcl-2 protein expression and significantly increased the cleaved PARP and Bax protein expression compared with TS I treatment alone. Furthermore, the ROS-mediated AKT signaling pathway has been reported to be an important regulator of apoptosis in cancer cells, and has been shown to affect cell proliferation (19,20). To elucidate the role of ROS production in the TS I-mediated inhibition of the AKT signaling pathway in U87 MG cells, the cells were treated with NAC for 1 h and subsequently treated with TS I (1.25 μM) for 12 h. As shown in Fig. 5D, western blot analysis revealed that TS I-induced reduction of p-AKT was reversed in the NAC pre-treatment group compared with the TS I alone group. These data indicated that TS I may induce apoptosis by regulating the ROS/AKT pathway in human glioma U87 MG cells.

TS I induces apoptosis via the ROS-mediated endoplasmic reticulum (ER) stress pathway in human glioma U87 MG cells. It has been previously reported that increased ROS generation can increase the expression of unfolded proteins and activate the ER stress response (19,21). Therefore, in the present study, the expression of the ER stress-associated proteins p-eIF2α, eIF2α, activating transcription factor (ATF)4 and CHOP was examined using western blot analysis. As shown in Fig. 6A,
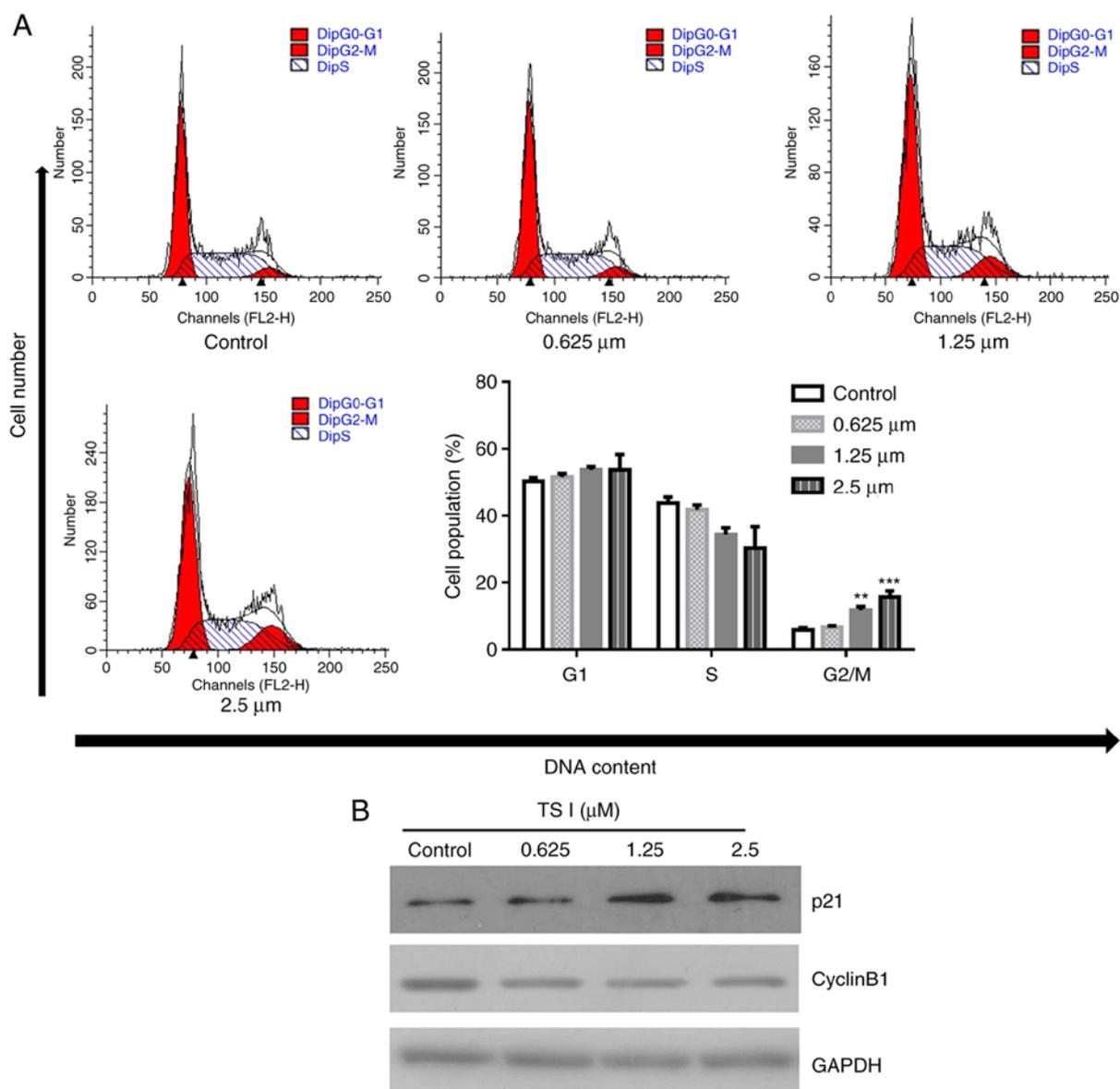
Figure 2. Effects of TS I on cell cycle distribution in U87 MG cells and the expression of cell cycle-associated proteins. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 µm) for 24 h, and cell cycle distribution was analyzed using a flow cytometer. (B) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 µm) for 12 h, and the protein levels of cyclin B1 and p21 were assessed using western blot analysis in U87 MG cells. GAPDH was used as a loading control. **P<0.01; ***P<0.001. TS-I, tanshinone I.

TS I significantly activated ER stress. To further verify the role of ROS production in the TS I-mediated activation of ER stress, cells were treated with 4 mM NAC for 1 h and subsequently treated with TS I (1.25 µM) for 12 h. The results indicated that 4 mM NAC pretreatment significantly attenuated TS I-mediated activation of ER stress (Fig. 6B). These results demonstrated that TS I may induce apoptosis via the ROS-mediated ER stress pathway in human glioma U87 MG cells.

TS I induces autophagy via ROS production in human glioma U87 MG cells. It has previously been suggested that TS I can induce autophagy in BGC823 and SGC7901 cells (10). Therefore, the present study assessed whether TS I induced autophagy in U87 MG cells. The expression of LC3B and beclin-1 was determined using western blot analysis. The

results demonstrated that the expression of LC3B-II and beclin-1 were markedly increased in U87 MG cells following treatment with TS I (Fig. 7A). To further determine the role of autophagy in TS I-induced cell apoptosis, cells were treated with 5 mM 3-MA for 1 h and subsequently treated with TS I (1.25 µM) for 24 h. Cell viability was analyzed using a CCK-8 assay. As shown in Fig. 6B, co-treatment with 5 mM 3-MA and TS I significantly enhanced the TS I-induced growth suppression of U87 MG cells (Fig. 7B). Additionally, increasing evidence has suggested that autophagy and ROS accumulation are closely associated events in a variety of cancer types (22-24). To elucidate the association between ROS production and TS I-mediated autophagy, cells were treated with 4 mM NAC for 1 h and subsequently treated with TS I (1.25 µM) for 12 h. LC3B and beclin-1 expression was determined using western blot analysis. The results of the
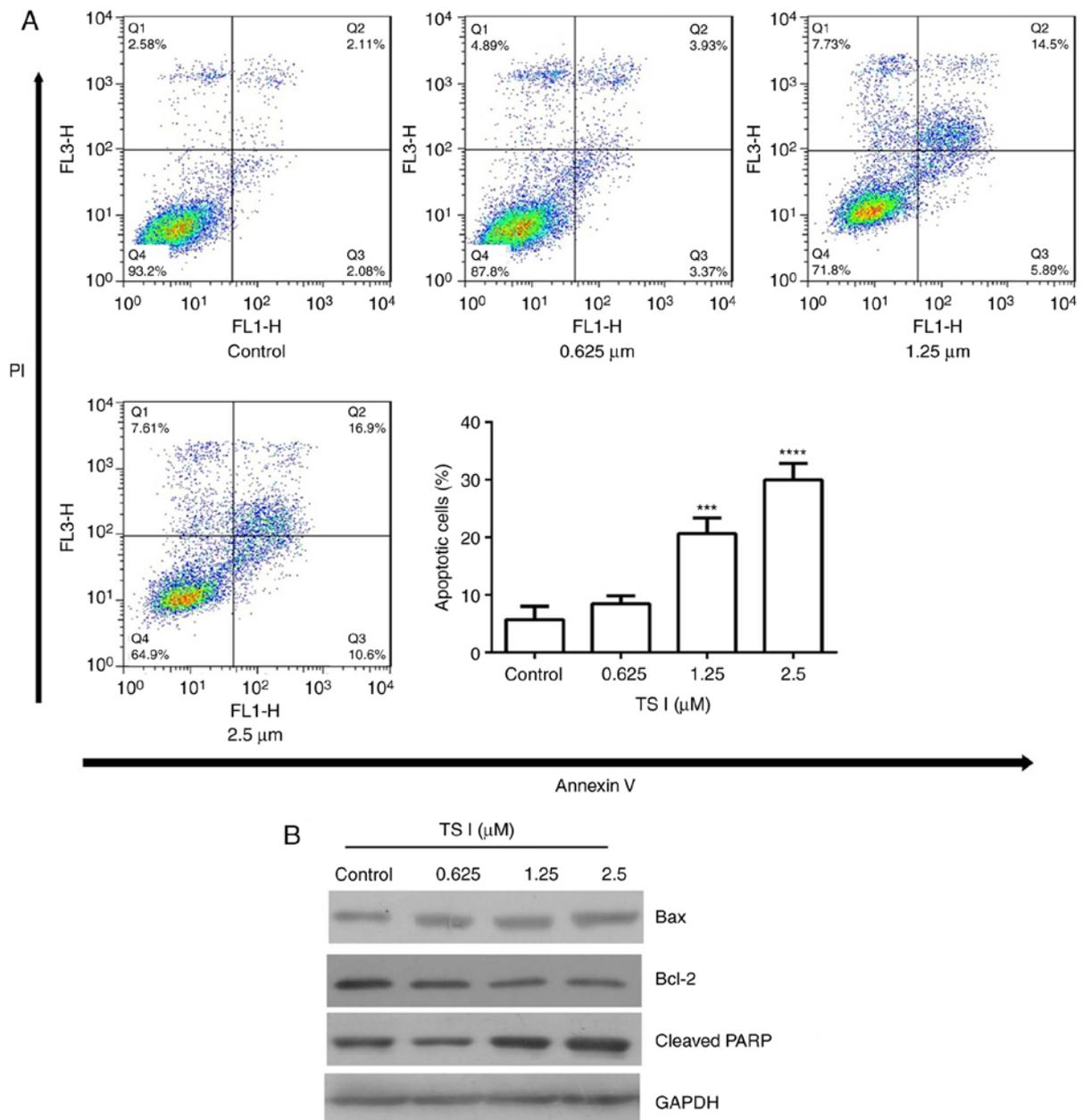
Figure 3. Effects of TS I on U87 MG cell apoptosis and the expression of cell apoptosis-associated proteins. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 24 h, and cell apoptosis was analyzed using flow cytometry in U87 MG cells. (B) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 12 h, and the protein levels of Bcl-2, Bax and cleaved PARP were assessed using western blot analysis in U87 MG cells. GAPDH was used as a loading control. ***$P<0.001$; ****$P<0.0001$. TS-I, tanshinone I; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly(ADP-ribose) polymerase.

present study indicated that NAC pretreatment significantly attenuated LC3B-II and beclin-1 expression compared with TS I treatment alone (Fig. 7C). Therefore, these findings suggest that TS I may promote protective autophagy via ROS generation in U87 MG cells.

Discussion

TS I has been demonstrated to exert antiproliferative effects on gastric cancers cell lines (including BGC823 and SGC7901) (10), human breast cancer cell lines (including MCF-7 and MDA-MB-453) (11,12) and human colon cancer cells (13,14). However, the effect of TS I on human glioma has not been extensively investigated. The present study investigated the anticancer effects of TS I on human glioma U87 MG cells. The data demonstrated that TS I inhibited cell proliferation, induced G2/M phase arrest and triggered apoptosis in a dose-dependent manner. The underlying molecular mechanism may involve increased ROS production, decreased phosphorylation of AKT, activated autophagy, or an activated ER stress pathway in human glioma U87 MG cells. TS I was indicated to induce apoptosis via activating the ER stress pathway and inhibiting AKT signaling, and was shown to induce protective autophagy via ROS production in U87 MG cells.

It is well established that abnormal cell cycle progression may cause uncontrolled growth, and that cell cycle arrest can inhibit the proliferation and induce apoptosis in cancer
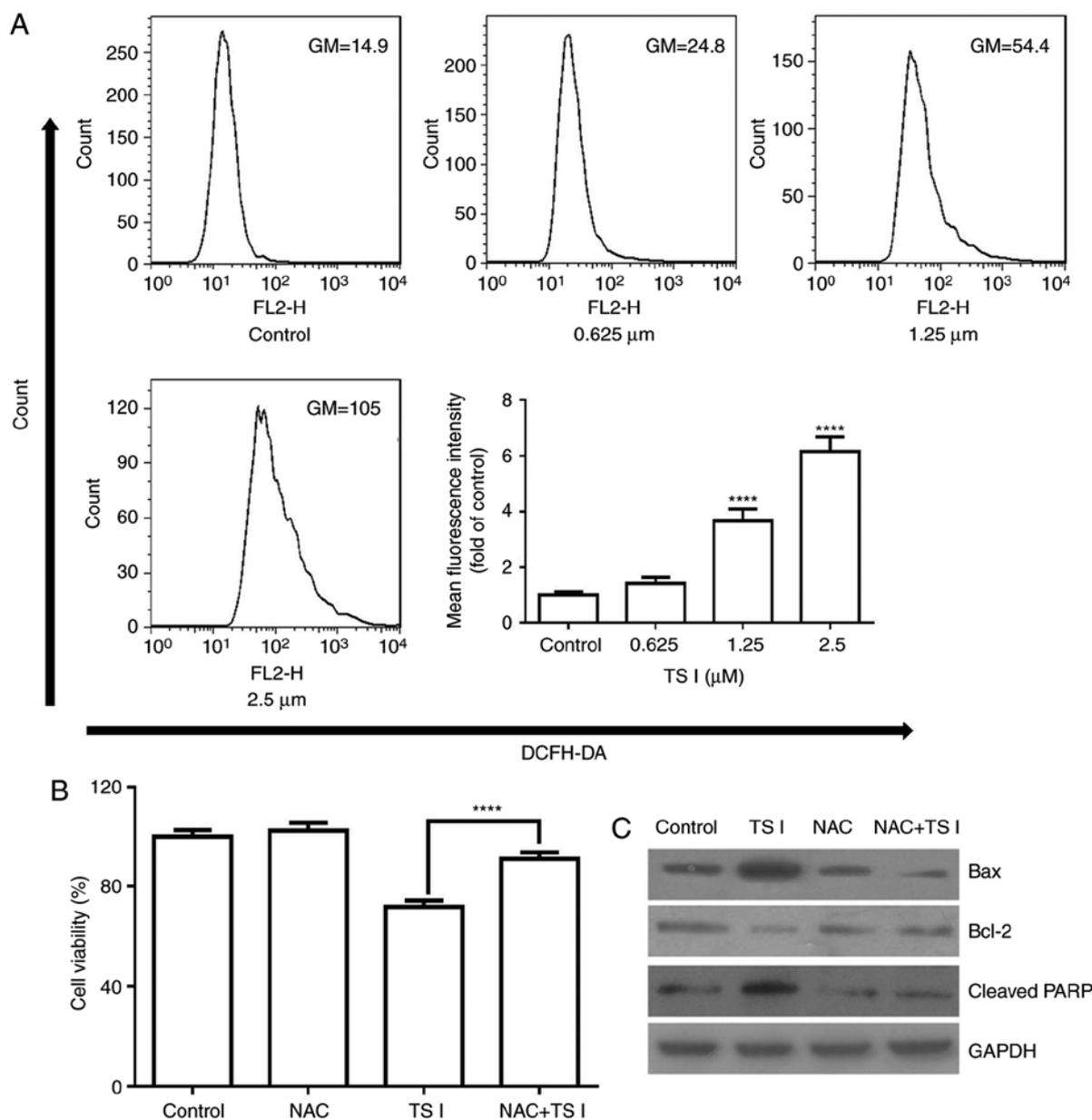
Figure 4. Effects of TS I on intracellular ROS generation in U87 MG cells. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 12 h, and the levels of ROS were examined using flow cytometry. Bar graphs indicate the quantification of ROS. (B) U87 MG cells were pretreated with NAC (4 mM) for 1 h, followed by treatment with TS I (1.25 μm) for 24 h. Cell viability was then detected using a Cell Counting Kit-8 assay. (C) U87 MG cells were pretreated with NAC (4 mM) for 1 h, followed by treatment with TS I (1.25 μm) for 12 h, and the protein levels of Bcl-2, Bax and cleaved PARP were subsequently assessed using western blot analysis. GAPDH was used as a loading control. [****]$P<0.0001$. TS-I, tanshinone I; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly(ADP-ribose) polymerase.

cells (25,26). Cell cycle progression is dependent on the activity of cyclin-dependent kinase (CDK) complexes (27,28). The downregulation of cyclin B1 may lead to the inhibition of the cell cycle at the G2/M phase (29,30). It has also been demonstrated that p21 is a broad-spectrum CDK inhibitor and may promote cell cycle arrest by inhibiting the activity of a number of cyclin-CDK complexes (31,32). Furthermore, a number of studies have revealed that the upregulated expression of p21 plays a key role in G2/M arrest (33,34). Additionally, it has been reported that TS I-induced inhibition of cyclin A and cyclin B decreases cell cycle progression through the S and G2/M phases (35). Wang *et al* (11) revealed that TS I inhibited cell cycle progression by decreasing

cyclin B and CDK2 protein levels. The results of the present study demonstrated that TS I upregulated the p21 level and decreased the levels of cyclin B1. These data revealed that TS I caused G2/M arrest by upregulating p21 and downregulating cyclin B1 expression.

Apoptosis is an important physiological process of programmed cell death and serves as an important homeostatic mechanism that balances cell growth and cell death (36,37). The induction of apoptosis in cancer cells is a strategy that may be used in the screening of new anticancer agents (38). A variety of studies have suggested that TS I can induce apoptosis in a variety of human cancer cells. TS I-induced apoptotic death of human breast cancer cells was indicated to
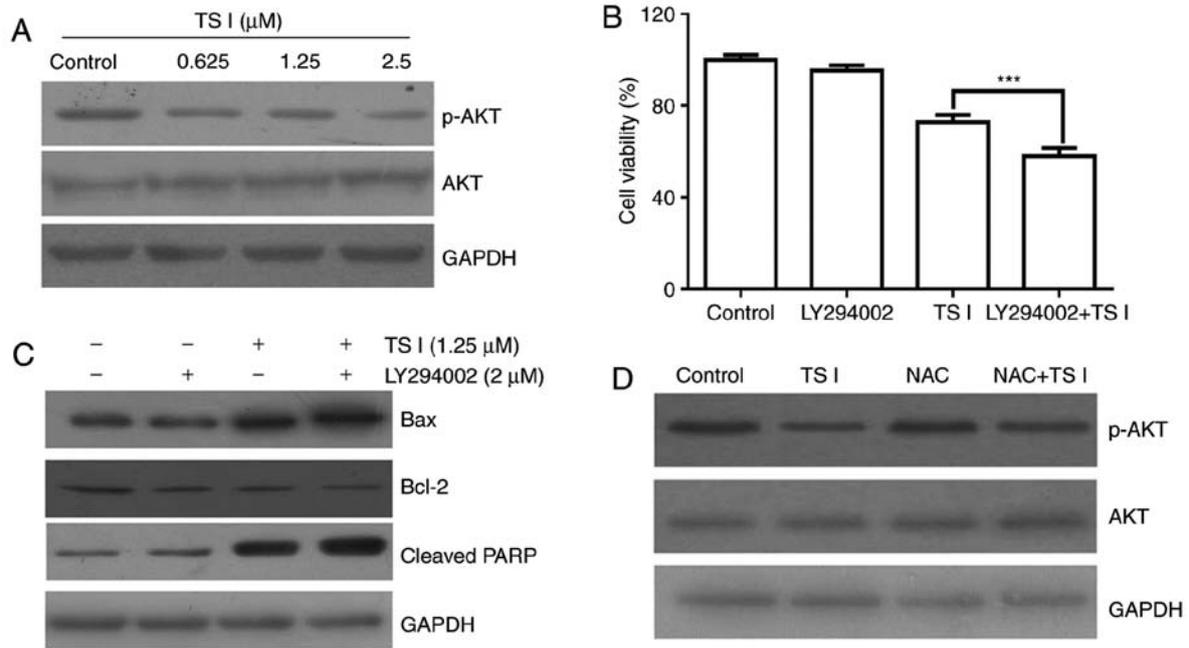
Figure 5. Effects of TS I on the AKT signaling pathway in U87 MG cells. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 24 h, and the expression of GAPDH, total and phosphorylated AKT were detected using western blot analysis. (B) U87 MG cells were pre-treated with LY294002 (2 μm) for 1 h, followed by treatment with TS I 1.25 μm for 24 h, and cell viability was subsequently detected using a cell counting kit-8 assay. (C) U87 MG cells were pre-treated with LY294002 (2 μm) for 1 h, followed by treatment with TS I 1.25 μm for 12 h, and protein levels of Bcl-2, Bax and cleaved PARP were then assessed using western blot analysis. (D) U87 MG cells were pre-treated with NAC (4 mM) for 1 h, followed by treatment with TS I 1.25 μm for 12 h, and the protein levels of GAPDH, total and phosphorylated AKT were then assessed using western blot analysis. GAPDH was used as a loading control. ***P<0.001; TS-I, tanshinone I; NAC, N-acetyl-L-cysteine; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly(ADP-ribose) polymerase.

be mediated by the activation of caspase 3, the downregulation of Bcl-2 and the upregulation of Bax expression (12). In human colon cancer COLO-205 cells, TS I was revealed to promote apoptosis by increasing the expression of Bax and caspase-3 proteins (13). In the present study, treatment with TS I was demonstrated to significantly induce apoptosis by upregulating the expression of cleaved PARP and Bax and downregulating the expression of Bcl-2 in U87 MG cells. These data indicate that apoptosis plays a key role in TS I-induced U87 MG cell death.

AKT kinase, which is a serine/threonine kinase, plays an important role in a number of biological processes, including cell proliferation, differentiation and apoptosis (39,40). A number of studies have demonstrated that natural compounds can induce cancer cell apoptosis through the suppression of the AKT signaling pathway (41-43). Additionally, Wang *et al* (11) revealed that TS I induced breast cancer cell apoptosis by regulating the PI3K/AKT/mammalian target of rapamycin signaling pathway. Furthermore, it has been demonstrated that the AKT signaling pathway mediates the mitochondrial apoptotic pathway by increasing the Bax/Bcl-2 expression ratio and activating PARP (44-46). The results of the present study demonstrated that TS I significantly decreased Bcl-2 and p-AKT protein expression, and significantly increased cleaved PARP protein expression. Furthermore, TS I and LY294002 enhanced the pro-apoptotic effects of TS I. These data suggest that the AKT pathway was associated with apoptosis in U87 MG cells that were treated with TS I. A number of studies have demonstrated that ROS serves as a mediator of apoptosis in a variety of types of therapeutic drug-induced apoptosis (23,47,48).
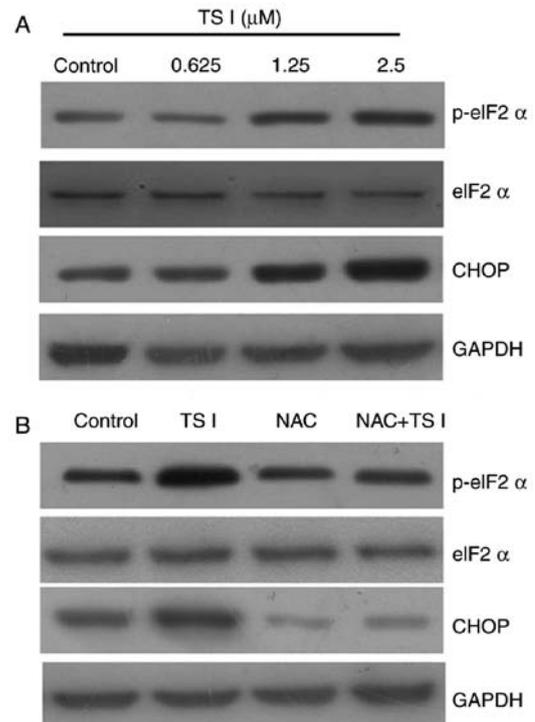
Figure 6. Effects of TS I on the ER stress signaling pathway in U87 MG cells. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 12 h, and the expressions of eIF2α, p-eIF2α and CHOP proteins were detected using western blot analysis. (B) U87 MG cells were pre-treated with NAC (4 mM) for 1 h, followed by treatment with TS I 1.25 μm for 12 h, and the protein levels of eIF2α, p-eIF2α and CHOP were then assessed using western blot analysis. GAPDH was used as a loading control. TS-I, tanshinone I; NAC, N-acetyl-L-cysteine ER, endoplasmic reticulum; eIF2α, eukaryotic initiation factor; CHOP, C/EBP homologous protein.
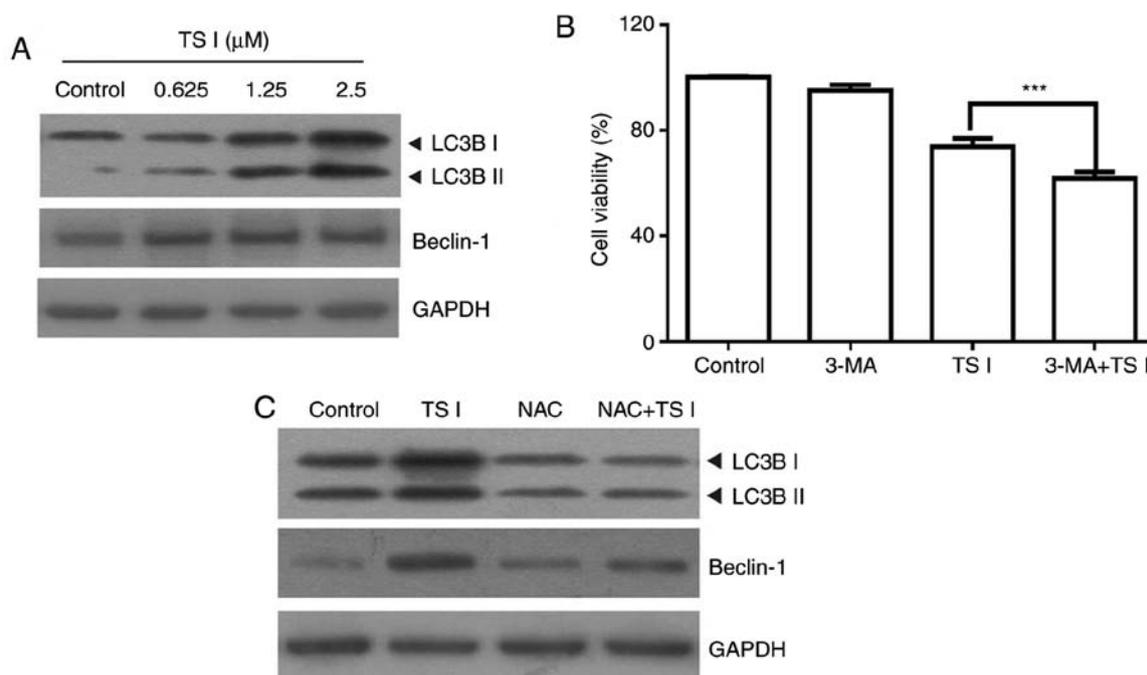
Figure 7. Effects of TS I on cell autophagy in U87 MG cells. (A) Cells were treated with TS I (0, 0.625, 1.25 and 2.5 μm) for 12 h, and the expression of LC3B and beclin-1 were detected using western blot analysis. (B) U87 MG cells were pretreated with 3-MA (2 mM) for 1 h, followed by treatment with TS I (1.25 μm) for 24 h, and then cell viability was detected using a Cell Counting Kit-8 assay. (C) U87 MG cells were pretreated with 3-MA (2 mM) for 1 h, followed by treatment with TS I (1.25 μm) for 12 h, and protein levels of LC3B and beclin-1 were then assayed using western blot analysis. GAPDH was used as a loading control. ***$P<0.001$; TS-I, tanshinone I; 3-MA, 3-methyladenine.

In human endometrial carcinoma HEC-1-A cells, TS I has been shown to increase ROS levels (49). In colon cancer cells, TS I has been demonstrated to induce apoptosis via the ROS-mediated p38 signaling pathway (9). In the present study, TS I significantly increased the intracellular levels of ROS in U87 MG cells. Additionally, previous studies have reported that ROS is a potential upstream regulator of PI3K/AKT inactivation, which are key molecules in cell apoptosis (19,20,50). In the present study, NAC was indicated to efficiently reverse the TS I-mediated cell apoptosis and inhibition of AKT phosphorylation in U87 MG cells. These data suggested that ROS may act as a mediator in TS I-induced U87 MG cell apoptosis via the inactivation of the AKT signaling pathway.

It has been widely reported that ER stress may lead to ER dysfunction and the activation of the ER stress-related signaling pathway, which is associated with ROS-mediated cell apoptosis in a variety of cancer types (51-53). TS IIA inhibited human prostate cancer cell proliferation via the induction of ER stress *in vitro* and *in vivo* (53). In human lung cancer cells, TS IIA increased TRAIL-induced cell death via the activation of ER stress (54). Consistently with these reports, the results of the present study indicated that TS I increased the expression of the ER stress-related proteins p-eIF2α, ATF4 and CHOP. Furthermore, the downregulation of CHOP markedly attenuated the inhibition of TS I-induced growth in human glioma U87 MG cells. These data suggested that ER stress may be associated with ROS-mediated TS I-induced cell apoptosis. To further determine the role of ROS in the activation of ER stress induced by TS I, cells were pretreated with NAC. The results revealed that NAC pretreatment significantly attenuated the TS 1-mediated activation of ER stress.

Overall, the results of the present study revealed that TS I promotes apoptosis of U87 MG cells via the ROS-mediated ER stress pathway.

Autophagy can be activated by a variety of different factors, including ROS accumulation and anticancer drugs, and is well-known to play an important role in determining cell fate (23,55,56). In lung cancer 95D cells, tanshinones induced protective autophagy by increasing ROS production (57). Jing *et al* (10) also reported that TS I induced protective autophagy in gastric cancer. In the present study, the results demonstrated that autophagy was activated by TS I in human glioma U87 MG cells. Furthermore, pretreatment with 3-MA effectively enhanced the TS I-induced inhibition of U87 MG cells. Additionally, NAC significantly reduced TS I-induced autophagy. Therefore, these results indicate that TS I induces protective autophagy via ROS production in U87 MG cells.

In summary, the results of the present study demonstrated that TS I inhibited the proliferation of human glioma U87 MG cells via the induction of apoptosis and G2/M cell cycle arrest. TS I was shown to mediate G2/M cell cycle arrest in U87 MG cells by upregulating p21 and downregulating cyclin B1 expression. TS I induced apoptosis by upregulating the expression of cleaved PARP and Bax, and downregulating the expression of Bcl-2. Furthermore, TS I induced apoptosis via the inactivation of the AKT signaling pathway. Therefore, these findings may be an important indication that TS I is a potential anticancer drug candidate that may prove useful in the treatment of human glioma. We aim to further investigate the specificity of TS I against malignant cells in a future study.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SJ, LC and GS conducted the experiments and analyzed the data. GS made substantial contributions to the design of the present study and prepared the manuscript. LM, CH, TR, FX and ZB performed the western blotting and analyzed the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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