Salvia miltiorrhiza extract dihydrotanshinone induces apoptosis and inhibits proliferation of glioma cells

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ABSTRACT

Dihydrotanshinone, a functional food in China, is an effective anti-cardiovascular disease substance isolated from *Salvia miltiorrhiza* (*S. miltiorrhiza*). Glioma is considered to be fatal due to its poor prognosis and few effective therapeutic options. In this study, we investigated the anticancer effects of *S. miltiorrhiza* extract dihydrotanshinone on human glioma SHG-44 cells, by using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay, Hoechst 33258 nuclear staining, Annexin V/propidium iodide double staining, as well as western blot analysis. The results showed that dihydrotanshinone effectively suppressed SHG-44 cells proliferation and induced apoptosis in both dose- and time-dependent manner. Moreover, we demonstrated that dihydrotanshinone increased the activation of caspases (caspase-3 and caspase-9) and the release of cytochrome c in SHG-44 cells. Overall, dihydrotanshinone could induce apoptosis and inhibit proliferation of glioma cells by regulating caspases and cytochrome c. This study suggests that dihydrotanshinone may serve as a potential treatment option for patients with glioma.

KEY WORDS: Dihydrotanshinone; glioma; SHG-44 cells; cell apoptosis; cell proliferation; MTT assay

INTRODUCTION

Native in China and Japan, *Salvia miltiorrhiza* Bunge, also called Chinese sage or danshen, is a perennial plant in the genus *Salvia* and has been considered as a functional food in Asia. In traditional Chinese herbal medicine, dried root of *S. miltiorrhiza* is famous for its high medicinal value and is widely used for promoting blood circulation, especially in angina pectoris and myocardial infarction [1,2]. Modern pharmacological studies demonstrated that *Salvia* has some antitumor effect, and it has been used as an auxiliary anticancer drug [3,4]. For example, it was demonstrated that *Salvia* alcohol extract significantly inhibited the growth of human colorectal cancer cells (HRT-18), colon cancer cells (HT-29), and liver cancer cells (HepG2) in a cell-culture setting [5,6]. The major ingredients isolated from *Salvia* ethanol extract include cryptotanshinone, dihydrotanshinone I, tanshinone IIA, and tanshinone IIB among others. While tanshinone IIA, IIB and cryptotanshinone have been reported for their effective

antitumor activity [2,6], the role of dihydrotanshinone in cancer treatment remains relatively unclear.

Cytotoxic effects of dihydrotanshinone have been reported in a variety of tumor cells [7]. For instance, dihydrotanshinone could interfere with the RNA-binding activity of HuR and thus inhibit viability, proliferation, and chemotaxis of breast cancer cells [8]. Dihydrotanshinone induced cell growth arrest during the S phase of the cell cycle and, subsequently, apoptosis following its application to human leukemia multidrug resistant K562/ADR cells [9]. However, the role of dihydrotanshinone in brain cancer remains completely unexplored.

Brain cancer has very limited treatment options due to the presence of the blood–brain barrier (BBB), which protects infiltrating glioma cells from the effect of chemotherapeutic agents [10]. It has been previously reported that tanshinones have the potential to penetrate the BBB [11], and based on this, anti-glioma effects of dihydrotanshinone have been proposed.

In this study, 0-100 μg/L of dihydrotanshinone was applied to human glioblastoma SHG-44 cells and the antitumor properties of dihydrotanshinone were observed. Specifically, dihydrotanshinone could remarkably inhibit SHG-44 cell growth and proliferation, as well as induce cellular apoptosis. Dihydrotanshinone increased the activation of caspase-3 and caspase-9, as well as promoted the release of cytochrome c.

This study may shed lights on the application of dihydrotanshinone in treating gliomas.

MATERIALS AND METHODS

Cell culture

Human glioma cell line SHG-44 was purchased from Shanghai Institute of Cell Bank (Shanghai, China) and grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Gibco) and 100 U/mL of penicillin-streptomycin (Gibco). The cells were incubated in a humidified incubator at 37°C with 5% CO_2. All cells used in the assays were in the logarithmic growth phase (adherent density of about 60-80%).

Treatment with dihydrotanshinone

Dihydrotanshinone, with purity of more than 98%, was purchased from Baoji Herbest Bio-Tech Co., Ltd. (Baoji, China). In all experiments, indicated drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO), and all original stock solutions were maintained at 100 mg/L and stored at 4°C. A series of different dihydrotanshinone concentrations (0, 10, 20, 40, 50, 60, 80, and 100 μg/L) were added upon attachment of the cells, to detect the effects of dihydrotanshinone on SHG-44 cells.

3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay

SHG-44 cells were seeded in 96-well plates with 1×10^4 cells/100 μL. The following groups were included: background group (blank), control group, and the experimental group in which increasing concentrations of dihydrotanshinone were used. Various concentrations (0, 10, 20, 40, 50, 60, 80, and 100 μg/L) of dihydrotanshinone were added upon attachment of the cells to the dishes, and four parallel wells were set up for each dihydrotanshinone concentration. After 24, 48 and 72 hours of incubation, 30 μL of MTT (5 mg/mL; Sigma-Aldrich) was added until formazan was formed. These 96-well plates were then placed upside down and dried, and 0.1 mL DMSO was added to dissolve formazan. Absorbance or optical density (OD) values of each well at λ = 595 nm were measured using a multi-plate reader, and the cell growth inhibition rate was calculated according to the formula as follows:

% inhibition of cell proliferation rate = (1−experimental group mean OD value/control group mean OD value) × 100%.

Flow cytometry analysis

SHG-44 cells were seeded in 6-well plates at 5×10^4 cells per well. After 24 hours, 10, 50 and 100 μg/L of dihydrotanshinone, and DMSO as the control, were added to the cells for 24 hours. The cells were collected and gently washed 2-3 times in phosphate-buffered saline (PBS), then fixed with 75% ethanol (4°C pre-cooled) overnight. After the cells were collected by centrifugation, 0.5 mL of PBS containing propidium iodide (PI) (0.02 mg/mL; Sigma-Aldrich) and RNase A (0.1 mg/mL; Sigma-Aldrich) were added and mixed gently. The samples were then incubated at 37°C in the dark for 30 minutes. Cell sieves with 8.47 μm pore size were used to prepare single cell suspension for measuring DNA content by Epics XL flow cytometry system (Beckman-Coulter, USA).

Hoechst 33258 staining

SHG-44 cells were seeded in 6-well plates at 5×10^4 cells per well. After 24 hours, 10, 50 and 100 μg/L of dihydrotanshinone, and DMSO as the control, were added to the cells for 24 hours. The cells were collected and gently washed 2-3 times in PBS, then stained with 1 mL of PBS solution containing 10 mg/mL of Hoechst 33258 (Sigma-Aldrich) and incubated at 37°C in the dark for 30 minutes. The cells were then washed 3 times with PBS and imaged using fluorescence microscopy at an excitation wavelength of 350 nm and an emission wavelength at 460 nm.

Annexin V-FITC/PI double staining

SHG-44 cells were seeded in 6-well plates at 5×10^4 cells per well. After 24 hours, 10, 50 and 100 μg/L of dihydrotanshinone, and DMSO as the control, were added to the cells for 24 hours. The cells were collected and gently washed 2-3 times in PBS, then resuspended in 0.5 mL of PBS containing 5 μL of Annexin V-FITC (Pharmingen, San Diego, CA) and 10 μL of PI (Sigma-Aldrich). The reactions were incubated at 37°C in the dark for 30 minutes. Cell sieves with 8.47 μm pore size were used to prepare single cell suspension for Epics XL flow cytometry analyses of cell apoptosis.

Western blot analysis

SHG-44 cells were seeded in 6-well plates at 5×10^4 cells per well. After 24 hours, 10, 50 and 100 μg/L of dihydrotanshinone, and DMSO as the control, were added to the cells for 24 hours. The cells were collected and lysed in RIPA lysis buffer (Beyotime, Beijing, China), and protein concentrations in whole cell lysates were measured by the bicinchoninic acid assay. Forty milligrams of total protein were loaded on 30% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Bedford, MA, USA). After blocking with 5% non-fat milk, indicated primary antibodies (1:1000, Cell Signaling Technology, USA) and secondary antibodies (1:5000, Cell Signaling Technology, USA) were incubated with

PVDF membrane, and the signals were visualized using the enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, UK).

Statistics

Data were presented as mean ± standard deviation (SD). GraphPad Prism 5.0 statistical software (GraphPad, San Diego, CA) and Student's *t*-test (two-tailed) were used to analyze the significant difference between the groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Dihydrotanshinone significantly inhibits SHG-44 cell growth *in vitro*

To investigate whether dihydrotanshinone can inhibit cell proliferation in human glioma, SHG-44 cells were exposed to various doses of dihydrotanshinone for 24-72 hours. As shown in Figure 1, dihydrotanshinone inhibited SHG-44 cell growth in a dose- and time-dependent manner, with an IC50 value of 50.32 ± 2.49 μg/L at 24 hours, 42.35 ± 2.25 μg/L at 48 hours, and 31.25 ± 2.82 μg/L at 72 hours. These data confirm a possible role of dihydrotanshinone in suppression of SHG-44 cell growth.

Dihydrotanshinone treatment induces DNA fragmentation in SHG-44 cells

Next, we examined the possible mechanism(s) responsible for dihydrotanshinone-mediated suppression of SHG-44 cell growth. Because the cleavage of chromosomal DNA by the DNA fragment factor is associated with cellular apoptosis [12], we first examined whether dihydrotanshinone treatment leads to DNA fragmentation in SHG-44 cells. Notably, we observed that the treatments with increased doses of dihydrotanshinone led to correspondingly increased amount of sub-G1 DNA content (<2N location apoptotic peaks in Figure 2A-D), a marker of cellular apoptosis [13]. Specifically, the number of cells with sub-G1 DNA content indicated that the apoptosis levels were 9.0%, 48.0%, and 52.3% for dihydrotanshinone concentrations at 10, 50, and 100 μg/L, respectively. This suggests that dihydrotanshinone treatment may induce cell apoptosis in SHG-44 cells in a dose-dependent manner.

Dihydrotanshinone treatment leads to nuclear condensation in SHG-44 cells

To further confirm the pro-apoptosis role of dihydrotanshinone in SHG-44 cells, Hoechst 33258 staining was applied
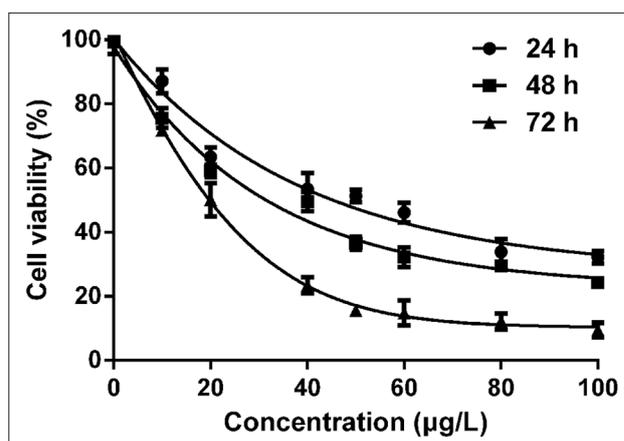
FIGURE 1. Dihydrotanshinone suppresses SHG-44 cell growth in time- and dose-dependent manner. SHG-44 cells were treated with various concentrations (0, 10, 20, 40, 50, 60, 80, and 100 μg/L) of dihydrotanshinone for 24, 48 and 72 hours, and analyzed with 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay.
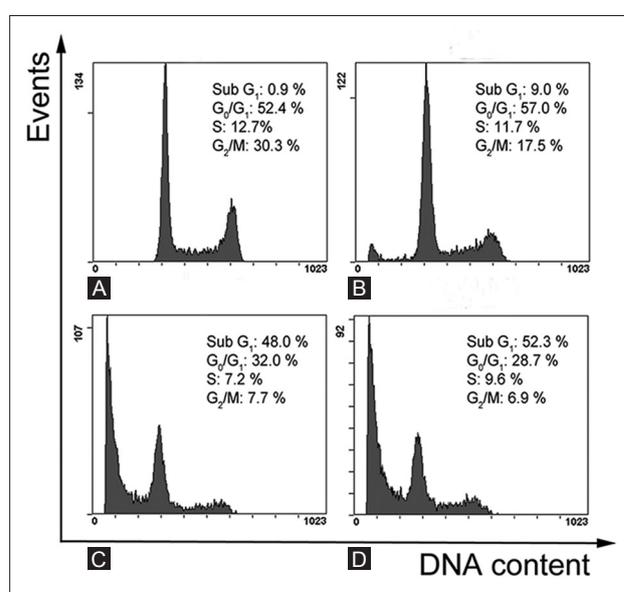
FIGURE 2. Dihydrotanshinone induces DNA fragmentation. SHG-44 cells were treated with dimethyl sulfoxide (A), and 10 μg/L (B), 50 μg/L (C) and 100 μg/L (D) of dihydrotanshinone for 24 hours, followed by propidium iodide staining and flow cytometry analysis.

to examine the nucleus morphology. After the staining, a blue fluorescent signal in normal cells is usually weak and evenly colored, while in apoptotic cells, fluorescent signals are unevenly distributed with increased fluorescence intensity. After the treatment of SHG-44 cells with different concentrations of dihydrotanshinone for 24 hours, the nuclei exhibited condensed and fragmented chromatin with increased staining intensity (Figure 3A-D), which is another feature of cellular apoptosis [13]. Moreover, the increase in staining intensity of condensed nuclei was positively correlated with the increasing doses of dihydrotanshinone used in the treatments. Taken together, these results suggest that dihydrotanshinone treatment results in nuclei condensation and fragmentation in SHG-44 cells.

Dihydrotanshinone-induced cellular apoptosis in SHG-44 cells

As both DNA fragmentation and nuclear condensation were observed in dihydrotanshinone-treated SHG-44 cells, we further examined whether these phenotypes were the result of the dihydrotanshinone-induced cellular apoptosis. We used the Annexin V-FITC/PI double staining method to check whether the cells undergo apoptosis. The cells stained only with Annexin V are shown in the lower right quadrant of the FACS histogram, and the cells stained with both dyes are presented in the upper right quadrant of the FACS histogram (Figure 4A-D). Notably, the early apoptotic population was positively increased with the increasing doses of dihydrotanshinone, from 2.6% in the control group gradually to 83.1% in the group treated with 100 μg/L of dihydrotanshinone (Figure 4A-D). However, the cell population in the late apoptosis stages (stained by both PI and Annexin V) did not change dramatically with the increasing doses of dihydrotanshinone (Figure 4B-D). These data suggest that dihydrotanshinone induces apoptosis in SHG-44 cells, and consequently triggers DNA fragmentation and nuclear condensation.

Dihydrotanshinone-induced cellular apoptosis is mediated by increased activation of caspases and release of cytochrome c

To investigate the possible molecular mechanism of dihydrotanshinone in suppressing SHG-44 cell growth through apoptosis, we performed western blot analyses using the dihydrotanshinone-treated cells. There was no significant change in the levels of caspase-8-mediated death receptor pathway, while the cleavage of caspase-9,

downstream of the mitochondrial pathway, was markedly increased, as well as the levels of cleaved caspase-3 and poly ADP-ribose polymerase (PARP) that are signs of apoptosis (Figure 5).

Because the cleavage-mediated activation of caspase-3 requires cytochrome c, we examined whether dihydrotanshinone also influences the cytoplasmic levels of cytochrome c. After the treatment with 10 μg/L and 50 μg/L of dihydrotanshinone, the cytoplasmic, but not the total levels of cytochrome c were markedly increased (Figure 6), further indicating the role of dihydrotanshinone in facilitating cytochrome c release, which activates caspase-3 and promotes apoptosis.

FIGURE 4. Dihydrotanshinone induces cell apoptosis in a dose-dependent manner. SHG-44 cells were treated with dimethyl sulfoxide (A), and 10 μg/L (B), 50 μg/L (C) and 100 μg/L (D) of dihydrotanshinone for 24 hours, followed by Annexin V/propidium iodide (PI) staining and flow cytometry analysis.

FIGURE 3. Dihydrotanshinone induces nuclear condensation. SHG-44 cells were treated with dimethyl sulfoxide (A), and 10 μg/L (B), 50 μg/L (C) and 100 μg/L (D) of dihydrotanshinone for 24 hours. The nuclei were stained with Hoechst 33258, and analyzed using a fluorescent microscopy. The representative images are shown. The arrowheads indicate Hoechst 33258 stained nucleus.
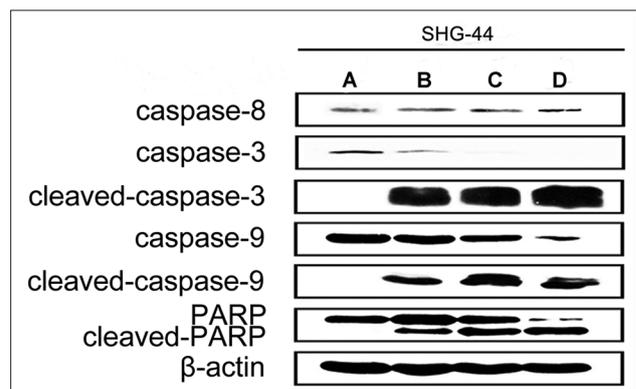
FIGURE 5. Dihydrotanshinone promotes caspase activation. SHG-44 glioma cells were treated with dimethyl sulfoxide (A), and 10 μg/L (B), 50 μg/L (C) and 100 μg/L (D) dihydrotanshinone for 24 hours, followed by cell lysis and western blot analyses with indicated antibodies.
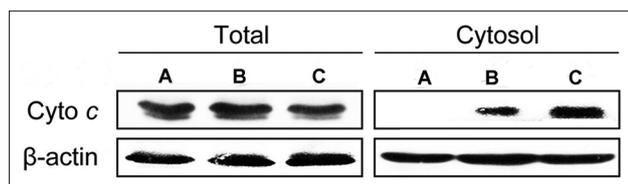
FIGURE 6. Dihydrotanshinone promotes cytochrome c release from mitochondria. SHG-44 cells were treated with dimethyl sulfoxide (A), and 10 μg/L (B) and 50 μg/L (C) of dihydrotanshinone for 24 hours, followed by cell lysis and western blot analyses with indicated antibodies.

DISCUSSION

Human glioma is one of the most frequent malignant neoplasms, accounting for over 70% of all primary brain tumors in adults [14]. Studies have reported that in more than 60% of adults over the age of 50 glioma relapsed, due to the absence of timely diagnosis and treatment [15,16]. The current treatment options for glioma patients include surgery, radiotherapy, chemotherapy and targeted therapy, most of which have profound side effects. Moreover, many anticancer chemotherapies kill a significant portion of healthy cells in addition to glioma cancer cells. On the other hand, the traditional Chinese herbal medicine has drawn more and more attention, due to its high efficiency and low toxicity effect. One effective way is to search for or isolate active ingredients from the plants that display antitumor activity, which would be helpful in discovery of new drugs but could also provide a molecular basis for the design of active lead compounds [17].

Salvia is one of the Chinese traditional medicinal plants that has been broadly studied. The active, fat-soluble ingredients such as quinone derivatives (i.e., tanshinone I, cryptotanshinone, isotanshinone I and others), have demonstrated broad antitumor activity [6]. Previous studies reported the cytotoxic effect of dihydrotanshinone from *Salvia* against various types of tumor cells [18]. To our knowledge, this is the first report of dihydrotanshinone antitumor activity in glioma cells. In this study, dihydrotanshinone showed a significant antiproliferative effect in the human SHG-44 metastatic glioma cells, in both time- and dose-dependent manner, with IC50 values of 50.32 ± 2.49 μg/L, 42.35 ± 2.25 μg/L, and 31.25 ± 2.82 μg/L at 24, 48, and 72 hours post treatment, respectively. More importantly, we further demonstrated that dihydrotanshinone triggers cellular apoptosis by releasing cytochrome c from mitochondria and thus activating caspases.

During cell apoptosis, changes in the cell morphology are observed including cell shrinkage, cytoplasmic vacuole formation, swelling of mitochondria, nuclear membrane shrinkage, chromatin aggregation, and appearance of apoptotic bodies [19]. In this study, dihydrotanshinone triggered cell shrinkage, nuclear chromatin aggregation and the movement of chromatin closer to the nuclear membrane, suggesting that

dihydrotanshinone might suppress SHG-44 cell growth by inducing cell apoptosis. Furthermore, cellular debris was identified by flow cytometry upon the treatment with increasing concentrations of dihydrotanshinone, which also suggests the role of dihydrotanshinone in inducing cell apoptosis. Finally, we performed Annexin V-FITC/PI double staining analysis to confirm that dihydrotanshinone can induce apoptosis in dihydrotanshinone dose-dependent manner. Cumulatively, our results confirm that dihydrotanshinone suppresses SHG-44 cell proliferation by inducing cell apoptosis.

There are two major apoptotic pathways, i.e., intracellular and extracellular apoptotic pathway [20]. The intracellular pathway, also known as mitochondrial pathway, is the major apoptotic signaling pathway in the cell. Apoptosis is the result of complex interactions between a variety of genes and proteins in cells where, in particular, caspase protein family plays an important role [21]. The proteins from caspase family are activated by cytochrome c, released from mitochondria, and by the apoptotic protease activating factor 1 upon apoptotic signals, through the formation of apoptotic bodies that ultimately induce apoptosis [22]. Once capase-3 and -9 are activated, they can cause PARP cleavage [23]. Our western blot analyses demonstrated that, upon the dihydrotanshinone treatment, the cytoplasmic level of cytochrome c was increased which activated caspase-9 and caspase-3, as well as PARP cleavage, and triggered the cell apoptosis. These findings are consistent with the results from previous studies [24,25].

CONCLUSION

Overall, our results suggest that dihydrotanshinone can effectively inhibit the proliferation of SHG-44 cells possibly through the activation of the mitochondrial apoptosis pathway. These results shed new light on the application of dihydrotanshinone as a new class of anticancer drugs in treating patients with glioma. As reactive oxygen species (ROS)-mediated p38 mitogen-activated protein kinases (MAPK) activation has been shown to play a key role in dihydrotanshinone-induced apoptosis in HepG2 cells [26], it warrants further studies to investigate molecular signaling pathways that are altered upon dihydrotanshinone treatment, to search for additional drug targets, or to overcome possible drug resistance triggered by dihydrotanshinone administration.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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