

Effects of Saikosaponin D on apoptosis in human U87 glioblastoma cells

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Abstract. Saikosaponin D (SSd) is a type of Saponin derivative, which is a component extracted from *Bupleurum falactum*. SSd has been reported to exert anticancer activities. However, the effects of SSd on gliomas have not been elucidated. The aim of the present study was to investigate the pharmacological functions and potential molecular mechanisms of SSd in human U87 glioblastoma cells. The cells were treated with SSd at various concentrations for 48 h, the cell viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, and the activation of Akt, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and caspase-3 was assessed by western blotting. In addition, apoptosis levels were analyzed with Hoechst 33258 and Annexin V staining. The results demonstrated that treatment of the U87 glioma cells with SSd markedly suppressed cell proliferation in a dose-dependent manner. Meanwhile, SSd treatment enhanced apoptosis in the U87 cells. Furthermore, SSd significantly inhibited the phosphorylation of Akt and ERK, and promoted phosphorylated-JNK and cleaved caspase-3 expression. The present study revealed the potential therapeutic effects of SSd in the treatment of gliomas, and the cytotoxic effects of SSd in U87 cells were at least partly attributed to the depression of phosphatidylinositol 3-kinase/Akt and ERK protein expression levels, and activation of JNK and caspase-3 expression.

Introduction

Gliomas are the most common and most fatal tumors of all types of brain malignancy (1,2). The general treatment measures against gliomas include surgery, radiotherapy and chemotherapy; however, the prognosis is poor, and the median

survival time of gliomas is only 6-14 months (3-5). Gliomas are often resistant to antitumoral chemotherapeutic strategies, thus limiting the efficacy of treatment (4). The pharmacological activities of traditional Chinese medicines have attracted increasing attention regarding their therapeutic effects against glioblastoma (6-8).

Saikosaponin D (SSd) is one of the major Saponin components derived from the dried roots of *Bupleurum falactum*, a traditional Chinese medicine plant. It has been reported that SSd exerts anticancer activities in cervical and lung cancer cells, and hepatocellular carcinoma cells (9-12). SSd may potentiate tumor necrosis factor (TNF)- α -mediated cell death via suppression of TNF- α -induced nuclear factor (NF)- κ B activation, while inducing apoptosis by enhancing the loss of mitochondrial membrane potential (13). In addition, SSd has been demonstrated as an inhibitor of cell survival signaling, and subsequently attenuates the expression of B-cell lymphoma-extra large (14). However, the molecular mechanisms remain unknown.

Although previous studies have indicated that SSd exerts anticancer activities in various tumor cell lines (9-12), the effects of it in central nervous system (CNS) malignant tumors remain unknown. In the present study, the growth potential, proliferation inhibition and apoptosis induction effects of SSd on human U87 glioma cells were investigated. In addition, the possible mechanisms underlying SSd-induced growth arrest and apoptosis in glioma cells were evaluated.

Materials and methods

Chemicals and reagents. SSd (purity, 96%) was purchased from the National Institutes for Food and Drug Control of China (Beijing, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Akt (cat. no. 4691), phosphorylated (p)-Akt (cat. no. 4060), extracellular signal-regulated kinases (ERK; cat. no. 4695), p-ERK (cat. no. 4370), c-Jun N-terminal kinases (JNK; cat. no. 9258), p-JNK (cat. no. 4668), cleaved caspase-3 (cat. no. 9664), β -actin (cat. no. 12620) and peroxidase-conjugated anti-rabbit (cat. no. 7074) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Chemicals for buffer

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preparations were purchased from Sigma-Aldrich (Merck KGaA).

Cell line and cell culture. Human U87 glioma cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and grown in DMEM containing 10% fetal bovine serum (Haoyang Biological Products Technology Co., Ltd., Tianjin, China), 100 U/ml penicillin, 100 µg/ml streptomycin, and incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability assay. Cell viability was assessed using an MTT bromide assay. U87 cells (200 µl; 3x10⁴ cells/ml) were seeded into 96-well tissue culture plates. Following overnight incubation, the cells were exposed to serial concentrations of SSd (1, 2, 3, 4, 5, 6, 7 and 8 µM) or control medium for 48 h. Subsequently, 10 µl MTT reagents was added to each well and incubated at 37°C for 4 h, followed by the addition of 150 µl dimethyl sulfoxide to each well. The plates were agitated for 10 min. Then, the absorbance was read at a wavelength of 490 nm using an iMark™ microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed with six replicate wells for each condition, and the data were obtained from three independent experiments. The half-maximal inhibitory concentration (IC₅₀) of SSd was calculated using the Logit method (15).

Hoechst 33258 staining and immunofluorescence. U87 cells in 6-well plates were treated with different concentrations of SSd for 48 h, washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. Subsequently, the cells were washed with PBS, and stained with Hoechst 33258 for 5 min at room temperature. The nuclear morphology was observed using a laser scanning confocal microscope. For quantification, three different fields were randomly selected and counted under the microscope. The apoptotic rates were calculated as the percentage of apoptotic cells relative to the total number of cells.

Flow cytometric evaluation of apoptosis. Cells treated with different concentrations of SSd were seeded into 6-well plates (1.8x10⁵ cells/well) for 48 h and isolated with trypsin. The cells were supplemented with 100 µl DMEM medium and analyzed according to the manufacturer's instructions using the Muse™ Annexin V and Dead Cell Assay kit (Muse™ Cell Analyzer; Merck KGaA).

Western blotting. Following SSd treatment for 48 h, U87 cells were washed with ice-cold PBS three times and resuspended in 100 µl radioimmune precipitation buffer [100 mM NaCl and 100 mM sodium fluoride, 20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 1% SDS, 1% Triton X-100 and 1% sodium deoxycholate]. The lysate was centrifuged at 12,000 x g for 20 min, and 50 µg cell lysate protein was used for western blotting. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight individually with primary antibodies at a dilution of 1:1,000 in TBS containing 0.1% Tween-20 at 4°C, and the membranes were incubated with peroxidase-conjugated anti-rabbit IgG (1:5,000) for 2 h at room temperature. All bands were detected

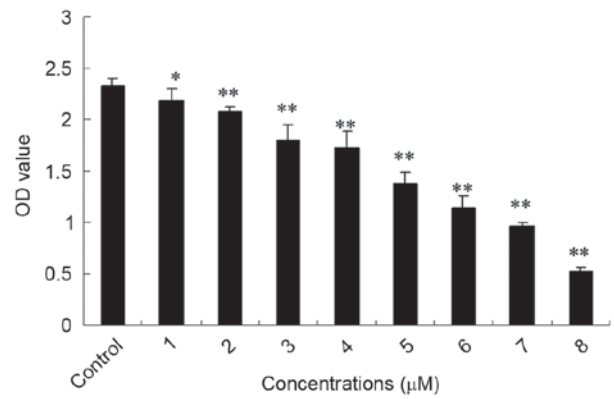


Figure 1. Effects of SSd exposure on the growth and proliferation of human U87 glioma cells *in vitro*. The U87 cells were treated with SSd at different concentrations for 48 h. Cell viability rate was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. Data are presented as means ± standard deviation. *P<0.05 and **P<0.01 vs. control. SSd, saikosaponin D; OD, optical density.

using the enhanced chemiluminescence (ECL) system (Tanon Science & Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions (16).

Statistical analysis. The data are presented as the mean ± standard deviations as indicated. Statistical analyses for comparison of mean values were performed by one-way analysis of variance followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference. All data were derived from three independent experiments.

Results

SSd treatment inhibited the proliferation of U87 cells. The mitochondria of living cells break down MTT to produce formazan, and the quantity of formazan corresponds with the living cell number. To determine whether SSd inhibits U87 glioma cell growth, the cell viability rate was assessed using the MTT method. In the present study, obvious inhibitory effects on the proliferation of U87 cells were observed with SSd exposure. As shown in Fig. 1, following treatment with 1-8 µM SSd for 48 h, the proliferation rate of U87 cells was significantly reduced in a dose-dependent manner when compared with the control group. Additionally, the half maximal inhibitory concentration (IC₅₀) value of SSd was 4.79 µM. The result indicates that SSd inhibited the viability of U87 glioma cells.

Effect of SSd treatment on changes of cellular morphology. When detecting nuclear morphological changes, U87 cells were stained with Hoechst 33258 and observed under a laser scanning confocal microscope. Intact nuclei in the living cells were stained blue, and the morphology was round or oval, while the condensed or fragmented nuclei in apoptotic cells were stained bright blue. As illustrated in Fig. 2, the cells treated with 2.5 µM SSd began to exhibit nuclear morphological changes when compared with the control cells, and the nuclei of cells treated with 7.5 µM SSd were markedly brighter, indicating a high prevalence of nuclear chromatin and fragmentation.

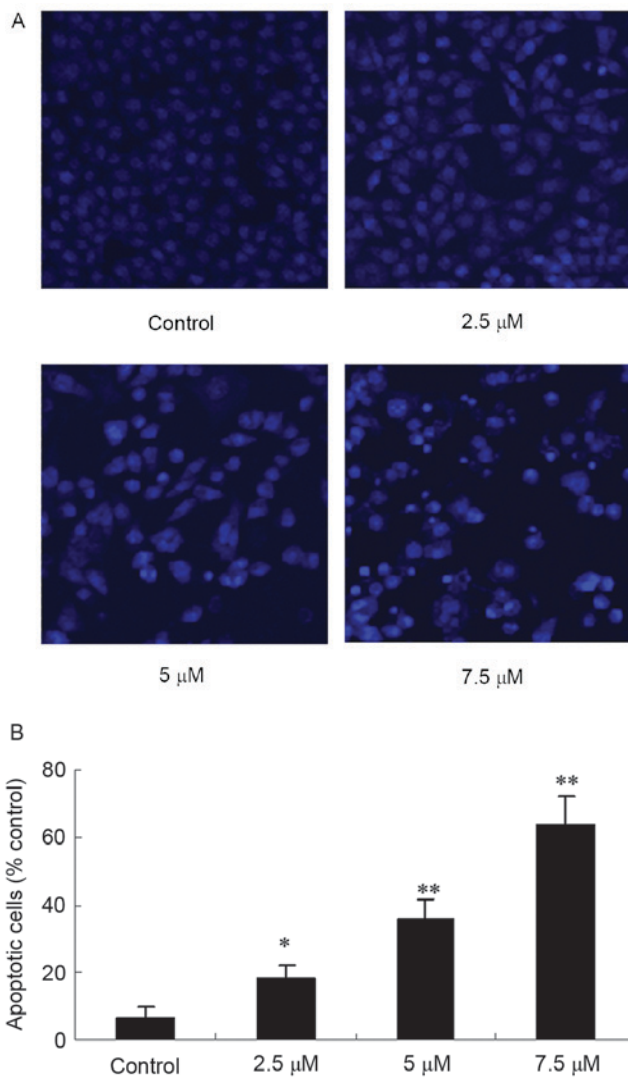


Figure 2. Hoechst 33258 staining of U87 cells observed under a laser scanning confocal microscope following treatment with saikosaponin D at different concentrations for 48 h (magnification, x200). (A) Nuclear morphological changes in each group were determined by Hoechst 33258 staining. Highly fragmented or condensed nuclei represent apoptotic cells and intact nuclei represent viable cells. (B) Bar graphs represent the percentage of nuclear fragments and condensation in the U87 cells. Data are presented as means \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control.

SSd treatment induced apoptosis in U87 cells. To further evaluate whether the inhibition of cell proliferation in U87 cells was associated with the induction of apoptosis, Annexin V staining was used to assess the rate of apoptotic cells following SSd treatment. As shown in Fig. 3, treatment with SSd significantly increased the proportion of Annexin V-positive cells in a dose-dependent manner. The results indicate that SSd treatment inhibited the viability of U87 cells by inducing apoptosis.

Effect of SSd treatment on phosphatidylinositol 3-kinases (PI3K)/Akt, ERK and JNK signaling pathways in U87 cells. To further elucidate the possible mechanisms underlying the effects of SSd treatment on U87 cells, the p-Akt/Akt, p-ERK/ERK, p-JNK/JNK protein expression levels with western blot analysis. The data demonstrated that SSd exposure induced a significant decrease of p-Akt and p-ERK

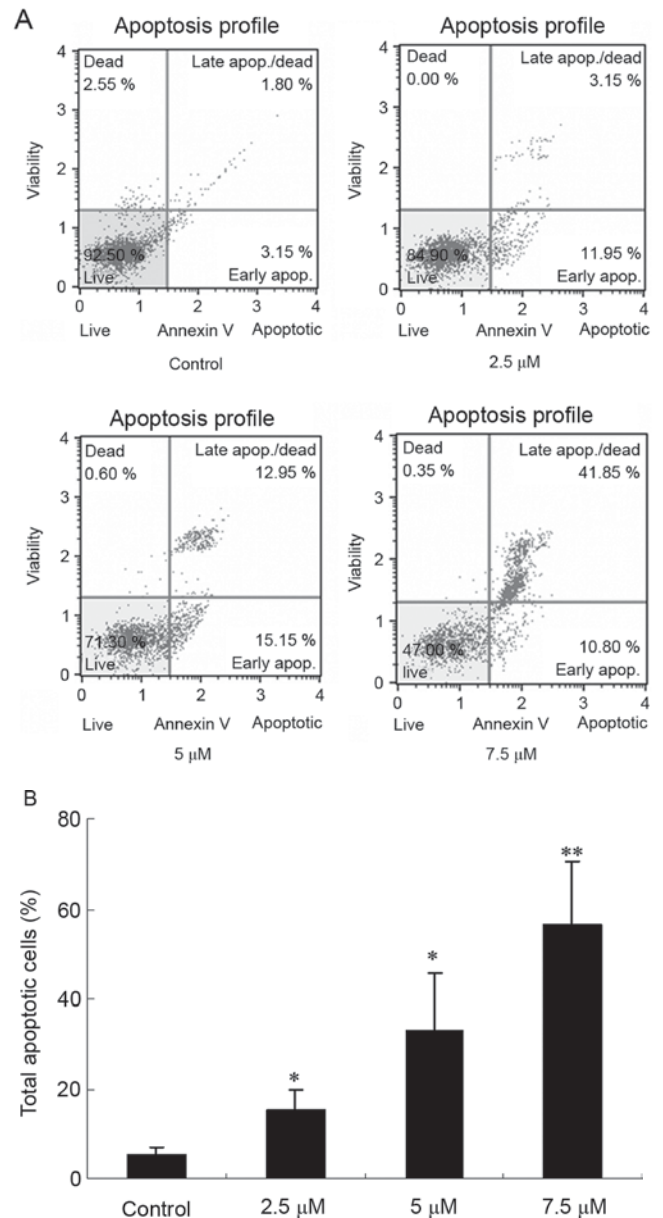


Figure 3. Effects of saikosaponin D treatment on the apoptotic rate of U87 cells as evaluated by Muse™ Annexin V and Dead Cell assay. (A) Muse™ Cell Analyzer analysis of Annexin V-stained cells. (B) Quantitative analysis of data from the total apoptotic cell ratio. Data are presented as means \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control.

relative protein expression levels, and increased p-JNK protein expression levels (Fig. 4; P<0.05). These results indicated that treatment with SSd potentially downregulated the PI3K/Akt and ERK signaling pathways, increased JNK activation and further enhanced apoptosis in U87 cells.

Activation of caspase-3 induced by SSd exposure. Caspases are critical mediators during the process of apoptosis. In all of apoptosis-associated caspases, caspase-3 is a key effector or executioner for programmed cell death (17). Thus, to determine whether caspase-3 was involved in SSd-induced cytotoxicity, the expression levels of cleaved caspase-3, an indicator of its activity, were examined. In the present study, SSd treatment significantly increased the expression level of cleaved caspase-3 (Fig. 5), indicating that SSd-induced

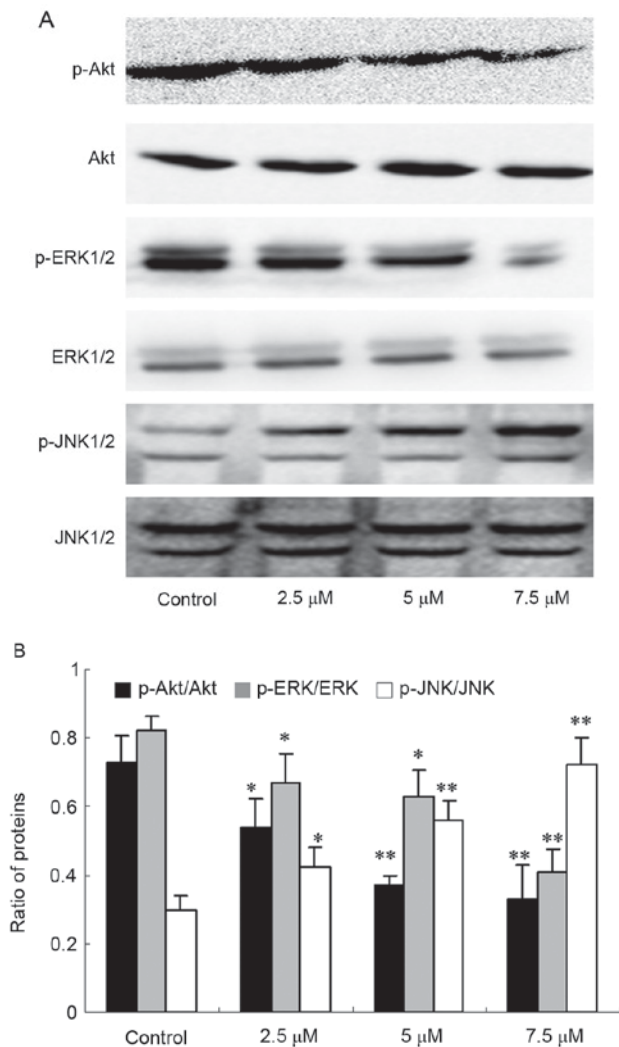


Figure 4. Effects of SSd treatment on phosphorylated and total Akt, ERK and JNK protein expression levels. (A) Western blot analysis of phosphorylated and total Akt, ERK and JNK levels with SSd administration at different concentrations for 48 h. Bands were visualized using the ECL detection method. (B) Relative expression levels of p-Akt/Akt, p-ERK/ERK and p-JNK/JNK ratios. Data are presented as means \pm standard deviation (n=3). *P<0.05 and **P<0.01 vs. control. SSd, saikosaponin D; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; p, phosphorylated.

apoptosis in U87 cells may be associated with the promoted activation of caspase-3.

Discussion

As a traditional Chinese medicine, *B. falcatum L* is commonly administered for the treatment of hepatopathy, inflammation and viral infection in Asia. Triterpene saponins are the major pharmaceutical ingredients in *B. falcatum L*, which are divided into saikosaponin-a, -b, -c and -d according to the different structures. SSd is one of the most active ingredients of triterpene saponins. During the past decades, studies have focused on SSd, as it demonstrates numerous beneficial properties, including anti-inflammatory, antioxidant and antitumor effects (12,18,19). In the present study, it was demonstrated that SSd treatment inhibited the proliferation of human malignant glioma U87 cells, indicating that SSd may exert potential beneficial effects in the treatment of malignant gliomas.

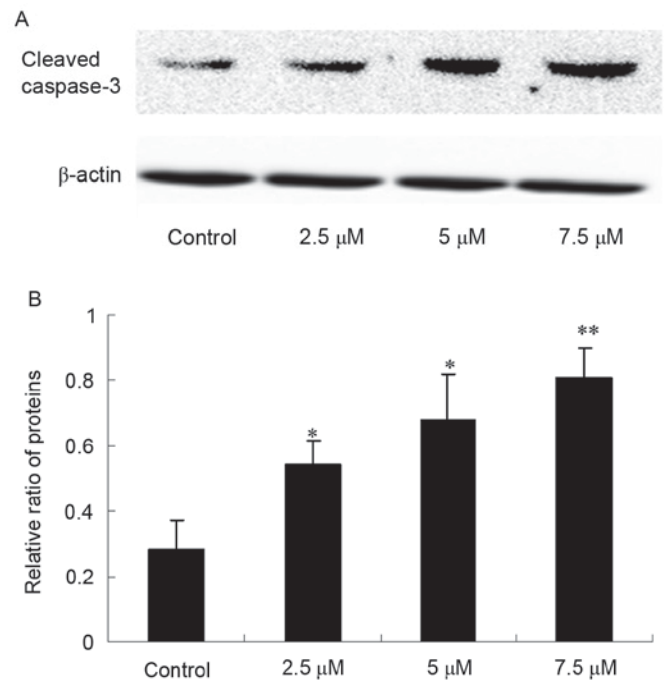


Figure 5. Saikosaponin D treatment upregulated the activity of caspase-3. (A) Cleaved caspase-3 expression levels were assayed by western blotting. (B) Relative densitometric analysis of the gels presented in (A). Data shown are representative of three different experiments. Values are presented as means \pm standard deviation of three separate experiments. *P<0.05 and **P<0.01 vs. control.

Apoptosis is recognized as the most important form of cell death in multicellular organisms, and occurs in physiological and pathological conditions. Certain types of cytotoxic stresses, such as hypoxia, UV, infrared irradiation and chemotherapeutic drugs, initiate apoptosis to remove target cells (20,21). Biochemical alterations of apoptosis include phosphatidylserine externalization, chromosomal DNA cleavage, and activation of a family of proteases (22). Apoptosis is important for the inhibition of cancer development (23,24). At present, drug-induced apoptosis is considered to be the primary strategy for curing tumors (25). Previous studies have confirmed that SSd exposure inhibits proliferation and promotes apoptosis in certain tumor cells (26-28). However, whether SSd induces apoptosis in glioma cells has yet to be elucidated. In the present study, the addition of SSd led to increased apoptosis in human U87 glioma cells, as measured by Hoechst 33258 staining and Annexin V staining assays, implying that SSd treatment exerts significant cytotoxic effects by increasing apoptosis in glioma cells.

In the current study, it was also found that SSd inhibited the PI3K/Akt signaling pathway in a dose-dependent manner, indicating that depression of the PI3K/AKT signaling pathway may be associated with SSd-mediated apoptosis and proliferation inhibition. Akt, also known as protein kinase B, is a primary downstream effector of the PI3K signal transduction pathway, with crucial functions in regulating cell proliferation and survival (29,30). Furthermore, it has been reported that the Akt signaling pathway is particularly important in preventing apoptosis (31,32) and inhibiting the activation of Akt may induce apoptosis (33). ERK and JNK are members of the mitogen-activated protein kinase

(MAPK) family, and have been shown to be key in cell proliferation, differentiation, development and programmed cell death (34). Numerous studies have indicated that abnormalities in MAPK signaling pathways were involved in the pathological processes of various types of cancer (35-37). In the current study, the aim was to investigate the effects of SSd exposure on the protein expression levels of ERK and JNK. The results demonstrated that SSd treatment significantly inhibited the activation of ERK and stimulated the phosphorylation of JNK.

There are three apoptotic pathways found in mammals: The extrinsic pathway (death receptor-mediated pathway), the intrinsic pathway (mitochondrial-mediated pathway) and the granzyme B pathway. The key regulatory factors in these three pathways are the caspases (cysteiny aspartate-specific proteinases), which are activated following cell damage and are responsible for regulating cell apoptosis (38). Accordingly, in recent years, much attention has been paid to developing anticancer therapeutic strategies that modulate the activation of caspases to inhibit tumor progression. Caspase-3 is a major mediator during the execution period of cell apoptosis. When activated by upstream signaling molecules, such as caspase-8, caspase-9 or caspase-10, caspase-3 would be either partially or totally responsible for the proteolysis of many downstream key proteins associated with apoptosis (39). In order to elucidate the molecular mechanisms responsible for SSd-induced apoptosis in U87 cells, the activity of caspase-3 was analyzed in the present study. The findings indicated that the addition of SSd to U87 cells promoted caspase-3 activity, demonstrating the role of caspase-3 activation in SSd-induced apoptosis.

In conclusion, the present study demonstrated that SSd treatment in human glioma cells inhibits cell proliferation, downregulates phosphorylation of Akt and ERK, upregulates JNK and caspase-3 activities and eventually causes cell apoptosis. Collectively, these observations provide further understanding of the pharmacological activity of SSd. However, future work is required to elucidate the underlying mechanisms of SSd-induced tumor cell apoptosis.

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