

Fatsioside A inhibits the growth of glioma cells via the induction of endoplasmic reticulum stress-mediated apoptosis

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Abstract. Malignant gliomas are a common type of primary tumor of the central nervous system. In spite of current intensive therapy, the prognosis of patients with malignant glioma remains poor, hence the development of novel therapeutic modalities is necessary. Cell apoptosis is a frequent target in the development of anti-cancer drugs. Fatsioside A, a novel baccharane-type triterpenoid glycoside, is extracted from the fruits of *Fatsia japonica*. Previous studies have shown that Fatsioside A induces growth inhibition, cell cycle arrest and apoptosis in C6 rat glioma cells and U251 human glioma cells. However, to the best of our knowledge, no detailed studies have reported its effect on U87MG glioma cells and its exact mechanisms remain unknown. In the current study, the growth inhibitory effect of Fatsioside A on U87MG cells was evaluated and the underlying molecular mechanisms were explored. Through the use of flow cytometry and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, it was determined that Fatsioside A markedly inhibits the growth of U87MG cells. Mechanistic studies demonstrated that Fatsioside A induces growth inhibition of U87MG cells via the induction of endoplasmic reticulum (ER) stress, which was supported by the upregulation of ER stress markers, including elevated levels of phosphorylation of PERK and eIF2 α , the increased expression levels of CHOP and the accelerated cleavage of caspase-4. The downregulation of CHOP via CHOP-specific siRNA reduced the growth-inhibitive effect of Fatsioside A on U87MG cells, further confirming the role of the ER stress response in mediating Fatsioside A-induced growth inhibition. In conclusion, Fatsioside A inhibits glioma

cell growth via the induction of ER stress-mediated apoptosis. This may provide a molecular basis for the development of Fatsioside A into a drug candidate for the treatment of malignant glioma.

Introduction

Glioblastoma is the most lethal type of primary adult brain tumor. It has a poor prognosis as it diffusely infiltrates other regions of the brain, which makes total surgical resection difficult (1-3). Hence, the majority of patients succumb to the disease within two years of diagnosis, in spite of current multi-disciplinary therapies, which include surgery, radiotherapy and chemotherapy. The ultimate aim of chemotherapy is the successful elimination of cancer cells through apoptosis (4,5). Apoptosis is an important process that controls the growth and development of organisms, and the perturbation of apoptosis is considered to be a promising strategy for the prevention and treatment of gliomas (6).

The endoplasmic reticulum (ER) is an important organelle involved in the secretory pathway, which possesses a vital role in membrane protein biosynthesis, protein folding and protein modification (7,8). However, homeostasis in the lumen of the ER is perturbed under a variety of toxic insults, including hypoxia, failure of protein synthesis, protein misfolding, and Ca²⁺ overload, which may result in a state of ER stress (9,10). The unfolded protein response (UPR) is induced to relieve this stress in eukaryotic cells in an attempt to restore and maintain normal ER homeostasis and function (11). If the UPR is unable to correct the balance of ER stress, the cellular apoptotic machinery may be triggered, ultimately leading to cell death. A major proapoptotic transcription factor induced during ER stress is CHOP, which is one of the main mediators of the apoptotic machinery (12,13). ER stress has been shown to occur in cancer cells *in vivo* and the ER stress response has been hypothesized to be a potential pathway that can be pharmacologically exploited to induce apoptosis in gliomas (14-17).

Fatsioside A, a novel baccharane-type triterpenoid glycoside, is extracted from the fruits of *Fatsia japonica*. Fatsioside A exerts growth inhibition, cell cycle arrest, and apoptosis in C6 rat glioma cells and U251 human glioma cells (18,19). Hence, Fatsioside A is a promising candidate for adjunctive therapy against human gliomas through activation of cell death.

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However to the best of our knowledge, no detailed studies have been reported on its action on U87MG glioma cells to date and its exact mechanisms are yet to be elucidated.

In the current study, the changes in growth of U87MG cells in response to Fatsioside A treatment were evaluated and the possible underlying molecular mechanisms were explored. The results of this study may be useful in identifying potential candidates for the targeted therapeutic intervention of glioma.

Materials and methods

Materials. Fatsioside A was obtained from the College of Pharmaceutical Sciences at Zhejiang University (Hangzhou, China). Fatsioside A was dissolved in 0.8 mM dimethylsulfoxide (Sigma, St. Louis, MO, USA) and diluted with fresh medium to obtain the desired concentration. Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse monoclonal antibodies specific for eIF2 α , p-eIF2 α , PERK and p-PERK, as well as rabbit polyclonal antibodies for cleaved caspase-4, CHOP and β -actin were purchased from Sigma. Secondary antibodies were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA).

Cell culture. U87MG cells were provided by the College of Pharmaceutical Sciences at Zhejiang University (Hangzhou, China) and were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma). When cells reached 80% confluence they were split into three plates. Experiments were performed once cells reached 50–60% confluence.

MTT assay. An MTT assay was employed to examine the effects of Fatsioside A on the proliferation of glioma cells. Briefly, the cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 200 μ l medium. Subsequently, the cells in the wells were treated with various concentrations of Fatsioside A and cultured for 24 or 48 h. At the end of the culture, MTT solution (0.5 mg/ml in 20 μ l phosphate-buffered saline; PBS) was added to each well and incubated for 4 h at 37°C. An ELISA instrument (Multiscan FC; Thermo Scientific, Waltham, MA, USA) was used to measure the absorbance of each well at a wavelength of 570 nm. Data were calculated from three independent experiments.

Flow cytometry. Flow cytometry was used to analyze the effects of Fatsioside A on the apoptosis of glioma cells. Briefly, U87MG cells were incubated with Fatsioside A at various concentrations for 24 h, and then the cells were harvested and washed twice with ice-cold PBS. Apoptotic cells were determined by Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI; Beyotime, Shanghai, China) double staining in a binding buffer (Beyotime) using flow cytometry (FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA). All experiments were performed in triplicate.

Transfection with small interfering RNA (siRNA). Cells were seeded at a density of 2×10^5 cells per well into 6-well plates

24 h prior to transfection with opti-MEM and Lipofectamine 2000 (Sigma). The cells were transfected with CHOP-specific siRNA or nontargeting siRNA using Lipofectamine 2000 (Sigma) according to the manufacturer's instructions. Four hours later, the opti-MEM was completely replaced by medium and the cells were incubated for an additional 48 h. Following the incubation, the cells were treated with various concentrations of Fatsioside A for 24 h and then they were used for subsequent experiments. The siRNAs were purchased from GenePharma (Shanghai, China) with the following sequences: CHOP-specific siRNA, 5'-AAGAACCA GCAGAGGUCACAA-3'; si-control, 5'-GAGCGCUAGACA AUGAAG-3'.

Western blot analysis. Whole cellular protein was extracted from the U87MG cells and prepared with lysis buffer for western blotting. Briefly, the cells were lysed in radioimmunoprecipitation assay buffer (Sigma) for 30 min on ice. Protein levels were quantified using the Lowry method. Equivalent amounts of protein (30 μ g per lane) were separated by 5–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose blotting membranes (Cell Signaling Technology). The membranes were blocked in Tris-buffered saline with Tween 20 (Cell Signaling Technology) containing 5% non-fat dry milk (w/v) for 2 h. Subsequently, the membranes were incubated overnight with primary antibodies at a 1:1,000 dilution at 4°C, followed by treatment with the corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Protein bands were visualized using chemiluminescence detection (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data are presented as the mean \pm standard deviation of three independent experiments. The difference between two mean values was evaluated using the Student's t-test and $P < 0.05$ was considered to indicate a statistically significant difference. The statistical analyses were performed using the SPSS software, version 19.0 (IBM, Armonk, New York, USA).

Results

Fatsioside A inhibits the growth of U87MG cells and induces apoptosis. The MTT assay revealed that Fatsioside A treatment markedly inhibited the growth of U87MG cells in a concentration-dependent manner; in addition, a time-dependent inhibition in cell viability was observed at 80 nM. Fatsioside A treatment that at 24 h. (Fig. 1A). Furthermore, Annexin V-FITC/PI double staining was applied to examine whether the Fatsioside A-induced reduction of the viability of glioma cells occurred via apoptosis. The flow cytometry results showed a marked concentration-dependent increase in the apoptotic rate of cells treated with 40 nM (apoptosis rate, 6.7%) and 80 nM (apoptosis rate, 11.9%) Fatsioside A compared with the control cells, which were not treated with Fatsioside A (apoptosis rate, 3.4%) (Fig. 1B and C).

ER stress-associated apoptosis is involved in Fatsioside A-induced cell death. A number of studies have demonstrated that ER stress-associated apoptosis may be involved in cell death

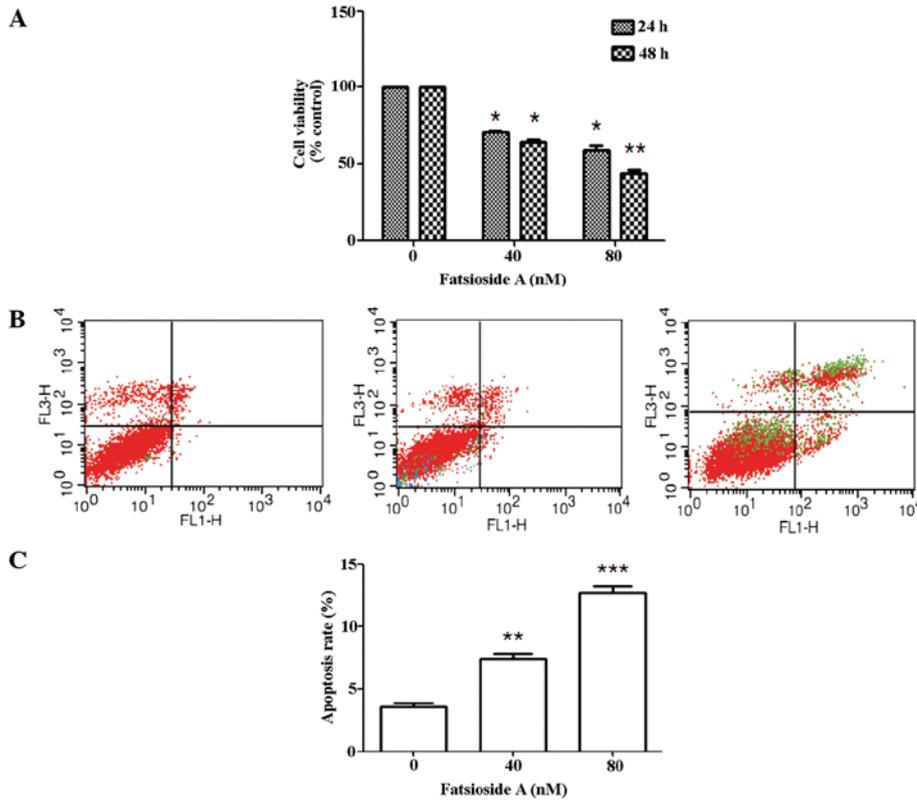


Figure 1. Fatsioside A inhibits growth and induces apoptosis of U87MG cells. U87MG cells were treated with various concentrations of Fatsioside A for 24 or 48 h. (A) Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as the mean \pm standard deviation (SD), n=3. (B) Apoptosis levels following a 24-h treatment with 0, 40 or 80 nM Fatsioside A was determined by Annexin V-fluorescein isothiocyanate/propidium iodide double staining and quantified using flow cytometry. Results are representative of three independent experiments. (C) The rate of apoptosis among different groups. Apoptosis rate was early apoptosis percentage plus late apoptosis percentage. The data are presented as the mean \pm SD. Columns, mean of three independent experiments; bars, SD. *P<0.05, **P<0.01 and ***P<0.001 versus untreated (0 nM) groups.

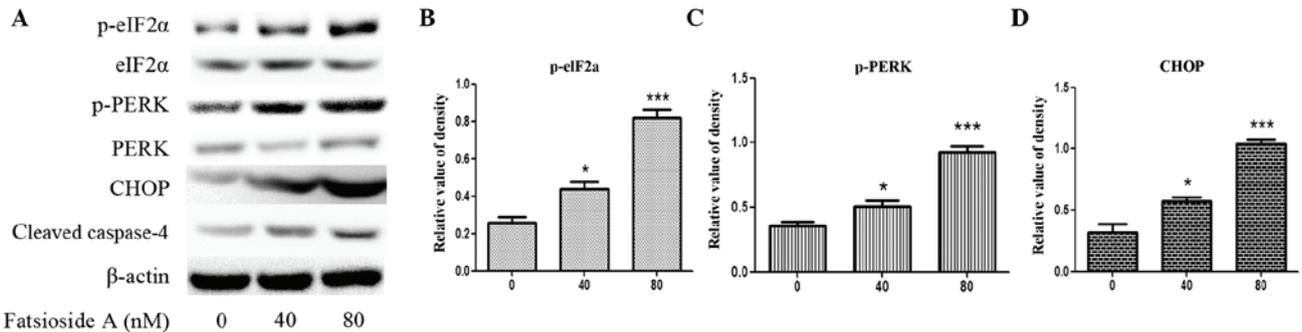


Figure 2. Endoplasmic reticulum (ER) stress-associated apoptosis is involved in Fatsioside A-induced cell death. U87MG cells were treated with various concentrations of Fatsioside A for 24 h. (A) The effect of Fatsioside A on the ER stress sensors p-eIF2 α , eIF2 α , p-PERK, PERK and CHOP, as well as cleavage of caspase-4 was determined by western blotting. Results are representative of three independent experiments. Quantification of (B) p-eIF2 α , (C) p-PERK and (D) CHOP band intensity measured against a β -actin protein loading control. Data are presented as the mean \pm standard deviation. Columns, mean of three independent experiments; bars, SD. *P<0.05 and ***P<0.001 versus the control group.

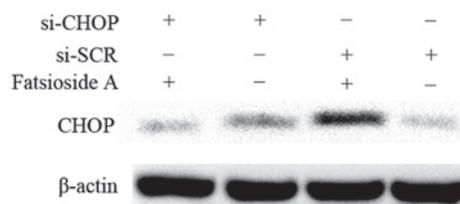


Figure 3. CHOP-specific siRNA inhibits the expression of CHOP induced by Fatsioside A treatment in U87MG cells. U87MG cells were transfected with CHOP-specific siRNA and scrambled (SCR) siRNA for 48 h. The cells were treated with Fatsioside A (80 nM) for 24 h. The expression of CHOP was determined by western blot analysis. Results are representative of three independent experiments.

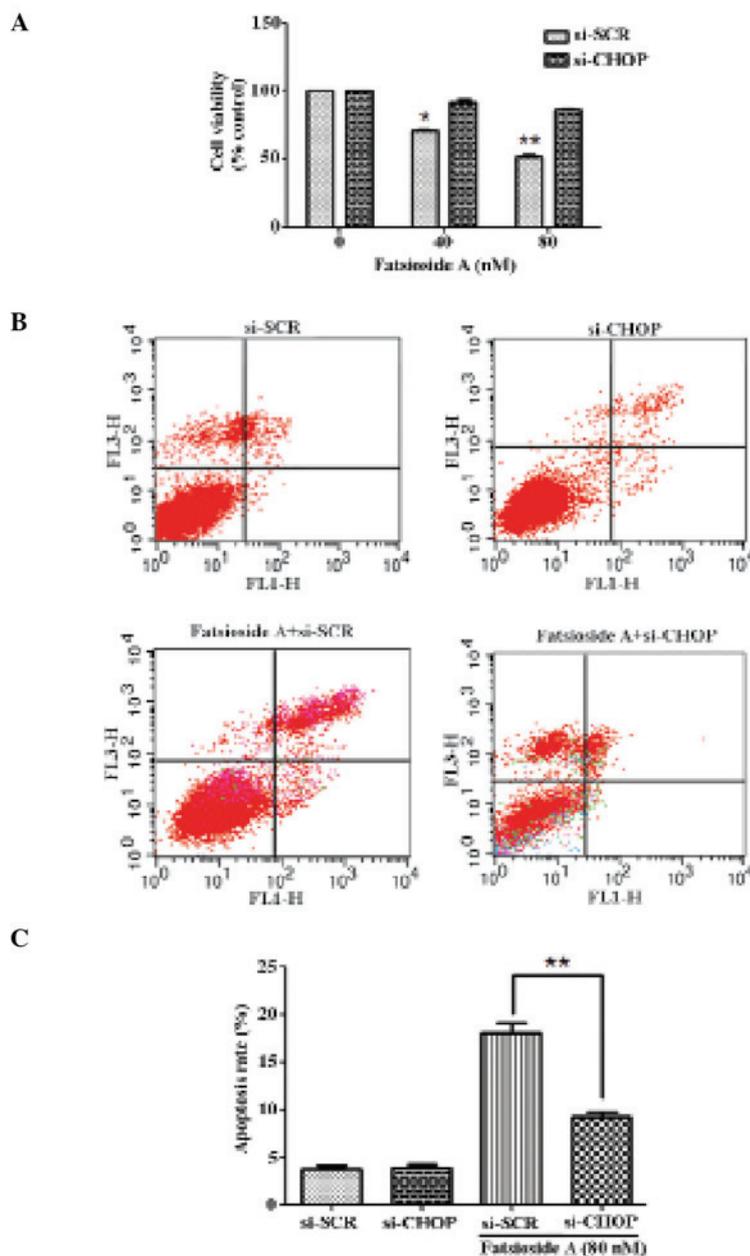


Figure 4. CHOP is a vital mediator in endoplasmic reticulum (ER) stress-mediated apoptosis induced by Fatsioside A. U87MG cells were transfected with CHOP-specific siRNA and scrambled (SCR) siRNA for 48 h and the cells were treated with Fatsioside A (80 nM) for 24 h. (A) Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are presented as the mean \pm standard deviation (SD), $n=3$. (B) Apoptosis was determined by Annexin V-fluorescein isothiocyanate/propidium iodide double staining and quantified using flow cytometry. Results are representative of three independent experiments. (C) The rate of apoptosis among different groups. Apoptosis rate was early apoptosis percentage plus late apoptosis percentage. The data are presented as mean \pm SD. Columns, mean of three independent experiments; bars, SD. * $P<0.05$ and ** $P<0.01$ versus the control groups.

induced by anti-tumor drugs (20,21). Therefore, to determine whether Fatsioside A induces ER stress in U87MG cells, the cells were exposed to various concentrations of Fatsioside A for 24 h, and then western blot analysis was used to identify ER stress markers. The results revealed that Fatsioside A significantly increases the phosphorylation of eIF2 α and PERK as well as the expression levels of CHOP, all of which are ER stress markers, in a concentration-dependent manner (Fig. 2A-D). A number of studies have demonstrated that caspase-4, which is an ER-resident caspase, is activated during ER stress to complete the execution of ER stress-induced apoptosis. Western blot analysis showed that Fatsioside A

markedly accelerated the cleavage of caspase-4 in a concentration-dependent manner in U87MG cells (Fig. 2A). Altogether, these results indicate that ER stress-associated apoptosis is involved in Fatsioside A-induced cell death in U87MG cells.

CHOP is a vital mediator in ER stress-mediated apoptosis induced by Fatsioside A. Previous studies have demonstrated that CHOP, as a major proapoptotic transcription factor induced during ER stress, is one of the main mediators in the apoptotic machinery (22-24). In order to confirm the role of ER stress in apoptosis induced by Fatsioside A in U87MG cells, CHOP-specific siRNA was applied to downregulate

the expression of CHOP. Western blot analysis revealed that CHOP-specific siRNA successfully downregulated the increased protein expression levels of CHOP induced by Fatsioside A (Fig. 3). The MTT assay showed that the knockdown of CHOP significantly reduced the cytotoxicity of Fatsioside A to U87MG cells (Fig. 4A). Following a 24-h exposure to 80 nM Fatsioside A, the average apoptotic percentage of U87MG cells was reduced from 17.6% in cells transfected with scrambled siRNA to 9.7% in cells transfected with CHOP-specific siRNA ($P < 0.05$) (Fig. 4B and C). These results indicate that CHOP is a vital mediator in ER stress-mediated apoptosis induced by Fatsioside A.

Discussion

Malignant gliomas are one of the most lethal types of tumor in humans and current anti-glioma therapies fail to obtain positive effects on these glioma cells (25,26). As the glioblastomas are resistant to apoptosis, patients with glioblastomas usually have a very poor prognosis (27-30). Thus, the development of novel therapies to target the inherent apoptosis-resistant phenotype of malignant gliomas is necessary.

The results of the present study revealed that Fatsioside A markedly inhibited the growth of U87MG cells and that this effect is exerted by inducing ER stress-mediated apoptosis. Furthermore, it was determined that CHOP is a vital mediator in the ER stress-mediated apoptosis induced by Fatsioside A.

In agreement with previous studies (18), the current study revealed that Fatsioside A significantly inhibits the proliferation of U87MG cells in a concentration- and time-dependent manner. One of the novel findings of the present study was the confirmation that Fatsioside A-induced reduction of the viability of glioma cells occurred via apoptosis, which was determined through the application of Annexin V-FITC/PI double staining. Apoptosis is a physiological phenomenon. The significance of apoptosis is to remove senescent and dysfunctional cells, such as activated T cells (31,32). Deregulation of apoptosis is associated with the pathogenesis of a number of disorders, including tumor cell growth (6,33,34). Thus, one of the predominant strategies to treat tumors is the induction of the apoptosis of tumor cells. The results of the current study indicate that Fatsioside A disturbs the inherent apoptosis-resistant ability of glioma cells.

Previous studies have demonstrated that ER stress is an important factor during tumorigenesis in response to oxidative stress, nutrient starvation and other metabolic dysregulations of cells (35,36). Numerous current anticancer therapies have been devised to induce ER stress in order to stimulate its pro-apoptotic function or block its pro-survival function. The results of the current study revealed that Fatsioside A induced ER stress in glioma cells, including increased phosphorylation of PERK and eIF2 α , in addition to increased expression levels of CHOP. Furthermore, the upregulation of CHOP accelerates the cleavage of caspase-4, thus leading to cell apoptosis (37). Downregulated CHOP protects cells from the lethal consequences of ER stress (38).

In conclusion, the current study determined that treatment with siRNA-targeting CHOP afforded U87MG cells an increased resistance to Fatsioside A-induced apoptosis. In summary, the results of the current study demonstrate the

ability of Fatsioside A to activate the key proteins of ER stress in addition to the ER-associated apoptotic proteins, CHOP and caspase-4. These data preliminarily indicate that Fatsioside A induces ER-mediated apoptosis in U87MG cells.

However, further studies are required to identify the underlying mechanisms of Fatsioside A in glioma therapy. For example, previous studies have shown that there is a crosstalk among the processes of apoptosis, autophagy and ER stress (39,40). Thus, further studies are required to determine whether autophagy is involved in ER-mediated apoptosis induced by Fatsioside A. Additionally, since ER stress is involved in pro-death and pro-survival mechanisms in glioma cells, further studies are required to define the optimal concentration of Fatsioside A to modulate ER stress for cancer treatment.

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