Saponin 6 derived from Anemone taipaiensis induces U87 human malignant glioblastoma cell apoptosis via regulation of Fas and Bcl-2 family proteins

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Abstract. Glioblastoma multiforme (GBM) is the most common and aggressive type of brain tumor, and is associated with a poor prognosis. Saponin 6, derived from Anemone taipaiensis, exerts potent cytotoxic effects against the human hepatocellular carcinoma HepG2 cell line and the human promyelocytic leukemia HL-60 cell line; however, the effects of saponin 6 on glioblastoma remain unknown. The present study aimed to evaluate the effects of saponin 6 on human U87 malignant glioblastoma (U87 MG) cells. The current study revealed that saponin 6 induced U87 MG cell death in a dose- and time-dependent manner, with a half maximal inhibitory concentration (IC50) value of 2.83 µM after treatment for 48 h. However, saponin 6 was needed to be used at a lesser potency in HT-22 cells, with an IC50 value of 6.24 µM. Cell apoptosis was assessed by flow cytometry using Annexin V-fluorescein isothiocyanate/propidium iodide double staining. DNA fragmentation and alterations in nuclear morphology were examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and transmission electron microscopy, respectively. The present study demonstrated that treatment with saponin 6 induced cell apoptosis in U87 MG cells, and resulted in DNA fragmentation and nuclear morphological alterations typical of apoptosis. In addition, flow cytometric analysis revealed that saponin 6 was able to induce cell cycle arrest. The present study also demonstrated that saponin 6-induced apoptosis of U87 MG cells was attributed to increases in the protein expression levels of Fas, Fas ligand, and cleaved caspase-3, -8 and -9, and decreases in the levels of B-cell lymphoma 2. The current study indicated that saponin 6 may exhibit selective cytotoxicity toward U87 MG cells by activating apoptosis via the extrinsic and intrinsic pathways. Therefore, saponin 6 derived from A. taipaiensis may possess therapeutic potential for the treatment of GBM.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain malignancy in adults. The prognosis of GBM is generally poor, with a median survival time of 14.6 months (1). GBM is currently treated with surgical resection, radiotherapy and adjuvant temozolomide (TMZ) chemotherapy. Although these treatment regimens have evolved over the years, the improvements have not translated into marked increases in patient survival. GBM is characterized by chemoresistance, and the clinical success of TMZ treatment is limited (2). Previous studies have demonstrated that >90% of recurrent gliomas do not respond to the second cycle of TMZ (3,4). In addition, TMZ may induce C>T/G>A transition mutations when DNA mismatch repair is deficient, which can potentially promote tumor progression (5,6). Therefore, there is an urgent requirement for the development of novel effective agents in the treatment of GBM.

Saponins, which are glycosides present in numerous plants, have been reported to exhibit marked cytotoxicity against various types of cancer (7,8). In particular, saponins derived from the rhizomes of Anemone taipaiensis (Ranunculaceae) exhibit potent cytotoxicity toward the human hepatocellular carcinoma cell line HepG2 and the human promyelocytic leukemia cell line HL-60 (9). Furthermore, our previous studies demonstrated that saponin 1 and saponin B, derived from A. taipaiensis, effectively inhibited brain tumor progression (10,11). Following these results, the present study aimed to determine the potential anti-glioma activities of other saponins derived from A. taipaiensis.
In the present study, the effects of A. taipaiensis-derived saponin 6 (3β-O-4-L-rhamnopyranosyl-(1→2)-4β-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl) oleanolic acid) on the viability and apoptosis of human U87 malignant glioblastoma (U87 MG) cells were evaluated. In addition, the molecular mechanisms underlying these effects were explored.

Materials and methods

Chemical reagents. Saponin 6 (>98% purity) was provided by Professor Tang (Institute of Materia Medica, School of Pharmacy, Fourth Military Medical University, Xi'an, China). The purity of saponin 6 was determined using a Dionex P680 high pressure liquid chromatography pump (Dionex; Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a UV 70 UV/Vis detector at 206 nm, and a YMC-Pack R&D ODS-A column (YMC Co., Ltd., Kyoto, Japan). Saponin 6 was dissolved in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Inc.) and diluted with water to generate the 111.6 µM stock solution, containing no more than 0.1% DMSO. The stock solution was stored in aliquots at -20°C. Prior to experimentation, the solution was thawed, sterilized by filtration through a 0.22-µm sterile filter (EMD Millipore, Billerica, MA, USA), and diluted in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) containing 0.1% DMSO. DMEM containing 0.1% DMSO was used as the vehicle control in subsequent experiments.

Cell culture. The human U87 MG cell line and the murine HT-22 hippocampal neuronal cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO2.

Cell viability. Cell viability was determined using the Cell Counting kit 8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) (12,13). Briefly, U87 MG and HT-22 cells were seeded in 96-well plates (2x104 cells/well) and incubated overnight. Subsequently, the cells were treated with saponin 6 at various concentrations (vehicle control, 1.79, 2.83 or 5.66 µM saponin 6 for 24 h). Following the treatment, the cells were harvested by trypsinization, washed with cold PBS, and double-stained with Annexin V-FITC and PI (Immunootech S.A., Marseilles, France) at room temperature for 15 min. The cells were then immediately subjected to flow cytometric analysis using a FACSCalibur™ system (BD Biosciences, Franklin Lakes, NJ, USA). Data were processed using ModFit software version 3.0 (Verity Software House, Topsham, ME, USA) and presented in a quadrantal diagram.

Ultrastructural studies by transmission electron microcopy (TEM). U87 MG cells were seeded in culture dishes (2x105 cells/ml) and incubated overnight. Subsequently, the cells were treated with vehicle only, 2.83 or 5.66 µM saponin 6 for 24 h. The cells were harvested by trypsinization and fixed with 2.5% glutaraldehyde (PBS, pH 7.4) at 4°C overnight. The cells were then post-fixed in 1% buffered osmium tetroxide at room temperature for 2 h, dehydrated through a graded series of ethanol solutions, and embedded in Poly/Bed (Polysciences, Warrington, PA, USA) for 24 h at 60°C. Ultrathin sections (70-90 nm) were cut on an ultramicrotome and double-stained with uranyl acetate and lead citrate. The cell TEM micrographs were acquired using a JEM-2000EX electron microscope (JEOL, Ltd., Tokyo, Japan).

Cell cycle analysis. U87 MG cells were seeded in 6-well plates (2x105 cells/ml) and incubated overnight. Subsequently, the cells were treated with vehicle only, 2.83 µM or 5.66 µM saponin 6 for 24 h. Following the treatment, the cells were collected by trypsinization, washed in cold PBS, and fixed in 75% ethanol at 4°C overnight. The fixed cells were subsequently washed with cold PBS and incubated with 50 µg/ml PI solution containing 50 µg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min in the dark. Cell cycle analysis was performed using a flow cytometer (FACSCalibur™; BD Biosciences) and ModFit software (Verity Software House).

Western blot analysis. U87 MG cells were treated with vehicle only, 2.83 µM or 5.66 µM saponin 6 for 24 h as previously described. The cells were then lysed in radiolabeled precipitation assay buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCL, pH 8.0] supplemented with a complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) at 4°C for 30 min. The cell lysates were centrifuged at 15,000 x g for 15 min, and the supernatants were collected. The protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein samples (25 µg) were then separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes.
(EMD Millipore). Subsequent to blocking the membranes in 5% nonfat dry milk containing 0.1% Tween-20 (Sigma-Aldrich) for 2 h, the membranes were incubated with polyclonal rabbit anti-Fas (1:500; cat. no. BS1461; Bioworld Technology, Inc., St. Louis Park, MN, USA), polyclonal rabbit anti-Fas ligand (FasL; 1:500; cat. no. BS1122; Bioworld Technology, Inc.), polyclonal rabbit anti-caspase-3 (1:1000; cat. no. ab90437; Abcam, Cambridge, MA, USA), monoclonal rabbit anti-caspase-8 (1:800; cat. no. 9496; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-caspase-9 (1:500; cat. no. BS1388; Bioworld Technology, Inc.), polyclonal rabbit anti-B-cell lymphoma 2 (Bcl-2; 1:500; cat. no. BS1511; Bioworld Technology, Inc.), polyclonal rabbit anti-Bcl-2-associated X protein (Bax; 1:500; cat. no. BS2538; Bioworld Technology, Inc.), and mouse monoclonal anti-β-actin (1:100; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies at 4°C overnight. The membranes were subsequently washed three times with 0.1% Tween-20 in PBS (10 min each wash) and incubated with the secondary antibodies bovine anti-mouse IgG-horse-radish peroxidase-conjugated (HRP) (cat. no. sc-2371) and bovine anti-rabbit IgG-HRP (cat. no. sc-2370) (both 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Uppsala, Sweden). Data were normalized to β-actin using ImageJ version 1.47 software (imagej.nih.gov/ij/).

Statistical analysis. All data are presented as the mean ± standard deviation calculated from at least three independent experiments. Data were analyzed using SPSS 20.0 (IBM SPSS, Armonk, NY, USA) or GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) software. Differences between groups were analyzed using Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Saponin 6 exerts cytotoxic effects against U87 MG cells. The chemical structure and high-performance liquid chromatography (HPLC) chromatograms of saponin 6 are presented in Fig. 2A. The percentage of cells at S phase in a concentration-dependent manner (Fig. 2B and C). In particular, ~70% of cells underwent apoptosis (early or late stage) following a 24 h treatment with 5.66 µM saponin 6 (Fig. 2D). Furthermore, Hoechst 33342 staining revealed the presence of pyknotic nuclei and apoptotic bodies in TUNEL-positive cells (Fig. 2E), further confirming the activation of apoptosis. Although treatment with saponin 6 also increased cell necrosis, this accounted for only a small fraction of cell death induced by saponin 6 (Fig. 2B and C). These results indicate that saponin 6 may induce U87 MG cell death, predominantly via the promotion of cell apoptosis.

Saponin 6 induces G1/G0 cell cycle arrest in U87 MG cells. To fully characterize the effects of saponin 6, cell cycle progression of the U87 MG cells was determined by flow cytometry using PI staining. Treatment with saponin 6 significantly increased the percentage of cells at G1/G0 phase, and decreased the percentage of cells at S phase in a concentration-dependent manner (Fig. 3). These results indicate that saponin 6 may induce G1/G0 arrest in U87 MG cells.

Saponin 6 activates both extrinsic and intrinsic apoptotic pathways in U87 MG cells. To explore the signaling pathways underlying saponin 6-induced cell apoptosis in U87 MG cells, the effects of saponin 6 were determined on the protein expression levels of: The Fas death receptor and its ligand FasL; proteins involved in the extrinsic (death receptor) apoptotic pathway; Bcl-2 family proteins, including Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic); and key regulators of the intrinsic (mitochondrial) apoptotic pathway. Treatment with saponin 6 for 24 h significantly increased the expression levels of Fas and FasL, and decreased Bcl-2 expression in a dose-dependent manner; however, there were no significant effects on Bax expression (Fig. 4A). These results suggest that saponin 6 may induce U87 MG cell apoptosis via activation of both the extrinsic and intrinsic apoptotic pathways. In addition, the protein expression levels of cleaved caspase-9 (an initiator caspase in the intrinsic pathway), cleaved caspase-8 (an initiator caspase in the extrinsic pathway), and cleaved caspase-3 (an effector caspase in both the extrinsic and intrinsic pathways) were detected. Treatment with saponin 6 significantly increased the expression levels of cleaved caspase-8, -9 and -3 in a concentration-dependent manner (Fig. 4B-C). These results are in agreement with the conclusion that saponin 6 may activate both the extrinsic and intrinsic apoptotic pathways in U87 MG cells.

Discussion

Saponins are natural glycosides that consist of a triterpene or steroid aglycone, with one or more sugar chains. Although these are primarily found in plants, saponins are also produced...
by certain marine organisms (14). Due to the great vari-
ability of their structures, saponins exert diverse biological
functions, including anticancer (15), anti-inflammatory (16),
antidiabetic (17), and cardioprotective (18) properties. In
particular, numerous saponins have recently been reported to
inhibit glioma cell proliferation in vitro (19-22) and suppress
glioblastoma progression in vivo (21). Our previous studies
demonstrated that saponin B and saponin 1 derived from
A. taipaiensis exhibited significant in vitro and in vivo anti-
glioma activity (10,11). In addition, our previous preliminary
structure-activity relationship studies demonstrated that the
cytotoxicity of these A. taipaiensis-derived saponins is mark-
edly affected by the length of their oligosaccharide chain at
C-3. The greatest potency was observed in compounds with a
chain of intermediate length (15). Therefore, the present study
hypothesized that saponin 6 derived from A. taipaiensis, which
has an oligosaccharide chain of intermediate length, may
possess potent antiglioma activity. The results of the present
study demonstrated that saponin 6 induced U87 MG cell death
in a concentration- and time-dependent manner. Furthermore,
saponin 6 had an IC_{50} value of 2.83 µM following a 48 h
treatment. Although saponin 6 also exerted cytotoxic effects
against noncancerous HT-22 hippocampal neuronal cells, its
potency toward HT-22 cells was much lower than that toward
U87 MG cells, thus indicating differential cytotoxicity against
cancer cells. Future studies on the in vivo efficacy and safety of
saponin 6 for the treatment of GBM are warranted.

Dysregulated cell apoptosis may lead to malignant trans-
formation, tumor progression and resistance to anticancer
drugs (23). Molecules targeting apoptotic pathways are
being actively developed for the treatment of cancer (24).
To further investigate saponin 6-induced U87 MG cell
death, cell apoptosis was assessed by flow cytometry using
Annexin V-FITC/PI double staining. The results revealed that
saponin 6 significantly induced U87 MG cell apoptosis in a
dose-dependent manner. In addition, the majority of apoptotic

Figure 1. Saponin 6 reduces human U87 MG malignant glioblastoma cell viability. (A) Chemical structure of saponin 6 derived from Anemone taipaiensis.
(B and C) Saponin 6 in different mobile phases was separated on a YMC Pack R&D ODS-A column (4.6x250 mm; YMC Co., Ltd., Japan) using a Dionex
P680 liquid chromatography system equipped with a UV170 UV/Vis detector. (D) U87 MG and (E) HT-22 murine hippocampal neuronal cells were treated
with saponin 6 at the indicated concentrations for 24 or 48 h. Cell viability was determined using the Cell Counting kit-8 assay. Data are presented as the
mean ± standard deviation; n=4. *P<0.01 vs. the vehicle control group in the 24-h treatment experiment; **P<0.01 vs. the vehicle control group in the 48-h treat-
ment experiment. MW, molecular weight.
cells were in the early stage of apoptosis following treatment with saponin 6 for 24 h. A TUNEL assay and ultrastructural TEM study of saponin 6-treated cells detected DNA fragmentation and other nuclear morphological changes typical of apoptosis, including condensed, fractured and marginalized chromatin. Furthermore, Hoechst 33342 staining revealed the presence of pyknotic nuclei and apoptotic bodies in TUNEL-positive cells. These results provide evidence to suggest that saponin 6 may induce apoptosis in U87 MG cells. In addition, increased levels of cell necrosis were detected following saponin 6 treatment; however, this accounted for only a very small fraction of cell death caused by saponin 6. Therefore, saponin 6-induced U87 MG cell death was predominantly attributed to apoptosis. Furthermore, cell cycle distribution was analyzed by flow cytometry, which revealed that saponin 6 induced cell cycle arrest at G0/G1 phase, suggesting that saponin 6 may inhibit DNA synthesis in U87 MG cells.

Figure 2. Saponin 6 causes DNA fragmentation, alters cell morphology, and induces apoptosis in human U87 MG malignant glioblastoma cells. Cells were treated with vehicle control, 2.83 µM or 5.66 µM saponin 6 for 24 h. (A) DNA fragmentation and subcellular morphological alterations were examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and transmission electron microscopy, respectively. Annexin V-FITC/PI (B) quadrant and (C) bar diagrams. (D) Cell apoptosis was determined by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining. (E) Fluorescence imaging with Hoechst 33342 staining. The red arrows indicate nuclear morphological changes characteristic of apoptosis, including chromatin condensation, boundary aggregation and splitting, and DNA fragmentation. B3, normal cells; B4, early apoptotic cells; B2, late apoptotic cells; B1, necrotic cells. Data are presented as the mean ± standard deviation; n=3. *P<0.05 and **P<0.01 vs. the vehicle control group.
Apoptosis can be triggered by extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The extrinsic apoptotic pathway is initiated by death ligand binding to death receptors, such as tumor necrosis factor receptor 1 and Fas (25). The intrinsic apoptotic pathway, which is characterized by permeabilization of the mitochondria, release of cytochrome c into
the cytoplasm and activation of caspases, is under the tight regulation of Bcl-2 family proteins (26). Fas is expressed in the majority of glioma tissues, but not in normal brain tissues (25). It has previously been reported that the Fas pathway has a pivotal role in the tumorigenesis and progression of glioma (27). To investigate the apoptotic pathways involved in saponin 6-induced U87 MG cell apoptosis, the protein expression levels of Fas and its ligand FasL, and the Bcl-2 family proteins Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) were detected by western blot analysis. The results demonstrated that the protein expression levels of Fas and FasL were significantly increased following saponin 6 treatment. Furthermore, the protein expression levels of Bcl-2 were decreased following treatment with saponin 6, whereas the protein expression levels of Bax remained similar. These results suggested that both the extrinsic and intrinsic pathways may be involved in saponin 6-induced U87 MG cell apoptosis. In addition, the present study examined the activation of caspase-8 and -9, the initiator caspases for the extrinsic and intrinsic pathways, respectively, and caspase-3, an effector caspase involved in both pathways (28). The results demonstrated that the expression levels of cleaved caspase-8, -9 and -3 were significantly increased following treatment with saponin 6. These results provide further evidence to suggest that saponin 6 is able to induce U87 MG cell apoptosis via both the extrinsic and intrinsic apoptotic pathways.

In conclusion, the present study demonstrated that saponin 6 derived from *A. taipaiensis* induced apoptosis of human glioblastoma U87 MG cells by inducing caspase activation via activation of both the intrinsic and extrinsic apoptotic pathways. Therefore, saponin 6 may have therapeutic potential for the treatment of GBM, and future studies regarding the in vivo efficacy and safety of saponin 6 are warranted.

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References


