

Saikosaponin D inhibits proliferation of human osteosarcoma cells via the p53 signaling pathway

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Abstract. Saikosaponin D (SSd), the major monomeric terpenoid extracted from *Radix bupleuri*, a traditional Chinese medicinal herb, exerts various pharmacological properties, including antitumor, anti-inflammatory and antiviral. The present study aimed to investigate the role of SSd in human osteosarcoma (OS) cell growth. In the investigation MTS and EdU assays were applied and flow cytometric analyses of cell cycle and apoptosis were performed. Western blotting and reverse transcription-quantitative polymerase chain reaction analyses were used to explore the underlying mechanisms of SSd on cell cycle transition and p53 signaling. Here, it was demonstrated that SSd administration at 80 $\mu\text{mol/l}$ significantly inhibited 143B and MG-63 proliferation. Furthermore, SSd significantly increased the percentage of 143B and MG-63 cells in G₀-G₁ phase and the number of apoptosis cells compared with the control group. Data further demonstrated that SSd treatment upregulated mRNA and protein levels of tumor protein 53 (p53) and its downstream targets, including p21, p27, B-cell lymphoma-2-like protein 4 and cleaved caspase-3, and downregulated mRNA and protein levels of cyclinD1. The results suggested that SSd was a functional tumor suppressor and inhibited OS proliferation via activation of the p53 signaling pathway and may be used in the treatment of osteosarcoma in future.

Introduction

Osteosarcoma (OS) is characterized by the direct formation of immature bone or osteoid tissue by malignant cells that occurs in children and adults (1). According to an estimate by the National Cancer Institute in 2017, OS accounted for 0.2% (3,260)

of all new cancer cases and 0.3% (1,550) of cancer worldwide mortality (2). Despite advance in diagnosis and treatment of OS, including magnetic resonance imaging, neoadjuvant chemotherapy and surgery, the overall survival rate remains poor (<30%) due to the invasiveness and distant metastasis largely affecting the lung (3,4). Therefore, novel effective and reliable methods are required in the treatment of OS.

Saikosaponin D (SSd) is one of the main bioactive ingredients extracted and purified from *Radix bupleuri* L, which is prescribed in traditional Chinese medicine for inflammatory, infectious and vascular diseases or as a hepatoprotective, antibacterial or antiviral (3,4). Previous research reported that SSd exhibits significant antitumor activities in cancer cells, including breast, lung, ovarian and pancreatic cancer and certain types of OS (5-12). SSd induces apoptosis in DU145 pancreatic cancer cells via intrinsic apoptotic pathways and sensitizes cancer cells to cisplatin through ROS-mediated apoptosis, which in combination with further saikosaponins may be an effective therapeutic strategy. Furthermore, SSd inhibited hepatocellular carcinoma (HCC) development and downregulated syndecan-2, matrix metalloproteinase (MMP)-2, MMP-13 and tissue inhibitor of metalloproteinase-2 expression in HCC liver tissue, suggesting SSd is a potential candidate in antitumor treatment in OS.

In previous studies, the potential antitumor mechanism of SSd influencing apoptosis and autophagic cell death was demonstrated (7-9,13). However, biological functions and associated molecular mechanisms of SSd in OS have not yet been elucidated. In the present research, tumor-suppressive functions and mechanisms of SSd in OS were evaluated in order to test the potential of using SSd as an antitumor drug in OS.

Materials and methods

Cell culture and chemicals. Human OS cells lines (143B and MG-63) were purchased from the American type culture collection (Manassas, VA, USA). Cell lines were cultured in RPMI-1640 containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO₂ humidified atmosphere, as previously described (14). SSd (purity, >95%; authenticated by Shanghai Academy of Life Sciences, Shanghai, China) was mixed with RPMI-1640 medium and stored at room temperature.

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Nucleic acid extraction. 143B and MG-63 cells (1×10^4 cells/well) were cultured in 6-well plates in RPMI-1640 medium supplemented with 10% FBS for 24 h at room temperature. The medium was replaced with RPMI-1640 supplemented with 10% FBS containing 80 $\mu\text{mol/l}$ SSd and cells were cultured for 48 h at 37°C prior to harvesting. RNA was extracted as reported previously (15). Total RNA was isolated from the cells using TRIzol reagent (Thermo Fisher Scientific, Inc.). Isolated RNA samples were quantified using spectrophotometry and stored at -80°C until further use. RNA concentrations were detected by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and 2 μl samples were separated using gel electrophoresis on a 5% agarose gel to examine the purity of the RNA as reported previously (15,16).

MTS assay. Cell viability was assessed by MTS assay (Beyotime Institute of Biotechnology, Haimen, China). Cells (143B and MG-63) were cultured in 96-well plates (2,000 cells/well) RMPI-1640 supplemented with 10% FBS for 24 h at room temperature (14,17). Cells were incubated with RMPI-1640 plus 10% FBS and varying concentrations of SSd (20, 40, 60 and 80 $\mu\text{mol/l}$) for 24, 48 and 72 h at room temperature. Following, 20 μl MTS solution was added to each well and samples were incubated at room temperature for 3 h prior to absorbance measurements at 450 nm using a microplate reader. Each experiment was repeated three times.

EdU assay. EdU assays were performed using the Click-iT EdU Imaging kit (Promega Corporation, Madison, WI, USA) and an apollo fluorescent dye (Guangzhou RiboBio Co., Ltd., Guangzhou, China) in accordance with the manufacturer's instructions. 143B and MG-63 cells (3×10^3 cells/well) were incubated in RMPI-1640 medium plus 10% FBS with 80 $\mu\text{mol/l}$ SSd for 24 and 48 h at room temperature. Cells were exposed to 100 μl EdU at 37°C for 3 h and fixed at room temperature for 20 min with 4% paraformaldehyde. The number of EdU-positive and total cells was counted using a fluorescence microscope (magnification in blue light, x20) with four non-overlapping fields per coverslip. Each experiment was repeated three times.

Flow cytometry analysis of cell cycle and apoptosis. Cell cycle and apoptosis were determined by flow cytometry as previously described (18). For cell cycle assays, cells were plated in six-well plates at 1×10^5 cells/well and cultured overnight at room temperature. RMPI-1640 medium plus 10% FBS with 80 $\mu\text{mol/l}$ SSd were added to the plates for 48 h and cells were collected, digested and centrifuged (800 x g at room temperature for 10 min). Following, cells were incubated with ice-cold 70% ethanol at room temperature for 24 h and treated with propidium iodide (PI) for 30 min at 4°C in the dark. Annexin V-fluorescein isothiocyanate (FITC)/PI staining was performed for apoptosis analysis following the manufacturer's guidelines (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 4°C in the dark. Samples were analyzed using a flow cytometer. Data were analyzed using CellQuest 16.0 software (BD Biosciences). Each experiment was repeated three times. Apoptosis

assays were performed using the described procedure and staining was based on FITC-Annexin V and PI using the FITC-Annexin V Apoptosis Detection kit (BD Biosciences) according to manufacturer's instructions. Experiments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RNA samples extracted from control and SSd-treated cells were reverse transcribed using a Go-Taq DNA polymerase kit (Promega Corporation) as reported previously (18,19). RT was performed with the following protocol: 37°C for 15 min and 85°C for 5 sec. β -actin functioned as the internal control. RT-qPCR reactions were performed using the HT7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and qPCR was performed using the SYBR-Green PCR Master mix (Thermo Fisher Scientific, Inc.). The PCR program began with an initial denaturation at 95°C for 2 min, followed by 40 amplification reaction cycles (95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec), with a final extension at 72°C for 3 min. Primer sequences are reported in Table I. Each sample was tested in triplicate and relative amounts of mRNA were determined using the $2^{-\Delta\Delta Ct}$ method (20).

Western blot analysis. Proteins from cells cultured in RMPI-1640 medium plus 10% FBS with 80 $\mu\text{mol/l}$ SSd were collected using RIPA buffer containing fresh protease and phosphatase inhibitor cocktails (all EMD Millipore, Billerica, MA, USA). To determine the concentration of proteins, a BCA protein kit (Thermo Fisher Scientific, Inc.) was used. Extracted proteins (50 $\mu\text{g/lane}$) were separated as previously described (21). Proteins were separated by SDS-PAGE on a 10% gel and transferred to polyvinylidene difluoride membranes (EMD Millipore). Aliquoted proteins were separated with 12% gels using SDS-PAGE and then transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk at room temperature for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. The following antibodies were included: Tumor protein 53 (p53; cat. no. ab131442); p21 (cat. no. ab188224); p27 (cat. no. ab190851); cyclinD1 (cat. no. ab137875); B-cell lymphoma (Bcl)-2-like protein 4 (Bax; cat. no. ab182733); cleaved caspase-3 (cat. no. ab198447; all 1:1,000); and β -actin (cat. no. ab8227; 1:2,000; all Abcam, Cambridge, UK) as a control. Membranes were then incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibodies (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. The bound antibodies were detected using enhanced chemiluminescence kit (EMD Millipore) and protein blots were visualized with the Las4000 Imaging system (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. SPSS 17.0 (SPSS, Inc. Chicago, IL, USA) was used for statistical analyses. All data are expressed as means \pm standard deviation. Differences between two samples were assessed by two-tailed Student's t-test and one-way analysis of variance followed by the Bonferroni post hoc test to compare between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Sequence (5'-3')	Fragment length (bp)
p53-F	TACTACTGCCAGGAGCCAGA	103
p53-R	TGGCACCAGTGTCCGGATTA	
p21-F	TGGAGACTCTCAGGGTTCGAAA	65
p21-R	GGCGTTTGGAGTGGTAGAAATC	
p27-F	CGTGCGAGTGTCTAACGG	206
p27-R	CGGATCAGTCTTTGGGTC	
CyclinD1-F	CTAGCAAGCTGCCGAACC	90
CyclinD1-R	TCCGAGCACAGGATGACC	
Bax-F	TCCACCAAGAAGCTGACGAG	275
Bax-R	GTCCAGCCCATGATGGTTCT	
Caspase-3-F	ATGGAGAACAATAAAACCCT	50
Caspase-3-R	CTAGTGATAAAAGTAGAGTTC	
β -actin-F	TCCTGTGGCATCCACGAAACT	315
β -actin-R	GAAGCATTTGCGGTGGACGAT	

P, tumor protein; Bax, B-cell lymphoma-2-like protein 4; F, forward; R, reverse.

Results

SSd inhibits cell proliferation. 143B and MG-63 cell viability and proliferation were tested by MTS and EdU assays. MTS assays assessed effects of varying concentration of SSd (20, 40, 60 and 80 μ mol/l) on cell viability and results demonstrated that SSd had no significant effects at 20-60 μ mol/l when compared with the control group ($P>0.05$; Fig. 1A). At 80 μ mol/l SSd significantly inhibited 143B and MG-63 cell viability following 24 and 48 h treatment compared with the control ($P<0.001$; Fig. 1A). EdU incorporation assays further suggested that SSd significantly inhibited cell proliferation at 80 μ mol/l compared with the control ($P<0.001$; Fig. 1B). In 143B and MG-63 lower doses of SSd (20, 40 and 60 μ mol/l) had no significant influence on proliferation following 24 or 48 h treatment compared with the control ($P>0.05$; Fig. 1B). Subsequent experiments were performed using 80 μ mol/l SSd as cell treatment.

SSd causes cell cycle arrest in G_0 - G_1 phase. Flow cytometric analyses of the cell cycle were used to evaluate the underlying mechanisms of proliferation suppression by SSd. It was observed that 80 μ mol/l SSd significantly increased the percentage of 143B and MG-63 cells in G_0 - G_1 phase by 18 and 23%, respectively ($P<0.001$; Fig. 2).

SSd induces apoptosis. A significant increase in apoptosis was observed compared with the control group following the treatment of 143B and MG-63 with 80 μ mol/l SSd for 48 h ($P<0.001$; Fig. 3), indicating that SSd may serve a vital role in the apoptosis of OS cells.

Effects of SSd on cell cycle and apoptosis are associated with p53 accumulation. While cell cycle arrest and apoptosis are known to be associated with the activation of the p53 signaling pathway, SSd has been demonstrated to lead to p53 accumulation in ovarian cancer cell lines, Hey and SKOV3 (9). A potential association of SSd and p53 was evaluated. p53 expression was assessed in 143B and MG-63 treated with 80 μ mol/l SSd. Western blot analysis revealed that SSd promoted p53 accumulation in OS cells. Furthermore, it was demonstrated that SSd treatment was associated with the upregulation of p53 downstream targets, including p21, p27, Bax, cleaved caspase-3 and the downregulation of cyclinD1 (Fig. 4A). RT-qPCR was performed to evaluate the mRNA expression of p53 signaling pathway targets. In 143B and MG-63, SSd treatment (80 μ mol/l) significantly decreased mRNA levels of cyclinD1 and significantly increased mRNA levels of p21, p27, Bax and cleaved caspase-3 ($P<0.001$; Fig. 4B). Thus, SSd increased the expression of p53 in protein and mRNA levels in 143B and MG-63.

Discussion

OS is the leading cause of death among primary bone malignancy, which originates from bone mesenchymal cells (22). It occurs mainly in adolescents and children (~90% of patients with OS are <20 years of age) (22,23). The OS survival rate in the US was ~20%, and the five-year OS survival rates for children and adolescents in Europe were similar (22,23). Long-term survival rates of patients who underwent surgical resection of OS were ~20% worldwide (23). In recent years, cisplatin-based combination chemotherapy has improved the prognosis of patients with OS and the 5-year overall survival rate for non-metastasis accounts for 60-70% (24). Resistance to chemotherapy describes an important factor influencing the treatment outcomes in OS (23,25).

Over the past decades, traditional Chinese medicines, based on herbs and botanicals, have been applied in the treatment of osteoarthritis, diabetes disease and malaria, and clinical evaluation deemed the formulations as extremely safe, efficient and to exhibit lower toxicity (25). For example, artemisinin was used in the treatment of malaria (24) and Chinese medicines, such as rosmarinic, were used as diabetic retinopathy therapies (26). SSd regulates T lymphocyte function, prevents the progression proteinuria, modulates macrophage function and enhances nonspecific resistance against aeruginosa infection (7,27,28). The compound further possesses anti-inflammatory effects and causes cell death in human HCC cell lines (29). Other studies reported that SSd sensitizes tumor cells to cisplatin via ROS-mediated apoptosis and the combination of SSd with cisplatin may describe an effective therapeutic strategy (29). Wang *et al* (30) suggested that SSd potentiates effects of radiation in SMMC-7721 hepatocytes; thus, it may be a promising radiosensitizer further affecting the cell cycle (30). However, functions and underlying mechanisms of SSd in OS remain to be investigated.

The current study focused on antitumor activities of SSd. The results suggested that 143B and MG-63 incubated with 80 μ mol/l SSd exhibited significantly reduced cell viability when compared with the control group. In addition, DNA

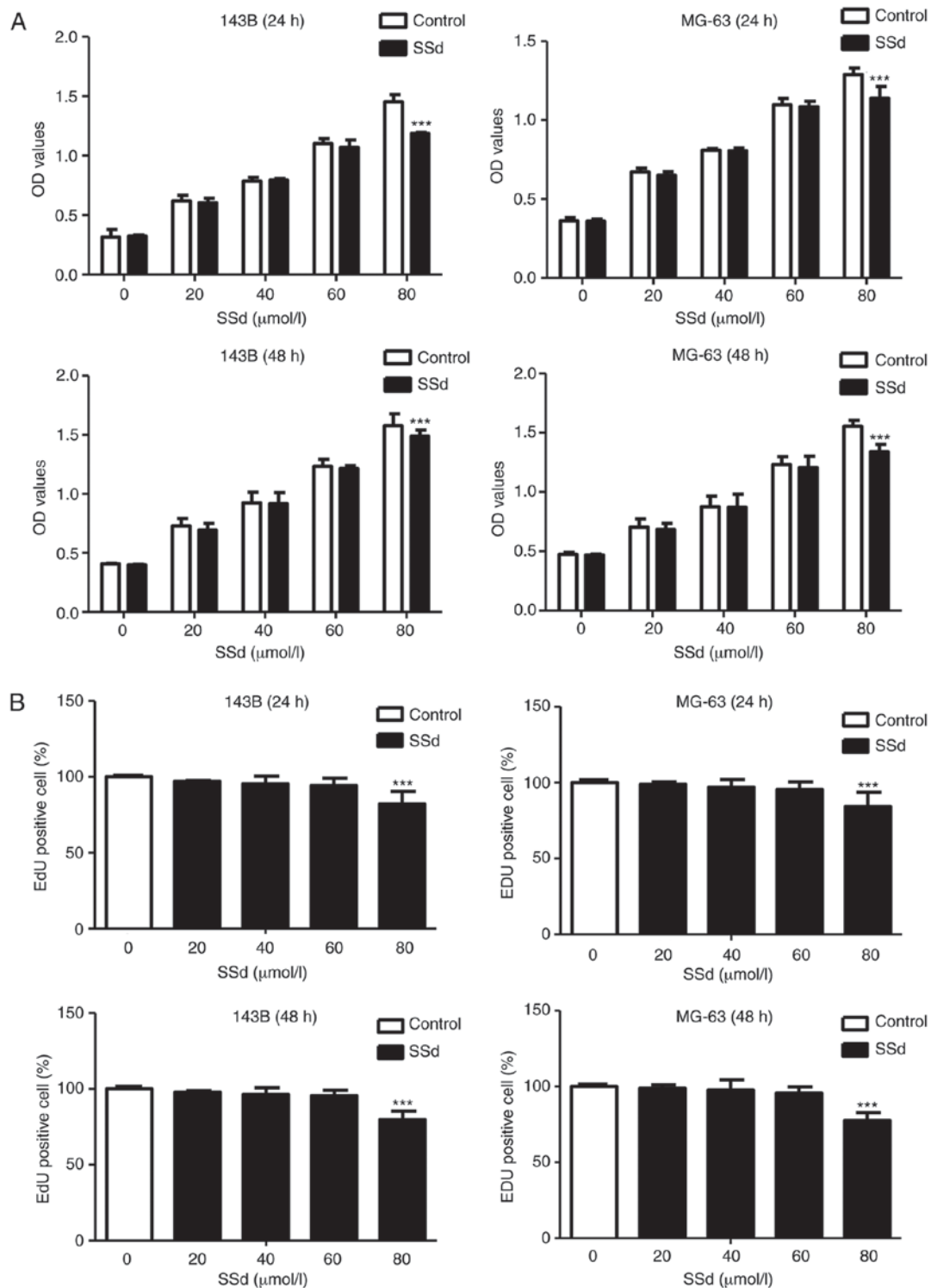


Figure 1. Inhibitory proliferation effects of SSd in 143B and MG-63. (A) MTS analysis was performed to detect proliferation at various SSd concentrations (20, 40, 60 and 80 $\mu\text{mol/l}$) following 24 and 48 h treatment at a wavelength of 450 nm. (B) EdU assays were performed to measure 143B and MG-63 viability at various SSd concentrations (20, 40, 60 and 80 $\mu\text{mol/l}$) following 24 and 48 h treatment. *** $P < 0.001$ vs. control. SSd, saikosaponin D; OD, optical density.

synthesis in 143B and MG-63 cells was significantly suppressed at the G_1 phase after incubation with high doses of SSd. It has been reported that SSd is associated with various anticancer functions, influencing cell proliferation, apoptosis and migration and invasion of various cancer types (7,27). The current study focused on antitumor activities of SSd in OS and it was demonstrated that SSd induced G_0/G_1 phase arrest and caused cells apoptosis, consistent with previous reports (30,31).

p53 serves a critical role in regulation of cell cycle checkpoints, DNA damage and the prevention of normal cell developing malignant phenotypes (32,33). Additionally, the tumor suppressor protein p53 is a vital component in the apoptotic pathway, and Bcl-2 and Bax are important transcriptional targets of p53. Bax, an anti-apoptotic protein, is essential for apoptosis and p53 can initiate cell death through activating target genes via the upregulation of Bax expression (31). Bcl-2

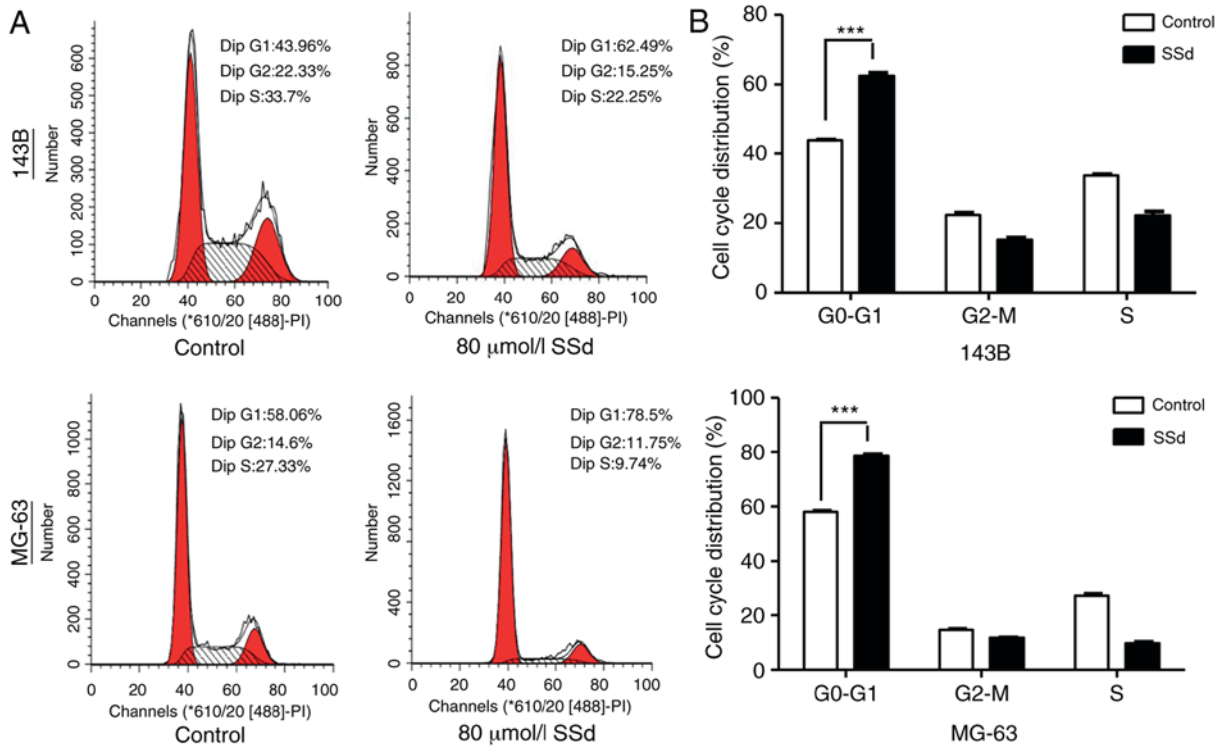


Figure 2. SSd induces cell cycle arrest at G₀-G₁ phase. Flow cytometry analysis of the cell cycle of 143B and MG-63 treated with 80 μmol/l SSq for 48 h. (A) Representative images of cell cycle distribution in 143B and MG-63 and (B) statistical analysis of the data. ***P<0.001 vs. control. SSd, saikosaponin D.

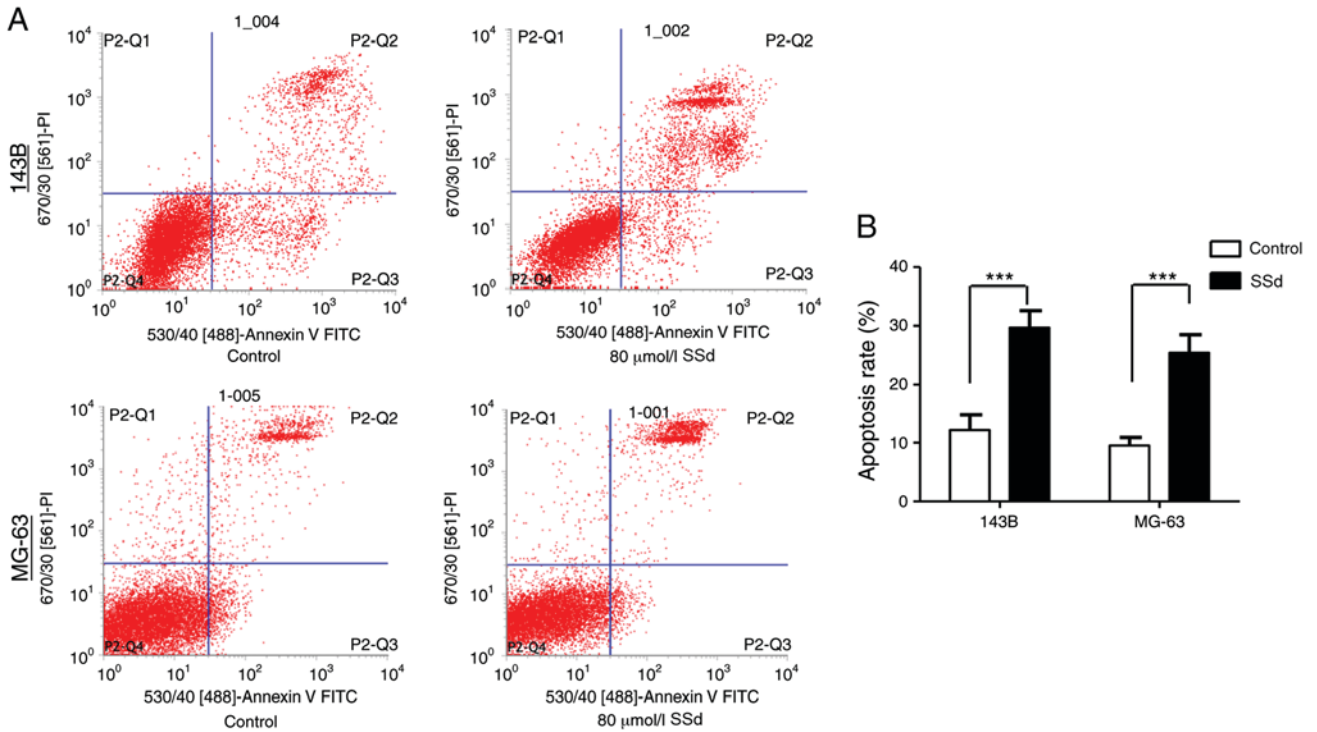


Figure 3. SSd induces apoptosis in osteosarcoma. Flow cytometry-based detection of apoptosis induced by SSd (80 μmol/l) treatment for 48 h. (A) Representative images of apoptosis analysis using Annexin V-FITC and PI double staining and (B) quantitative analysis of flow cytometry data. ***P<0.001. SSd, saikosaponin D; FITC, fluorescein isothiocyanate; PI, propidium iodide.

family proteins serve central roles in mitochondria-mediated apoptosis and are divided into three groups: BH3-only proteins, BAX/bcl-2 homologous antagonist/killer (BAK) proteins and pro-survival proteins (34). Activated BAX/BAK forms

homo-oligomers that permeabilize mitochondria and allow the release of pro-apoptotic factors, such as cytochrome c, which promote the activation of caspases (35,36). In the present study, SSd significantly increased transcription of p53 and its

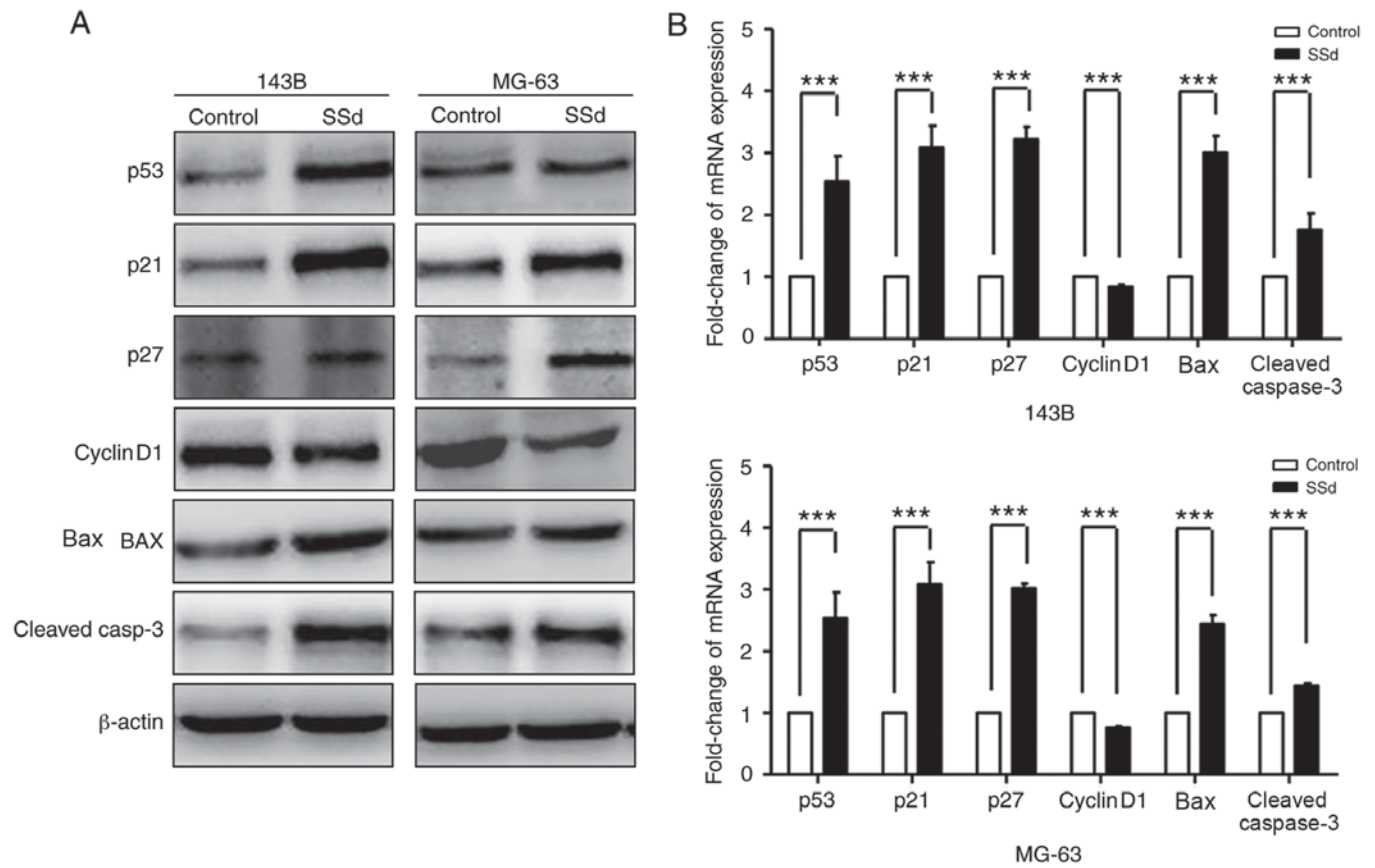


Figure 4. SSd affects p53 expression and downstream targets in 143B and MG-63. Cells were treated with SSd (80 $\mu\text{mol/l}$) for 48 h and (A) protein and (B) mRNA levels of p53, p21, p27, cyclinD1, Bax and cleaved-caspase-3 were determined by western blot and reverse transcription-quantitative polymerase chain reaction, respectively. *** $P < 0.001$. SSd, saikosaponin D; p, tumor protein; Bax, B-cell lymphoma-2-like protein 4.

downstream targets, including p21, p27 and Bax, and decreased cyclinD1 expression. CyclinD1 has been demonstrated to activate the G1-S transition of the cell cycle (35,36). Gumz *et al* (37) reported that cyclinD1 was up-regulated in clear cell RCC and that an antagonist of the Wnt signaling pathway, sFRP1, inhibited cyclinD1 expression. The results of the current study demonstrated that SSD had a similar effect on cyclinD1.

In conclusion, the current study describes the role of SSd in inhibiting cell growth and inducing apoptosis through activation of the p53 signaling pathway in OS. SSd may serve as a potential tumor inhibitor in OS. However, large-scale *in vivo* experiments may be needed to validate its mechanism.

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Availability of data and materials

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

Authors' contributions

LZ and JL conducted the nucleic acid extraction, MTS and EdU assays and western blot analyses, and wrote the manuscript. Z-BS, CS and Z-HY collected the data and performed the cell culture, flow cytometry and the reverse transcription-quantitative polymerase chain reaction analysis. XG designed the study. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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