Sodium cantharidinate suppresses human osteosarcoma MG-63 cell proliferation and induces cell cycle arrest by inhibition of PI3K/AKT activation

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Received June 8, 2018; Accepted October 30, 2018

DOI: 10.3892/or.2018.6906

Abstract. The function and mechanism of sodium cantharidininate (SC) underlying its suppression of human osteosarcoma (OS) MG-63 cells were investigated for the first time in the present study. MG-63 cell proliferation was determined by WST-1 assay post SC treatment at 0, 12, 24, 48 and 72 h. The results showed that SC effectively inhibited MG-63 cell proliferation and induced cell cycle arrest at the G0/G1 phase in a dose-dependent manner. Western blotting revealed that *SC* induced MG-63 cell cycle arrest at the G0/G1 phase by means of inhibition of cyclin D1, CDK4 and CDK6 expression. The expression of MAPK and AKT were evaluated using western blotting and FACS experiments to determine whether such signaling pathways are involved in the antiproliferative action of SC on MG-63 cells. SC significantly inhibited the phosphorylation of AKT, but not mTOR, JNK or P38. PI3K/AKT stimulator, IGF-1, reversed the SC-induced cell cycle arrest in the MG-63 cells. Collectively, our data indicate that the phosphorylation of AKT was inhibited by SC, consequently decreasing the expression of cyclin D1, CDK4 and CDK6 and inducing MG-63 cell G0/G1 phase arrest. SC has potential as a treatment agent for human osteosarcoma.

Introduction

Osteosarcoma (OS) is the most common type of bone cancer in children and adolescence. OS accounts for 2.4% of all cancer-related mortality in children and 20% of all primary bone cancers (1,2). Due to the lack of effective treatment options to date, 50-70% of osteosarcoma patients survive no >5 years (3). Osteosarcoma, which is derived from malignant mesenchymal stem cells, is believed to be a primary tumor of the bone (4). The tumor usually develops in the metaphyses of the long bones; thus, the proximal tibia, the proximal humerus and the distal femur are high risk areas for tumor development (5,6). At present, chemotherapeutics is the first choice of treatment along with surgical treatment. Yet, there are numerous side-effects associated with chemotherapeutic drugs, such as cisplatin, ifosfamide and high-dose methotrexate. Acquirement of drug resistance is the most serious problem in osteosarcoma patients following treatment with chemotherapeutic drugs (7). Therefore, the development of novel effective therapeutic agents with moderate side-effects for the treatment of osteosarcoma is urgent (8,9).

Natural or herbal medicines are often used as alternative forms of chemotherapy, due to low mortality and side effects (10). Currently, novel anticancer agents from natural products have become increasingly popular (11). In the history of Traditional Chinese Medicine (TCM), medicinal plants and their extracts have been used to treat various diseases. Accumulative data concerning TCM have shown remarkable activity in influencing the tumor cell death pathway, which can guide tumor treatment decisions and clinical management (12). Natural products from TCM, with unique and diverse chemical entities, are a considerable resource for developing novel medications. Sodium cantharidinate (SC) has powerful antitumor activity that has been confirmed in clinical practice in recent years (13). This compound directly inhibits multiple malignant tumors, and has low toxic/adverse effects to date (14). In recent years, researchers have confirmed through *in vitro* experiments that (SC) and its derivatives directly kill liver cancer cells (15). SC induces HepG2 cell apoptosis through the LC3 autophagy pathway, which has potential for the treatment of human hepatocellular carcinoma (HCC) (16). Yet, no study exists concerning SC activity in OS to date.

Cell cycle control is a major regulatory mechanism of cell proliferation. Therefore, inhibition of cancer cell growth is the most effective method for cancer treatment in the clinic (17). Cytotoxic agents and/or DNA damaging agents, which arrest the cell cycle at the G0/G1, S or G2/M phase, induce cancer cell

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Key words: sodium cantharidinate, MG-63 cells, cell cycle, *AKT*

apoptosis (18). The cyclin-dependent kinases (Cdks), highly conserved protein kinases, closely mediate the cell cycle (19). Cyclins form complexes with Cdks to activate Cdks to regulate the cell cycle. Cyclin D1, CDK4 and CDK6 are related closely to the G0/G1 phase, cyclin B1 and CDK1 are closely related to the G2/M phase, while cyclin A and CDK2 are closely related to the S phase (20). Mitogen-activated protein kinase (MAPK) and Akt pathways play an important role in the antiproliferative actions in certain cells (21). Extracellular signal-regulated kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK) are main MAPK family members. Akt (also known as Akt1) may promote cell proliferation via phosphorylation, which acts as a mediator of growth factors (22). The present study aimed to investigate the antiproliferation effect of SC on the cell growth and cell cycle arrest of human OS MG-63 cells, to evaluate whether SC may be a potential antitumor agent for the treatment of this disease.

Materials and methods

Reagents. Sodium cantharidinate [(1R,2S,3R,4S)-rel-2, 3-dimethyl-7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic acid, disodium salt, SC] was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) (cat. no. 1465-77-6) (Fig. 1). Water soluble tetrazolium (WST-1) cell proliferation reagent was purchased from Roche (Shanghai, China). Antibodies used in FACS were all purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies used in western blotting were all purchased from [Cell Signaling Technology (CST), Inc., Danvers, MA, USA]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin, penicillin and streptomycin were obtained from Gibco-BRL Life Sciences/Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Preparation of SC. SC was dissolved in PBS (pH 7.2) to prepare a stock solution (1.0 mM) and was stored at -20˚C. Appropriate concentrations of SC were prepared by dilution with DMEM complete medium prior to use.

Cell cultivation and treatments. MG-63 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM. DMEM full cell culture medium was prepared and supplemented with 1% antibiotics (penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and 10% FBS (HyClone; GE Healthcare Life Sciences, Beijing, China). MG-63 cells were cultured at 37˚C with a constant air flow of 5% CO_2 in a humidified incubator.

Cell proliferation assay. MG-63 cells ($5x10^4$) were seeded in a 96-well plate and then treated without or with SC (0.2, 1.0 and 5.0 μM) for 24 h in the dose-dependent experiment. MG-63 cells were treated with SC (5.0 μM) for 12, 24, 48 or 72 h in the time-dependent experiment. WST-1 cell proliferation reagent was applied to determine the cell proliferation. Under the manufacturer's instructions, 20 μl WST-1 was initially added to 200 μl of MG-63 cell, and then incubated in the dark for 2 h in the original incubator. Subsequently, the absorbance at 450 and 630 nm were measured using a microplate reader

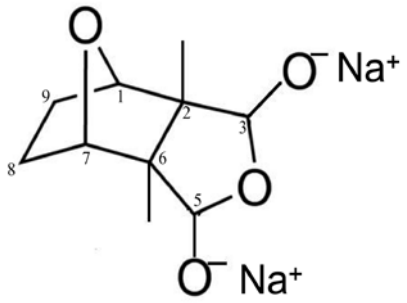

Figure 1. The chemical structure of sodium cantharidinate (SC).

(Bioteck, Beijing, China). Final the optical density (OD) was designated as OD_{450} - OD_{630} - OD_{blank}.

Cell cycle assay. MG-63 cells ($4x10^3$) treated with or without SC were collected after the appropriate time, and then washed with 1 ml cold PBS twice to remove the residual trypsin and serum. Cells were pelleted and re-suspended in 1 ml fixation solution (PBS:ethanol = 3:7). After incubation at 4˚C for 4 h, the cells were centrifuged at 300 x g for 5 min and fixation solution was removed. After washing twice with 1 ml PBS, the cells were pelleted and suspended in 0.5 ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) staining solution (50 μg/ml PI, 20 μg/ml RNase A and 0.2% Triton X-100) and incubated in the dark at 37˚C for 30 min. Cell suspensions were filtered through a 400-mesh sieve before being analyzed by a BD FACSCalibur flow cytometer (BD Biosciences, Sparks, MD, USA).

Western blotting. MG-63 cells treated without or with SC lysates were separated by SDS-PAGE under nonreducing conditions on a 10% polyacrylamide gel. The proteins were then transferred onto PVDF membranes by electroblotting. The membranes were blocked with blocking buffer overnight at 4˚C and then incubated with the cyclin A (dilution 1:2,000; cat. no. 4656), cyclin B (dilution 1:1,000; cat. no. 4138), cyclin D1 (dilution 1:1,000; cat. no. 2922), CDK1 (dilution 1:1,000; cat. no. 9868), CDK2 (dilution 1:1,000; cat. no. 78B2), CDK4 (dilution 1:1,000; cat. no. D9G3E), CDK6 (dilution 1:1,000; cat. no. D4S8S), AKT (dilution 1:1,000; cat. no. 2966), p-AKT (Ser-473, dilution 1:1,000; cat. no. 4060), mTOR (dilution 1:1,000; cat. no. 2972), p-mTOR (Ser-2448, dilution 1:1,000; cat. no. 5536), JNK (dilution 1:1,000; cat. no. 9252), p-JNK (Tyr-185, dilution 1:1,000; cat. no. 9251), P38 (dilution 1:1,000; cat. no. 8690), p-P38 (Thr180/Tyr182, dilution 1:1,000; cat. no. 9211) and β-actin (dilution 1:1,000; cat. no. 3700) antibodies for 1.5 h at room temperature. The membranes were then washed with TBS washing buffer [Tris-buffered saline with Tween-20 (0.1%)] six times and incubated with HRP-conjugated secondary antibodies for another 1 h. After washing, protein bands were visualized using an enhanced chemiluminescent system (Thermo Fisher Scientific, Inc., Shanghai, China). The primary antibodies used were all obtained from Cell Signalling Technology, Inc., (Danvers, MA, USA). Western blot images were quantified by optical density analysis. Protein expression levels were determined semi-quantitatively by densitometric analysis with

Quantity One software (v4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

FCM for cell signal detection. Approximately 2x10^6 MG-63 cells treated without or with SC were collected and washed with 1 ml cold PBS twice to remove the residual trypsin and serum. Cells were pelleted and resuspended in FACS tubes. Broken membrane buffer solution was used initially, and incubation was carried out for 30 min, followed by the addition of 1 ml washing buffer. The samples were then incubated with p-AKT (PE), p-JNK (APC), p-P38 (FITC) and p-mTOR (Percp) for another 15 min and immediately analyzed using a flow cytometer (FACScan; BD Biosciences) with Flowjo 7.6 FACS analysis software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. All data and results were calculated from at least three replicate measurements and are presented as the mean ± SD. Mean values were compared using paired t-tests (two groups) followed by the Bonferroni correction for multiple comparison tests. P-values <0.05 were considered significant. All statistical tests were performed with GraphPad Prism software (v5.0; GraphPad Software Inc., San Diego, CA, USA).

Results

SC inhibits cell growth in MG-63 cells. In order to investigate the function of SC on human OS MG-63 cells, we established three different doses of SC including 0.2, 1.0 and 5.0 μM. Cell proliferation was determined by WST-1 assay 24 h post-treatment. We found that 1.0 and 5.0 μM doses of SC significantly inhibited the growth of MG-63 cells when compared to the control group (Fig. 2A, P<0.05 and P<0.01, respectively). The 5.0 μM dose of SC had the most significant effect. We compared the cell growth in MG-63 cells at different time points following treatment with 5.0 μM of SC. Cell proliferation was determined by WST-1 assay at 0, 12, 24, 48 and 72 h post-treatment. Apparently, MG-63 cells showed a decelerated proliferation between 24 and 72 h after treatment with 5.0 μM of SC (Fig. 2B, P<0.05).

SC arrests the cell cycle at G0/G1 in MG-63 cells. Flow cytometry was utilized to analyze cell cycle distribution at 24 h post SC treatment. We found that after treatment with 5.0 μM SC, MG-63 cells showed a significant G0/G1 phase arrest compared to the control group (Fig. 3A). Cell percentages in the different phase analysis showed that the percentage of cells in the G0/G1 phase was increased from 42±2.5 to 63±3.5% in the SC treatment groups comparing to the control (Fig. 3B, P<0.05). At the same time, the percentage of cells in the S phase was significantly decreased (P<0.05), while the G2/M phase cell percentage did not change. This finding indicated that SC significantly induced MG-63 cell cycle arrest at the G0/G1 phase.

SC inhibits cyclin D1 expression in MG-63 cells. The cell cycle is precisely regulated by cyclins and kinases. Cyclin D1, CDK4 and CDK6 are closely related to the G0/G1 phase, cyclin B1 and CDK1 are closely related to the G2/M phase, while Cyclin A and CDK2 are closely related to the S phase.

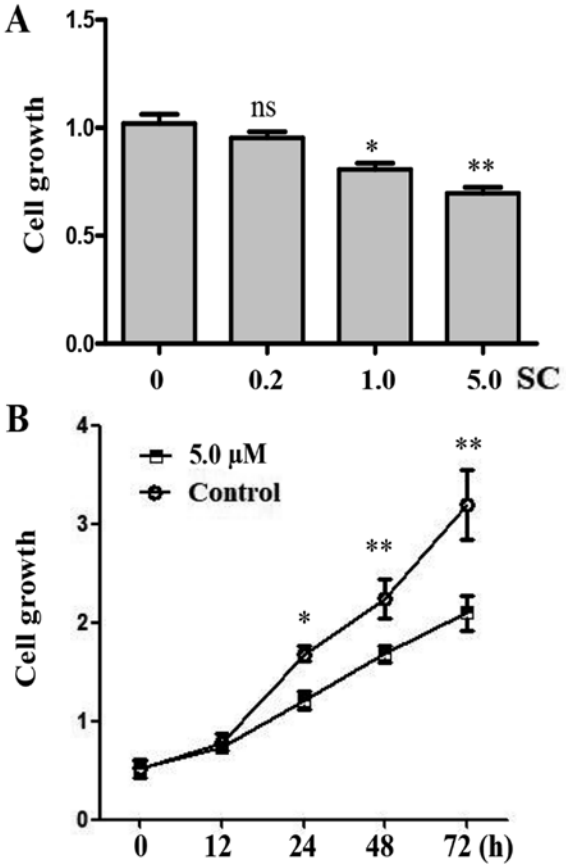

Figure 2. Rates of the inhibition of OS MG-63 cell growth following treatment with 0.2, 1.0 and 5.0 μM concentrations of SC. (A) MG-63 cells were treated with varying doses of SC. Cell growth was determined by WST-1 assay at 24 h post treatment. (B) Growth of MG-63 cells at 12, 24, 48, 72 h post treatment with 5.0 μM of SC. The growth of MG-63 cells after 5 μM of SC was recorded and cell proliferation at different time points was determined by WST-1 assay. The experiments were repeated at least three times. Data are expressed as the mean ± SD of the three experiments (*P<0.05, **P<0.01 vs. the control). OS, osteosarcoma; SC, sodium cantharidinate.

To examine whether SC exhibits functions on cyclins and kinases in the MG-63 cells, western blotting was performed to assess the levels of cyclin A, cyclin B, cyclin D1, CDK1, CDK2, CDK4 and CDK6. It was found that SC significantly inhibited cyclin D1 (Fig. 3A and B, P<0.05), CDK4 (Fig. 4A and B, P<0.05) and CDK6 (Fig. 4A and B, P<0.05) expression, consistent with the cell cycle distribution assay. In contrast, the expression of cyclin A, cyclin B, CDK1 and CDK2 did not show a significant difference between the SC treatment group and the control group (Fig. 4A and B, P>0.05).

PI3K/AKT pathway participates in the inhibition of MG-63 cell proliferation by SC. The expression of MAPK components and AKT was evaluated using western blotting and FACS experiments. As shown in Fig. 5A and B, SC significantly inhibited the phosphorylation of AKT (P<0.05), but not mTOR (P>0.05), JNK (P>0.05) or P38 (P>0.05). Fluorescent-labeled flow cytometry was also applied to test the MG-63 cell signaling pathway activation. The phosphorylation of AKT fluorescence intensity was significantly inhibited by SC (Fig. 5C, P<0.05), consistent with the western blot results.

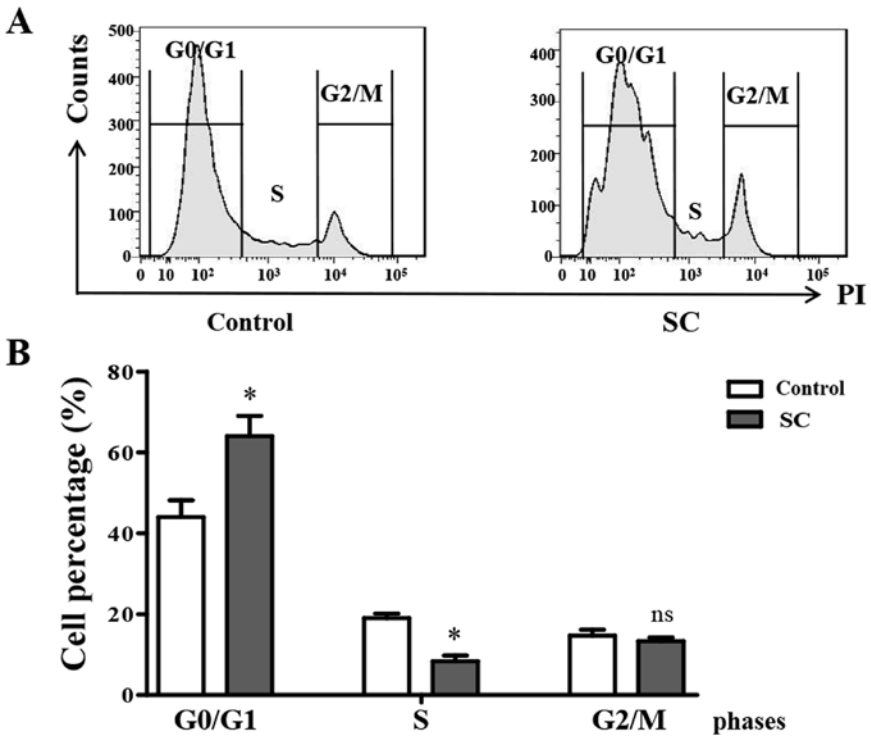

Figure 3. SC arrests the cell cycle at the G0/G1 phase in OS MG-63 cells. Cell cycle distribution of MG-63 cells. Following SC treatment, cell cycle distribution was determined by flow cytometry at 24 h. (A) Cell cycle distributions of MG-63 cells. (B) Average percentages of cells in each cell cycle phase. The experiments were repeated at least three times. Data are expressed as the mean ± SD of the three experiments (*P<0.05, vs. the control; ns, not significant). OS, osteosarcoma; SC, sodium cantharidinate.

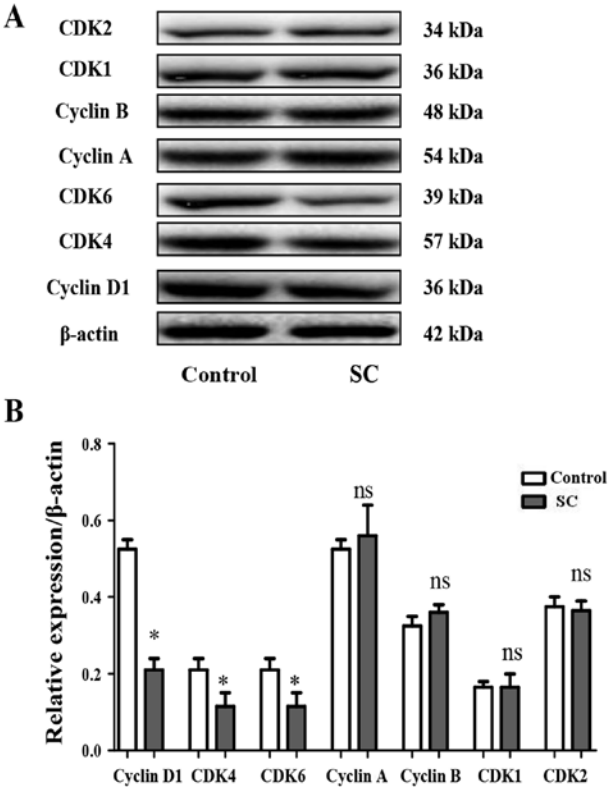

Figure 4. SC inhibits cyclin D1 expression in OS MG-63 cells. (A) MG-63 cells were treated with 5 μM of SC and total proteins were extracted. The expression levels of cyclin A, cyclin B, cyclin D1, CDK1, CDK2, CDK4 and CDK6 were determined by western blot analysis. (B) Quantification of blots via assessment of the relative band density. The experiments were repeated at least three times. Data are expressed as the mean ± SD of the three experiments (*P<0.05 vs. the control; ns, not significant). OS, osteosarcoma; SC, sodium cantharidinate.

Stimulation of the PI3K/AKT signaling pathway reverses SC-induced cell cycle arrest. To further test whether stimulation of the PI3K/AKT signaling pathway reverses SC-induced cell cycle arrest in MG-63 cells, the cells were treated with IGF-1 (50 μM), or SC for 24 h alone, and in combination, to effectively stimulate the PI3K/AKT signaling pathway in MG-63 cells, compared with the control cells. IGF-1 significantly increased the phosphorylation level of AKT, comparing with the SC treated alone group (Fig. 6A, P<0.05). Cyclin D1 (P<0.05), CDK4 (P<0.05) and CDK6 (P<0.05) expression was also observably reversed, consistent with the phosphorylation level of AKT (Fig. 6B). Cell cycle detection by FACS showed that IGF-1 reversed the SC-induced cell cycle arrest, decreased the G0/G1 phase percentage and increased the S phase percentage (Fig. 6C and D, P<0.05). These findings indicated that the PI3K/AKT pathway participates in the inhibition of MG-63 cell proliferation by SC. Together, these data suggest that SC inhibits the phosphorylation of AKT, consequently decreasing the expression of cyclin D1, CDK4 and CDK6, and inducing MG-63 cell G0/G1 phase arrest (illustrated in Fig. 7).

Discussion

Osteosarcoma is a common malignant cancer that has threatened the health of children worldwide over the last few decades. At present, a number of adverse side-effects are associated with chemotherapeutic drugs, such as cisplatin, ifosfamide and high-dose methotrexate. Drug resistance may be acquired by osteosarcoma cells after treatment for an extended period

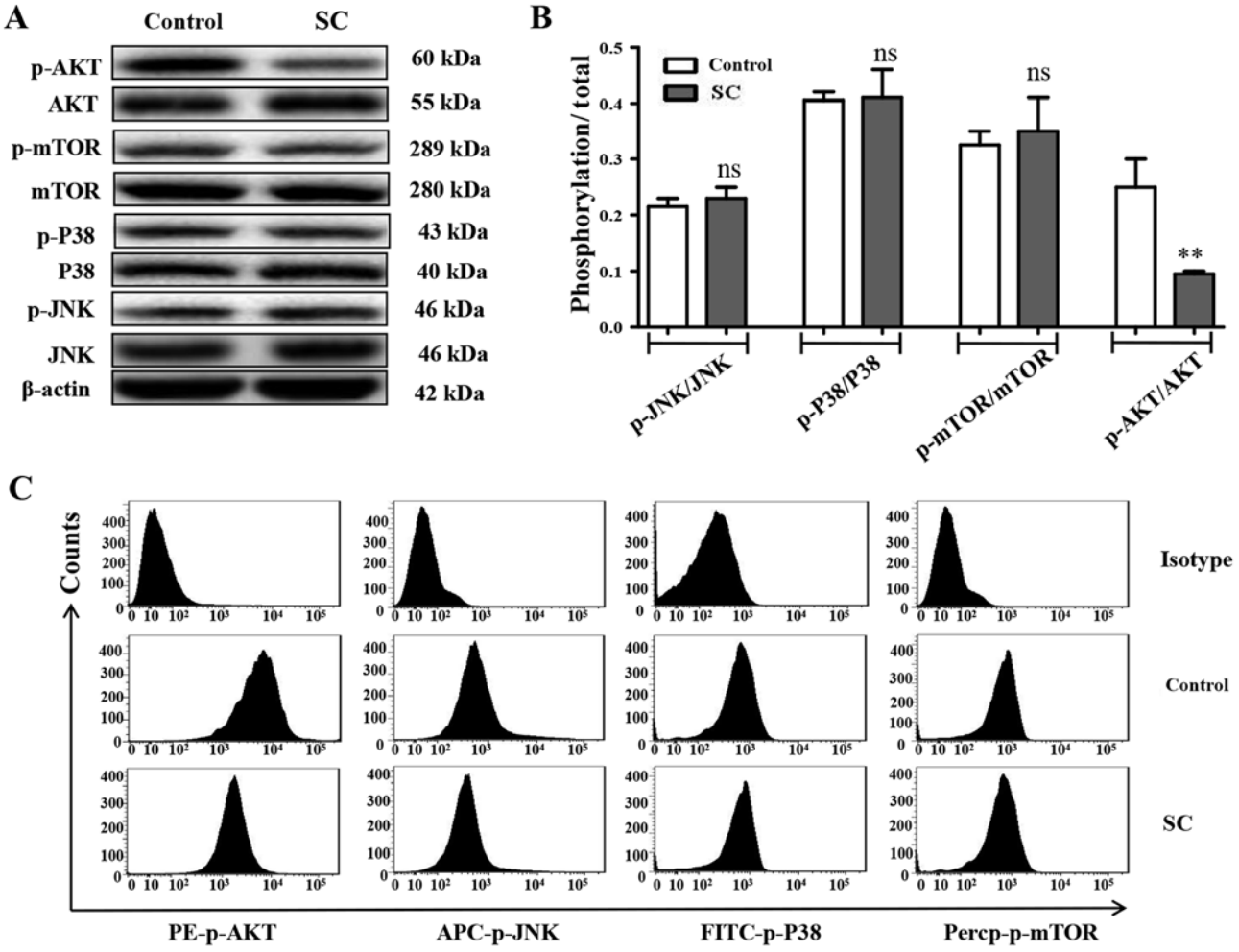

Figure 5. PI3K/AKT pathway participates in the inhibition of OS MG-63 cell growth by SC. (A) Western blot analysis of AKT, mTOR, JNK, P38 and β-actin in MG-63 cells. (B) Quantification of blots via measuring the relative band density. (C) Fluorescent-labeled flow cytometry was applied to test the MG-63 cell signaling pathway activation. The phosphorylation of AKT, mTOR, JNK and P38 fluorescence intensity was detected. Data are expressed as the mean ± SD of the three experiments. (**P<0.01 vs. the control; ns, not significant). OS, osteosarcoma; SC, sodium cantharidinate.

with such chemotherapeutic drugs. Therefore, development of novel effective therapeutic drugs with moderate side-effects for the treatment of osteosarcoma is urgent (23). There are many dangerous factors affecting the biology of tumor cells during the occurrence and progression of osteosarcoma (24). Cell cycle control is a major regulatory mechanism of cell proliferation. Thus, reprogramming of the cell cycle is the most effective method for cancer treatment in the clinic (25). There are numerous natural compounds that display significant inhibitory effects on osteosarcoma. Shangguan *et al* demonstrated that a natural product from ginseng, ginsenoside Rf, displays powerful cytotoxicity to human osteosarcoma MG-63 cells, in a dose-dependent manner. Additionally, ginsenoside Rf induced MG-63 cell cycle arrest at the G2/M phase and then apoptosis (26). Liu *et al* found that melatonin inhibits the ERK1/2 signaling pathway to display antiproliferative action, but did not affect the p38, JNK, or Akt pathways (27).

Although some studies have focused on the antitumor activity of sodium cantharidinate (SC) in clinical practice in recent years, there is no study concerning the activity of SC in osteosarcoma to date. There are several human osteosarcoma cell lines, such as MG-63, U2OS and 143B. SC was assessed using the 3 different cell lines in a pre-experiment, but no difference was found. Thus, the MG-63 cell line was used in the following mechanistic experiment. The present study investigated the antiproliferation and cell cycle arrest effects of SC on MG-63 cells for the first time. We found that 1.0 and 5.0 μM doses of SC significantly inhibited the growth of MG-63 cells. We then compared the cell growth in MG-63 cells at different time points following treatment with 5.0 μM of SC. Apparently, MG-63 cells showed a decelerated proliferation between 24 and 72 h after 5.0 μM of SC treatment. Cell cycle arrest is closely related to inhibition of cell proliferation (28). Thus, FACS experiment was established to analyze cell cycle distribution after SC treatment. We found that MG-63 cells showed a significant G0/G1 phase arrest compared to the control group after 5.0 μM SC treatment. Analysis of the percentages of cells in the different cell cycle phases showed that the G0/G1 phase percentage increased from 42±2.5 to 63±3.5% in the SC treatment group comparing to the control. At the same time, the S phase percentage decreased significantly, while the G2/M phase percentage did not change. This finding indicated that SC significantly induced MG-63 cell cycle arrest at the G0/G1 phase. Cytotoxic agents and/or DNA damaging agents, which arrest the cell cycle at the G0/G1, S or G2/M phase, induce cancer cell apoptosis (18). PI staining

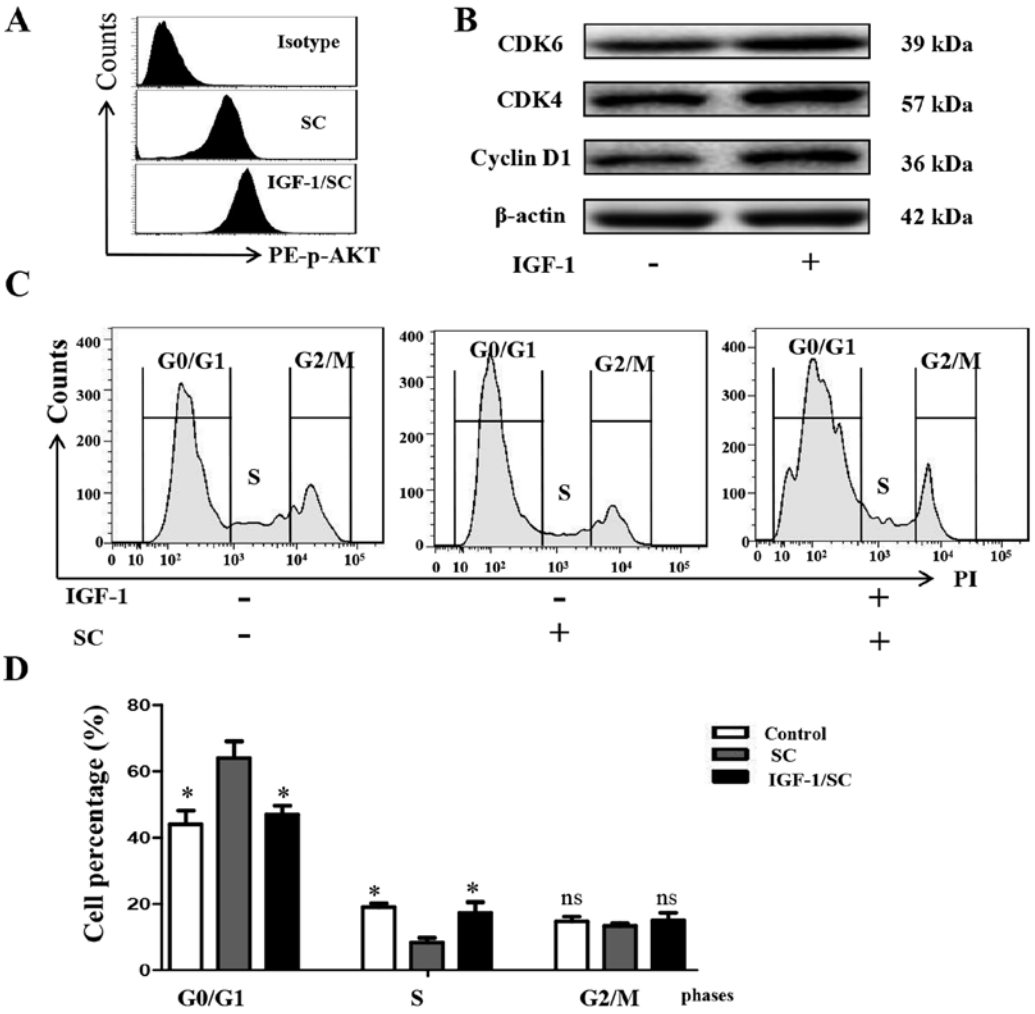

Figure 6. Stimulation of the PI3K/AKT signaling pathway reverses SC-induced cell cycle arrest. (A) Flow cytometric analysis of the phosphorylation of AKT detection in OS MG-63 cells treated by IGF-1, SC or the combination. (B) Cyclin D1, CDK4 and CDK6 were determined by western blot analysis. (C) Cell cycle distributions of MG-63 cells. (D) Average percentages of cells in each cell cycle phase, SC treatment alone as a control. The experiments were repeated at least three times. Data are expressed as the mean ± SD. (*P<0.05 vs. the control; ns, not significant). OS, osteosarcoma; SC, sodium cantharidinate.

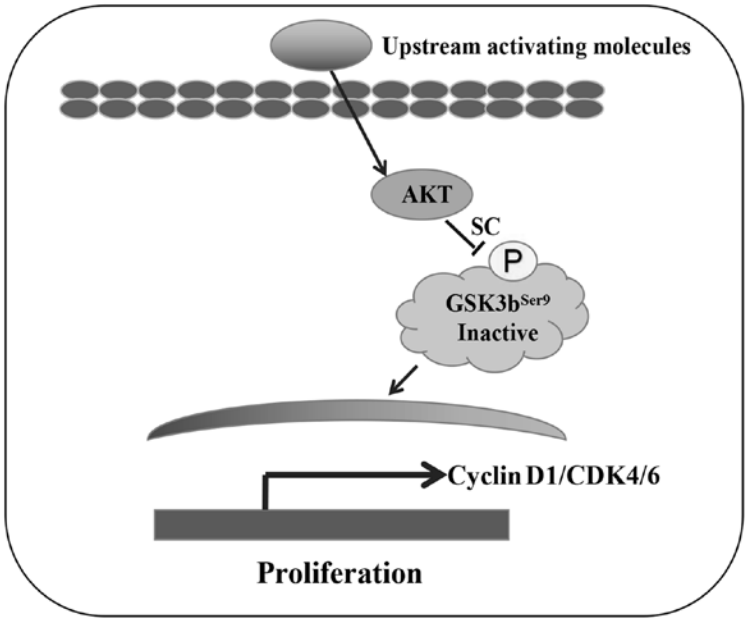

Figure 7. PI3K/AKT pathway participates in the inhibition of OS MG-63 cell proliferation by SC. The present data suggest that SC inhibits the phosphorylation (P) of AKT, consequently decreasing the expression of cyclin D1, CDK4 and CDK6, and finally inducing MG-63 cell G0/G1 phase arrest. OS, osteosarcoma; SC, sodium cantharidinate.

is a classical methods for the cell cycle, the peak width represents the different phases, although BrdU staining is an efficient methods for assessment of the cell cycle. Cyclins form complexes with Cdks to activate Cdks to regulate the cell cycle. To examine whether SC has functions on cyclins and kinases in MG-63 cells, western blotting was performed. SC significantly inhibited cyclin D1, CDK4 and CDK6 expression, consistent with the cell cycle detection assay. In contrast, expression of cyclin A, cyclin B, CDK1 and CDK2 did not show a significant difference between the SC treatment group and control group, which showed similar mechanisms as in a previous study (29).

Akt (also known as Akt1) may promote cell proliferation via phosphorylation, which acts as a mediator of growth factors (21). In order to further verify which signaling molecules is related to the MG-63 cell cycle arrest, we detected the expression of MAPK components and AKT using western blotting and FACS experiments. SC significantly inhibited phosphorylation of AKT, but not mTOR, JNK, or P38. To confirm the importance of the AKT phosphorylation in the SC-induced cell cycle arrest, we applied PI3K/AKT stimulator IGF-1 to pre-incubate MG-63 cells before SC treatment. IGF-1 significantly increased the phosphorylation level of AKT, compared with the SC treated alone group. Cyclin D1, CDK4 and CDK6 expression were also significantly reversed. Cell cycle detection by FACS showed that IGF-1 reversed SC-induced cell cycle arrest, decreased G0/G1 phase percentage and increased S phase percentage. These findings indicated that the PI3K/AKT pathway participated in the inhibition of MG-63 cells by SC. In a previous study, Liu *et al* found that that melatonin display antiproliferative action, which is mediated by inhibition of the ERK1/2 signaling pathway rather than the p38, JNK or Akt pathways (27). When cells were stimulated by upstream activating molecules, PI3K/AKT were phosphorylated, and then activated $GSK3b^{Ser9}$ phosphorylation for migration into the nucleus and regulation of the cell cycle (30). In the present study, we revealed a different mechanism: SC inhibited the phosphorylation of AKT, then decreased the expression of cyclin D1, CDK4 and CDK6, and induced MG-63 cell G0/G1 phase arrest. To the best of our knowledge, this is the first study to reveal the exact mechanism of SC in the induction of MG-63 cell inhibition. SC has potential for development as a new drug for the treatment of human osteosarcoma, although the results were only verified *in vitro*. Experiments *in vivo* in mice or rats will potentially be utilized for further investigation of the efficacy of SC for osteosarcoma treatment.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DLK and YL designed and performed the experiments, analyzed the data and wrote the manuscript. JYW and GL performed the experiments. MLZ designed, interpreted and funded the study, and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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