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Original Article

Wogonoside induces cell cycle arrest and mitochondrial mediated apoptosis by modulation of Bcl-2 and Bax in osteosarcoma cancer cells

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Abstract: Osteosarcoma (OS) is the most common bone cancer with a great tendency for local invasion and distant metastasis. Restricted by the severe toxicity of conventional drugs, the therapeutic challenge of osteosarcoma still remains unconquered. The objective of the present research work was to investigate the antiproliferative activity of wogonoside against human osteosarcoma (SaOS-2) cell line. Cell viability after wogonoside treatment was evaluated by MTT assay. Phase contrast microscopy was used to evaluate the change in cell morphology following drug treatment. The effect of wogonoside on cell cycle phase distribution and mitochondrial membrane potential was investigated by flow cytometry using propidium iodide (PI) and rhodamine-123 DNA-binding fluorescent dyes respectively. Western blotting was used to evaluate the effect of wogonoside on cell cycle-related proteins as well as on the expression levels of Bcl-2, Bax, cytosolic and mitochondrial cytochrome *c* and apoptotic protease activating factor-1 (Apaf-1). Wogonoside induced a dose-dependent as well as time-dependent growth inhibitory effects on cell proliferation of SaOS-2 cancer cells. Wogonoside induced G2/M cell cycle arrest as well as loss in mitochondrial membrane potential in these cells. Within 48 h of incubation, approximately 4.36%, 6.72%, 11.54%, 21.88% and 15.54% of the cells underwent early apoptosis after treatment with 0, 5, 10, 25 and 75 μM of wogonoside respectively. Wogonoside led to reduced Bcl-2 expression and increased Bax expression, while as it led to s decrease in the levels of mitochondrial cytochrome c and an increase in cytosolic fraction and expressions of cytosolic apoptotic protease activating factor-1 (Apaf-1).

Keywords: Osteosarcoma, wogonoside, apoptosis, cell cycle, mitochondria

Introduction

Osteosarcoma is one of the most frequent malignant cancers of the bones prevalent mostly in adolescents and children. The precise mechanisms of osteosarcoma are mainly unidentified, but numerous evidences have suggested that multiple genetic and environmental factors might play significant roles in the carcinogenesis of osteosarcoma. Treatment strategies such as surgery, chemotherapy and radiotherapy are primarily efficacious in patients with confined cancers improving patient survival rate. Chemotherapeutic agents include methotrexate, cisplatin, 5-fluorouracil (5-FU), doxorubicin, Taxol and etoposide [1, 2].

However, in contrast to confined malignancy, many of the patients (10-20%) possess clinically evident metastatic osteosarcoma with more than one third of patients developing recurrent high-grade osteosarcoma. The metastatic stage typically affect lung, liver and bone itself resulting in 5-year survival rates not more than 50-60%. In addition, the frequent acquisition of drug resistance coupled with toxic side effects of chemotherapy still remain a major loophole in the treatment of osteosarcoma. Thus, there is an urgent need for the development of new and alternative therapeutic approaches to the treatment of osteosarcoma [3, 4]. It is important to explore more effective chemotherapeutic agents especially natural products, for treating aggressive osteosarcoma. As a new source of chemotherapy, natural products and their derivatives exert their anticancer effects against cancer cells by inducing apoptosis. Due to this tendency, natural products have

gained significant attention in order to reduce chemotherapy-linked serious side-effects [5].

Natural products especially plant-derived agents have played a key role in new drug discovery for centuries, with over 55% of approved anticancer agents being of natural origin. These compounds can be used as antioxidants and cancer preventing agents or cancer chemotherapeutic drugs [6].

The objective of the present research work was to investigate the potential application of wogonoside, a natural product, in the treatment of osteosarcoma. The study examined the effects of wogonoside on SaOS-2 human osteosarcoma cancer cells using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flow cytometry and fluorescence microscopy were used to examine the effect of wogonoside on the induction of apoptosis, cell cycle arrest and the loss of mitochondrial membrane potential (ΛΨm) in osteosarcoma cells. The effect of wogonoside on DNA damage as well as on Bcl-2 and Bax expression levels were also investigated.

Materials and methods

Cell line, natural product, and other reagents

Human osteosarcoma cell line, SaOS-2 was procured from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were maintained in a humidified environment comprised of 5% CO_2 and 95% air at 37°C in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA). The medium was changed every 2-3 days. Mouse monoclonal antibodies against Bax, Bcl-2, cytochrome c, Cdk2, Cdk6, p27, C-myc, pRb, GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, US). MTT was purchased from Sigma Chemical Co., (St. Louis, MO, USA). Wogonoside and cisplatin were purchased from (> 98% purity) Langze Pharmaceutical Co, Ltd, Nanjing, China) and dissolved in DMSO (Sigma Chemical Co.). All other chemicals and reagents were of the highest quality and obtained from Sigma-Aldrich (St. Louis, MO, US).

Cell viability assay

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to measure the inhibition of cell proliferation induced by wogonoside. Briefly, cells were plated in 96-well culture plates (1×10^6 cells/well) separately. After incubating for 24 h, SaOS-2 cells were treated with wogonoside (2.5, 5, 10, 25 and 75 μM) for 24 and 48 h, MTT solution (5 mg/mL) was then added to each well. After 3 h incubation, the formazan precipitate was dissolved in 100 μL dimethyl sulfoxide, and then the absorbance was measured in a microplate reader (Bio-Rad).

The cell viability ratio was calculated by the following formula: Inhibitory ratio (%) = (OD control - OD treated)/OD control × 100%.

Cytotoxicity was expressed as the concentration of capillarisin inhibiting cell growth by 50% (IC_{50} value).

Assessment of cell morphology by phase contrast microscopy

SaOS-2 cells at a density of 2×10^5 cells/well were placed in 12-well plates and incubated at 37°C overnight. Different concentrations of wogonoside (0, 5, 10, 25, and 75 μM) in dimethyl sulfoxide (DMSO) were added to each well and the cells were incubated for 48 h. DMSO (solvent) only was used for the control regimen. For cell morphology, the cells in the plate were examined under a phase contrast microscope and photographed.

Flow cytometry analysis for cell cycle phase distribution

SaOS-2 cells at a density of 2×10^6 cells/well were placed in 12-well plates and were incubated with different concentrations of wogonoside (0, 5, 10, 25, and 75 μM) for 48 h before the cells were harvested by centrifuging. The cells were then trypsinized, washed with PBS and treated with 50 μg/ml cold propidium iodide solution containing 0.1 mg/ml RNase A in PBS (pH 7.4) for 30 min in the dark. Flow cytometric analysis was performed on a FACS Calibur instrument (Becton-Dickinson, San Jose, CA, USA). The percentage of cells in the sub-G1 (apoptosis), G0/G1-, S- and G2/M-phases were determined by flow cytometry.

Annexin V-FITC and Propidium Iodide Staining Assay for detection and quantification of apoptosis

SaOS-2 cells were stained with FITC-conjugated Annexin V and propidium iodide (Bestbio,

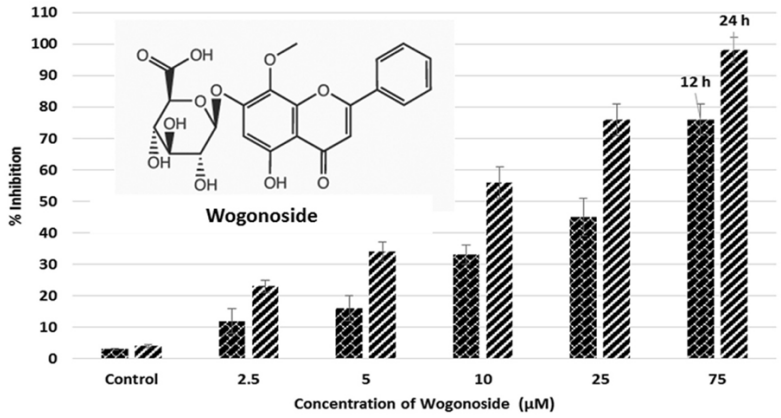

Figure 1. Dose-dependent and time-dependent growth inhibitory effects of wogonoside on the cancer cell proliferation of human osteosarcoma (SaOS-2) cells in vitro.

Shanghai, China). The cells were seeded in 96-well plates at density of 1×10^5 cells/mL. The cells were treated with different concentrations of wogonoside (0, 5, 10, 25, and 75 μM) for 48 h. The cells were then harvested using 0.2% trypsin-EDTA and cell plates were resuspended in ice-cold 1X binding buffer. One microliter of Annexin V-FITC solution and 5 μL of propidium iodide were added to 100 μL of cells suspension. The tube was incubated on ice for 15 min in the dark followed by addition of 200 μL ice-cold 1X binding buffer and mixing gently. The samples were analyzed using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software. Cells grown in media containing an equivalent amount of DMSO without any drug were served as control.

Effect of wogonoside on mitochondrial membrane potential loss (ΛΨm) in SaOS-2 cells

Mitochondrial membrane potential (ΛΨm) in osteosarcoma cells (SaOS-2) was measured by Rhodamine-123 dye (Zouping Mingxing Chemical Co., Ltd.). SaOS-2 cells (5×10^6) were treated with cisplatin (2.5 μM, positive control), and different concentrations of wogonoside (0, 5, 10, 25, and 75 μM) for 48 hours and mitochondrial membrane potential was measured by flow-cytometry. Rhodamine-123 (20 mM) was added 2 h before the end of experiment. After it, the cells were washed with PBS and incubated with PI (10 μg/ml) for 30 min. Finally the cells were analyzed by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

DNA fragmentation assay

After wogonoside treatment at various concentrations (0, 5, 10, 25, and 75 μM), both adherent and floating cells were collected and washed with PBS. Pellets were then lysed with DNA lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% SDS) at room temperature for 25 min. After centrifugation for 10 min at 15 000 × *g*, the supernatants were collected and treated with RNase A (final concentration, 250 μg/ml) for 30 min at 37°C, followed by digestion with proteinase K (final concentration 250 μg/ml) for 2.5 h at 50°C. The DNA was extracted using the phenol/chloroform/isoamylol (25:24:1), precipitated with ethanol, dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis.

Western blot analysis

The wogonoside-treated cells were collected and washed with ice-cold PBS, then treated with ice-cold RIPA lysis buffer (Beyotime Inst. Biotech, Beijing, China) with 1 mM phenylmethyl sulfonyl fluoride (PMSF). Cell lysates were centrifuged at 15 000 × *g* at 4°C for 10 min. The proteins of mitochondrial and cytosolic fraction were isolated using the Mitochondria/cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA, US) according to the manufacturer in-structions. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, US). Equivalent amounts of proteins samples were uploaded and separated by 12% SDS-PAGE and then electro transferred to polyvinylidene difluouride (PVDF) membrane (Millipore Corp, Atlanta, GA, US). The membranes were blocked in 5% nonfat dry milk in PBS-T at room temperature for 1 h, and then incubated with indicated primary antibodies over night at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The signal was detected by enhanced cheniluminescence (ECL) reagents (Pierce, Rockford, IL, US).

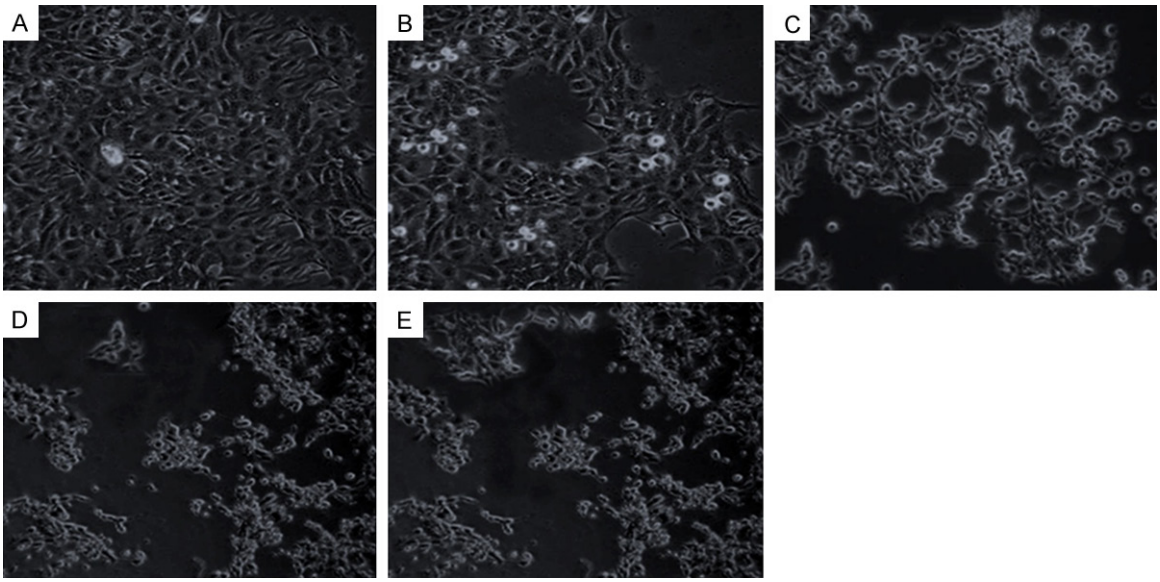

Figure 2. Effects of wogonoside on cell morphology change and percentage of viable SaOS-2 cells. SaOS-2 cells were cultured in DMEM + 10% FBS with different concentrations of wogonoside for 48 h. The cells were observed and photographed by phase-contrast microscopy (× 200 magnification). A. Represents control (untreated cells), B-E. Represent 5, 10, 25 and 75 μM dose of wogonoside. The number of viable cells decreases significantly as well as the cell morphology gets significantly altered as the wogonoside dose increases.

Statistical analysis

Data are given as the mean ± standard deviation (SD). Statistical analysis was performed by using one-way analysis of variance (ANOVA), followed by a Dunnett's multiple-comparison test with 95% confidence intervals. A *P*-value < 0.05 was considered to indicate a statistically significant difference.

Results

Wogonoside induced potent cytotoxicity in human osteosarcoma (SaOS-2) cells

The anticancer activity of wogonoside against Human osteosarcoma (SaOS-2) cells was evaluated by using MTT assay. Five different concentrations of wogonoside (2.5, 5, 10, 25 and 75 μM) were tested against the cancer cells. The osteosarcoma cancer cells showed considerable and dose-dependent susceptibility to the treatment of different concentrations of wogonoside. The compound also showed time-dependent inhibition of the cancer cell growth as shown in **Figure 1** at 24 hours and 48 hour time intervals. The number of viable osteosarcoma cells exposed to wogonoside treatment reduced considerably. The IC_{50} value of the compound after 12 h and 24 h time intervals was found to be 27.1 and 8.5 μM respectively.

Effect of wogonoside on cell morphology and apoptosis induction in human osteosarcoma (SaOS-2) cells

SaOS-2 cells were used for examining the possible cytotoxic effects of wogonoside on human osteosarcoma cells *in vitro*. The control cells (without wogonoside treatment) appeared in a well spread normal shape when observed and photographed with a phase-contrast microscope (**Figure 2**). After being treated with different concentrations (0, 5, 10, 25, and 75 μM) of wogonoside for 48 h, a substantial proportion of cells were of a round shape and the cell density was reduced in a dose-dependent manner (**Figure 2**). SaOS-2 cells incubated with higher doses of wogonoside for 48 h resulted in a significant decrease in cell viability and significant alteration in cell morphology. The change in cell morphology is a characteristic feature of apoptosis especially the appearance of apoptotic body formation after drug treatment.

Wogonoside induced G2/M cell cycle arrest in (SaOS-2) cells

The inhibition of cancer cell growth by wogonoside was further studied with flow cytometric analysis of cellular DNA content using propidium iodide (PI) as a staining agent. As shown in **Figure 3A-E**, the percentage of cells in the

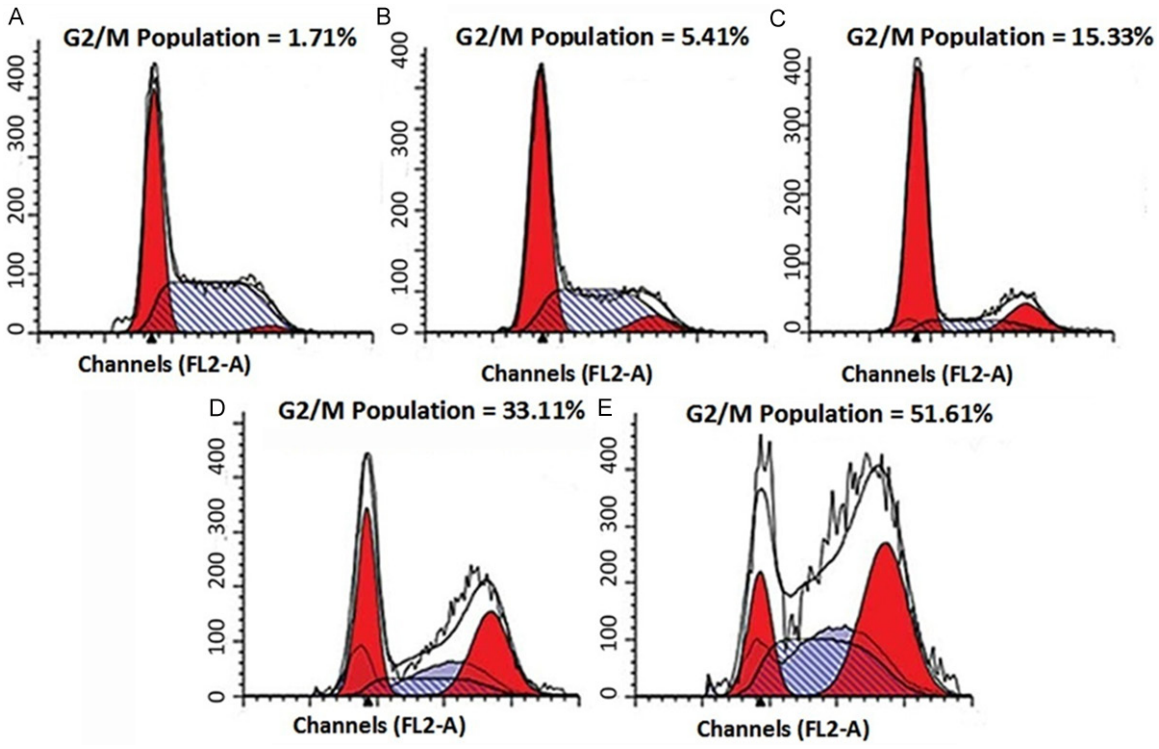

Figure 3. Effects of wogonoside on the cell cycle arrest in SaOS-2 cells. SaOS-2 cells were exposed to different concentrations of wogonoside for 48 h, and the cells were harvested and analyzed for cell cycle analysis by flow cytometry, as described in Materials and Methods. A. Represents control (untreated cells), B-E. Represent 5, 10, 25 and 75 μM dose of wogonoside.

G2/M-phase was 1.71% in control cells (without wogonoside treatment), whereas it significantly increased to 5.41%, 15.33%, 33.1% and 51.61% when treated with 5, 10, 25 and 75 μM concentration of wogonoside for 48 h. The results also showed that there was a slight increase in the percentage of sub-G1 apoptotic peak and a decrease in G1 peak as the dose of wogonoside was increased from 5 to 75 μM.

Effect of wogonoside on SaOS-2 cell apoptosis and its quantification

Since cell cycle arrest may lead to apoptosis, in the next series of experiments we analyzed whether necrosis or apoptosis was involved in the mechanism of action of these compounds. Early events in the apoptotic process are loss of plasma membrane asymmetry accompanied by translocation of PS from the inner to the outer membrane leaflet exposing PS to the external environment [7]. To verify the molecular mechanism (apoptosis or necrosis) of wogonoside on SaOS-2 cells, the apoptotic effects of wogonoside were tested using Annexin V-FITC and PI apoptosis kit. After cells are stained with annexin V in combination with propidium iodide (PI), this reagent enters the cell only when the plasma cell membrane is damaged. The results of this annexin V assay are shown in **Figure 4A-E**. The results of the flow cytometry study with Annexin V/FITC and PI showed that within 48 h of incubation, approximately 4.36%, 6.72%, 11.54%, 21.88% and 15.54% of the cells underwent early apoptosis after treatment with 0 (no wogonoside treatment), 5, 10, 25 and 75 μM of wogonoside respectively. Similarly, 6.07%, 18.32%, 25.11%, 29.34% and 43.14% of the cells underwent late apoptosis after treatment with 0 (no wogonoside treatment), 5, 10, 25 and 75 μM of wogonoside respectively (**Figure 4**). The percentage of necrotic cell death was almost similar in all treatments indicating wogonoside induces apoptotic cell death and not necrotic cell death in SaOS-2 cells.

Wogonoside induces mitochondrial membrane potential loss (ΛΨm) in SaOS-2 cells

One key and indicative stage in the intrinsic apoptosis pathway is the depolarization of the

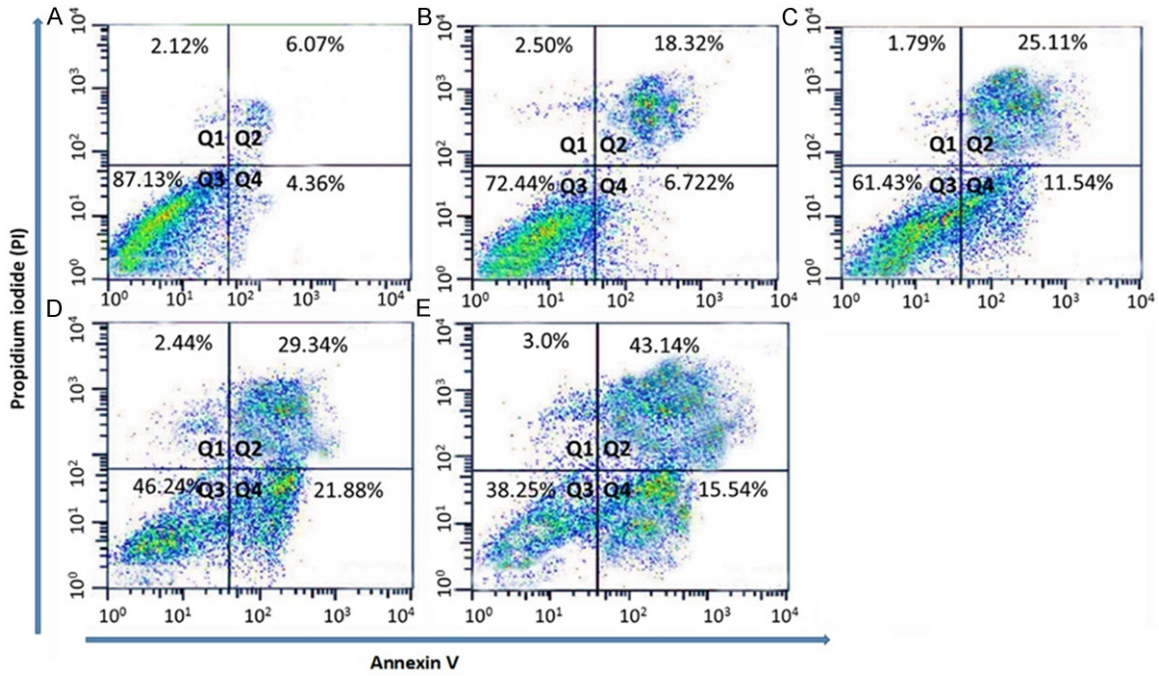

Figure 4. Induction of apoptosis by wogonoside and confirmation by Annexin V-FITC/PI dual staining. SaOS-2 cells were treated with 0 (A, control), 5 μM (B), 10 μM (C), 25 μM (D), and 75 μM (E) of wogonoside for 48 h and analysed using FACS Calibur flow cytometer as described in "Materials and Methods". Normal healthy, early apoptotic, late apoptotic and dead/necrotic cell populations are shown as percentage of total cells in the quadrants Q3, Q4, Q2 and Q1, respectively.

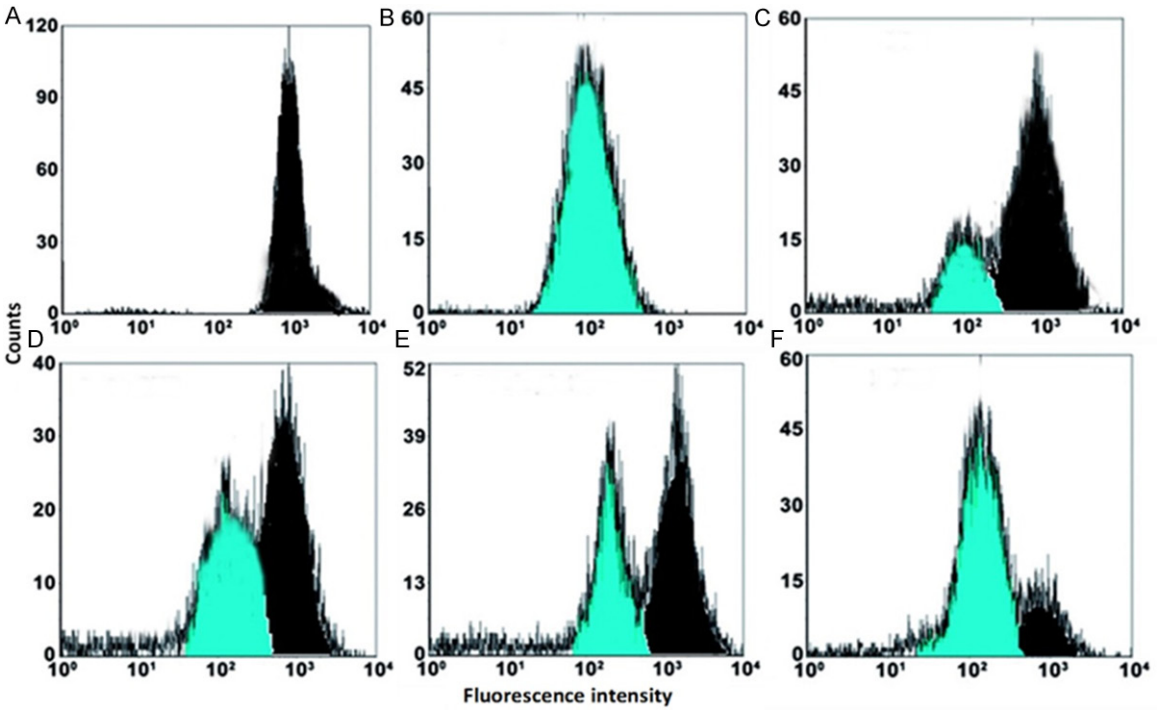

Figure 5. Effect of wogonoside on the mitochondrial membrane potential loss in osteosarcoma (SaOS-2) cancer cells. A. Represents negative control (only solvent) cells. B. Represents effect of cisplatin (positive control) on ΛΨm, C-F. represent effect of 5, 10, 25 and 75 μM of wogonoside on ΛΨm respectively. Compared to control cells, wogonoside treatment indicate a significant loss of mitochondrial membrane potential.

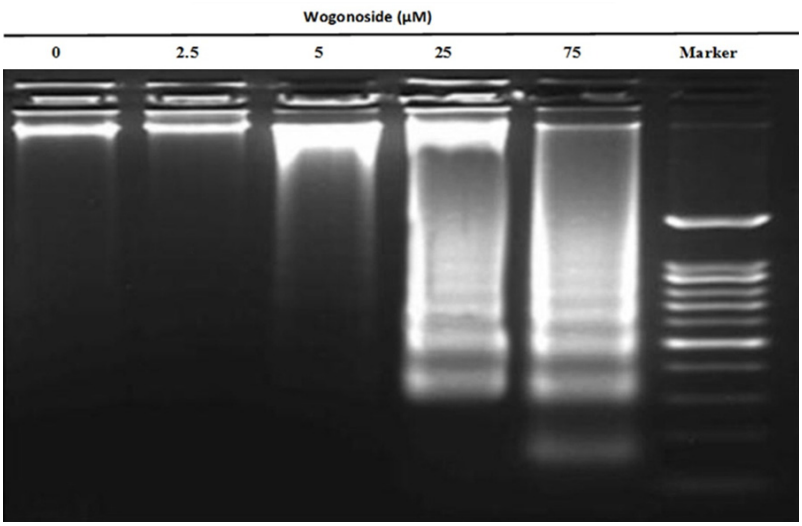

Figure 6. Induction of DNA fragmentation following exposure to different concentrations of wogonoside (0, 2.5, 5, 25 and 75 μM) in osteosarcoma (SaOS-2) cancer cells. DNA isolated from wogonoside-treated cells was subjected to 2% agarose gel electrophoresis, followed by visualization of bands and photography. No DNA fragments were observed in the control groups (left panel).

mitochondrial membrane and the successive leakage of the outer membrane by pore formation. This is accompanied by release of pro-apoptotic molecules and cytochrome C [8]. The fluorescent dye, rhodamine-123 (Rh-123) is a specific probe for the detection of alterations in mitochondrial membrane potential in living cells. Our results revealed that the wogonoside induced a potent and dose-dependent loss of mitochondrial membrane potential by decreasing the number of cells with intact membrane potential and increasing the number of cells with low potential after 48 h exposure to varying concentrations (0, 5, 10, 25, and 75 μM) of wogonoside. As can be seen in **Figure 5A-F**, as compared to control cells, wogonoside treatment led to a substantial loss of membrane potential. Cisplatin was used as a positive control.

Effect of wogonoside on DNA fragmentation inSaOS-2 cells

DNA fragmentation of wogonoside-treated cells was also examined by observation of the formation of DNA ladder. As shown in **Figure 6**, DNA ladder appeared to be more evident with the increasing wogonoside concentration, however, no DNA fragments were observed in the control groups (**Figure 6**, left panel). Even lower doses of wogonoside (2.5 μM) also did not induce DNA fragmentation. However, 25 and 75 μM doses of wogonoside after 48 exposure led to a substantial increase in DNA fragmentation (**Figure 6**, right panel).

Effects of wogonoside on the expression levels of Bcl-2, Bax, cytochrome c and cytosolic Apaf-1 in SaOS-2 cells

Wogonoside induces mitochondrial membrane potential loss in SaOS-2 cells, but this is not the only apoptotic pathway involving mitochondria. The mitochondrial pathway of apoptosis also involves signaling by Bcl-2 family proteins as well as cytochrome c [9-11]. Therefore, it was necessary to investigate the expression of Bcl-2 family proteins in these cell lines and to see whether wogonoside triggers the release of cytochrome c. For this, we investigated the cytosolic and mitochondrial levels of cytochrome c as shown in **Figure 7**. As observed in the **Figure 7**, Bcl-2 expression reduced after treatment with wogonoside at different concentrations, whereas Bax expression improved. At the same time, the levels of cytochrome *c* in the mitochondrial fraction declined, whereas the levels in the cytosolic fraction increased and the expression of cytosolic apoptotic protease activating factor-1 (Apaf-1) also increased (**Figure 7**). This advocates a key role for the mitochondria in wogonoside-induced apoptosis.

Effect of wogonoside on the expression of cell cycle-related proteins

To examine the apoptotic mechanisms through which wogonoside obstructs with cell cycle progression, we studied the effect of wogonoside on the cell cycle-related protein content. The levels of p27, c-myc, pRb, Cdk2 and Cdk6 were measured by western blot analysis using specific antibodies against these proteins. As presented in **Figure 8**, the levels of Cdk2, Cdk6, pRb and c-myc decreased, whereas the p27 level in the nuclear fraction increased.

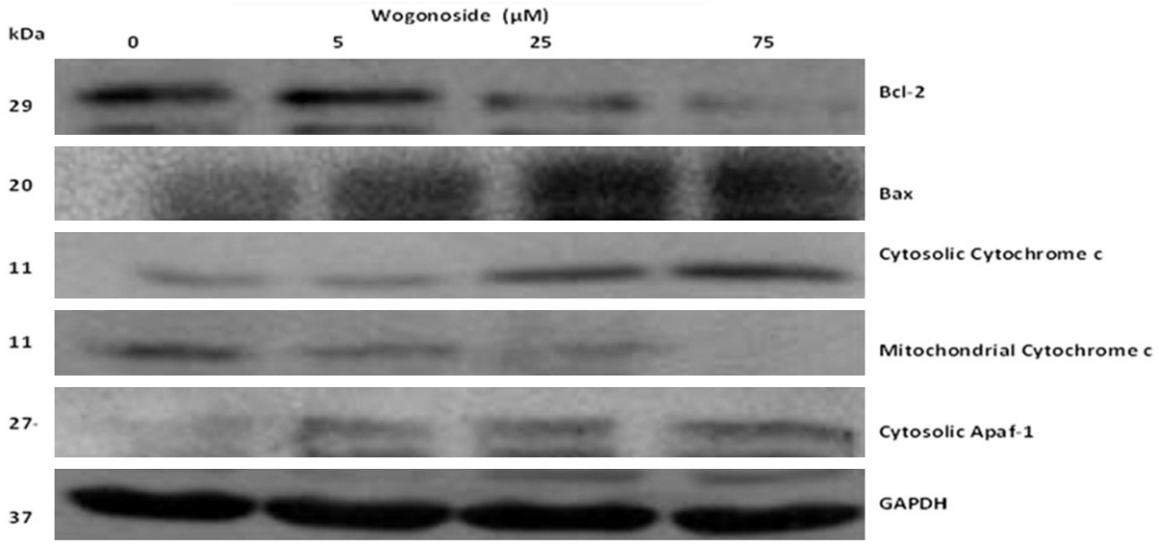

Figure 7. Effects of wogonoside on the expression levels of Bcl-2, Bax, cytosolic and mitochondrial cytochrome c and apoptotic protease activating factor-1 (Apaf-1) in osteosarcoma (SaOS-2) cancer cells. Western blot analysis of Bcl-2, Bax protein expression. The cells were treated with wogonoside (0, 5, 25 and 75 μM).

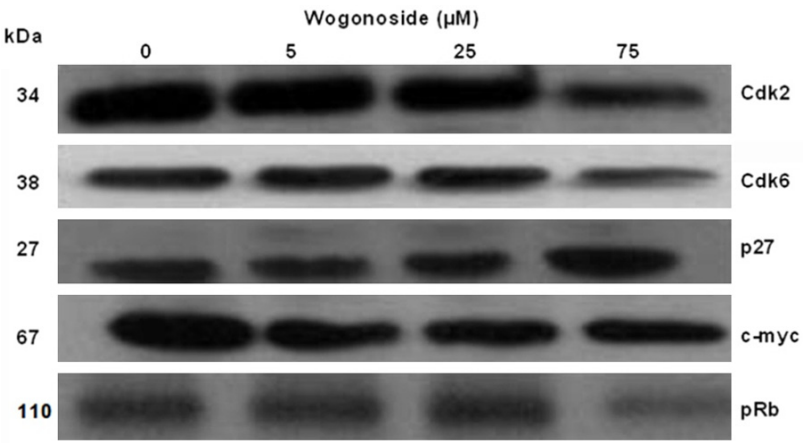

Figure 8. Effects of wogonoside on the levels of cell cycle-related proteins in osteosarcoma (SaOS-2) cancer cells. SaOS-2 cancer cells were treated with wogonoside (0, 5, 25 and 75 μM) for 48 h. Cells were lysed and then total proteins were separated by SDS-PAGE. Proteins were visualized by western blot analysis using antibodies against Cdk2, Cdk6, pRb, p27 and c-myc.

Discussion

Wogonoside is the main flavonoid component derived from the root of *Scutellaria baicalensis* Georgi. It is a popular Chinese herbal medicine with the potential to treat hematologic malignancies. The flavonoid wogonoside can be derived from *S. baicalensis,* as it is a metabolite of wogonin. Wogonoside has been reported to induce cell cycle arrest and differentiation by affecting expression and sub cellular localization of PLSCR1 in acute myeloid leukemia cells [12]. In another study, wogonoside was also reported to induce autophagy in human breast cancer cells (MDA-MB-231) by regulating MAPK-m TOR pathway [13]. So far the anticancer activity of wogonoside against osteosarcoma cancer cells has not been reported nor is the detailed mechanism of action of this flavonoid reported so far. As such the objective of the present study was to investigate the anticancer effects of wogonoside on SaOS-2 cancer cells. Wogonoside induced a dose-dependent as well as time-dependent growth inhibitory effects on the proliferation of SaOS-2 cancer cells. Further, phase contrast microscopy revealed that wogonoside induced a significant loss of viable cells along with alterations in cell morphology. Flow cy-tometry analysis using propidium iodide as a fluorescent probe revealed that wogonoside induced a G2/M phase cell cycle arrest. The fact that wogonoside induced an apoptotic cell death and not necrotic cell death was confirmed by annexin V-FITC binding assay.

Wogonoside induced both early as well as late apoptosis in these cell lines. The apoptotic cell death induced by wogonoside was mediated through mitochondrial pathway. The mitochondrial pathway of apoptosis also involves signaling by Bcl-2 family proteins as well as cytochrome c. Bcl-2 family proteins regulate apoptosis and the release of pro-apoptotic factors [9-11]. Therefore, it was necessary to investigate the expression of Bcl-2 family proteins in these cell lines and to see whether wogonoside triggers the release of cytochrome c. For this, we investigated the cytosolic and mitochondrial levels of cytochrome c. Wogonoside led to a reduction in Bcl-2 expression whereas the expression levels of Bax were increased on wogonoside treatment. Bax is an apoptotic molecule while as Bcl-2 is an anti-apoptotic molecule. At the same time, the levels of cytochrome *c* in the mitochondrial fraction declined, whereas the levels in the cytosolic fraction increased and the expression of cytosolic apoptotic protease activating factor-1 (Apaf-1) also increased (**Figure 7**). This advocates a key role for the mitochondria in wogonoside-induced apoptosis. A loss of mitochondrial membrane potential is associated with apoptosis following the release of cytochrome *c* [14]. The induction of Bax is associated with the release of cytochrome c from the mitochondria to the cytosol and the cleavage of poly (ADP-ribose) polymerase [15].

Further, wogonoside affected the expression levels of cell cycle related proteins including p27, c-myc, pRb, Cdk2 and Cdk6. As presented in **Figure 8**, the levels of Cdk2, Cdk6, pRb and c-myc decreased, whereas the p27 level in the nuclear fraction increased. To our knowledge, this report provides the first evidence that wogonoside affects Bcl-2 family protein expression as well as the expression levels of cell cycle related proteins.

In conclusion, our study reveals that wogonoside is a potential natural product which can inhibit cancer growth in osteosarcoma cells by inducing apoptosis, cell cycle arrest at G2/M phase, loss of mitochondrial membrane potential and DNA fragmentation. Wogonoside also increased the expression of apoptotic proteins and decreased the expression of anti-apoptotic proteins. Wogonoside also affected the cell cycle related proteins which results in cell cycle arrest at G2/M phase.

Disclosure of conflict of interest

None.

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