

***Xiao Jin Wan*, a traditional Chinese herbal formula, inhibits proliferation via arresting cell cycle progression at the G2/M phase and promoting apoptosis via activating the mitochondrial-dependent pathway in U-2OS human osteosarcoma cells**

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Abstract. *Xiao Jin Wan* (XJW) is a well-known traditional Chinese folk-medicine, which is commonly used for the treatment of various types of diseases including cancers. However, the mechanism of the anticancer activity of XJW against U-2OS human osteosarcoma cells, have not yet been reported. In the present study, we investigated the cellular effects of the XJW on the U-2OS human osteosarcoma cell line. Our results showed that XJW induced cell morphological changes, reduced cell viability in a dose- and time-dependent manner and arrested in the G2/M phase of the cell cycle suggesting that XJW inhibited the proliferation of U-2OS cells. Hoechst 33258 staining and Annexin V/propidium iodide double staining exhibited the typical nuclear features of apoptosis and increased the proportion of apoptotic Annexin V-positive cells in a dose-dependent manner, respectively. In addition, XJW treatment caused loss of plasma membrane asymmetry, collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, and

increase of the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2. Taken together, the results indicate that the U-2OS cell growth inhibitory activity of XJW was due to cell cycle arrested and mitochondrial-mediated apoptosis, which may partly explain the anticancer activity of *Xiao Jin Wan*.

Introduction

Human osteosarcoma (OS) is the most common primary malignant bone tumor, accounting for approximately 20% of all primary sarcomas in bone (1). Well-known for its metastasis and high local recurrence rate (2,3), osteosarcoma is a type of cancer whose treatment requires an extensive multimodal approach including surgery, radiotherapy and chemotherapy. Currently, chemotherapeutic regimens for human osteosarcoma treatment use the combination of multiple chemotherapeutic agents including high-dose methotrexate (HD-MTX) with leucovorin rescue, doxorubicin (adriamycin), cisplatin and ifosfamide either with or without etoposide (4). Although new therapies consisting of aggressive adjuvant chemotherapy and wide tumor excision have led to a significant benefit in terms of patients' survival, the frequent acquisition of drug-resistant phenotypes and unwanted side-effects are often associated with chemotherapy and remain as serious problems (5). It is therefore urgent that new therapeutic strategies which can improve the effect of current chemotherapy be developed.

Chinese herbal medicine, a major modality in traditional Chinese medicine (TCM) and practiced for thousands of years in China and other Asian countries, is used for treating cancers (6-8). Herbal formulations are the common form of administration in Chinese herbal practice, and herbal formulas are well documented in ancient and modern literature (9,10). According to Chinese herbal theory, interactions among the different herbs in a formula exert a synergistic effect and neutralize potential

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Abbreviations: OS, osteosarcoma; XJW, *Xiao Jin Wan*; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide

Key words: osteosarcoma cell, *Xiao Jin Wan*, proliferation, apoptosis, mitochondria

toxicity and side-effects of the individual constituents (11,12). However, there is as yet a lack of rigorous scientific evaluation of such formulas.

The classical formula *Xiao Jin Wan* (XJW), formerly known as *Xiao Jin Dan* (XJD), first documented in the book *Wai Ke Zheng Zhi Quan Sheng Ji* (13), consists of ten component herbs, *She Xiang* (*Moschus*), *Mu Bie Zi* (*Cochinchina momordica seed*), *Zhi Cao Wu* (*Radic aconiti Kusnezoffii preparata*), *Feng Xiang Zhi* (*Resina liquidambaris*), *Ru Xiang* (*Frankincense*), *Mo Yao* (*Myrrh*), *Dang Gui* (*Chinese angelica*), *Wu Ling Zhi* (*Trogopterus dung*), *Di Long* (*Pheretima*) and *Xiang Mo* (*Pine-soot ink*). As a well-known traditional Chinese folk-medicine, it is used for eliminating stagnation, removing of blood stasis, promoting of blood circulation and alleviating pain (14), which is commonly used for treatment of various types of diseases including cancers, such as breast cancer. However, the mechanism of XJW's anticancer activity of human osteosarcoma, have not yet been reported.

The cell cycle is the series of events that take place in a cell leading to its division and duplication (replication), which is monitored and regulated by cell cycle checkpoints which establish the timing and strength of arrest, repair and apoptotic responses to a damaging agent (15). Molecules regulating cell division, such as cyclin-dependent kinases (CDKs) and inhibitors for CDKs, are also implicated in regulating apoptosis. The tumor suppressor p53 and its downstream transcriptional target p21^{Cip1/Waf1} are also essential to sustain G2/M phase arrest after DNA damage through the inhibition of cdc2 (16). In addition, recent studies suggest that caspase-mediated cleavage of p21^{Cip1/Waf1} is a critical step in converting cancer cells from growth arrest to apoptosis (17).

Apoptosis is a genetically mediated mechanism by which individual cells orchestrate their own deletion in normal and diseased tissues. It is a complex process which includes signal transduction (18) and the degradation of cellular protein and DNA (19). Disturbed regulation of this vital process represents a major causative factor in the pathogenesis of cancers including osteosarcoma (20,21). The Bcl-2 family proteins are important regulators of apoptosis including both anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax (22,23). One possible mechanism by which Bcl-2 family proteins regulate apoptosis is through their influence on the permeability of mitochondrial outer membrane (MOM) following homo- or hetero-association. It has been demonstrated that after activation, the pro-apoptotic Bax or Bak is sufficient to induce mitochondrial outer membrane permeabilization (MOMP) (20-23), releasing apoptogenic proteins such as cytochrome *c*, Smac/DIABLO and apoptosis inducing factor (AIF) (24,25). The released cytochrome *c* leads to apoptotic protease-activating factor (Apaf-1)-mediated activation of initiator caspase-9, which in turn activates effector caspases (26). Meanwhile, anti-apoptotic Bcl-2 proteins have been reported to protect cells from many different apoptotic stimuli and are important for cell survival (27,28) and may bind to active Bax to prevent it from damaging the MOM (22,29). Thus, the balance of active anti- and pro-apoptotic Bcl-2 family members determines the fate of cells and alteration of the ratio by aberrant expression of these proteins impairs the normal apoptotic program contributing to various apoptosis-related diseases (30). Therefore, promoting cell apoptosis through regulating the Bcl-2 family proteins has been the main focus in

the development of anticancer therapies. In order to extend the clinical observations of the potential anticancer effect of XJW and help to elucidate the mechanism of its anticancer activity, in this study, we investigated the cellular effect of the XJW on the proliferation and apoptosis of U-2OS human osteosarcoma cells. We found that XJW inhibited the growth through arresting in the G2/M phase of the cell cycle and promoted apoptosis of U-2OS cells by loss of mitochondrial membrane potential ($\Delta\psi_m$), activation of caspase-9 and caspase-3 and upregulation of Bax to Bcl-2 ratio, suggesting that inhibition the proliferation via blocking cell cycle progression at the G2/M phase and promotion of apoptosis via activating of the mitochondrion-dependent pathway may be one of the mechanisms by which XJW can be effective in the treatment of cancer.

Materials and methods

Materials and reagents. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and trypsin were purchased from Hyclone Laboratories Inc. (Logan, UT, USA). A cell cycle assay kit and an apoptosis assay (Annexin V-FITC Apoptosis Detection Kit II) were provided by Becton-Dickinson (San Jose, CA, USA). A JC-1 mitochondrial membrane potential detection assay was obtained from Biotium Inc. (Hayward, CA, USA). Caspase-9 and caspase-3 colorimetric protease assays and Hoechst 33258 were obtained from Invitrogen Inc. (Grand Island, NY, USA). The Bcl-2, Bax and GAPDH primers were purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Bcl-2, Bax antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies and antibody against β -actin were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA).

Herbal preparation. *Moschus*, *Cochinchina momordica seed*, *Radic aconiti Kusnezoffii preparata*, *Resina liquidambaris*, *Frankincense*, *Myrrh*, *Chinese angelica*, *Trogopterus dung*, *Pheretima* and *Pine-soot ink* used in XJW were prepared with traditional methods after harvest (12) and purchased from the Tong Ren Tang pharmaceutical company (Beijing, China). The ten herbs were ground into powder, respectively. XJW was formulated by mixing herbal powders in relative proportions according to the Chinese pharmacopoeia (14) (see Table I). Stock solutions of XJW were prepared by dissolving the XJW powder in water to a concentration of 30 mg/ml and stored at -20°C. The working concentrations of XJW were made by diluting the stock solution in the culture medium.

Cell culture. Human osteosarcoma cell lines U-2OS were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% (v/v) FBS and 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂. The cells were subcultured at 80-90% confluency. Cells used in this study were subjected to no more than 20 cell passages.

Evaluation of cell viability by MTT assay. Cell viability was assessed by the MTT colorimetric assay. U-2OS cells were seeded into 96-well plates (Corning Costar Corporation, Corning, NY, USA) at a density of 1.0x10⁵ cells/ml in 0.1 ml of medium. After 24 h of incubation, the cells were treated with various concentrations of XJW for 48 h. At the end of the

Table I. Composition of Xiao Jin Wan (XJW) formula.

Herb name	Relative proportion
<i>Moschus</i>	10
<i>Cochinchina momordica seed</i>	50
<i>Radic aconiti Kusnezoffii preparata</i>	50
<i>Resina liquidambaris</i>	50
<i>Frankincense</i>	25
<i>Myrrh</i>	25
<i>Chinese angelica</i>	50
<i>Trogopterus dung</i>	25
<i>Pheretima</i>	50
<i>Pine-soot ink</i>	4

treatment, 100 μ l MTT [0.5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 μ l DMSO. The absorbance was measured at 570 nm using an Elx808™ absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The relative cell viability was expressed as the ratio (%) of the absorbance in the experimental wells to that of the control wells (normal culture without treatment). Following this, the IC₅₀ (cytotoxic concentration for 50% cell death) was determined from the dose-response curve.

Observation of morphologic changes. U-2OS cells were seeded into 6-well plates at a density of 2.0x10⁵ cells/well in 2 ml medium. The cells were treated with various dose of XJW for 48 h. Cell morphology was observed using a phase-contrast microscope (Olympus, Japan). The photographs were taken at a magnification, x100.

Detection of cell cycle by flow cytometry analysis with propidium iodide (PI) staining. U-2OS cells were digested with 0.25% trypsin and incubated in 25-cm² culture flasks at a density of 1x10⁵ cells/ml in 4 ml of medium for 24 h and starved for 24 h in serum-free DMEM medium and were treated with various concentration of XJW for 48 h. After treatment, the cell cycle of U-2OS cells were determined by flow cytometric analysis using a fluorescence-activated cell sorting FACSCalibur cytometer and a cell cycle assay kit. PI staining was performed according to the manufacturer's instructions. The percentage of cells in the different phases was calculated by the ModFit LT Version 3.0 Software, and the cell numbers in the G0/G1, S and G2/M phases were obtained.

Assessment of apoptotic morphology by Hoechst 33258 staining. After treatment with various concentrations of XJW, trypsinized adherent cells were collected, washed once with ice-cold PBS, fixed with 1 ml of 4% paraformaldehyde for 20 min, and washed once with ice-cold PBS. Then, the cells were incubated in 1 ml PBS containing 10 μ mol/l Hoechst 33258 at 37°C for 30 min, washed twice and observed using a fluorescence microscopy with standard excitation filters (Leica Dmirb) in random microscopic fields at x200 magnification.

Detection of apoptosis by flow cytometry analysis with Annexin V/PI staining. Following incubated with various doses of XJW, apoptosis of U-2OS cells was determined by flow cytometric (FCM) analysis using a fluorescence-activated cell sorting (FACS) caliber (FACSCalibur, Becton-Dickinson) and the Annexin V-FITC Apoptosis Detection Kit II. Staining was performed according to the manufacturer's instructions and as we previously described (31). The percentage of cells in early apoptosis was calculated by Annexin V-positivity and PI-negativity, while the percentage of cells in late apoptosis was calculated by Annexin V-positivity and PI-positivity.

Measurement of mitochondrial membrane potential ($\Delta\psi$ m) by flow cytometry analysis with JC-1 staining. To evaluate for the loss of mitochondrial membrane potential, a hallmark of apoptosis, cells were stained with the fluorescent dye JC-1, which is a cationic dye that exhibits potential mitochondria-dependent accumulation, indicated by a fluorescence emission shift from green to red. In this experiment, 1x10⁶ treated U-2OS cells were resuspended after trypsinization in 0.5 ml of medium and incubated with 10 μ g/ml of JC-1 at 37°C, 5% CO₂, for 15 min. Both red and green fluorescence emissions were analyzed by flow cytometry.

Analysis of caspase activation. The activity of caspase-9 and caspase-3 were determined with a colorimetric assay using a colorimetric protease assay, following the manufacturer's instructions and our previous description (31). Briefly, after treated with various dose of XJW for 48 h, U-2OS cells were lysed with the manufacturer's provided lysis buffer for 10 min on ice. The lysed cells were centrifuged at 10,000 x g for 1 min. An aliquot (150 μ g) of the protein was incubated with 50 μ l of the colorimetric tetrapeptides, Leu-Glu-His-Asp (LEHD)-pNA (specific substrate of caspase-9) or Asp-Glu-Val-Asp (DEVD)-pNA (specific substrate of caspase-3) at 37°C in the dark for 2 h. Samples were read at 405 nm in an absorbance microplate reader (Elx808, BioTek Instruments Inc.). The data were normalized to the activity of the caspases in control cells and represented as 'fold of control'.

RNA extraction and RT-PCR analysis. U-2OS cells were seeded into 25-cm² culture flasks at a density of 1x10⁵ cells/ml in 4 ml of medium and treated with various doses of XJW for 48 h. Total RNA from U-2OS cells was isolated with TRIzol reagent (Invitrogen). Oligo(dT)-primed RNA (1 μ g) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Bcl-2 or Bax by PCR with Taq DNA polymerase (Fermentas). GAPDH was used as an internal control. The primers and the annealing temperature (°C) used for amplification of Bcl-2, Bax and GAPDH transcripts are as follows: Bcl-2 forward 5'-CAG CTG CAC CTG ACG CCC TT-3', reverse 5'-GCC TCC GTT ATC CTG GAT CC-3', 55°C; Bax forward 5'-TGC TTC AGG GTT TCA TCC AGG-3', reverse 5'-TGG CAA AGT AGA AAA GGG CGA-3', 55°C; GAPDH forward 5'-GT CAT CCA TGA CAA CTT TGG-3', reverse 5'-GA GCT TGA CAA AGT GGT CGT-3', 60°C.

Western blot analysis. U-2OS cells were seeded into 25-cm² culture flasks at a density of 1x10⁵ cells/ml in 4 ml of medium

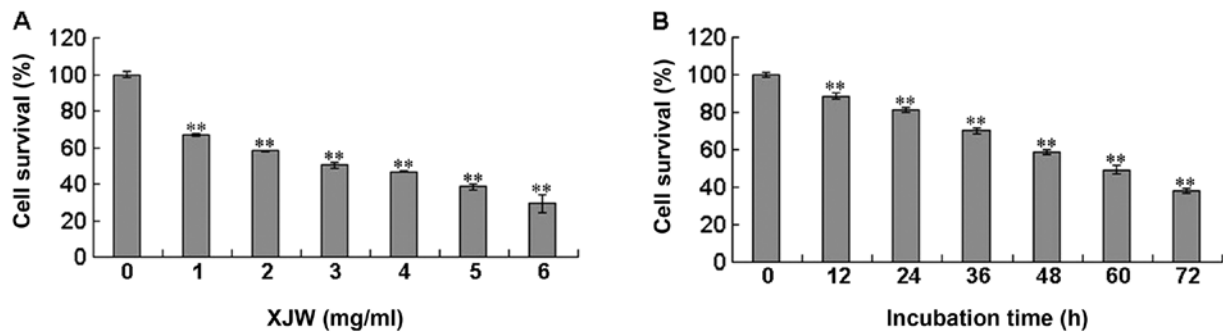


Figure 1. Effect of XJW on cell viability of U-2OS cells. (A) U-2OS cells were treated with the indicated concentrations of XJW for 48 h. (B) Cells were treated with 2.75 mg/ml of XJW for the indicated time periods. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%). Data are averages with SD (error bars) from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, significant vs. control cells.

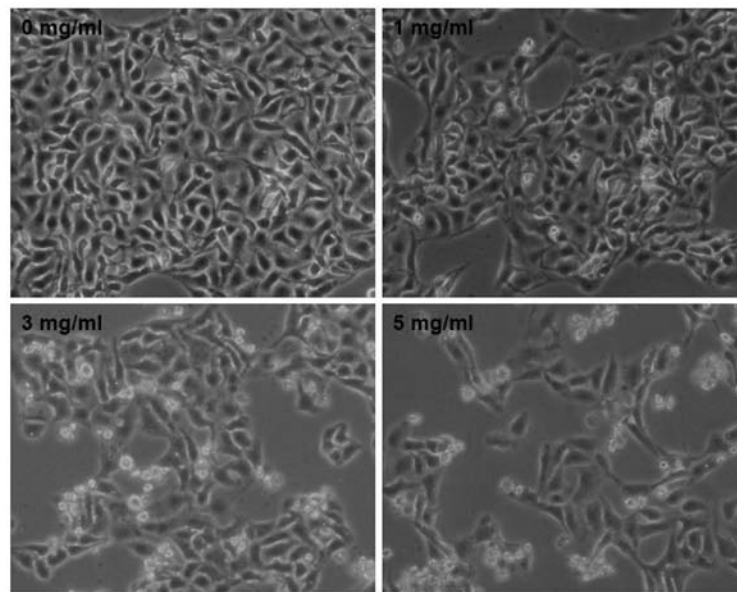


Figure 2. Effect of XJW on the morphological changes of U-2OS cells. U-2OS cells were treated with the indicated concentrations of XJW for 48 h and morphological changes were observed using phase-contrast microscopy. The photographs were taken at a magnification, x200. Images are representative of three independent experiments.

and treated with various doses of XJW for 48 h. The treated cells were lysed with mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails, and the lysates were separated by 12% SDS-PAGE gel under a reducing condition using 100 V for 1 h. The proteins were then electrophoretically transferred onto nitrocellulose membranes using the iBlot western detection stack/iBlot dry blotting system (Invitrogen). Membranes were blocked for 30 min with agitation at RT in SuperBlock T20 (TBS) blocking buffer (Thermo Scientific, Rockford, IL, USA). Membranes were washed in TBS with 0.25% Tween-20 (TBST) and exposed to primary antibodies against Bcl-2 (1:1,000) or Bax (1:500) overnight at 4°C with rocking. β -actin (1:1,000) was also measured as an internal control for protein loading. After membranes were washed in TBST, secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-rabbit) were added at 1:2,500 dilution for 1 h at room temperature and the membranes were washed again in TBST. Finally, the antibody-bound protein bands were detected with ECL, and images were taken using a Bio-Rad ChemiDoc

XRS+ (Bio-Rad Laboratories Inc., Hercules, CA, USA). The grayscale value ratio of the target protein to the internal control was used to measure the relative amount of Bcl-2 and Bax.

Statistical analysis. Data were analyzed using the statistical software SPSS13.0. Statistical analysis of the data was performed with Student's t-test and one-way analysis of variance (ANOVA). P -values < 0.05 was considered as significant.

Results

XJW inhibits the growth of U-2OS cells. The effect of XJW on the viability of U-2OS cells was determined by MTT assay. As shown in Fig. 1A, treatment with 1-6 mg/ml of XJW for 48 h dose-dependently reduced cell viability by 33.24-70.71% compared to untreated control cells ($P < 0.01$), with an estimated half-maximal inhibitory concentration (IC_{50}) value of 2.75 mg/ml. The cell viability was decreased to 29.29% at the highest concentration of XJW (6 mg/ml) in this study. We also

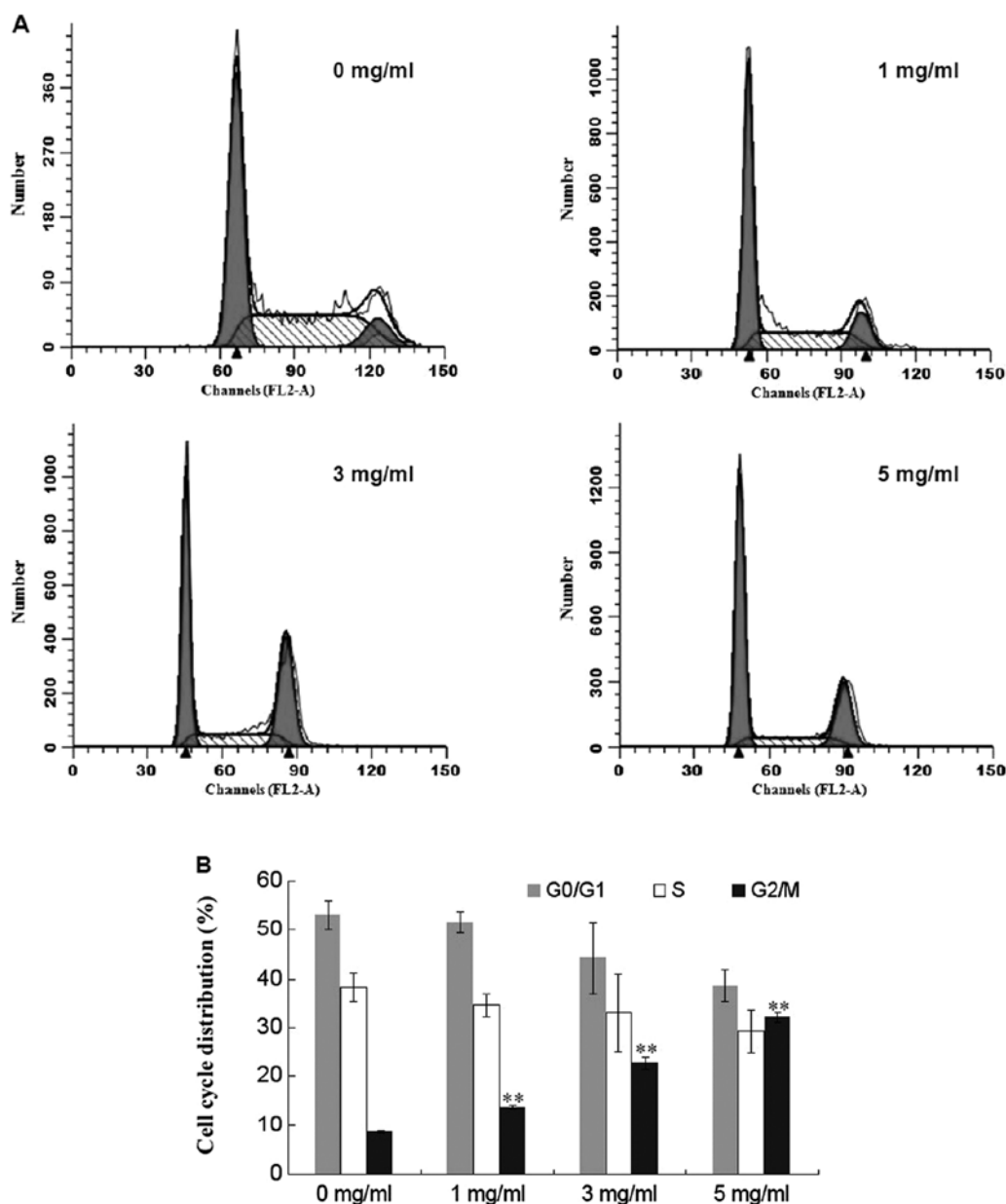


Figure 3. Effect of XJW treatment on the cell cycle of U-2OS cells. (A) After treatment with the indicated concentrations of XJW for 48 h, U-2OS cells were collected and stained with PI staining followed by FACS analysis. (B) The cell cycle distribution (%) of U-2OS cells is shown after treatment with different concentrations of XJW for 48 h. The data shown are averages with SD (error bars); * $P < 0.05$, ** $P < 0.01$, significant vs. untreated cells.

evaluated the effect of 2.75 mg/ml of XJW on cell viability with incubation for different periods of time. As shown in Fig. 1B, treatment with 2.75 mg/ml of XJW led to a gradual decrease in cell viability with the increase of exposure time. These results suggest that XJW inhibits U-2OS cell growth and viability in a dose- and time-dependent manner. To further verify these results, we evaluated the effect of XJW on U-2OS cell morphology via phase-contrast microscopy, since cell morphology in culture is indicative of the healthy status of the cells. As shown in Fig. 2, untreated U-2OS cells appeared as cobblestone, whereas after treatment with various doses of XJW for 48 h many of the cells became bright and shrunken, and detached from each other or floated in the medium. The phenomenon was much more obviously in the higher concentration of XJW. In addition, we evaluated the effect of XJW on the cell cycle of U-2OS cells,

since cell cycle plays an important role in a cell leading to its division and duplication, which is monitored and regulated by cell cycle checkpoints which establish the timing and strength of arrest, repair and apoptotic responses to a damaging agent (15). The G2/M transition is one of the two main checkpoints used by the cell to regulate the progression of the cell cycle. As shown in Fig. 3A and B, the percentage of G2 phase cells following treatment with 1, 3 and 5 mg/ml of XJW, was 13.76 ± 0.41 , 22.64 ± 1.34 and $32.14 \pm 1.02\%$, all of which were significantly higher than that of untreated cells ($8.59 \pm 0.26\%$; $P < 0.01$). Consistently, the percentage of G1-phase cells showed the opposite trend after XJW treatment, suggesting that XJW treatment can inhibit cell cycle of U-2OS cells by inhibiting the G2 to M transition. Taken together, these data demonstrate that MW inhibits the proliferation of U-2OS cells.

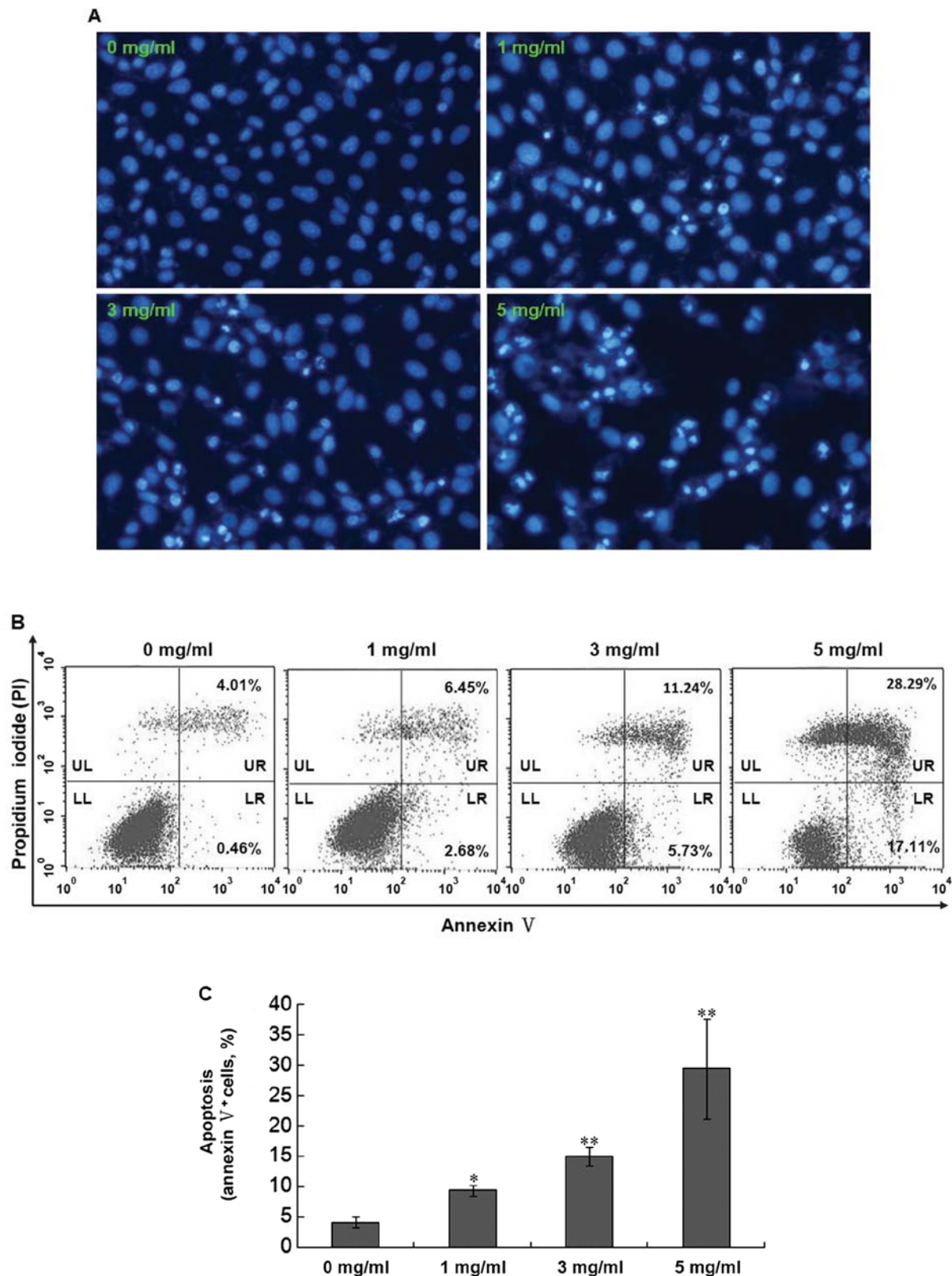


Figure 4. Effect of XJW on the apoptosis of U-2OS cells. After treatment with the indicated concentrations of XJW for 48 h, U-2OS cells were collected and stained with Hoechst 33258 staining and observed under a fluorescence microscope and Annexin V/PI followed by FCM analysis. (A) XJW-mediated cell apoptosis morphologic changes were examined by Hoechst 33258 staining and observed under a fluorescence microscope at x200 magnification. The apoptotic cells detected by the fluorescence microscopy displayed condensed and fragmented nuclei, shrinkage of cell volume in a concentration-dependent manner. (B) Apoptosis analysis in U-2OS cells was assessed by Annexin V/PI double staining. After cells were exposed to four desired concentrations of XJW for 48 h, respectively, the attached and detached cells were collected. Following staining with Annexin V and PI, cells were subjected to flow cytometry analysis. Representative FCM analysis scatter-grams of Annexin V/PI staining display four different cell populations labeled as: double-negative stained cells (LL, lower left) representing the live cell population; Annexin V-positive/PI-negative stained cells (LR, lower right) and Annexin V/PI double-positive stained cells (UR, upper right) representing early apoptosis and late apoptosis, respectively; Annexin V-negative and PI-positive stained cells (UL, upper left) representing dead cells. (C) FCM results are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with the control group.

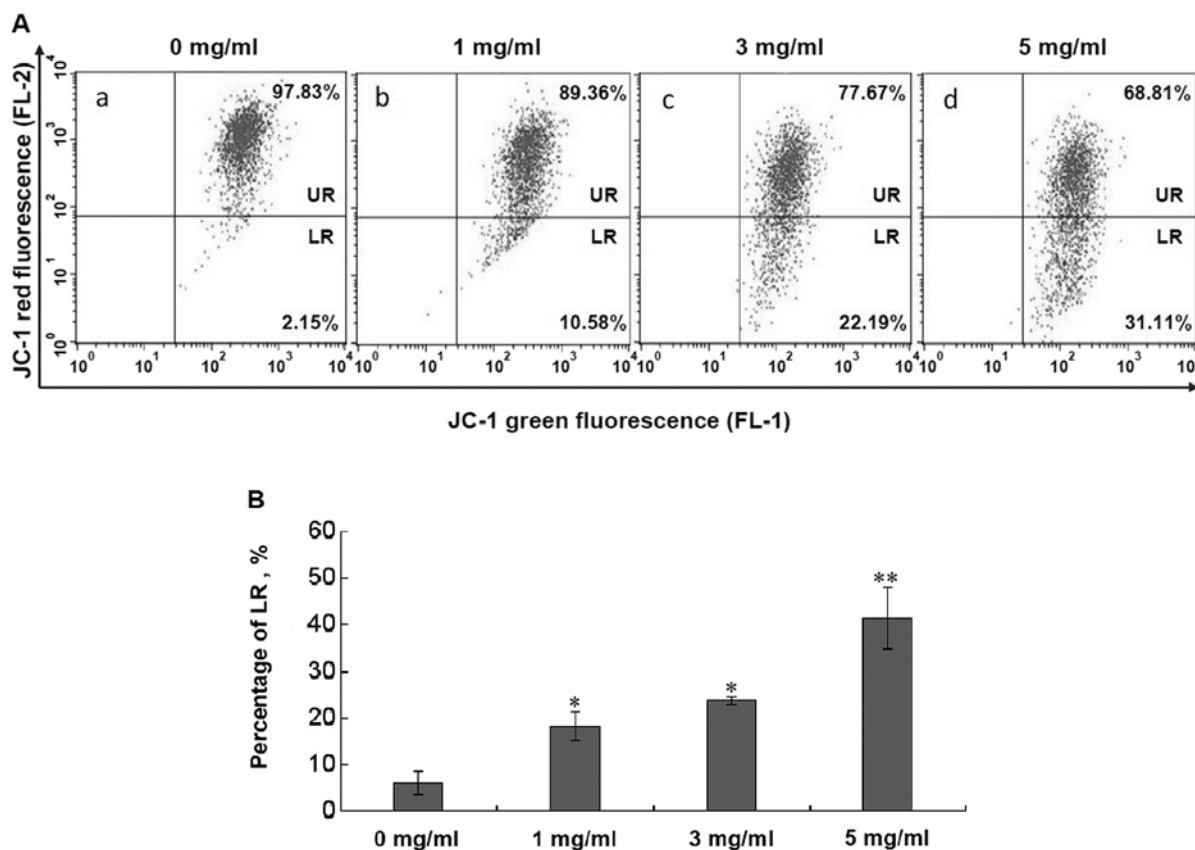


Figure 5. Effect of XJW on the loss of mitochondrial membrane potential in U-2OS cells. U-2OS cells were treated with the indicated concentrations of XJW for 48 h and stained with JC-1. (A) Representative results from JC-1 FCM analysis. (a) JC-1 fluorescence was seen in both the FL-2 and FL-1 channels (UR) in the majority of untreated control cells. A small percentage of the control cells fluoresced in the FL-1 channel and lacked fluorescence in the FL-2 channel (LR). (b-d) XJW treatment groups resulted in an increased number of cells with low red fluorescence (LR) as compared to the untreated cells, indicative of promotion of the alteration in $\Delta\psi_m$. (B) Results are expressed as mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with the control group.

XJW induces apoptosis in U-2OS cells. To determine whether XJW inhibits the growth of U-2OS cells also by inducing apoptosis, the morphologic characteristics of apoptosis was observed. Cells were stained with Hoechst 33258 after treated with XJW for 48 h and detected by fluorescence microscopy. We found that control cells showed distribution of the stain and round homogeneous nuclei, while apoptotic cells increased gradually in a dose-dependent manner and displayed typical changes including condensed and fragmented nucleus (Fig. 4A). For a further assessment of apoptosis induced by XJW, we examined the exposure of phosphatidylserine on the cell surface by Annexin V/PI staining followed by FACS analysis. In this assay, Annexin V/PI double-negative population (labeled as LL in the FACS diagram) indicates viable cells; Annexin V-positive/PI-negative or Annexin V/PI double-positive population (labeled as LR or UR in the FACS diagram) represents cells undergoing early or late apoptosis, respectively. As shown in Fig. 4B and C, the percent of cells undergoing apoptosis following treatment with 0, 1, 3 and 5 mg/ml of XJW (including the early and late apoptotic cells) was 4.16 ± 0.902 , 9.38 ± 0.866 , 15.06 ± 1.553 and $29.45 \pm 8.178\%$, respectively ($P < 0.01$ or 0.05 vs. untreated control cells). This indicates that XJW treatment induces U-2OS cell apoptosis in a dose-dependent manner.

Effect of XJW on the loss of mitochondrial potential ($\Delta\psi_m$) and the activation of caspase-9 and caspase-3. The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate animal cells. The mitochondrial membrane permeabilization, accompanied by the collapse of electrochemical gradient across the mitochondrial membrane, is one of the key events during cellular apoptosis (32). This results in the release of numerous apoptogenic proteins, such as cytochrome c, from the mitochondria triggering the activation of caspases-9 and -3, and eventually inducing apoptosis. To investigate the mechanism of XJW's inducing U-2OS cell apoptosis, we used FACS analysis with JC-1 staining to examine the change in mitochondrial membrane potential after XJW treatment. JC-1 is a lipophilic, cationic dye that selectively enters into mitochondria. In healthy cells with high mitochondrial potential, JC-1 forms J-aggregates with intense red fluorescence (590 nm, FL-2), whereas under apoptotic condition, the mitochondrial membrane potential collapses, so that JC-1 does not accumulate within the mitochondria but remains in the cytoplasm in monomeric form showing green fluorescence (529 nm, FL-1). These fluorescence differences can be detected by FACS analysis using JC-1 green and red channels. As shown in Fig. 5A and B, JC-1 fluorescence was shifted from a JC-1-green-bright/JC-1-red-bright signal in

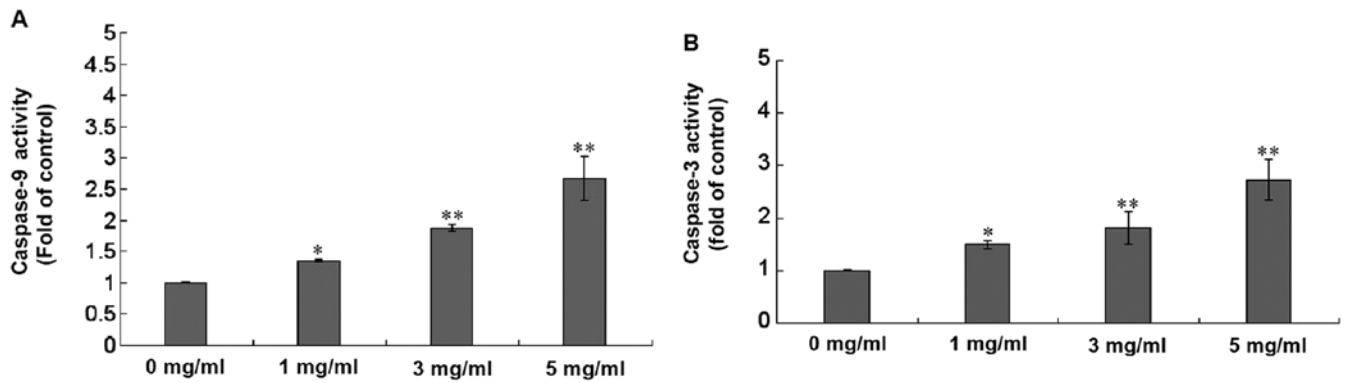


Figure 6. Effect of XJW on the activity of caspases in U-2OS cells. Cells were treated with the indicated concentrations of XJW for 48 h. Caspase-9 and -3 activities were determined by a colorimetric assay. The data were normalized to the caspase activities with untreated control cells and represented as 'fold of control'. Data are averages with SD (error bars) from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, compared with the control group.

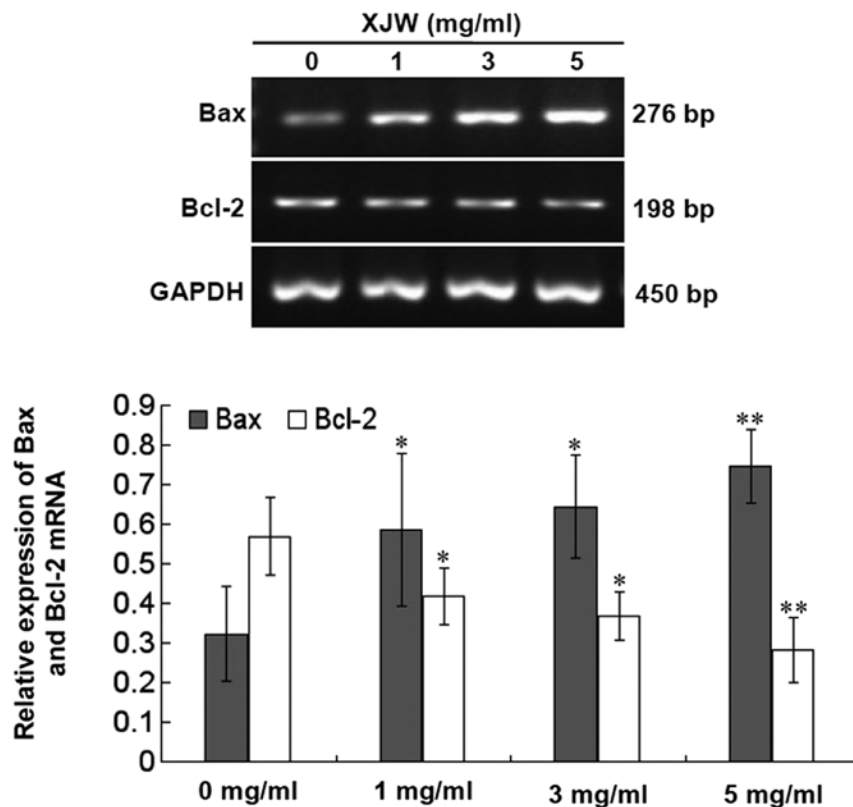


Figure 7. Effect of XJW on the mRNA expression of Bax and Bcl-2 in U-2OS cells. Cells were treated with the indicated concentrations of XJW for 48 h. The mRNA levels of Bax and Bcl-2 in XJW-treated and untreated cells were determined by RT-PCR. GAPDH was used as the internal control. Data shown are averages with SD (error bars) from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, significant vs. control group.

untreated U-2OS cells to a JC-1-green-bright/JC-1-red-dim signal in cells treated with XJW in a dose-dependent fashion, indicating XJW-induced loss of mitochondrial membrane potential in U-2OS cells. To identify the downstream effectors in the apoptotic signaling pathway, the activation of caspases-9 and caspases-3 were examined by a colorimetric assay using specific chromophores, LEHD-pNA (specific substrate of caspase-9) and DEVD-pNA (specific substrate of caspase-3). As shown in Fig. 6A and B, XJW treatment significantly and dose-dependently induced activation of both caspase-9 and

caspase-3 in U-2OS ($P < 0.01$ or 0.05 vs. untreated control cells). These data suggest that XJW promotes U-2OS cell apoptosis via the mitochondrion-dependent pathway.

XJW regulates the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax. Bcl-2 family proteins are key regulators of mitochondrion-mediated apoptosis, including anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax. Tissue homeostasis is maintained by controlling the ratio of active anti- and pro-apoptotic Bcl-2 family proteins. Higher

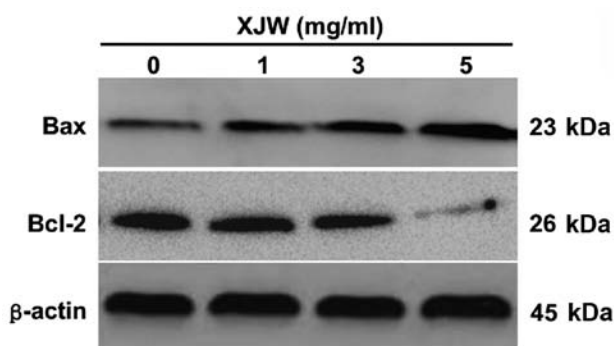


Figure 8. Effect of XJW treatment on the protein expression of Bax and Bcl-2 in U-2OS cells. Cells were treated with the indicated concentrations of XJW for 48 h. The protein expression level of Bax and Bcl-2 in XJW-treated and untreated cells were analyzed by western blotting. β -actin was used as the internal controls. Data are representative of three independent experiments.

Bcl-2-to-Bax ratio by aberrant expression of the proteins is found commonly in various cancers. To further study the mechanism of XJW inducing apoptosis activity, we performed RT-PCR and western blot analysis to examine the mRNA and protein expression of Bcl-2 and Bax in XJW-treated U-2OS cells. The results of the RT-PCR assay showed that XJW treatment profoundly increased Bax and reduced Bcl-2 mRNA expression in U-2OS cells (Fig. 7; $P < 0.01$ or 0.05 vs. untreated control cells); and the pattern of protein expression of Bax and Bcl-2 was similar to their respective mRNA levels (Fig. 8) suggesting that XJW induces mitochondrion-dependent apoptosis in U-2OS cells through the regulation of expression of Bcl-2 family proteins.

Discussion

Cancer cells are characterized by an unregulated increase in cell proliferation and/or a reduction in cell apoptosis (16). In addition, disrupted apoptosis contributes to drug resistance of tumor cells, which has become a significant obstacle for the successful management of patients with malignant tumors including osteosarcoma (5). Moreover, many currently used anticancer agents contain intrinsic and potent cytotoxicity to normal cells, which limits their long-term use and their therapeutic effectiveness (33). These problems highlight the urgent need for the development of novel cancer chemotherapies. Since natural products, such as traditional Chinese herbal medicines, have relatively fewer side-effects as compared to modern chemotherapeutics and have long been used clinically to treat various types of diseases including cancer (34-36), discovering naturally occurring agents with antiproliferative activity is a promising approach for anticancer treatment.

XJW is a well-known traditional Chinese folk-medicine used for eliminating stagnation, removing of blood stasis, promoting of blood circulation and alleviating pain (14), which is commonly used for treatment of various types of diseases including cancers, such as breast cancer. However, the mode of action for its antitumor is still largely unknown. Therefore, before XJW can be further developed as an anticancer agent,

its antitumor activity and underlying molecular mechanism should be elucidated.

Cell cycle plays an important role in U-2OS cells leading to its division and duplication. Moreover, the G2/M transition is one of the two main checkpoints used by the cell to regulate the progression of the cell cycle. Once the checkpoint late in G2 phase is passed, further progression through the cell cycle occurs with little or no interference from extracellular stimuli followed by the decision to continue cell division. To determine the mechanism of the inhibition of XJW, we examined its effect on the G2 to M transition in U-2OS cells via PI staining followed by FACS analysis. We demonstrated that XJW treatment dose-dependently increased the percentage of G2 phase in U-2OS cells after treated with 1, 3 and 5 mg/ml of XJW (Fig. 3B; $P < 0.01$ vs. untreated control cells). The percentage of G1-phase cells showed the opposite trend after XJW treatment, suggesting that XJW treatment can inhibit cell cycle of U-2OS cells by blocking the G2 to M transition. Taken together, these data demonstrate that XJW inhibits the growth of U-2OS cells.

Apoptosis is activated through two major pathways. For the intrinsic pathway, death signals are integrated at the level of the mitochondria. For the extrinsic pathway, death signals are mediated through cell surface receptors. Both pathways eventually lead to the activation of caspases and nucleases, resulting in the destruction of the cell (16). Our experimental results showed that apoptotic cells induced by XJW displayed condensed and fragmented nuclei by Hoechst 33258 staining (Fig. 4A). For the loss of plasma membrane asymmetry is one of the morphologic characteristics of the apoptotic program. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cell environment. Annexin V is a 35-36 kDa Ca^{2+} -dependent phospholipid-binding protein that has a high affinity for PS. Annexin V binds to cells with exposed PS. Therefore, flow cytometry with Annexin V staining was used to further confirmed the results of Hoechst 33258 staining by showing that the important membrane alterations relating to apoptosis in U-2OS cells and the percent apoptosis increased in dose-corresponding manner (Fig. 4B and C). Taken together, these results suggested that XJW indeed induced apoptosis in U-2OS cells. The loss of mitochondrial membrane potential is a hallmark of apoptosis. It is an early event preceding phosphatidylserine externalization and coincides with caspase activation (32,37). In healthy cells, the JC-1 dye stains the mitochondria fluorescent red (38). The negative charge established by the intact mitochondrial membrane potential allows this lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, the J-aggregates are formed. These aggregates are fluorescent red (590 nm). In apoptotic cells, the mitochondrial membrane potential collapses, and JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm in the green fluorescent monomeric form. JC-1-stained apoptotic cells, having primarily green fluorescence (530 nm), are easily differentiated from healthy cells that have red and green fluorescence (39). Using FCM, healthy cells with red JC-1 aggregates are detected in the FL-2 channel and apoptotic cells with green JC-1 monomers are detected in the FL-1 channel. Thus, JC-1-

stained cells that fluoresce in the FL-2 and FL-1 channels (UR quadrant) carry mitochondria with a polarized $\Delta\psi$, whereas JC-1-stained cells that fluoresce in the FL-1 channel and not in the FL-2 channel (LR quadrant) carry mitochondria with a depolarized $\Delta\psi$. Therefore, JC-1 dye-based assay was used to evaluate mitochondrial membrane potential in the study. Our data clearly showed that treatment with XJW leads to a collapse of mitochondrial membrane potential (Fig. 5A and B).

The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate animal cells. Mitochondrial outer membrane permeabilization (MOMP) accompanied by the collapse of electrochemical gradient across the mitochondrial membrane is a key commitment step in the induction of mitochondrion-dependent apoptosis. This is the point of convergence for a large variety of intracellular apoptotic signaling pathways that eventually lead to the release of pro-apoptotic proteins from the mitochondrial intermembrane space, including cytochrome *c*, Smac/DIABLO, and Omi/HtrA2. Released cytochrome *c* activates APAF-1, which oligomerizes to form an apoptosome. This structure, in turn, recruits and activates caspase-9. Activated caspase-9 cleaves and activates executioner caspases, such as caspase-3, and eventually results in apoptosis (32,37,40). Therefore, to evaluate the effect of XJW on the mitochondrion-dependent apoptosis pathway, we evaluated the activation of caspase-9 and caspase-3. In this study, we found that XJW activated both caspase-9 and caspase-3 in U-2OS cells in a dose-dependent manner (Fig. 6A and B). Thus, XJW-induced U-2OS cell death is accompanied by the activities of caspases-9 and caspase-3, which then stimulates the molecular cascade for apoptosis.

Occurrence of mitochondrial-dependent apoptosis is typically governed by contradicting the Bcl-2 family (41). Bcl-2 is a well-known anti-apoptotic protein that can prevent cytochrome *c* release whereas Bax is a pro-apoptotic protein that enhance cytochrome *c* release from mitochondria into cytosol (42), which is responsible for activating caspase-9, caspase-3 and facilitates apoptosis (43). Therefore, the ratio of Bax to Bcl-2 is a critical for determining the fate of cells. In this study, we demonstrated that XJW treatment dose-dependently enhances Bax mRNA expression and reduces Bcl-2 mRNA expression in U-2OS cells (Fig. 7; $P < 0.01$ or 0.05 vs. untreated control cells). This indicates that XJW induces apoptosis by affecting the ratio of Bax/Bcl-2 at transcriptional level. We further studied the role of XJW on the expression of proteins involved in the mitochondrial pathway. The results showed that XJW treatment upregulates Bax protein expression and downregulates Bcl-2 protein expression (Fig. 8), which is in accordance with the pattern of their mRNA expression after XJW treatment.

In conclusion, our data for the first time demonstrate that XJW inhibits U-2OS cell proliferation via cell cycle G2/M arrest and promotes apoptosis via the mitochondrion-dependent pathway. This study may provide a mechanistic background for the introduction of this new type of promising therapeutic agent in the study of cancer chemotherapy.

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