Antiproliferation potential of withaferin A on human osteosarcoma cells via the inhibition of G2/M checkpoint proteins

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Abstract. Withaferin A (WA) is a well-known steroidal lactone of the medicinally important plant, *Withania somnifera*. This secondary metabolite has been noted for its anticancer effects against a number of human cancer cell lines. However, there are a limited number of studies investigating the growth inhibitory potential of WA against human osteosarcoma cells and the underlying molecular mechanisms. Thus, in the present study, the antiproliferative activities of WA, along with the underlying mechanisms of action, were investigated using flow cytometry for cell cycle distribution and western blot analysis for the assessment of various checkpoint proteins. In addition, the antiproliferative activity was evaluated using a sulforhodamine B assay, where MG-63 and U2OS human osteosarcoma cell lines were treated with different concentrations of WA. Furthermore, the mRNA expression levels of the checkpoint proteins in the WA-treated MG-63 and U2OS cells were examined. The results obtained corresponded with the western blot analysis results. Furthermore, WA was shown to significantly inhibit the proliferation of the two types of treated cell lines (MG-63 and U2OS). Flow cytometric analysis revealed that WA induced cell cycle arrest at the G2/M phase, which was associated with the inhibition of cyclin B1, cyclin A, Cdk2 and p-Cdc2 (Tyr15) expression and an increase in the levels of p-Chk1 (Ser345) and p-Chk2 (Thr68). In conclusion, the present study found that the antiproliferative potential of WA was associated with the induction of cell cycle arrest at the G2/M phase, which was a result of the attenuation of the expression levels of G2/M checkpoint proteins.

Introduction

Cancer is a major human disease that causes a considerable economic burden to health institutions worldwide. Although significant progress has been made in cancer treatment, the incidence and mortality rates for almost every type of cancer remain high (1). Thus, further research is required to develop safe and effective drugs for the treatment of human cancers. Among the various types of cancer, primary malignant osteosarcoma predominantly affects the growing bones of adolescents and children, and is characterized by locally aggressive growth and an early metastatic potential (2). The incidence rate of osteosarcoma among younger individuals (<20 years-old) is 8.7 per million, and this rate is higher in males compared with females (3). Respiratory failure due to lung metastasis is the major cause of mortality in patients, despite significant improvements in clinical treatments with combination intensive chemotherapy and surgical resection (4,5). However, patients frequently suffer from intolerable, acute and long-term toxicities following the administration of approved chemotherapeutic agents (6). Thus, more effective and improved treatment strategies are required to prevent the progression of osteosarcoma.

Withanolides (steroidal lactones) have been isolated from various species of the Solanaceae family, including *Withania somnifera (W. somnifera)*, which is widely distributed across the South Asian subcontinent (7). In the Ayurveda system of medicine, *Withania* is noted for its aphrodisiac, sedative and life-prolonging properties (8). Extracts from different parts of *W. somnifera* have been evaluated for various biological activities, such as cholinesterase inhibition (9,10), anti-inflammatory properties via cyclooxygenase-2 enzyme inhibition from leaf extracts (11), antibacterial properties (12) and sex hormone deficiency regulation in rats with diabetes (13). The isolation and identification of several withanolides with anti-glycation (14) and antipyretic activities (15) from *Withania* has been reported. Among the reported withanolides, withaferin A (WA) (16) (as seen in Fig. 1) and ashwagandhanolide (17), obtained from the leaves and roots, respectively, are well-known potent secondary metabolites of *W. somnifera* and are reported to possess anticancer properties. Antiproliferative, antimetastatic, antiangiogenic, anti-invasive and proapoptotic activities of withanolides have been reported, with the underlying mechanism found to be associated with the suppression of nuclear factor (NF)-κB and NF-κB-regulated gene products (18). Previous studies have demonstrated the anticancer activity of WA in prostate (19,20), breast (21), leukemia (22) and melanoma cancer cells (23). In addition, a previous study demonstrated that WA induces apoptosis in

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prostate cancer cells through Par-4 induction (19) and inhibits IκB kinase activation via a thioalkylation-sensitive redox mechanism (24); by covalently modifying the cysteine residue, WA targets the intermediate filament protein, vimentin (25). Furthermore, WA has been shown to induce actin microfilament aggregation by targeting annexin II (26). The aim of the present study was to investigate the antiproliferative effects of WA on two osteosarcoma cell lines (U2OS and MG-63) and determine the mechanism underlying the induction of cell cycle arrest.

Materials and methods

Reagents. WA, bovine serum albumin (BSA), ribonuclease A (RNase A), propidium iodide (PI), sulforhodamine B (SRB) and a rabbit polyclonal anti-β-actin antibody (1:1,000; cat. no. A2668) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI 1640 medium, trypsin-EDTA and antibiotic-antimycotic were purchased from Gibco Life Technologies (Grand Island, NY, USA). The following antibodies: Rabbit polyclonal Cdk2 (1:1,000; cat. no. sc-163), rabbit polyclonal cyclin A (1:1,000; sc-751), mouse monoclonal cyclin B1 (1:1,000; cat. no. sc-245) and mouse monoclonal Cdc2 (1:1,000; cat. no. sc-54) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The following antibodies: Rabbit polyclonal p-Cdc2 (Tyr15; 1:1,000; cat. no. P06493), rabbit polyclonal p-Chk1 (Ser345; 1:1,000; cat. no. 2341S), rabbit polyclonal p-Chk2 (Thr68; 1:1,000; cat. no. 2661), rabbit polyclonal Chk1 (1:1,000; cat. no. 2344S) and rabbit polyclonal Chk2 (1:1,000; cat. no. 2662) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Ethics and cell lines. U2OS and MG-63 human osteosarcoma cell lines were obtained from the China Center for Type Culture Collection (Wuhan, China), following standard ethical procedures (27). Following isolation, the osteosarcoma cells were identified based on histological type and grade. The cell lines were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin G and 100 U/ml streptomycin (Sigma-Aldrich). The cells were subsequently incubated in a humidified incubator at 37˚C with 5% CO_2. All cells used in the experiments were in the logarithmic phase.

Cell proliferation assay. The antiproliferation potential of WA in U2OS and MG-63 cells was examined using an SRB assay (11). Cell lines were seeded at a density of $9x10^3$ cells/well into 96-well plates and allowed to adhere for 72 h. The cells were subsequently treated with WA at various concentrations (0.1, 1 and 10 μM). Dimethyl sulfoxide (DMSO) was used as the experimental control. The results were presented as percentages relative to the solvent-treated control incubations. Using nonlinear regression analysis, the IC_{50} values were calculated.

Cell cycle analysis. U2OS and MG-63 cell lines were plated into 35-mm culture dishes at a density of $1x10^6$ cells/100 mm and incubated for 24 h. The medium was replaced with fresh medium containing WA (at concentrations of 0.1, 1 and 10 μM) and 0.1% DMSO alone. Following incubation for 24 h at 37˚C, the cells were harvested, washed with phosphate-buffered saline and fixed with 70% (v/v) ice-cold ethanol for 1 h at 4˚C. Subsequently, 50 μg/ml RNase A and 50 μg/ml PI were added to the fixed cells. In total, ~10,000 cells were analyzed by flow cytometry (BD Accuri™ C6; BD Biosciences, Franklin Lakes, NJ, USA). All experiments were repeated three times and data analysis was recorded using BD CellQuest™ cell cycle analysis software (BD Biosciences).

Western blot analysis. U2OS and MG-63 cell lines were incybated with varying concentrations of WA (0.1, 1 and 10 μM) for 24 h. The cell samples were lysed in sample buffer [150 mM Tris (pH 6.8), 8 M urea, 50 mM DTT, 2% sodium dodecyl sulfate, 15% sucrose, 2 mM EDTA, 0.01% bromophenol blue and 1% protease and phosphatase inhibitor cocktails], sonicated (Ultrasonic Homogenizer 300MP; BioLogics, Manassas, VA, USA) and the protein concentrations were recorded using the Lowry protein assay method (28). Subsequently, samples of 40-45 μg were refluxed, run through an 8-12% Bis/Tris gel (Invitrogen Life Technologies, Carlsbad, CA, USA) using 5X 2-(N-morpholino)ethanesulfonic buffer (Sigma-Aldrich) and transferred to an Immobilon-nitrocellulose membrane (Sigma-Aldrich). The membrane was blocked with 5% skimmed milk or 3% BSA in Tris-buffered saline Tween-20 [TBST; 150 mM NaCl, 50 mM Tris (pH 7.5) and 0.1% Tween-20] (blocking buffer). Next, the membrane was probed with antibodies targeted against cyclin A, cyclin B1, Cdk2, p-Cdc2 (Tyr15), Cdc2, p-Chk1 (Ser345), Chk1, p-Chk2 (Thr68), Chk2 and β-actin (1:1,000) overnight at 4˚C in blocking buffer. Following washing with TBST, the membranes were probed with anti-rabbit (cat. no. 7074; Cell Signalling Technology, Inc.) and goat anti-mouse (cat. no. 11011MP; Life Technologies, Grand Island, NY, USA) immunoglobulin G secondary antibodies (1:5,000) in blocking buffer at room temperature for 90 min, and after washing again, the fluorescence was subsequently detected using a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay. In the RT-qPCR analysis, the two cell lines were treated with 10 μM WA for 12 h. Extraction of the total cellular RNA was performed using TRIzol® reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. For reverse transcription of the RNA into cDNA, a SuperScript III First-Strand Synthesis kit (Invitrogen Life Technologies) was used. Subsequently, the qPCR was conducted using an ABI Prism 7900HT Real-Time PCR system (PerkinElmer, Inc., Waltham, MA, USA) with SYBR Green PCR Master Mix (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers used in the RT-qPCR analysis are presented in Table I. The mRNA expression levels were recorded as the fold change relative to the control. Following completion of the RT-qPCR, cycle numbers (Ct values) in which the signal intensity was equal to the threshold value were obtained from the software (SDS Plate Utility v2.3; Applied Biosystems Life Technologies). The relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (29).

Table I. Primer sequences used for reverse transcription quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Cdk2	GAAACTCTGAAGCCGACCAG	GCCCTCTCAGTGTCCAGAAG
Cyclin B1	CGGGAAGTCACTGGAAACAT	AAACATGGCAGTGACACCAA
Cyclin A1	GTCAGAGAGGGGATGGCAT	CCAGTCCACCAGAATCGTG
Cdc2	GGTTCCTAGTACTGCAATTCG	TTTGCCAGAAATTCGTTTGG
Chk1	GGTGCCTATGGAGAAGTTCAA	TCTACGGCACGCTTCATATC
Chk2	CGGATGTTGAGGCTCACGA	TATGCCCTGGGACTGTGAGG
β-actin	GCTCGTCGTCGACAACGGCTC	CAAACATGCTCTGGGTCATCTTCTC

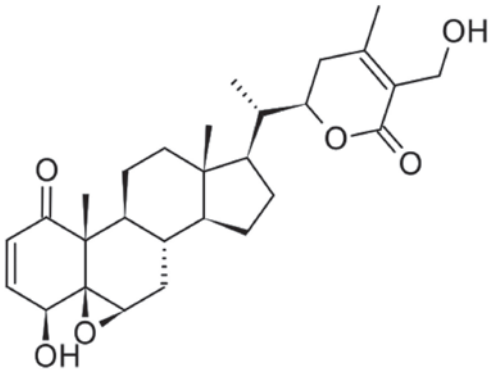

Figure 1. Chemical structure of withaferin A (molecular formula, $C_{28}H_{38}O_6$; molecular mass, 470.60 g/mol).

Statistical analysis. All experiments were repeated a minimum of three times. Data are expressed as the mean ± standard deviation. The treated groups were compared using one-way analysis of variance with SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA), where $P<0.05$ was considered to indicate a statistically significant difference. Relative percentages were calculated using GraphPad Prism software, version 4.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

WA prevents the proliferation of MG-63 and U2OS osteosarcoma cells. Two human osteosarcoma cells lines, MG-63 (Fig. 2A) and U2OS (Fig. 2B), were treated with varying concentrations of WA for 72 h to assess the antiproliferative effect of WA. The cell proliferation of each of the tested samples was recorded using an SRB assay. Higher concentrations of WA were shown to exhibit potent antiproliferative effects. Thus, the results revealed that WA produced a potent antiproliferative effect by significantly inhibiting the growth of the tested cell lines.

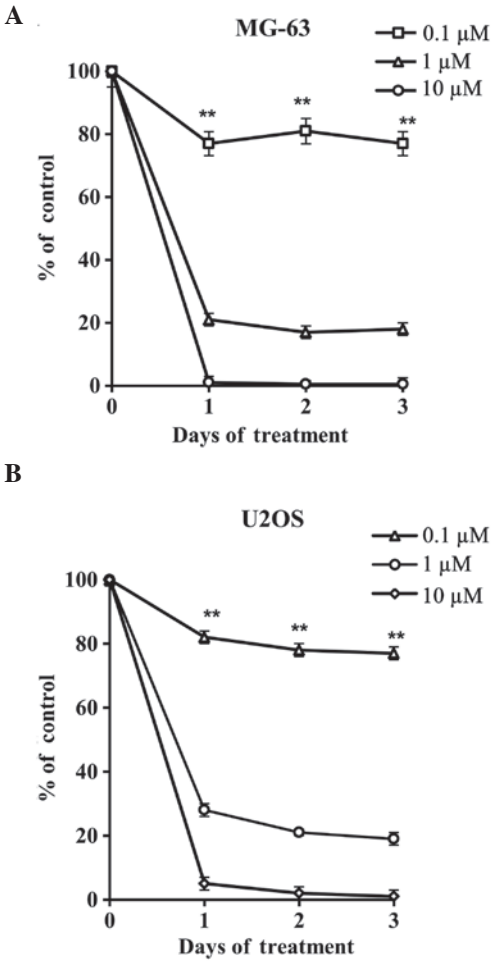

Figure 2. Effect of withaferin A on the proliferation of MG-63 and U2OS osteosarcoma cells. MG-63 and U2OS cells were plated on 96-well plates in RPMI medium supplemented with 10% fetal bovine serum, and incubated with different concentrations of the test compound for 72 h. The antiproliferative effect was determined using a sulforhodamine B assay. The total percentage of cell growth was calculated as the mean absorbance of samples/absorbance of the vehicle-treated control. Data are presented as the mean ± standard deviation (n=3). $^{**}P<0.01$, as compared with the control.

Effect of WA on cell cycle distribution. MG-63 and U2OS cells were treated with varying concentrations of WA for 24 h and the underlying antiproliferative mechanisms were investigated via flow cytometric analysis with PI staining. Cell cycle distribution analysis revealed that WA administration resulted in the significant arrest of MG-63 and U2OS human osteosarcoma cells at the G2/M phase in a dose-dependent manner (Fig. 3). At a concentration of 10 μM WA, more than half of the cells were arrested at the G2/M phase.

Western blot analysis on the expression levels of G2/M phase cell cycle checkpoint proteins. Western blot analysis was used to examine the expression levels of G2/M cell cycle regula-

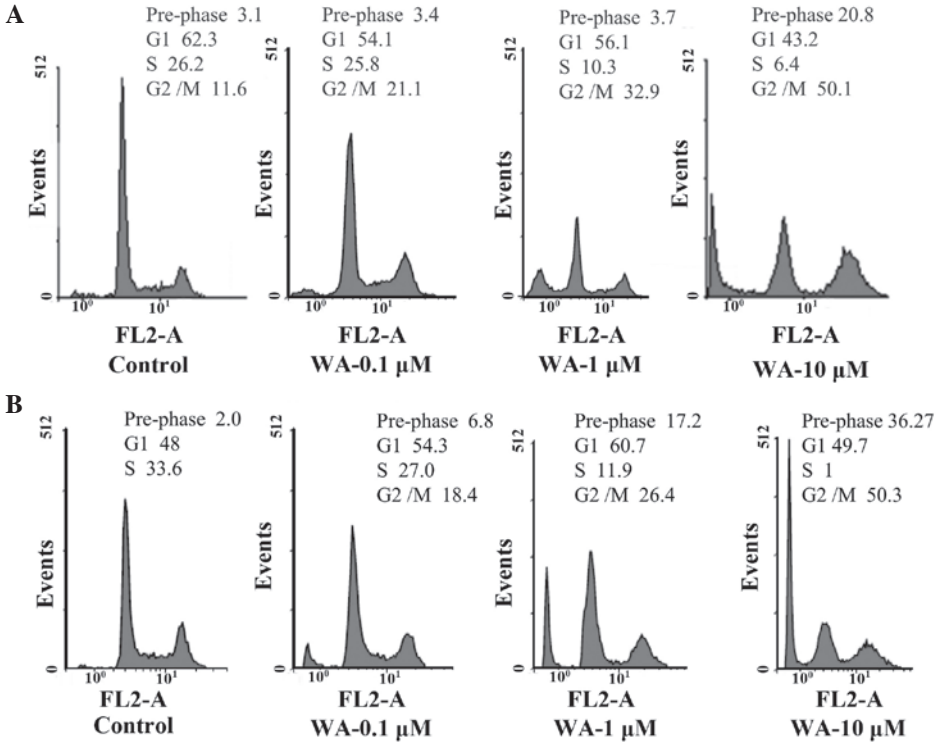

Figure 3. Effect of WA on the regulation of cell cycle distribution in MG-63 and U2OS cells. Cells were seeded at a density of $1x10^6$ cells/100 mm dish in RPMI medium supplemented with 10% fetal bovine serum, and treated with various concentrations of WA for 24 h. The cell cycle distribution was analyzed by flow cytometry. WA, withaferin A.

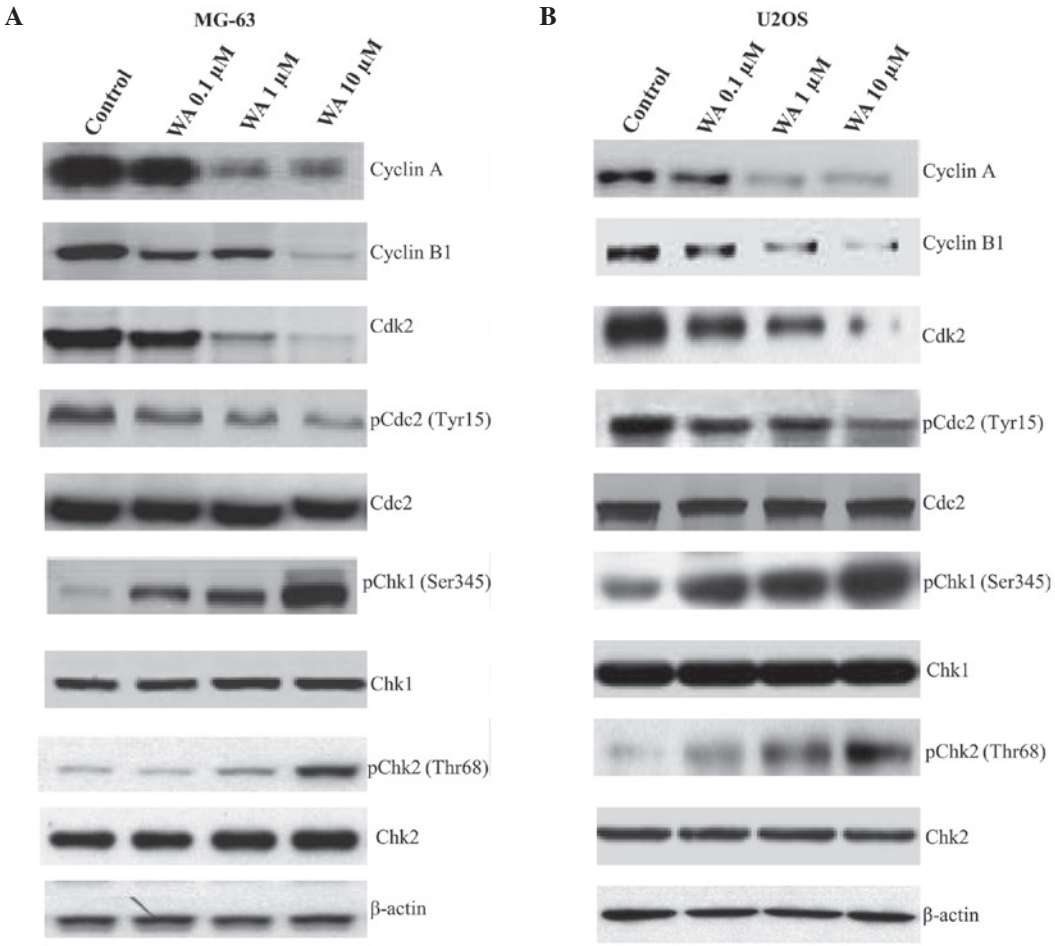

Figure 4. Effect of WA on the expression levels of cell cycle-associated proteins in MG-63 and U2OS cells. Cells were treated with the indicated concentrations of WA for 24 h. The expression levels of the proteins were analyzed by western blot analysis. WA, withaferin A.

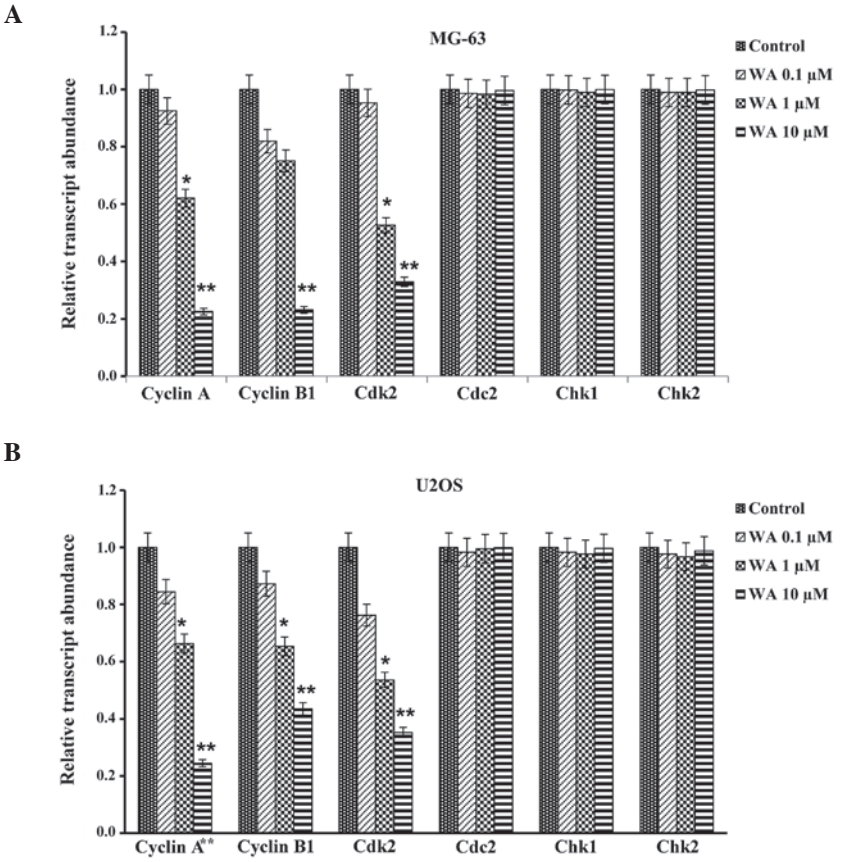

Figure 5. Effect of WA on the mRNA expression levels of cell cycle-associated proteins. MG-63 and U2OS cells were treated for 24 h with various concentrations of WA, and the mRNA expression levels were measured by reverse transcription quantitative polymerase chain reaction. The results obtained were in agreement with the western blot analysis results, with a maximum decrease in mRNA expression levels observed with 10 μM WA treatment in the two cell lines. WA, withaferin A. $^{*}P<0.05$ and $^{**}P<0.01$, as compared with the control.

tory proteins in MG-63 (Fig. 4A) and U2OS (Fig. 4B) human osteosarcoma cell lines treated with WA, in order to determine whether the cell cycle arrest was associated with the regulation of cell cycle checkpoint proteins. There was a significant decrease in the levels of cyclin A, cyclin B1, Cdk2 and p-Cdc2 (Tyr15) in the MG-63 and U2OS cells; however, the protein expression levels of p-Chk1 (Ser345) and p-Chk2 (Thr68) increased. No statistically significant changes were observed in the levels of Cdc2 and Chk2 in the two cell lines following WA treatment.

Effect of WA on the mRNA expression levels of the cell cycle checkpoint proteins. In order to determine the effect of WA on the mRNA expression levels of cell cycle checkpoint proteins, the expression levels of G2/M cell cycle regulatory proteins, including cyclin A, cyclin B1, Cdk2, Cdc2, Chk1 and Chk2, were elucidated by RT-qPCR analysis for the MG-63 (Fig. 5A) and U2OS (Fig. 5B) cell lines. There was a significant decrease in the mRNA expression levels of cyclin A, cyclin B1 and Cdk2, while no marked changes were observed in the mRNA expression levels of Cdc2, Chk1 and Chk2 in the two cell lines.

Discussion

WA is a well-known steroidal lactone from the withanolides group. These are major constituents identified from the important medicinal plant, *Withania somnifera*, and the related Solanaceae family (30). A number of biological properties of withanolides have been reported, including anti-inflammatory, antitumor, antibacterial, antidepressant, antioxidant, antiulcer, cytotoxic, quinone reductase inducive, antileishmanial, antitrypanosomal, immunosuppressive, cognition-enhancing and memory-improving effects, as well as hypotensive, bradycardic and respiratory-stimulating properties (31-33). Withanolides have been studied extensively, and >130 withanolides are known and >40 withanolides have been isolated (34). Certain newly-isolated withanolides, including withangulatins B, C, G, H and I (33), have been shown to exert cytotoxic activity. Withanolide A possesses a reactive enone moiety in the A ring, an epoxy alcohol in the B ring, as well as a tetrasubstituted unsaturated lactone side chain. The main synthetic challenges arise from the stereoselective construction of the side chain, the oxidation pattern of the A and B ring, and the diastereoselective instalment of a tertiary alcohol at C20 (35). Furthermore, structure-activity relationship studies have confirmed that the unsaturated ring A and epoxide play an important role in the cytotoxic activity of withanolides (32). Withangulatin A has been shown to inhibit topoisomerase II and induce a heat shock response (36,37). Zaarur *et al* (38) suggested that withangulatin A may induce the heat shock response by inhibiting Hsp90 activity.

The present study investigated the anticancer potential of WA in MG-63 and U2OS human osteosarcoma cancer cell

lines. The results revealed that WA exhibited potent antiproliferative effects. Data from the cell cycle distribution analysis showed that WA treatment at a concentration of 10 μM caused a significant accumulation of treated cells that were arrested in the G2/M phase; this effect was shown to be dose-dependent. Furthermore, western blot analysis revealed that WA significantly regulated the protein expression levels of G2/M cell cycle regulatory proteins in the two cell lines.

The key component involved in G2 to M phase transition is cyclin B1 (39). In addition, cyclin A/Cdk2 complexes are known to participate in the initiation of mitosis in human cancer cells (40). The DNA damage sensors, Chk1 and Chk2, participate in G2/M checkpoint control through the ataxia-telangiectasia mutated (ATM)/ATM-RAD3-related (ATR) pathway. In the current study, a significant decrease in the expression levels of cyclin A, cyclin B1, Cdk2 and p-Cdc2 (Tyr15) were observed, whereas the expression levels of p-Chk1 (Ser345) and p-Chk2 (Thr68) increased significantly in the two treated cell lines. At a concentration of 10 μM, WA was found to exhibit the maximal effect on protein expression and cell cycle arrest. At a transcription level, decreases in the mRNA expression levels of cyclin A, cyclin B1 and Cdk2 were observed, while no change was observed in the mRNA expression levels of Cdc2, Chk1 and Chk2 in the two treated cell lines.

In conclusion, the results of the present study demonstrated that the antiproliferative effects of WA on the human osteosarcoma cell lines, MG-63 and U2OS, at the G2/M phase were primarily associated with cell cycle arrest. These observations indicate that WA may be used in human osteosarcoma therapy following further clinical investigation. The main limitation of the present study was the recruitment of all participants from a single centre. This study may serve as a basis for future prospective studies. Future clinical investigation should be conducted prospectively, and include a large number of patients from various centres, so that results can be generalized.

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