ORIGINAL ARTICLE

Withaferin-A Induces Apoptosis in Osteosarcoma U2OS Cell Line via Generation of ROS and Disruption of Mitochondrial Membrane Potential

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ABSTRACT

Background: Withaferin-A (WF-A) is a well-known dietary compound isolated from *Withania sominifera*. It has tremendous pharmacological potential and has been shown to exhibit antiproliferative activity against several types of cancerous cells. Currently, the main focus of anti-cancer therapeutic development is to identify apoptosis inducing drug-like molecules. Osteosarcoma is a rare type of osteocancer, affecting human. The present study therefore focused on the evaluation of antitumor potential of WF-A against several osteosarcoma cell lines. **Materials and Methods:** MTT assay was used to evaluate WF-A against osteosarcoma cell lines and to calculate the IC_{50}. DAPI staining was used to confirm the apoptosis inducing potential of WF-A. Mitochondrial membrane potential, reactive oxygen species (ROS) assay, and Western blotting were used to confirm the basis of apoptosis. **Results:** The results revealed that that WF-A exhibited strong antiproliferative activity against all the cells lines, with IC_{50} ranging from 0.32 to 7.6 µM. The lowest IC_{50} (0.32 µM) was observed against U2OS cell line and therefore it was selected for further analysis. DAPI staining indicated that WF-A exhibited antiproliferative activity via induction of apoptosis. Moreover, WF-A induced ROS-mediated reduction in mitochondrial membrane potential ΔΨm) in a dose-dependent manner and activation of caspase-3 in osteosarcoma cells. **Conclusion**: We propose that WF-A may prove a potent therapeutic agent for inducing apoptosis in osteosarcoma cell lines via generation of ROS and disruption of mitochondrial membrane potential.

Key words: Osteosarcoma, WF-A, caspase-3, apoptosis, U2OS

SUMMARY
- WF-A exhibits strong anticancer activity against osteosarcoma cell lines
- Antiproliferative activity of WF-A is via induction of apoptosis
- WF-A induced ROS-mediated reduction in mitochondrial membrane potential
- WF-A induced expression of caspase-3 in osteosarcoma cells.
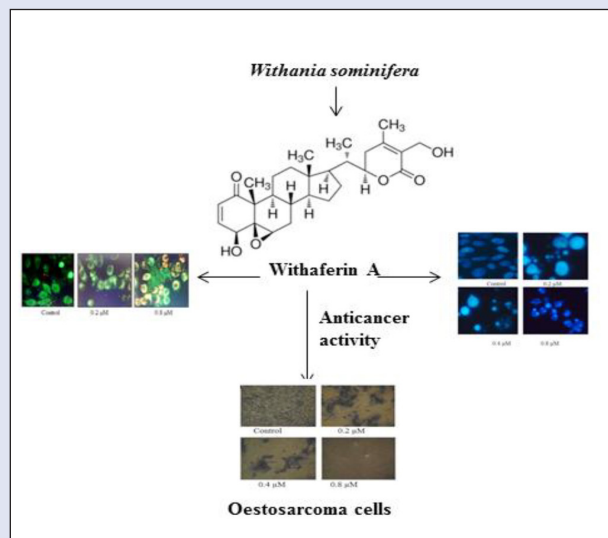
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INTRODUCTION

Osteosarcoma (OS) is one of the most common primary osteotumor affecting young adults with an incidence of approximately 0.4 per 100,000 population per year.[1] OS is an aggressive malignant cancer of mesenchymal origin that[2] shows high variability in therapy response between patients. Therefore, a single therapeutic agent cannot be used for curing all OS patients. OS is currently being treated using the pre- and postoperative chemotherapy after surgical removal of tumor.[3] The survival rate of conventional treated OS patients is only 50-60%, while that for recurrent OS patients is 20-30%.[4] OS is generally characterized by several genetic alterations at varying frequencies. However, OS-specific mutation or pathway is still a big challenge and is yet to be fully explored. Therefore, it is imperative to identify the effective and novel drug-like molecules for improving the survival rate of patients and

control the metastasis of OS. Although, in the last few decades, many novel molecules have been developed as the therapeutic agent to cure OS patients. These molecules are tested in multimodal therapies with dose variations of conventional drugs, but the vast majority of molecules
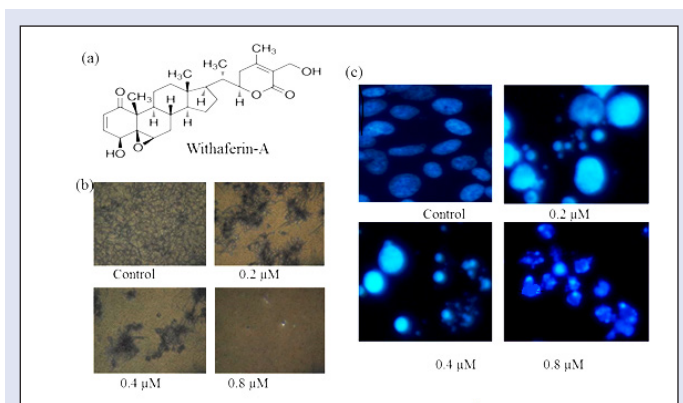
Figure 1: Antiproliferative effect of WF-A on U2OS cells. (a) Structure of WF-A phytomolecule (b) Formazen crystal assay. WF-A inhibits the formazen crystal formation and showed cell death in a dose-dependent manner. (C) DAPI staining showing WF-A induces apoptosis in a dose-dependent manner

failed to enter in phase III clinical trials due lack efficacy and selectivity. The rarity of OS disease, resources, and a limited number of patients are the major factors that limit the OS-related studies.[5,6] Currently, most of the research has focused to design the drug-like molecules for targeting the single mutation of kinases deregulated in OS such as insulin-like growth factor 1 receptor (IGF-1R). IGF-1 and -2 involved in cell growth as well as in cell survival pathways.[7,8] Phytomolecules are the vast variety of medically active compounds that are being frequently used as drugs against several types of diseases, which include, but are limited to, cancer, diabetes, and malaria.[9-13] *Withania somnifera* L. Dunal (Solanaceae) is an ancient Indian medicinal plant known as Ashwagandha. It is well known for its therapeutic properties and widely used in traditional systems of Indian medicine including, Siddha, Unani, and Ayurveda.[14] Withaferin-A (WF-A) is one of the dietary compounds [Figure 1A] isolated from *Withania* plant and medicinally used as antioxidant, antitumor, Cox-2 inhibitor, apoptosis inducer, and cell cycle modulator.[14,15] WF-A has been reported to arrest the cell cycle in G2/M in breast tumor through modulating the phosphorylation of CDK-1 and histone 3 as well as downregulating the expression of CDC25C and securin.[15] However, the antitumor potential of WA is demonstrated against various cancer types *in vivo* and *in vitro* such as the prostate (induction of Par-4 and inhibition of HSP-90),[16,17] breast (induction of G2/M arrest, FOXO3a and Bim regulation, decrease in the level of estrogen receptor-α, inhibition of STAT3),[14,18-23] colon (inhibition of Notch-1),[16] and cervical (downregulation of HPV E6 and E7 proteins, induction of p53 and apoptotic genes, and downregulation of STAT3 expression).[22] Moreover, WF-A activates the p38MAPK, leading to apoptosis through the marked reduction in mitochondrial membrane potential ($\Delta\Psi$m) and caspase-3 activation in lymphoblastic and myeloid leukemia.[23,24] Likewise, WF-A inhibited the NF-κB nuclear transcriptional factor in a cellular model of cystic fibrosis inflammation, induced by *Pseudomonas aeruginosa*.[24] However, the antitumor potential of WF-A against OS has not been evaluated as yet. The present study was designed to determine the antiproliferative potential of WF-A against the OS and investigate the possible mechanism of action. In this study, we examined the antiproliferative potential of WF-A against low- and high-proliferative OS cell lines. We report that WF-A triggered apoptosis through induction of caspase-3 and reduced the level of mitochondrial membrane potential $\Delta\Psi$m) in osteosarcoma cell lines.

MATERIALS AND METHODS

Cell lines and culture condition

HOS (ATCC CRL-1543, human), Saos-2 (ATCC HTB-85, human), 143B (ATCC CRL-8303, human), MG-63(ATCC CRL-1427, human), MNNG (ATCC CRL-1547, human), U2OS (ATCC 40432, human), and Vero cell line (VERO C1008; ATCC CRL-1586, valvet monkey kidney epithelial cells) cell cultures were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cells were grown in 75-cm2 flasks in RPMI-1640 (Cat-23400-62, Invitrogen, Germany) with 10% FBS (Gibco BRL), penicillin (10,000 U), and streptomycin (10,000 µg/mL) of stock (Gibco BRL) at 37°C in an atmosphere of 95% humidity and 5% CO_2 atmosphere.

Reagents

WF-A was purchased from natural products company ChromoDex, Irvine, CA (ABS-00023249-010), MTT (Sigma-Aldrich, M-2128), DMSO (Merck-61857125001730, 80291225001730), caspase-3 Assay Kit (Sigma-Aldrich, CASP3F), active caspase-3 antibody (Sigma-Aldrich, C8487), cyt-c antibody (SC-13561, Santa Cruz, USA), B-actin antibody (Sigma-Aldrich, A2228), H2DCFDA (Sigma-Aldrich, D6883), and JC-1 (Sigma-Aldrich, T4069). Active PARP antibody (Abcam, ab32064), doxorubicin (Sigma-Aldrich, D-1515), and all other secondary antibodies were purchased from Santa Cruz, USA.

Cytotoxicity assay

In vitro anticancer activity of WF-A was carried out by MTT assay as described previously.[20] In total, 1–2 × 104 cells/well were cultured with 5% CO_2 for 24 h in 96-well microplate (Grenier, Germany). WF-A was dissolved in 100% DMSO Gr. (Merck, Germany) at different concentrations. Cells were then treated with WF-A for 48 h at 37°C. Afterward, 10 µL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added and the plates were incubated for more 4 h at 37°C. One hundred microliters of DMSO was added to all the wells and mixed thoroughly to dissolve blue formazen crystals for 5 min at room temperature. The plates were read on a Fluostar Omega microplate reader (BMG LABTECH, Australia) at 570 nm. The experiment was done in triplicates and the inhibitory concentration (IC_{50}) was calculated as follows:

Inhibition (%) = [1 - OD (570 nm) of sample well/OD (570 nm) of control well] × 100.

DAPI staining

U2OS cells were seeded at the density 1-2 × 104 cells into the six-well plates and treated with varied concentrations of WF-A for 48 h. In brief, cells were harvested and fixed in methanol and acetic acid in the ratio of 3:1 and kept overnight at 4°C. After incubation for overnight, the cells were centrifuged, resuspended in methanol and acetic acid, and then plated on chilled glass slides. After drying the slides, cells were stained with DAPI at the concentration of 1 µg/mL in dark for 30 min and then slides were washed. Finally, images were taken using fluorescence microscope.

Reactive oxygen species activation assay

Intracellular reactive oxygen species (ROS) activation was measured by a Fluostar Omega microplate reader (BMG LABTECH, Australia) following staining with H2DCFDA as described elsewhere.[26] A total of 2 × 104 cells were added in each well of 96-well microplate and grown

Table 1: IC$_{50}$ (µM) value of WF-A against a panel of osteosarcoma cancer cell lines

Cell lines	Withaferin-A	Doxorubicin
HOS	0.56	4.8
Saos-2	0.88	5.2
143B	0.48	6.0
MG-63	0.36	4.2
MNNG	0.44	6.3
U2OS	0.32	5.4
Veru	7.6	5.3

overnight at 37°C and treated with WF-A at different concentrations (0.2, 0.4, and 0.8 µM). The cells were then stained with 5 µM H2DCFDA for 30 min at 37°C. The cells were collected and fluorescence was measured using a fluorescence plate reader.

Mitochondrial membrane disruption and potential assay

Mitochondrial membrane disruption assay was performed using JC-1 dye in accordance to the manufacture's instructions. U2OS cells were treated with or without WF-A for different time periods. Treated and untreated cells were collected and washed with PBS and then centrifuged at 300*g* at 4°C for 5 min. Treated and untreated cells were incubated with JC-1 dye for 30 min at 37°C. The cells were then visualized under fluorescence microscope. Mitochondrial membrane potential (ΔΨm) assay was performed with WF-A-treated and untreated cell lysate as per manufacturer's instructions using JC-1 and fluorescence was read at 540 and 490 nm using plate reader.

Caspase-3 activation

A total of 2 × 104 cells/well were incubated with or without WF-A in a time-dependent manner. Cells were collected and lysed in cell lysis buffer. Caspase-3 activation was measured fluorometrically using caspase-3 fluorescent assay kits (Sigma). Active caspase-3 cleaved substrate peptides Ac-DEVD-AMC and released of AMC (7-amido-4-methyl coumarin). Fluorescence was analyzed at 354 nm.

Western blot analysis

Cell lysate is prepared in lysis buffer 1 M Tris (7.4), 150 mM NaCl, 1% NP-40, 10% Triton-X, 0.5 M EDTA, 50% glycerol, 50 mM NaF, 1 mM Na$_3$VO$_4$, 15 mM glycerophosphate, and 200 X phosphatase inhibitors PMSF (Sigma, cat-7626), pepstatin A (Sigma, cat-P4265), leupeptin (Sigma, cat-L0649), and benzamidine (Sigma, cat-B6506) and were separated onto SDS-PAGE. The separated proteins were electrotransferred to PVDF membranes and blocked with 10% nonfat skim milk in 1 × TBST for 1 h. The blot membrane incubated with primary antibodies for overnight at 4°C. Blots were washed three times in 1 × TBST and incubated with HRP-conjugated secondary antibody for 2 h. Protein bands were spotted using enhanced chemiluminescence's reagent (ECL kit, Amersham Biosciences).

Statistical analysis

All experiments were carried out in triplicates and expressed as plus minus standard deviation (SD). Differences were considered significant at *P* less than 0.05.

RESULTS

Antiproliferative potential of WF-A on OS cell lines

To identify the antiproliferative role of WF-A on OS cell lines. Cells were treated with WF-A concentration range 0.01–10 µM for 48 h. The WF-A
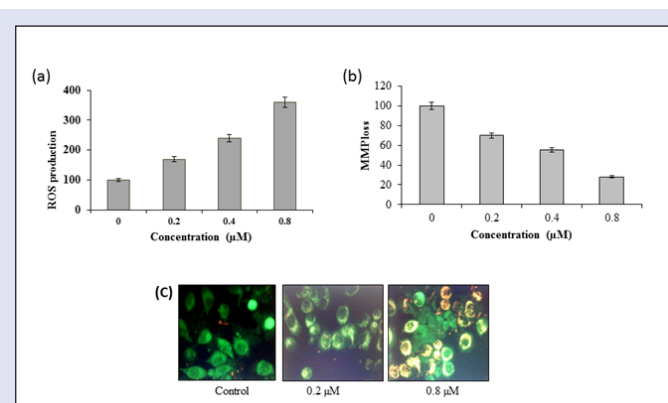

Figure 2: (A) Production of ROS by WF-A in a dose-dependent manner. (B) WF-A reduces the mitochondrial membrane potential (MMP) in U2OS cells. (C) WF-A-treated cells showed a significant reduction in MMP (yellowish-red fluorescence) as compared to untreated cells (green fluorescence)
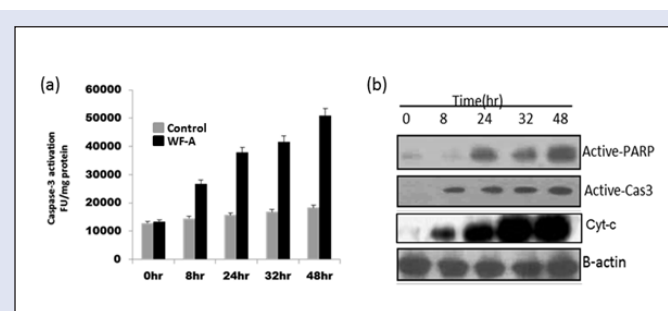

Figure 3: WF-A trigger the apoptosis through caspase-3, cyt-c, and PARP activation. (A) WF-A activates the caspase-3 in a dose-dependent manner leading to apoptosis at IC$_{50}$. (B) In western blot analysis, WF-A-treated cells showed the activation of caspase-3, cytosolic cyt-c, and PARP in a time-dependent manner

showed a dose-dependent inhibition of OS cell lines with IC$_{50}$ ranging from 0.32 to 7.6 µM [Table 1]. WF-A displayed the potent antiproliferative effect against U2OS with an IC$_{50}$ 0.32 µM. In the formazen crystal assay, we observed that WF-A-treated OS cells reduced the number of formazen crystals in a dose-dependent manner [Figure 1B].

WF-A trigger the apoptosis in U2OS cells

In order to examine whether the antiproliferative potential of WF-A is due to apoptosis induction, U2OS cells were incubated with different concentrations (0.2, 0.4, and 0.8 µM) of WF-A and subjected to DAPI staining for 48 h. The treated cells showed condensed and prominent fragmented nuclei in a dose-dependent manner. At 0.8 µM, most of cells underwent apoptosis and apoptic corps were observed in treated cells as compared with untreated control [Figure 1c].

WF-A trigger the ROS activation in U2OS cells

The proapoptotic potential of WF-A observed through DAPI staining study suggested that WF-A might induce generation of intracellular ROS. Therefore, we calculated the ROS level at varied concentrations of WF-A for 48 h. The results showed that the intracellular ROS levels of treated cells increased 70, 140, and 260% as compared with untreated cells [Figure 2A]. Our result suggested that WF-A is a potent molecule for activating ROS in U2OS cells to trigger the apoptosis.

AWF-A reduces the mitochondrial membrane potential (ΔΨm)

ROS generation is related to mitochondrial dysfunction. It disrupts the outer mitochondrial potential to release the death-promoting proteins like cytochrome C.[22] Therefore, we examined whether WF-A reduces the ΔΨm in U2OS cells treated with WF-A at varied concentrations (0.2, 0.4, and 0.8 μM) and measured the ΔΨm fluorometrically using JC-1 dye. WF-A-treated U2OS cells showed a significant reduction in ΔΨm in a dose-dependent manner. The ΔΨm reduced by 30, 45, and 72% at 0.2, 0.4, and 0.8 μM of WF-A, respectively, as compared to untreated control [Figure 2B]. The fluorescence microscopic analysis demonstrated that WF-A-treated cells displayed fluorescence (yellowish-red) indicating apoptotic cells as compared to control [Figure 2C]. Taken together, these results suggested that reduction in ΔΨm by ROS generation may be one of the reasons for induction of apoptosis in U2OS cells.

WF-A trigger apoptotic proteins caspase-3, cytosolic cyt-c, and PARP

To observe whether WF-A activates apoptosis-related proteins. We treated U2OS cells with WF-A at IC_{50} (0.32 μM) at different time points. Results indicated that WF-A treatment triggered the caspase-3 activation in U2OS cells in a time-dependent manner as compared to control (solvent), fluorometrically [Figure 3A]. Western blot analysis revealed that WF-A induced caspase-3, cytosolic cyt-c, and PARP protein expression at IC_{50} in a time-dependent manner [Figure 3B].

DISCUSSION

Osteosarcoma is one of the rare type of bone tumor affecting children. The limited availability of biopsy samples is a one of the obstacles for studying the OS on the genetic and molecular level and to identify the OS-specific druggable target. In this study, we demonstrate that WF-A is a potent candidate for treating OS. WF-A showed potential growth inhibition activity against U2OS cells, as is evident from the proliferation assay. As reported previously, several drugs exhibit antiproliferative effects via induction of apoptosis. For instance, several chemotherapeutic drugs, such as cisplatin, 5-fluorouracil, and taxol[25-31] have been reported to induce specific apoptotic pathways. Additionally, resistance to drug is partly explained by the ability of cancer cells to flee apoptosis.[33] To asses weather WF-A induces apoptosis in U2OS, we carried out the DAPI staining of the treated cells. It was observed that WAF-A induces apoptosis in a concentration-dependent manner. Further, it was observed that WF-A-treated cells displayed ROS-mediated ΔΨm reduction and caspase-3 induction. Our results are in agreement with studies carried out previously.[22] Therefore, the results suggest that WF-A may induce apoptosis through increasing intracellular ROS and reduction in ΔΨm. Many anticancer drugs target cancer cells partly by generating high levels of intracellular ROS.[32] Moreover, mitochondria play a key role in ROS and apoptosis.[33] For example, capsaicin disrupts mitochondrial membrane potential ΔΨm and mediates oxidative stress resulting in apoptosis in pancreatic cancer cells.[34] Moreover, ΔΨm reduction is required for triggering the release of cyt-c. Released cyt-c is well-known protein that binds to apoptotic protease activating factor 1 (Apaf-1) in the cytosol and make a heterodimeric complex, namely, apoptosome. Apoptosomes activate the procaspase-9 for activating its downstream target caspase-3.[35,36] WF-A-treated cells indicated an increase in the level of cytosolic cyt-c in U2OS, suggesting that WF-A-treated cells undergo apoptotic cell death via activation of cyt-c.

CONCLUSION

We conclude that WF-A is a potential candidate for treating osteosarcoma. It induces apoptosis in U2OS cells via ROS-mediated ΔΨm reduction and activation of caspase-3. Further, mechanistic studies are required to establish WF-A as a lead molecule.

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Conflicts of interest

There are no conflicts of interest

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