JBUON 2017; 22(1): 244-250
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Withaferin A induces mitochondrial-dependent apoptosis in non-small cell lung cancer cells via generation of reactive oxygen species

Xi Liu*, Lei Chen*,Tao Liang, Xiao-dong Tian, Yang Liu, Tao Zhang
Department of Thoracic Surgery, Chinese PLA General Hospital, Beijing, 100853, P.R.China
*These authors contributed equally to this work

Summary

Purpose: *Withaferin A (WA) is a bioactive lactone, isolated from natural sources, mainly found in Withania somnifera, and was known to be highly effective against a variety of tumor cells both in vitro and in vivo. Accumulating experimental evidence suggests the involvement of reactive oxygen species (ROS) in WA-mediated cytotoxicity against cancer cells. Hence, the purpose of this study was to investigate the effect of WA in non-small cell lung cancer (NSCLC) cells and also the role of ROC in WA-mediated cytotoxicity.*

Methods: *In the present study we investigated the cytotoxic potential of WA against NSCLC cell line A549 and also highlighted the mechanism of cytotoxicity of this compound. Non-carcinoma WI-38 and PBMC cell lines were used as controls.*

Results: *WA treatment resulted in a dose-dependent cytotoxicity in A549 cells, while the non-carcinoma cells WI-38 and PBMC were unaffected. Further experimental approaches revealed that ROS plays a major role in WA-induced apoptosis in NSCLC cells.*

Conclusion: *WA induces oxidative damage to NSCLC cells with minimum toxicity to normal cells.*

Key words: *apoptosis, non-small cell lung cancer, oxidative stress, reactive oxygen species*

Introduction

Lung cancer is one of the malignancies with very high mortality worldwide and NSCLC is responsible for 85% of the total mortality, while small cell lung cancer (SCLC) accounts for the remaining 15% of the cases [1-3]. Despite the improvements in treatment strategies, poor prognosis, reduced survival rate and accelerated metastasis of NSCLC contribute to high lethality of this tumor [4]. Although the incidence of lung cancer had decreased to a certain extent in the developed countries, recent epidemiological studies suggest that the incidence of NSCLC had increased significantly in Asian countries including China [5,6]. Hence development of effective drug candidates against NSCLC is an urgent requirement for better treatment of this disease.

WA, a bioactive steroidal lactone (Figure 1A), reported to be present in the Ayurvedic medicinal plant *Withania somnifera*, is known to be effective against various tumor cells both *in vitro* and *in vivo* [7-10]. There are several reports which suggest the involvement of ROS in WA-mediated cytotoxicity against cancer cells [8,9,11]. This naturally occurring lactone had become a popular anticancer agent to the researchers due to its limited toxicity in noncancerous cells [12]. Although there is a report on WA action on lung cancer cells [13], how ROS plays its

Correspondence to: Xi Liu, MD. Department of Thoracic Surgery, Chinese PLA General Hospital, 28 Fuxing Road, Beijing, 100853, P.R. China. Tel & Fax: +86 010 66938314, E-mail: liuxi12@hotmail.com
Received: 12/05/2016; Accepted: 02/06/2016

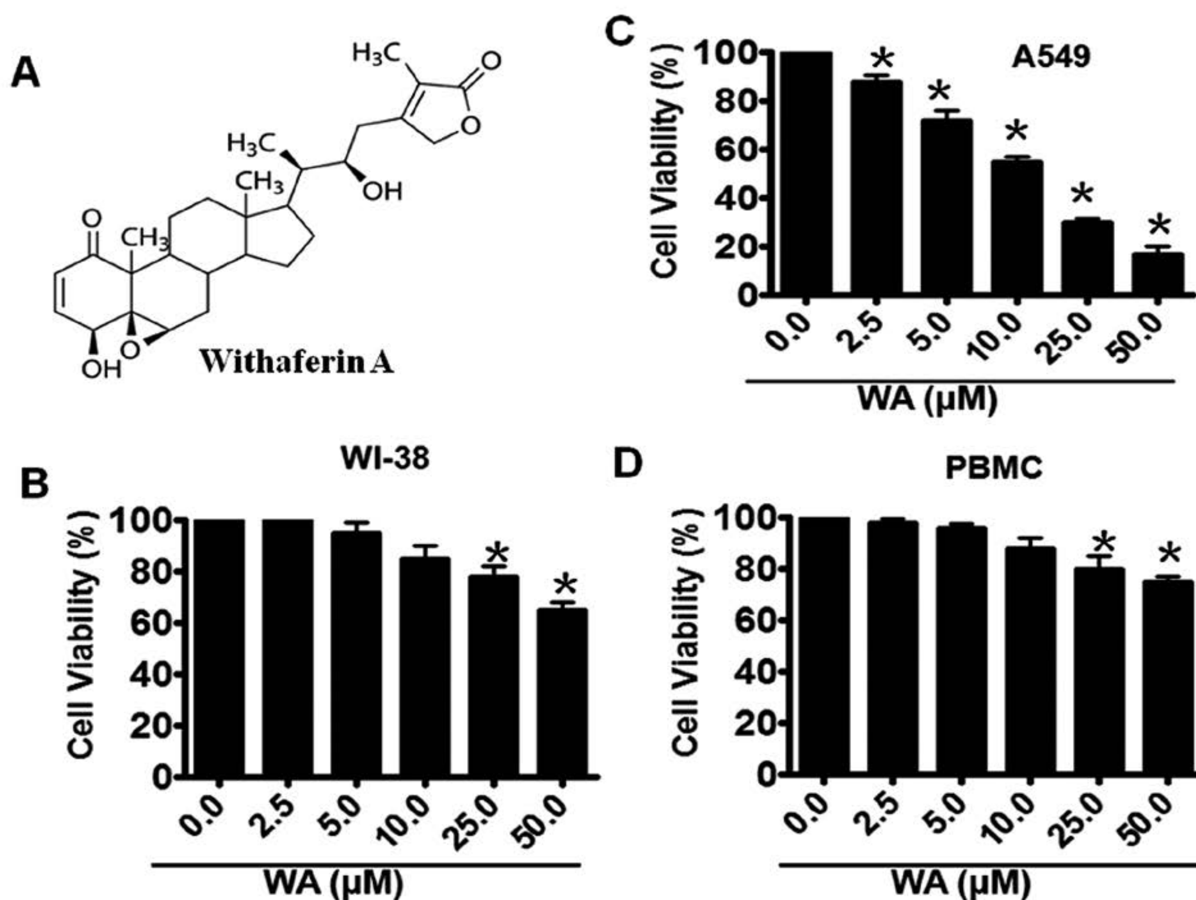

Figure 1. A: Chemical structure of Withaferin A. **B:** Cell viability of human non-small cell lung cancer cells A549 in the presence of different concentration of WA. **C:** Cell viability of lung fibroblasts WI-38 in the presence of different concentration of WA. **D:** Cell viability of human peripheral blood mononuclear cells (PBMC) in the presence of different concentration of WA. The results are expressed as mean±standard deviation of three independent experiments (*p<0.05 vs WA-treated cells).

role in WA-mediated apoptosis in NSCLC has not been highlighted.

Hence we tried to investigate in detail the mechanism of WA-induced apoptosis in NSCLC cells A549 and also monitored the effect of WA on the noncancerous lung fibroblasts WI-38 cells and also in human peripheral blood mononuclear cells (PBMC).

Methods

Cell culture and maintenance

Human NSCLC cells A549 and noncancerous lung cells WI-38 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics (streptomycin 100 μg/ml and penicillin 100 U/ml) and 10% fetal bovine serum (FBS).

PBMC were isolated from healthy donors by density gradient technique, using Ficoll-Hypaque (Histopaque 1077, Sigma Aldrich-USA) [11]. Briefly, whole blood was collected from a healthy donor and carefully mixed with Ficoll-Hypaque (1:1). The mixture was centrifuged at 400 × g for 30 min and PBMC were collected from the plasma/ Ficoll interphase and propagated accordingly in DMEM media.

Cell viability assay

Cultured A549, WI-38 and PBMC were grown to a density of $1X10^4$ cells/ml and treated with different concentrations of WA (0-50 μM) for 24 hrs. After treatment, the cell viability was determined by MTT assay.

Apoptosis assay

Apoptosis was monitored by BD Fluorescence Activated Cell Sorter (ARIA II) using FACS Diva software for analysis. Cultured A549 cells ($1x10^4$ cells/ml) were exposed to different concentrations of WA (0-10 μM) for 24 hrs or with 10 μM WA for different time intervals (0-24 hrs). Apoptosis was monitored by flow cytometry using Annexin V-FITC apoptosis kit (Cayman Chemicals, Ann Arbor, USA) following the protocol supplied by the manufacturers.

Mitochondrial membrane potential (MMP) by JC-1 assay

Change in the MMP was determined by fluores-

cence spectroscopy using the fluorogenic probe JC-1 assay kit (Cayman Chemicals, Ann Arbor, USA). Cultured A549 cells (1x10^4 cells/ml) were treated with different concentrations of WA (0-10 μM) for 24 hrs and after treatment cells were incubated with JC-1 staining solution (manufacturers' recommendation), at 37°C for 30 min. The ratio of J-aggregates to J-monomers were determined by monitoring the fluorescence at excitation/ emission: 540/570 and excitation/ emission: 485/535, respectively.

ROS-determination by DCF-DA staining

Cultured A549 cells (1x10^4 cells/ml) were treated 10 μM WA for different time intervals (0-24 hrs). After treatment, cells were washed with PBS and incubated with 25 μM 2',7'- dichlorodihydrofluorescin diacetate (DCF-DA) for 30 min at 37 °C. Nuclei were stained with DAPI and images were taken by a confocal microscope (Olympus FluoView-1000, NY, USA). Fluorescence intensities were calculated from approximately 500 cells from different fields using Olympus analysis software.

Western blot analysis

Cultured A549 cells (1x10^4 cells/ml) were treated with different concentrations of WA (0-10 μM) for 24 hrs and the total protein was isolated in lysis buffer and estimated by Bradford method [14]. The primary antibodies such as rabbit polyclonal anti-Bax antibody (sc-493) and mouse monoclonal anti- Bcl-2 antibody (sc-7382), purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), were used with 1:500 dilution. The primary antibodies such as rabbit polyclonal anti-cleaved caspase-3 (9661), rabbit monoclonal cleaved anti-caspase-9 (7237), purchased from Cell Signaling Technologies (Beverly, MA, USA). were used with a 1:1000 dilution.

Statistics

Results were analyzed by using GraphPad Software, San Diego California USA. All results are expressed as the mean ± SD. Statistical significance was determined by one way ANOVA using Newman-Keuls Multiple Comparison Test. A p value <0.05 was considered as statistically significant.

Results

Selective cytotoxicity of WA in lung cancer cells

WA treatment resulted in a dose-dependent cytotoxicity in A549 cells after 24 hrs of treatment, with the IC_{50} observed at ~ 10 μM (Figure

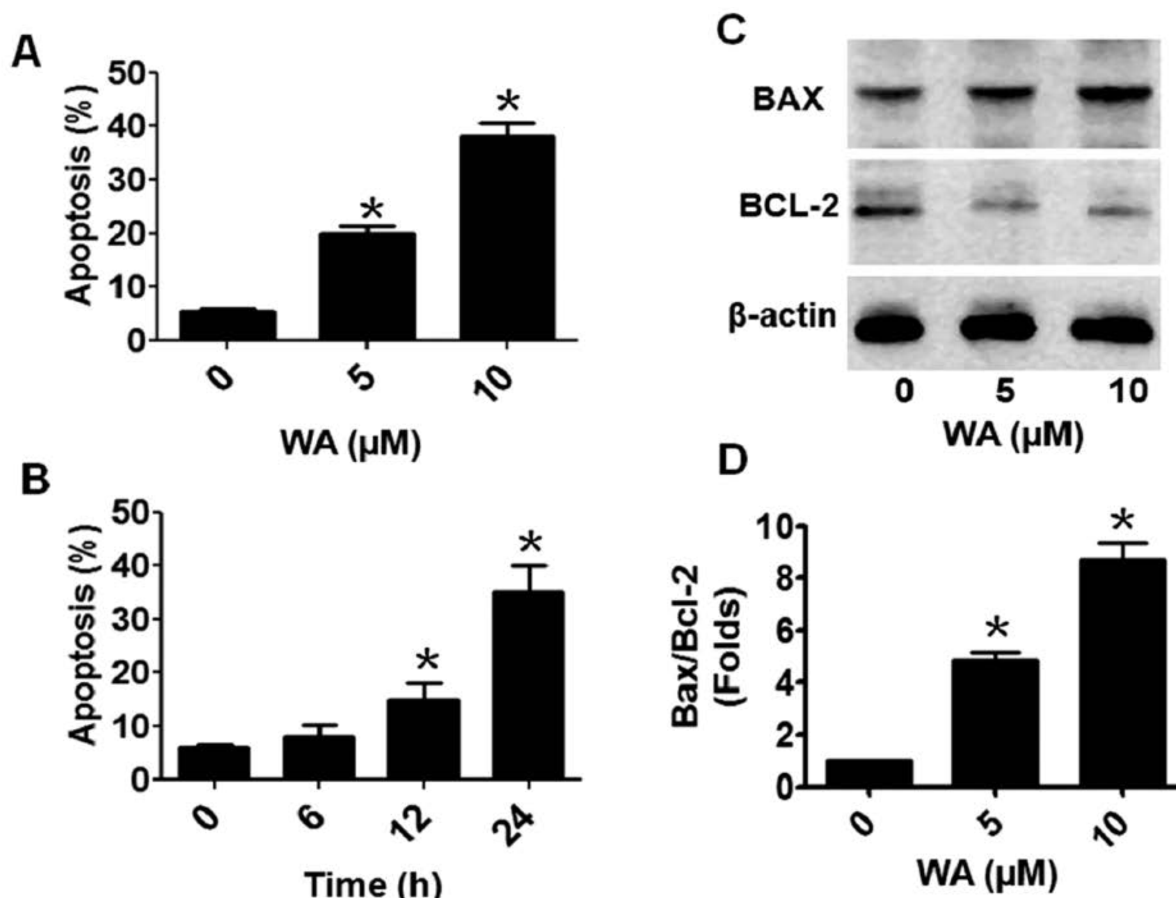

Figure 2. Induction of apoptosis by Withaferin A in A549 cells. **A:** Dose-dependent induction of apoptosis by WA in A549 cells. **B:** Time-dependent induction of apoptosis in A549 cells by WA. **C:** Modulation of pro- and anti-apoptotic markers Bax and Bcl-2 WA in A549 cells. **D:** Bax/Bcl-2 ratio in WA-treated A549 cells. The results are expressed as mean ± standard deviation of three independent experiments (*p<0.05 vs WA-treated cells).

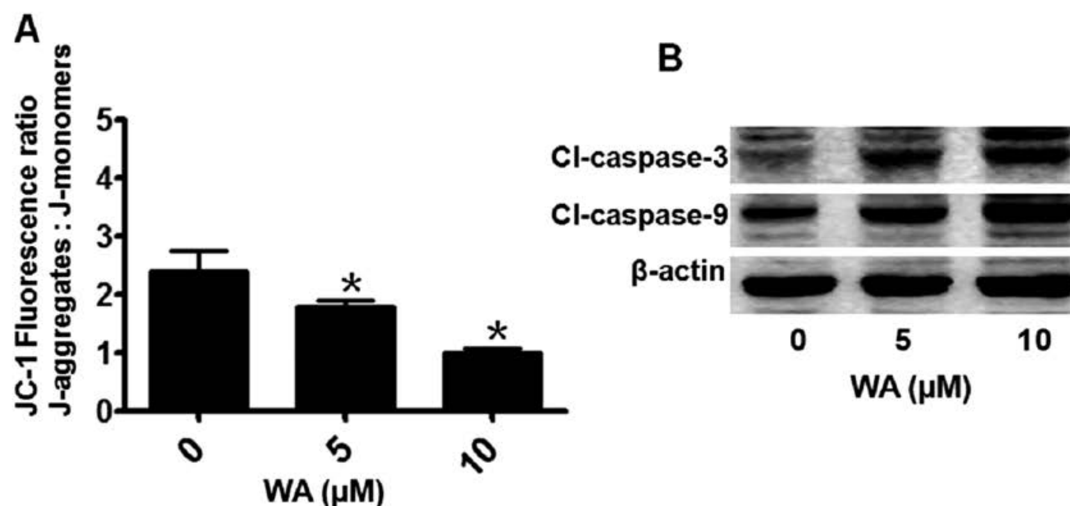

Figure 3. WA induced mitochondrial-dependent activation of caspase-3 in A549 cells. **A:** Alterations in mitochondrial membrane potential by WA in A549 cells as determined by JC-1 staining. The results are expressed as mean±standard deviation of three independent experiments (*p<0.05 vs WA-treated cells). **B:** Dose-dependent increase in the expression levels of caspase-3 and caspase-9 in WA-treated A549 cells.

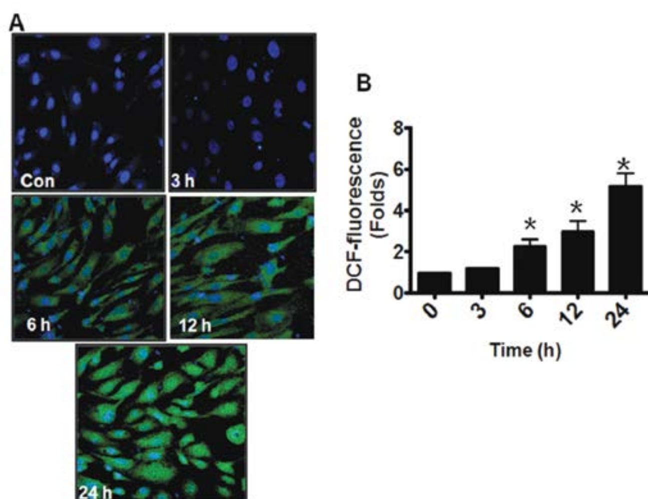

Figure 4. Generation of ROS Withaferin A in A549 cells. **A:** Time-dependent ROS generation in WA-treated A549 cells, as determined by DCF-DA-staining. **B:** Measurement of DCF-fluorescence intensity in WA-treated A549 cells. The results are expressed as mean ± standard deviation of three independent experiments (*p<0.05 vs WA-treated cells).

1B). But very interestingly, WA showed negligible cytotoxicity against the normal lung cells WI-38 and also human PBMC (Figure 1C-1D). In both WI-38 and PBMC cell lines no IC_{50} was observed even at 50 μM WA after 24 hrs of treatment.

WA induces apoptosis in A549 cells via modulation of Bax/Bcl-2 ratio

Treatment of A549 with WA resulted in significant induction of apoptosis in both dose-and time-dependent manner, as determined by FACS after annexin V/PI dual staining . After 24 hrs of treatment with 5 μM WA , ~20 % of the cells were found to be apoptotic while in the presence of 10 μM WA, the apoptotic population was increased to 38% (Figure 2A). To track the onset of apoptosis in WA (10 μM)-treated cells, we determined the time-dependent apoptosis and we observed that significant apoptotic population appeared only after 12 hrs of treatment and reached the maximum after 24 hrs of treatment (Figure 2B). In addition, we also observed that Bax/Bcl-2 ratio was also altered in WA-treated cells, deciding the cell fate towards apoptosis (Figure 2C-2D).

WA damages mitochondrial membrane potential in A549 cells and activates caspase-3

To investigate the involvement of mitochondrial-dependent pathway in WA-mediated apoptosis in A549 cells, we measured the MMP in control and WA-treated cells by fluorescence spectroscopy using the fluorogenic probe JC-1. In the mitochondria of the healthy cells, JC-1 persisted as J-aggregates and emitted green fluorescence, whereas in the apoptotic mitochondria JC-1 remained as monomers and emitted red fluorescence. Alterations in MMP could be determined from the ratio of red:green fluorescence which directly reflected the apoptotic or healthy conditions of the cells. We observed that red:green ratio reflecting the ratio of J-aggregates:J-monomers, decreased in a dose-dependent manner in WA-treated cells, indicating loss in MMP (Figure 3A). Further investigations revealed that WA treatment resulted in the activation of caspase-3 and caspase-9 (Figure 3B), thus confirming the role of mitochondrial–dependent activation of cell death cascade.

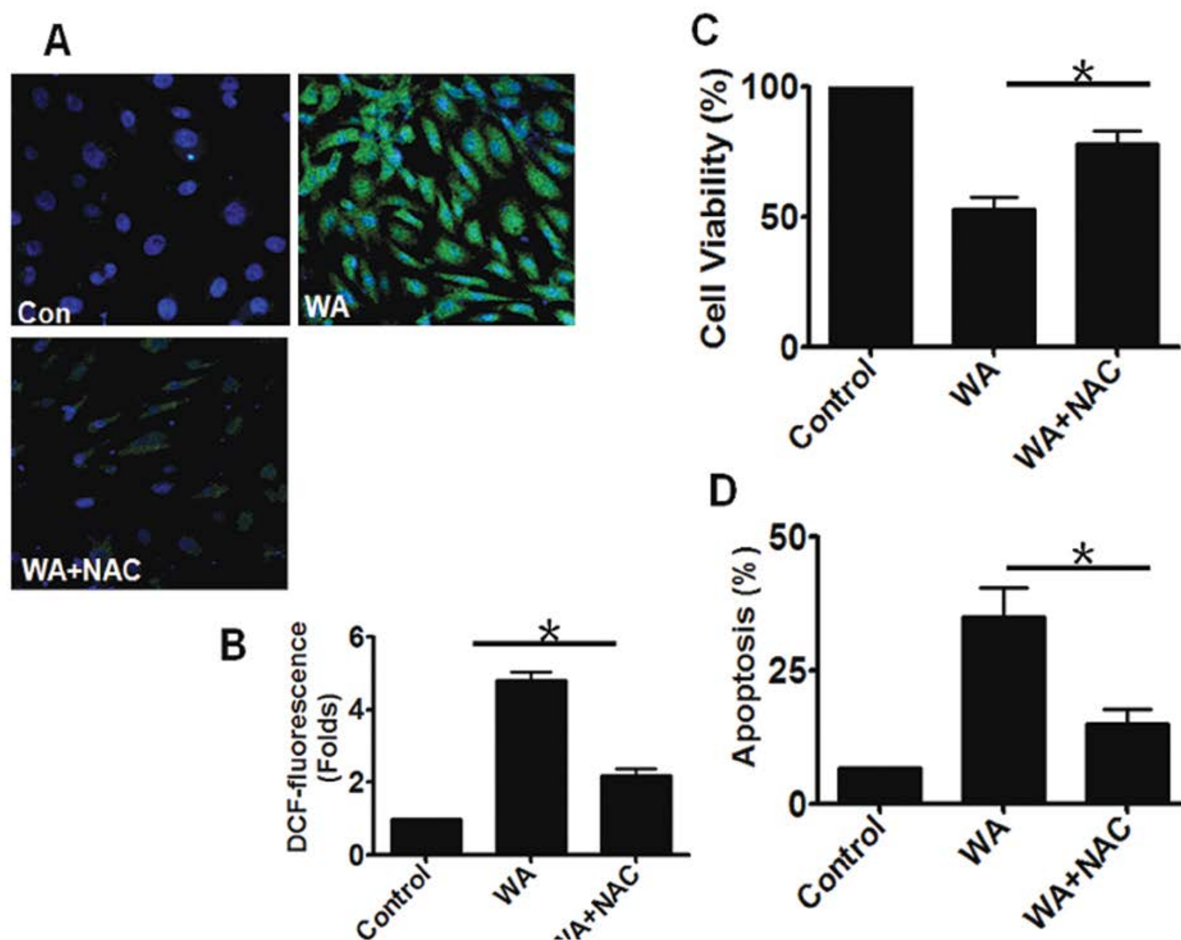

Figure 5: N-acetyl cysteine attenuates ROS-induced cytotoxicity in WA-treated A549 cells. **A:** ROS generation in WA-treated A549 cells co-incubated with 500 μM NAC, as determined by DCF-DA staining. **B:** Measurement of DCF-fluorescence intensity in WA-treated A549 cells co-incubated with 500 μM NAC. **C:** Cell viability of WA-treated A549 cells co-incubated with 500 μM NAC. **D:** Apoptosis of WA-treated A549 cells co-incubated with 500 μM NAC. The results are expressed as mean±standard deviation of three independent experiments (*p<0.05 vs WA+NAC treated cells).

Generation of ROS in A549 cells by WA

Time-dependent generation of ROS in A549 cells treated with 10 μM WA was investigated by staining the cells with DCF-DA (Figure 4). No significant enhancement in ROS levels was observed in the treated cells up to 3 hrs of incubation. But after 6 and 12 hrs of treatment ROS generation was increased by 2.2 and 3-fold, respectively. After 24 hrs of treatment ROS levels were elevated by around 5-fold compared to the untreated cells. Thus, the results indicated that WA treatment leads to ROS generation in A549 cells to a significant level and ROS generation occurred prior to apoptosis. Thus ROS might play an important role in WA-induced apoptosis in A549 cells.

The antioxidant N-acetyl cysteine (NAC) augments ROS-induced cytotoxicity in WA-treated A549 cells

Since ROS generation was found to an early response even in WA-treated A549 cells, we further evaluated whether WA-induced ROS was responsible for its cytotoxicity in cancer cells. Co-incubation of WA-treated A549 cells with 1mM NAC significantly abolished the ROS generation as determined by the DCF-fluorescence (Figure 5A-5B). In addition, we observed that administration of NAC also restored the cell viability and inhibited apoptosis in WA-treated A549 cells (Figure 5C-5D). Therefore we might conclude that ROS plays an important role in WA-mediated cytotoxicity in NSCLC cells.

Discussion

Withaferin-A [(4β,5β,6β,22R)-4,27-Dihydroxy-5,6-22,26-diepoxyergosta-2,24-diene-1,26-di one] (Figure 1A) is a bioactive steroidal lactone found in the medicinal plant *Withania somnifera*, commonly known as Ashwagandha or Indian winter

cherry [10]. The anticancer potential of *W. somnifera* was discovered 4 decades ago only after the isolation of WA from the leaves of this plant [12]. Up until today WA has been reported to be very effective against various types of cancer such as prostate, melanoma, thyroid, breast etc. to name a few [10,13]. Generation of ROS has been known to be one of the mechanisms by which WA induces cytotoxic effects on cancer cells [10]. WA was reported to induce oxidative stress in a variety of cancer cells such as leukemia, breast, cervical, and renal cancer cells [8,9,15,16]. Involvement of ROS in WA-mediated apoptosis in prostate cancer and cervical carcinoma cells have also been documented [10]. Hence, in the present study we have investigated whether oxidative stress plays any role in WA-mediated cytotoxicity in human NSCLC A549. Treatment of A549 cells with WA resulted in a dose-dependent loss of cell viability, while the non cancerous WI-38 and PBMC cells remained unaffected (Figure 1). Furthermore, we also observed that WA treatment resulted in induction of apoptosis in both dose- and time-dependent manner (Figure 2). JC-1 staining of WA-treated cells revealed that the MMP was declined in a concentration-dependent manner, and was accompanied by activation of caspase-9 and caspase-3, the terminal players of apoptosis (Figure 3). To check whether ROS plays any role in WA-induced cytotoxicity, we monitored ROS generation at different time intervals after WA treatment of A549 cells. Interestingly enough we observed that a significant amount of ROS was formed in WA-treated cells as early as 6 hrs of treatment and the ROS levels further went up in a time-dependent manner (Figure 4). To confirm the role of oxidative stress in WA-induced cytotoxicity in NSCLC cells, cell viability and apoptosis of WA-challenged cells were determined in the presence of the antioxidant NAC. Administration of NAC significantly attenuated WA-induced cytotoxicity, as evident from the restoration of the cell viability and inhibition of apoptosis in WA-treated cells (Figure 5).

In conclusion, oxidative stress plays a key role in WA-mediated cytotoxicity against human NSCLC cells.

Acknowledgement

This work was supported by Chinese PLA General Hospital and research grant from Beijing Natural Science Foundation (7142152).

Conflict of interests

The authors declare no confict of interests.

References

1. Jemal A, Ward E, Thun MJ. Contemporary lung cancer trends among U.S. women. Cancer Epidemiol Biomarkers Prev 2005;14:582-585.
2. Smith RA, Cokkinides V, Brawley OW. Cancer screening in the United States, 2009: a review of current American Cancer Society guidelines and issues in cancer screening. CA: Cancer J Clin 2009;59:27-41.
3. Yang J, Kuang XR, Lv PT, Yan XX. Thymoquinone inhibits proliferation and invasion of human nonsmall-cell lung cancer cells via ERK pathway. Tumour Biol 2015;36:259-269.
4. Chan DC, Earle KA, Zhao TL et al. Exisulind in combination with docetaxel inhibits growth and metastasis of human lung cancer and prolongs survival in athymic nude rats with orthotopic lung tumors. Clin Cancer Res 2002;8:904-912.
5. Fan H, Shao ZY, Xiao YY et al. Incidence and survival of non-small cell lung cancer in Shanghai: a population-based cohort study. BMJ open 2015;5(12):e009419.
6. Zhou C. Lung cancer molecular epidemiology in China: recent trends. Transl Lung Cancer Res 2014;:270-279.
7. Choi MJ, Park EJ, Min KJ, Park JW, Kwon TK. Endoplasmic reticulum stress mediates withaferin A-induced apoptosis in human renal carcinoma cells. Toxicol In Vitro 2011;25:692-698.
8. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. PloS One 2011;6(8):e23354.
9. Yang ES, Choi MJ, Kim JH, Choi KS, Kwon TK. Withaferin A enhances radiation-induced apoptosis in Caki cells through induction of reactive oxygen species, Bcl-2 downregulation and Akt inhibition. Chem Biol Interactions 2011;190:9-15.
10. Vyas AR, Singh SV. Molecular targets and mechanisms of cancer prevention and treatment by withaferin a, a naturally occurring steroidal lactone. AAPS J 2014;16:1-10.
11. Bhattacharya S, Kumar NM, Ganguli A, Tantak MP, Kumar D, Chakrabarti G. NMK-TD-100, a novel microtubule modulating agent, blocks mitosis and induces apoptosis in HeLa cells by binding to tubulin. PloS One 2013;8(10):e76286.
12. Shohat B, Gitter S, Abraham A, Lavie D. Antitumor activity of withaferin A (NSC-101088). Cancer Chemo-

ther Rep; Part 1. 1967;51:271-276.

13. Lee IC, Choi BY. Withaferin-A - A Natural Anticancer Agent with Pleitropic Mechanisms of Action. Intern J Mol Sci 2016;17:290.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 1976;72:248-254.
15. Mayola E, Gallerne C, Esposti DD et al. Withaferin A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. Apoptosis 2011;16:1014-1027.
16. Malik F, Kumar A, Bhushan S et al. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concomitant protection by N-acetyl cysteine. Apoptosis 2007;12:2115-2133.