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ORIGINAL PAPER

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

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Abstract Induction of apoptosis in cancer cells has become the major focus of anti-cancer therapeutics development. WithaferinA, a major chemical constituent of *Withania somnifera*, reportedly shows cytotoxicity in a variety of tumor cell lines while its molecular mechanisms of action are not fully understood. We observed that withaferinA primarily induces oxidative stress in human leukemia HL-60 cells and in several other cancer cell lines. The withanolide induced early ROS generation and mitochondrial membrane potential ($\Delta\psi_{mt}$) loss, which preceded release of cytochrome c, translocation of Bax to mitochondria and apoptosis inducing factor to cell nuclei. These events paralleled activation of caspases –9, –3 and PARP cleavage. WA also activated extrinsic pathway significantly as evidenced by time dependent increase in caspase-8 activity vis-à-vis TNFR-1 over expression. WA mediated decreased expression of Bid may be an important event for cross talk between intrinsic and extrinsic signaling. Furthermore, withaferinA inhibited DNA binding of NF-κB and caused nuclear cleavage of p65/Rel by activated caspase-3. *N*-acetyl-cysteine rescued all these events suggesting thereby a pro-oxidant effect of withaferinA. The results of our studies demonstrate that withaferinA induced early ROS generation and mitochondrial dysfunction in cancer cells trigger events responsible for mitochondrial -dependent and -independent apoptosis pathways.

Keywords Withaferin A · ROS · NAC · AIF · NF-κB · Caspases · Apoptosis

Abbreviations

AIF	Apoptosis inducing factor
DCFH-DA	Dichlorofluorescein diacetate
EMSA	Electrophoretic mobility shift assay
HPLC	High Performance Liquid Chromatography
IR	Infra red
NAC	*N*-acetyl-cysteine
NF-κB	Nuclear factor κB
PI	Propidium iodide
ROS	Reactive oxygen species
TNFR	Tumor necrosis factor receptor
WA	Withaferin A

Introduction

Dysregulation of apoptosis is the hallmark of all cancer cells and agents that activate programmed cell death could be valuable anticancer therapeutics [1]. Most of the current anti-cancer drugs are derived from plant sources, which act through different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity [2]. Anti-neoplastic agents therefore, act through several pathways in the death of cancer cells. Recent studies have amply documented that two major pathways are involved in the regulation of apoptosis [3]. One pathway is mediated via cell surface death receptors, such as Fas/CD95 and TNFR1, which upon activation

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recruit cytoplasmic tail of the receptors and down stream associated signaling complex leading to the activation of caspase-8. The second pathway is mitochondrial-dependent, which is regulated by signaling cascade-involving members of Bcl-2 family. A loss of mitochondrial membrane potential ($\Delta\psi_m$) brings about translocation of pro-apoptotic Bax to mitochondria and cytochrome c from mitochondria to cytosol resulting in caspase-9 activation [4]. Members of Bcl-2 family therefore play a crucial role in the regulation of apoptosis. For instance, overexpression of anti-apoptotic Bcl-2 prevents the release of cytochrome c while overexpression of pro-apoptotic Bcl-2 member Bax facilitates the formation of mitochondrial pores and release of cytochrome c [5] after depolarisation of mitochondrial membranes [6]. Many anti-cancer drugs would act as pro-oxidant, which target mitochondria [7] and may initially involve generation of free radicals such as reactive oxygen/nitrogen species [8, 9] eventually leading to the activation of apoptosis. Currently, natural plant based products are increasingly investigated for their cytotoxicity in cancer cells targeting apoptosis activation for the development of anti-cancer leads [2].

Withania somnifera plant has found extensive uses in the Indian traditional system of medicine and also as dietary supplement [10, 11]. It has also been reported for its tumor cell growth inhibitory activity, antitumor and radiosensitizing effect on transplantable mouse tumor [12]. This herbal plant yields a host of steroidal lactones called withanolides, some of which have shown growth inhibition of human tumor cell lines [13]. WithaferinA (WA) amongst these withanolides reportedly is very active in impairing metastasis and angiogenesis [14] while it was also shown to suppress nuclear factor-kB (NF-kB) activation and its regulated genes expression in cancer cells [15, 16]. Current studies further demonstrated that WA also acts like a proteosome inhibitor and that inhibition of proteosomal chymotrypsin-like activity may contribute to anti-tumor action in vivo [17]. In an another recent study, WA has been reported to induce Par-4 selective apoptosis of prostrate cancer cells in both androgen responsive and androgen refractory prostrate cancer cells, and causes regression of PC-3 xenografts in nude mice [18]. WA thus appears to exert at multi-targets proteins in the cancer cell. This is also observed from studies where WA interferes with actin cytoskeleton and disrupts F-actin organization via interaction with annexin II [19] and thus markedly limits the migratory and invasive capabilities of cancer cells. WA has also been proposed as a new generation molecule capable of eliciting growth inhibitory effect on cancer cells [13].

The aim of this study therefore is to further broaden and understand the molecular mechanisms of WA action on cancer cells cytotoxicity. A central point to alteration of plethora of proteins phenotypes in cancer cells by WA appeared to involve the role of free radicals in altering the redox balance of the cells. We first postulated that WA might be inhibiting cell proliferation by initiating oxidative stress through generation of reactive oxygen species (ROS) in cancer cells. We used human promyelocytic leukemia HL-60 cells as a model for investigating in details the analysis of oxidative stress mediated pathways involved in cancer cell killing because some anticancer therapies are known to mediate apoptosis through oxidative stress within the cancer cells [20]. Moreover, some anticancer therapies may add to the oxidative stress within cancer cells. For instance, the chemotherapeutic agents doxorubicin, mitomycin C, etoposide and cisplatin are superoxide -generating agents [21]. We here describe for the first time that WA alters the redox potential of cells by inducing oxidative stress and demonstrate that initial events involve generation of ROS and loss of mitochondrial membrane potential. Because WA appeared to elicit a pro-oxidant effect, we used antioxidants ascorbate, trolox and *N*-acetyl cysteine (NAC) as the ROS scavenger to rescue cells from oxidative stress. Amongst these only NAC was found highly effective against WA induced cytotoxicity. WA was found to induce early ROS formation, disrupt mitochondrial membrane functions, translocate cytochrome c, apoptosis inducing factor (AIF) and Bax with concomitant activation of caspases leading to cleavage of NF-kB and PARP, all of which were rescued efficiently by *N*-acetyl cysteine. Our studies have provided a deeper insight into one of the mechanisms of action of WA induced apoptosis in cancer cells. The observed apoptotic activity of WA is associated with ROS generation not only in HL-60 cells but also in other cancer cell lines too.

Materials and methods

Isolation and structural elucidation of withaferin A (5β, 6β-epoxy-4β, 27-dihydroxy-1-*oxo*-witha-2, 24 dienolide) from *withania somnifera*

Withaferin A (WA) was isolated from 1:1 aqueous ethanol extract of leaves of *Withania somnifera* [22]. The compound was identified as WA on the basis of mp. 252.5°C, $[\alpha]_D^{28}$ + 125° (c 1.30, $CHCl_3$), IR, NMR and MS spectral data. Further, HPLC analysis of isolated WA [23] confirmed its purity to almost 100% as shown in Fig. 1.

Reagents and antibodies

RPMI-1640, 2′, 7′-dichlorofluoresceine diacetate (DCFH-DA), D, L-buthionine-*S*, *R*-sulfoximine (BSO), propidium

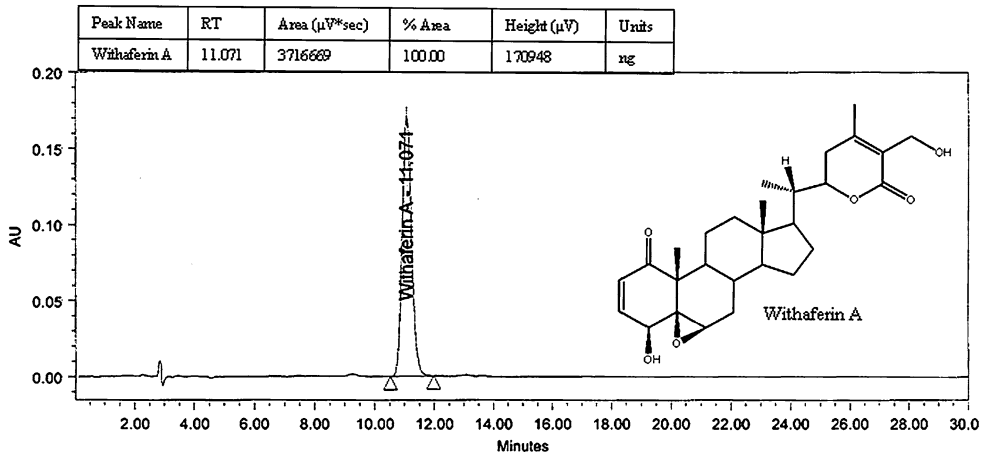


Fig. 1 HPLC chromatogram of WA. The purity of isolated WA was analyzed by HPLC employing Shimadzu HPLC system consisting of a Diode Array detector and phenomene ×C18 column (5 μm, 250 × 4.0 mm I.D.) by UV detection at 237 nm. WA was resolved isocratically on a mobile phase consisting of methanol: water (60:40) at a flow rate 0.7 ml/min. Other conditions were same as described in Materials and methods. The chromatogram is representative of one of three independent analyses

iodide (PI), DNase-free RNase, proteinaseK, Hoechst-33258, 3-(4,5, -dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), NAC, penicillin, streptomycin, L-glutamine, pyruvic acid, eukaryotic protease inhibitors cocktail and camptothecin were purchased from Sigma chemical Co. St. Louis. Fetal bovine serum was obtained from GIBCO Invitrogen Corporation (#16000-044, lot No. 1237517) USA. AnnexinV-FITC apoptosis detection kit and Cycle Test™ Plus DNA reagent Kit were from BD Biosciences while Apoalert caspases assay kits were from B.D. Clontech. Mouse anti-human antibodies to Bax (#SC20067), PARP-1 (#SC8007), Bcl-2 (SC7382), actin (#SC-8432), TNFR1 (#SC8436), Bid (#SC 6538) and goat anti-rabbit IgG-HRP (#SC2030) and goat anti-mouse IgG-HRP (#SC2031) were from Santa Cruz, USA. Rabbit anti-AIF (#PC536) was from Calbiochem, USA while mouse anti-NFkB (#554184, clone G96-337) and cytochrome c (#556433, clone 7H8.2C12) from BD, Pharmingin. Electrophoresis reagents, protein markers were from Bio-RAD, USA: Hyper film and ECL reagents from Amersham Biosciences, UK.

Cell culture, growth conditions and treatment

Human promyelocytic leukemia cells HL-60, acute lymphoblastic leukemia cell line Molt-4, prostrate carcinoma PC-3 and DU 145, T cell lymphoma HuT-78 and cervical carcinoma HeLa cells were obtained from National Cancer Institute (NCI), Bethesda, USA. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), and 0.37% $NaHCO_3$ at 37°C in an atmosphere of 95% air and 5% CO_2 with 98% humidity. WithaferinA was dissolved in dimethylsulfoxide and delivered to cell cultures in complete medium while the controls received only DMSO (<0.2%, v/v).

Assay of cell proliferation

The cells were plated in 96-well plates at a density of 2.5×10^4 cells/200 μl of medium. Cultures were incubated with different concentrations of WA for indicated time periods. The MTT assay was performed as described earlier [24]. Cell growth was calculated by comparing the absorbance of treated versus untreated cells.

Flow cytometric analysis of apoptosis and necrosis

WA treated HL-60 cells were washed and resuspended in 100 μl of the binding buffer provided with the apoptosis detection kit (BD Pharmingin). Cells were stained with annexinV-FITC antibody and PI as per the instructions given by the manufacturer, and scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels.

TUNEL assay for detection of DNA fragmentation

DNA fragmentation is a late event because of interplay of large number of molecules involved in signaling cascades in apoptotic cell death in contrast to the annexinV binding assay of early apoptosis. DNA strand breaks were evaluated by labeling 3′-hydroxyl (OH) termini of double stranded or single stranded DNA breaks employing

instructions as described in the Apo-Direct assay kit (BD Biosciences). The process involves end-labelling of DNA fragments with FITC-tagged deoxyuridine triphosphate nucleotide (FITC-dUTP). The preparations were analyzed for end-labeled DNA content using BD-LSR flow cytometer equipped with electronic doublet discrimination capability using blue (488 nm) excitation from argon laser. Data were collected in list mode on 10,000 events for FL1 fluorescence intensity where an increase in fluorescence intensity indicated apoptotic cell fraction.

Hoechst 33258 staining of cells for nuclear morphology

WA treated HL-60 cells (2×10^6 cells/3 ml) were washed twice with PBS, fixed and stained with Hoechst 33258 as described earlier [24]. The slides were observed for any nuclear morphological alterations and apoptotic bodies under inverted fluorescence microscope.

DNA content and cell cycle phase distribution

Cells were treated with WA, collected, washed in PBS, fixed in 70% cold ethanol and placed at –20°C overnight. Cells were washed with PBS, subjected to proteinase-K and RNase digestion followed by staining of clean nuclear materials (nuclei) with propidium iodide using procedures and reagents as described in the instruction manual of the Cycle Test plus DNA reagent kit (Becton Dickinson, USA). The preparations were analyzed for DNA content using BD-LSR flow cytometer. Data were collected in list mode on 10,000 events for FL2-A versus FL2-W.

Flow cytometric analysis of reactive oxygen species (ROS)

Influence of WA on the endogenous generation of reactive oxygen species was measured with DCFH-DA probe as described earlier [24].

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by using a Mitochondrial Membrane Sensor Kit containing JC-1, as described by the manufacturer (BD Bioscience, CA). Briefly, cells after treatment were washed twice with PBS and centrifuged at 300*g* at 4°C for 5 min. Each cell pellet was suspended in 1 ml of diluted BD Mito-Sensor reagent and incubated at 37°C/5% CO_2 for 15 min. The cells were washed and suspended in 1 ml incubation buffer and analyzed by Flow cytometry for FL-1 versus FL-2 fluorescence.

Caspase assays

Cells (2×10^6/2 ml/well, 6-well plate) were incubated with WA for the indicated time periods. At the end of treatment cells were washed in PBS and pellet lysed in cell lysis buffer. Activities of caspase-3, -8 and -9 in the cell lysates were determined fluorometrically using BD Apoalert caspase fluorescent assay kits. Caspase-3 and -8 employed fluorochome conjugated peptides DEVD-AFC and IETD-AFC as substrates, respectively while caspase-9 employed LEHD-AMC. Release of AFC (7-amino-4-trifluoromethyl coumarin) and AMC (7-aminomethylcoumarin) were assayed according to the instructions provided in the Manual by the supplier. Specific inhibitors were used as negative control to determine whether fluorescence intensity changes were specific for the activity of caspases. The peptide based inhibitors used were DEVD-CHO for caspase-3, IETD-fmk for caspase-8 and LEHD-CHO for caspase-9.

Measurement of GSH contents in Cells

Intracellular levels of GSH were estimated using the fluorescent reagent *ortho*-phthalaldehyde (OPT) [25]. Briefly HL-60 cells (1×10^6/ml) were treated with WA (4 μM) along with and without NAC (5 Mm) for different time periods. GSH was measured fluorometrically at excitation and emission wavelengths of 350 nm and 420 nm, respectively.

Preparation of cytosolic and mitochondrial lysates of HL-60 cells

Cells were collected and washed twice with PBS. The cytosolic and mitochondrial fractions were obtained after selective plasma membrane permeabilization with digitonin [26]. The cell lysates were transferred to fresh tubes and stored at –80°C for immunoblotting of proteins.

Preparation of total cell lysates for expression of NF-kB, PARP and Bcl2

HL-60 cells (3×10^6) after treatment with WA were harvested and resuspended in 0.2 ml of RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 30 mM Na_2HPO_4, 50 mM NaF,

0.5 mM $NaVO_4$, 2 mM phenylmethylsulfonyl fluoride, and 10% protease cocktail inhibitor). Cells were incubated on ice for 30 min, vortexed and centrifuged at 12,000*g* for 15 min. Supernatants were collected and stored at –80°C [27].

Preparation of cytosolic and nuclear extracts

HL-60 cells (5×10^6) were washed with ice-cold phosphate-buffered saline after WA treatment and centrifuged. All steps of fractionation were carried out at 4°C [28]. Cell pellets were homogenized in 200 µl of buffer A (10 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1 mM $NaVO_4$ and 10% protease cocktail inhibitor). The tubes were placed in ice for 10 min. Nonidet P-40 was added (0.5%, v/v), tubes vortexed briefly and centrifuged at 8,000*g* for 15 min. The cytosolic supernatants were stored at –80°C. The pellets obtained were resuspended in 50 µl of buffer A supplemented with 20% glycerol, 0.4 M KCl, kept on ice for 30 min and centrifuged at 13,000*g* for 15 min. The supernatants were stored at –80°C for analysis of nuclear NF-kB and AIF.

Electrophoretic mobility shift assay (EMSA) for NF-κB activation

To determine NF-κB activation, EMSA was conducted essentially as described [29]. Briefly, nuclear extracts prepared from WA treated and untreated HL60 and HUT-78 cells were incubated with ^{32}P-end-labeled 22-mer (5′AGTTGAGGGGACTTTCCCAGCC-3′), underlining indicates NF-κB binding site) double-stranded NF-κB oligonucleotide (Promega, USA). The incubation of the protein lysate with the oligomer (8 µg of protein with 10 fmoles DNA) was carried out for 30 min at 37°C. The DNA–protein complex formed was separated from the free oligonucleotide on 6.6% native polyacrylamide gels. The specificity of the binding was examined by competition with unlabelled oligonucleotide. The dried gels were visualized, and the radioactive bands quantitated by Phosphor Imager (Bio-RAD, USA) using Quantity One software.

Western blot analysis

Proteins from the mitochondrial, nuclear and cytosolic lysates were analyzed on SDS-PAGE. The resolved proteins were electro transferred to polyvinylidene difluoride (PVDF) membranes (Bio-RAD) over night at 30 V, 4°C. The membranes were blocked in blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20) containing 5% milk for 1 h and blotted with respective mouse anti- human primary antibodies for 2 h. Blots were washed in TBS and incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were detected using enhanced chemiluminescence's reagent (ECL kit, Amersham Biosciences). The density of the bands was arbitrarily quantified using Quantity One software of Bio-RAD gel documentation system. The protein contents were determined using Bradford reagent (Bio-Rad protein assay kit) and aliquots normalized to equal quantities before loading.

Statistical analysis

Data are presented as mean ± S.D. of the number of experiments indicated. The comparisons were made with '*t*' test and the difference was considered to be statistically significant if the *P* value was <0.05.

Results

Purity of WA

The purity of WA used in the present study was almost 100% as confirmed by HPLC (Fig. 1).

WA inhibits cancer cell proliferation

In order to determine the effect of WA on cell proliferation; HL-60 cells were treated with WA at indicated concentrations (0.1–10 µM) for 24 and 48 h. The withanolide produced concentration dependent inhibition of cell proliferation with 24 h IC_{50} value of ~ 2 µM and 48 h IC_{50} value of ~ 1 µM (Fig. 2A). No inhibition in the proliferation was obtained in cultures treated with the vehicle only (DMSO, $<0.2\%$, v/v).

WA induces apoptosis in HL-60 cells

HL-60 cells were incubated with different concentrations of WA for 12 h, and the percentages of cells undergoing apoptosis/necrosis were determined by staining with annexinV-FITC and PI (Fig. 2B). WA produced concentration dependent increase in apoptosis, which was 34, 49 and 70% at 2, 4, and 10 µM, respectively. The PI positive post-apoptotic/necrotic cell population however, was relatively small suggesting that WA induced cytotoxicity is predominantly through apoptotic pathways (Fig. 2B).

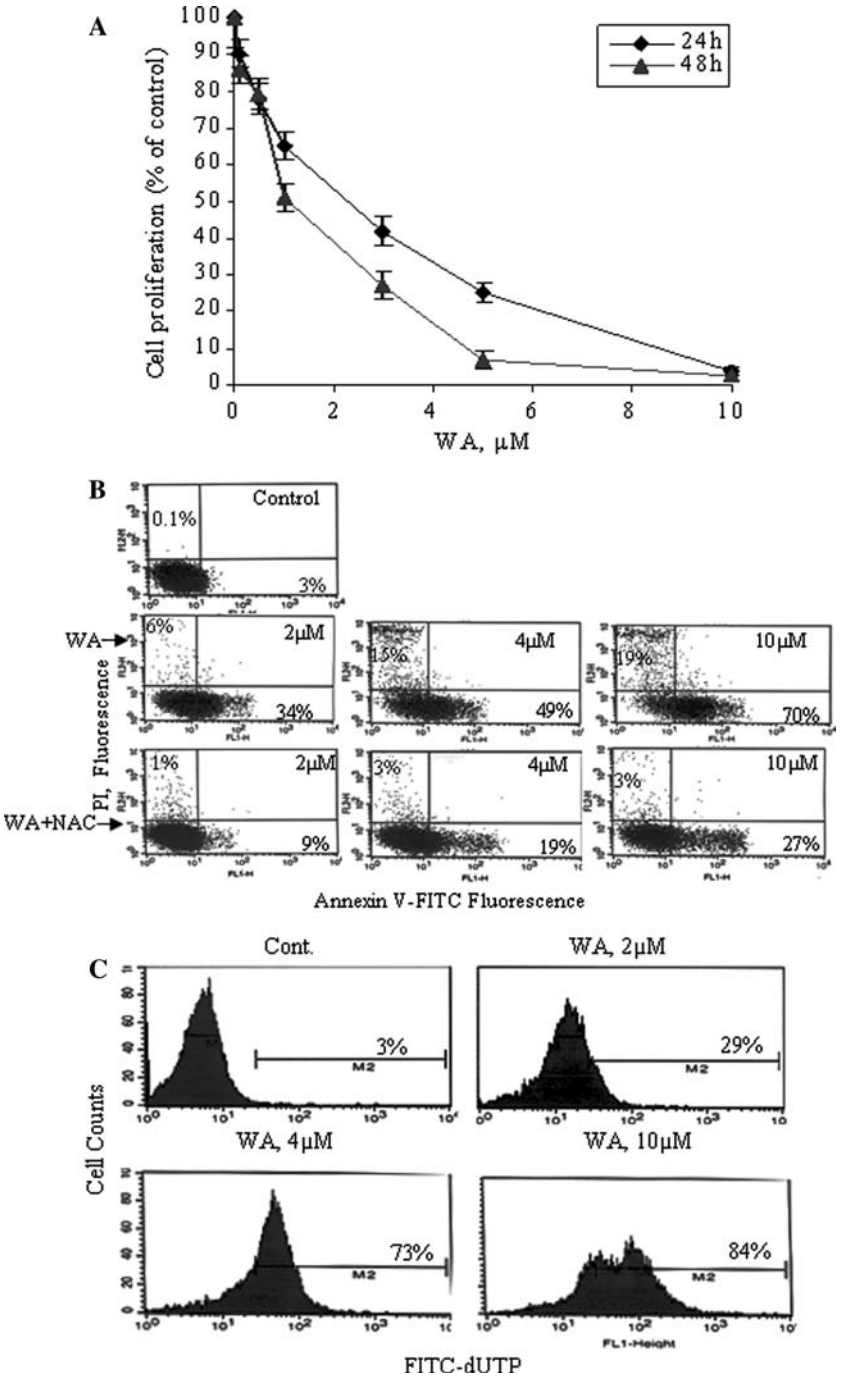


Fig. 2 WA inhibits cell proliferation and induces apoptosis in HL-60 cells. (**A**) For cell proliferation assay, HL-60 cells (2.5×10^4/well) grown in 96-well culture plate were incubated with indicated concentrations of WA. Cell proliferation was assessed by MTT reduction assay. Data are mean value ±S.D. ($n = 8$ wells) and representative of one of two similar experiments. (**B**) Flow cytometric analysis of WA induced apoptosis and necrosis in HL-60 cells using annexinV-FITC and PI double staining. HL-60 cells (1×10^6/ml) were incubated with indicated concentrations of WA for 12 h and stained with Annexin V-FITC/PI as described in Materials and methods. Quadrant analysis of fluorescence intensity of ungated cells in FL-1 versus FL-2 channels was from 10,000 events. Cells in the lower right quadrant represented apoptosis while in the upper right quadrant indicated post-apoptotic necrosis. FACSCan is representative one of two similar experiments. (**C**) DNA fragmentation determined by TUNEL assay. HL-60 cells were treated for 12 h with indicated concentrations of WA. Cells were incubated with FITC-dUTP in the presence of terminal deoxynucleotidyltransferase, which incorporates FITC-dUTP into 3′-hydroxyl-DNA ends found in apoptotic cells. The cells were analyzed by flow cytometry. The presence of apoptotic cells is demonstrated by histogram statistical analysis indicating increase in fluorescence intensity (M2 gate). A representative result of three independent experiments is shown

WA induces DNA fragmentation measured by TUNEL assay

To further verify the extent of apoptosis induced by WA in HL-60 cells, we applied a single-step staining method for labeling DNA strand breaks with FITC-dUTP to detect apoptotic cells by flow cytometry. It may be mentioned that in contrast to phosphatidylserine translocation measuring the immediate early onset of apoptosis, the tunnel assay on the contrary represents late events of apoptosis as a result of engagement of several signaling cascades leading to DNA fragmentation. The TUNEL assay was therefore, performed in samples treated with different concentrations of WA for 12 h (Fig. 2C). The highest number of apoptotic cells with DNA strand breaks were detected as a fraction of FITC-positive cell population depicting concentration dependent increase in fluorescence intensity. These results bear close correspondence to the AnnexinV/PI positive cell populations. The studies again supplement our above claims that cell death by WA is by way of activation of apoptosis signaling pathways.

WA induces early generation of ROS in HL-60 cells

The strong pro-apoptotic effect of WA observed with annexinV binding suggested that WA might be producing a rapid potential oxidant stress by attenuating the redox status of the cells. Therefore, we measured the effect of WA on ROS production in HL-60 and other cells also such as PC-3, Molt-4, DU-145 and Hela cells in the presence and absence of antioxidant NAC by flow cytometry. After the treatment cells were collected and stained with DCFH-DA. We used D, L-buthionine-*S*, and *R*-sulfoximine (BSO, 100 μM) treated cells, as positive control. Our studies demonstrated that WA stimulates ROS generation in all the cell lines examined as evidenced by increase in cell population of DCF-derived fluorescence when cells were incubated with indicated concentration of WA for 6 h. (Fig. 3A).

ROS generation is protected by NAC against WA induced oxidative stress

Because WA treatment led to the enhancement of ROS generation, it is possible that alterations in the cellular

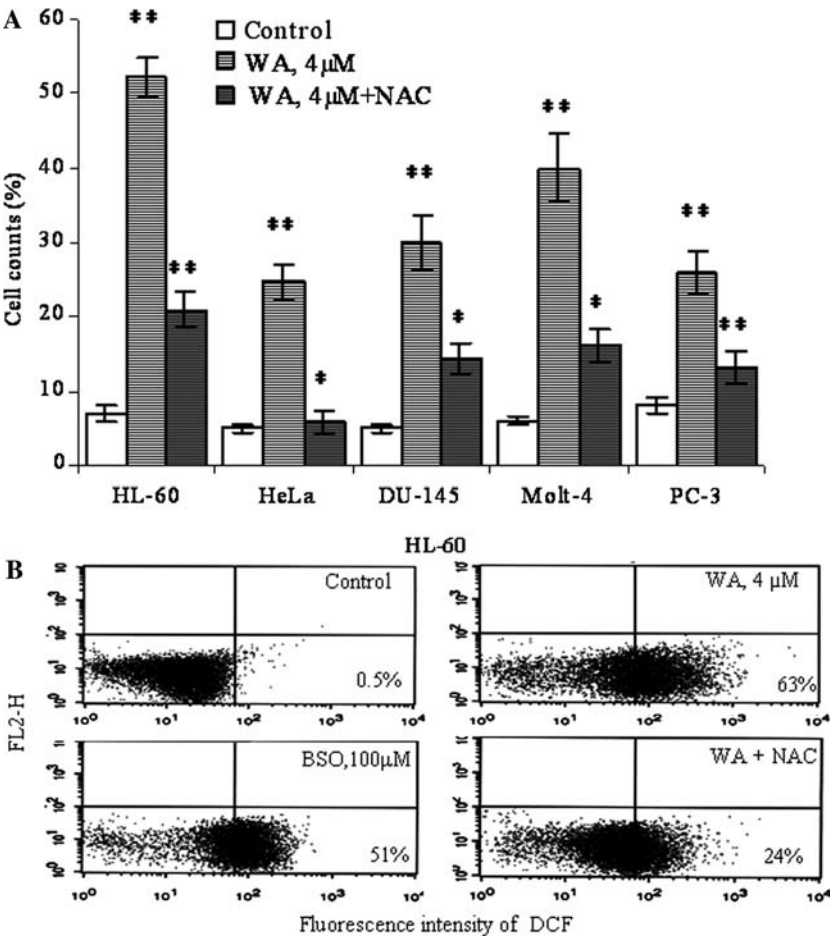


Fig. 3 WA induced generation of ROS. (**A**) HL-60, PC-3, Molt-4, DU-145 and Hela cells (1×10^6/ml) were treated with WA (4 μM) in 12-well culture plates for 6 h. NAC (5 mM) was added 1 h before the treatment of WA. BSO (100 μM) was used as a positive control. Cells were stained with DCFH-DA and 10,000 events analyzed in BD-SLR flow cytometer. Other conditions are same as described in Materials and methods. The data are representative one of three similar experiments. (**B**) A representative result of one of the three independent experiments of HL-60 cells is shown

redox state could play a role in WA induced apoptosis. To examine this, the antioxidant agent's ascorbate, trolox and NAC were used to counter the WA-induced ROS generation and its attendant consequent events. NAC is known as a thiol antioxidant and functions as both redox buffer and reactive oxygen intermediate scavenger [30]. Our results clearly demonstrated that WA is able to generate a strong oxidative stress not only in HL-60 cells but also in other cancer cells and pretreatment with NAC resulted in marked protection against ROS generation (Fig. 3A, B) and hence WA induced apoptosis measured by annexinV binding (Fig. 2B). However, ascorbate and trolox used as ROS scavengers in WA treated cells did not show any significant protection (data not shown).

WA disrupts mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial outer membrane permeabilization (MOMP) is considered the 'point of no return' as this event is responsible for engaging the apoptotic cascade in numerous cell death pathways While the inner mitochondrial membrane may control MOMP by regulating oxidative phosphorylation and mitochondrial transmembrane potential, $\Delta\psi_m$ [31]. The onset of MOMP is often associated with a loss of $\Delta\psi_m$, that may be caused by incomplete reduction of molecular oxygen during mitochondrial electron transport leading to superoxide formation. As ROS generation is related to mitochondrial dysfunctions, we therefore, examined the effect of WA on mitochondrial membrane potential ($\Delta\psi_m$) loss in HL-60 cells. Cells were treated with WA (4 μM) for different time periods and $\Delta\psi_m$ was measured by Flow cytometry using specific fluorescent MitoSensor JC-1 dye. WA caused time dependent increase in depolarization of mitochondrial membrane as evidenced by increase in green fluorescence intensity (FL1) due to the monomeric JC-1 dye with simultaneous decrease in red fluorescence (FL-2, not shown). Almost all cells after 6 h exposure to WA appeared to have suffered a potential loss of $\Delta\psi_m$ with parallel increase in ROS formation (Fig. 4A).

Loss of mitochondrial membrane potential is an early event elicited by WA

We asked if ROS generation is an early event in the induction of apoptosis. In fact, ROS generation appeared to parallel $\Delta\psi_m$ loss and onset of apoptosis happened to be a late subsequent event (Fig. 4B). The relationship between these three important critical events was determined in the gated cell population exposed to WA for different time periods and measured by flow cytometery. At 30 min there was a significant loss of $\Delta\psi_m$ and an increase in ROS generation while apoptotic cells were at basal minimal levels. However after 1 h, the extent of ROS formation and $\Delta\psi_m$ loss were almost similar and increased with time of exposure. A high degree of apoptotic population appeared only after 6 h when most of the cells had lost their $\Delta\psi_m$ (Fig. 4B). The increase in apoptotic population on the contrary was gradual and lower compared to ROS formation and $\Delta\psi_m$ loss.

N-acetyl cysteine protects WA induced cell damage

In order to verify that generation of ROS and loss of $\Delta\psi_m$ are critical events responsible for cell cytotoxicity, we incubated cells with NAC before treating with WA. Consequences of abrogation of ROS generation by NAC on WA altered functions are described in terms of cytotoxicity assay, GSH depletion in the cells, restoration of altered nuclear morphology, and impairment of formation of sub-G_0 cell fraction (Fig. 5 A–D).

NAC protects WA induced cell cytotoxicity

HL-60 cells were treated with NAC before exposure to various concentrations of WA (Fig. 5A). Cell proliferation was assayed in terms of the mitochondrial reduction of MTT by viable cells. WA alone produced concentration dependent cytotoxicity, which was rescued, completely to untreated control cultures by NAC.

WA depleted GSH level in HL-60 cells is a late event

GSH level in the untreated cells is usually very high while in WA treated cells the GSH pool observed a gradual and time-related decline (Fig. 5B). WA produced time dependent decrease in GSH content with significant decline starting after 3 h of treatment when at this period cells were already overwhelmed with ROS formation. NAC, a potent scavanger of ROS enabled to protect cellular GSH from depletion by WA.

WA induced altered nuclear morphology is rescued by NAC

We further sought to examine whether the abrogation of intracellular ROS by NAC could rescue the WA induced apoptotic death. After treatment for 24 h with WA alone and along with NAC, the Hoechst-33258 stained cells were observed under fluorescence microscopy for nuclear morphology and apoptotic bodies (Fig. 5C). Marked morphological changes were observed in WA treated cells such as nuclear condensation, formation of apoptotic and

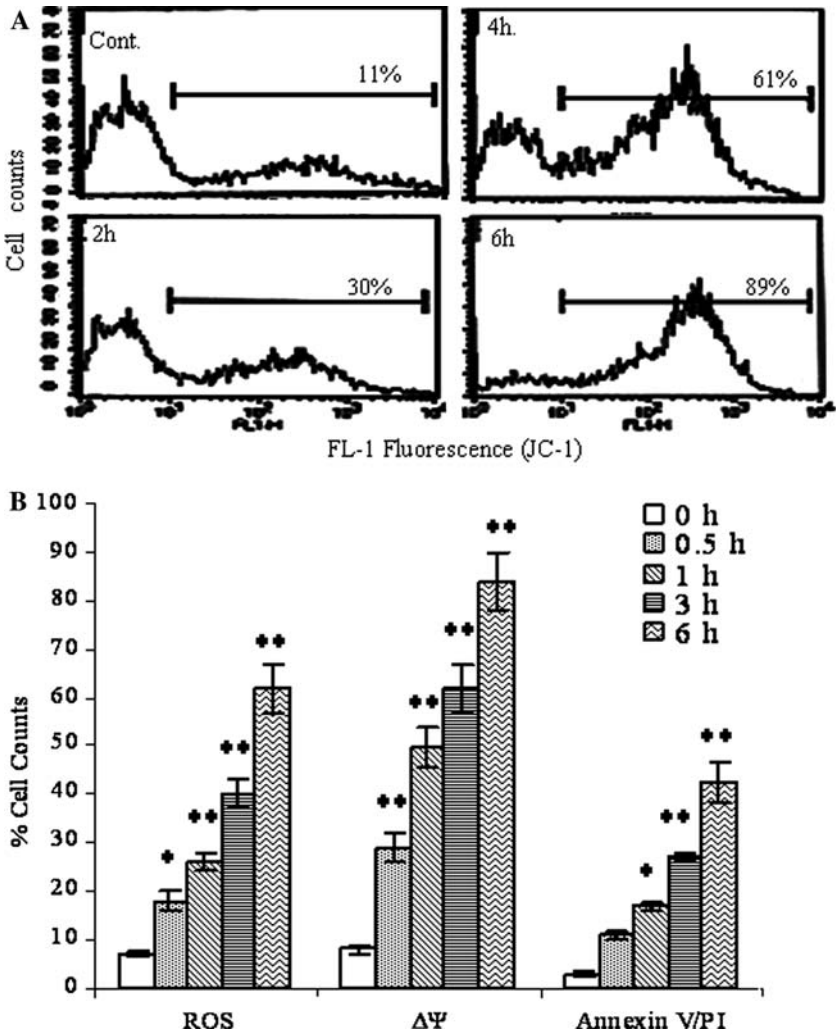
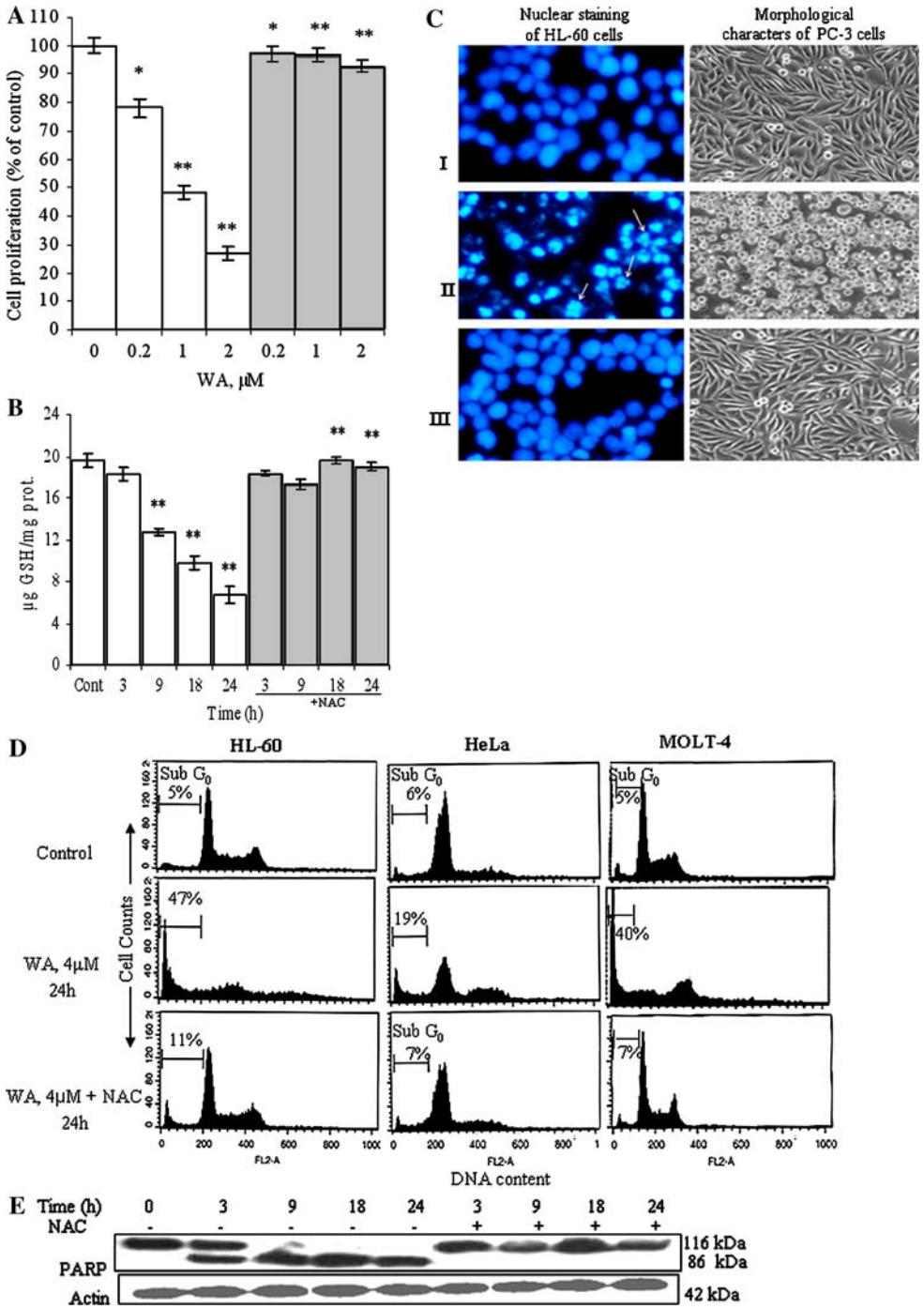


Fig. 4 Flow cytometeric analysis of WA mediated temporal events in the early onset of ROS generation and related pro-oxidant events. (**A**) HL-60 cells (1×10^6/ml) grown in the 24 well culture plates were exposed to the WA (4 μM) for indicated time periods. Cells were stained with JC-1 and analyzed by flow cytometery as described in Material and methods. FACScan analysis of a typical histogram of $\Delta\psi_m$ loss is shown. A decrease in FL-2 fluorescence (not shown) and a concurrent increase in FL-1 fluorescence are indicative of mitochondrial membrane depolarization. (**B**) HL-60 cells (1×10^6/ml) grown in the 24 well culture plates were exposed to the WA (4 μM) for indicated time periods. Cells were stained with DCFH-DA, JC-1 and annexin V-FITC/PI and analyzed by flow cytometry for their respective fluorochromes fluorescence as described in Material and methods. Data are Mean ± S.D. of three independent experiments. (*$P < 0.05$; **$P < 0.001$); statistically significant when WA treated cells compared to respective controls)

scattered apoptotic bodies. However, WA exposed cultures pretreated with NAC exhibited features that were comparable to the untreated control cells. The gross morphological changes were also observed under light microscopy in WA treated PC-3 cells, which again were rescued by NAC (Fig. 5C).

NAC protects WA induced hypo-diploid sub-G_0 DNA population in HL-60 cells

Induction of apoptosis through ROS and protection by antioxidant NAC in HL-60 cells was also confirmed by FACScan analysis of cell cycle phase distribution studies. The results of the cell cycle analysis of HL-60 cells treated with WA for 24 h resulted in increase in hypo-diploid sub-G_0 DNA fraction. As Sub- G_0 peak is reported to be a qualitative indicator of apoptosis [32], increase in this fraction therefore not only supports apoptotic mode of cell death by WA, but also that apoptosis is the result of ROS formed as the cells pretreated with NAC exhibited marked decline in Sub G_0 population. Similar findings were also observed in Molt-4 and HeLa cells (Fig. 5D). The increase in annexin V binding is also protected by NAC as observed earlier (Fig. 2B).



WA causes early cleavage of PARP protein

PARP (poly (ADP ribose) polymerase), an enzyme involved in DNA repair, is a preferential substrate for caspase-3. We investigated PARP protein cleavage in WA treated HL-60 cells. WA treatment caused early cleavage of PARP, 116 kDa into 89 kDa fragment beginning in less than 3 h that was almost complete after 9 h. This corresponded with the activation of caspase-3. Again pre-treatment with NAC resulted in a complete protection against WA-induced PARP cleavage (Fig. 5E).

WA is a potent activator of caspases

After validating cell death by apoptosis measured by several end-points, we asked what types of signaling cascades cells followed during programmed cell death

◀**Fig. 5** Protective effect of NAC on WA induced apoptotic alterations in HL-60. (**A**) Protection against WA inhibition of cell proliferation. HL-60 cells were treated with NAC (5 mM) 1 h before treatment with various concentrations of WA for 48 h and the cell proliferation was determined by MTT reduction assay. Control wells received medium containing DMSO (<0.2%, v/v). Other conditions were same as described in Fig. 2A. Data are mean value ± S.D. (n = 8 wells) and representative of one of two similar experiments and statistically significant. P values: $^{*}P < 0.05$; $^{**}P < 0.001$; WA treated versus control cells; WA+ NAC versus WA treated cells. (**B**) Protection against WA induced GSH depletion- HL-60 cells (3×10^6/2 ml, 6-well plate) were treated with WA (4 μM) for indicated time periods with and without 5 mM NAC. NAC was added 1 h before exposure of cells to WA. Cells were collected and washed thrice with PBS to remove NAC before the determination of reduced glutathione contents. Data are Mean ± SD (n = 4 wells) and represent one of two similar experiments. The statistically significance is similar to as shown in Fig. 4A. (**C**) NAC rescued WA induced nuclear morphological changes. Hoechst 33258 staining of HL-60 cells observed under fluorescence microscopy as described in Materials and methods detected influences of WA on nuclear changes. (i) Untreated control cells show rounded nuclei; (ii) cells treated with WA (4 μM) for 24 h show condensed chromatin/nuclei, apoptotic (arrows) and scattered apoptotic bodies; (iii) cells incubated with NAC (5 mM) 1 h before the treatment with WA show protection against WA-mediated nuclear alterations. WA induced morphological characteristics of the PC3 cells were restored by NAC (5 mM) after 24 h exposure to WA (4 μM). Cells were subjected to the same treatment as that of HL60 cells. Photographs were taken under phase contrast microscope (30×) to observe the characteristic morphological changes in the cells. Data are one of two similar experiments. I, control; II, WA treated cells 24 h; III, NAC + WA treated cells. (**D**) NAC protects increase in hypodiploid Sub-G_0 cell population in WA treated cells. HL-60 cells (1×10^6/ml) in culture were treated with WA (4 μM) for indicated time period. NAC (5 mM) was added 1 h before WA treatment. Cells were stained with PI to determine DNA fluorescence by flow cytometery as described in Materials and methods. Sub-G_0 population indicative of DNA damage was analyzed from the hypo diploid sub-G_0 fraction (<2n DNA) of DNA cell cycle analysis. Data on HL-60, HeLa and Molt-4 cells are representative one of two similar experiments. (**E**) WA induced PARP cleavage is protected by NAC. HL-60 cells were treated with WA (4 μM) for different time periods in the presence and absence of NAC. Equal amounts of total cell lysate protein were resolved on 10% SDS-PAGE, then transferred to PVDF membrane and probed with anti-PARP antibody. Anti-body to actin served as sample loading control for protein level. Other conditions were same as described in Materials and methods. Western blot is representative from one of two similar experiments

induced by WA, because activation of caspase-9 and -8 suggests engagement of both intrinsic and extrinsic pathways of apoptosis. We therefore examined the activation of caspase 3, 8 and 9 in HL-60 cells treated with WA for different time periods in the presence and absence of NAC. WA produced remarkable early activation of executioner caspase-3 by more than 3-fold at 3 h and an optimal activation of almost 6-fold after 9 h of treatment. This activation exhibited correspondence with ROS generation. With prolonged treatment through 24 h, the caspase activity decreased to the level of 3 h treatment possibly due to the inactivation because of increasing population of cells undergoing post-apoptotic necrosis (Fig. 6A). Simultaneous treatment with NAC however, recovered substantially the activity through 3 and 9 h treatment while prolonged treatment with NAC returned back the activity to almost untreated control level (Fig. 6A). Caspase-9 exhibited similar activation profile as that of caspase-3 when cells were treated with WA and in the presence of NAC (Fig. 6B). On the contrary, WA produced slow but time-related induction of caspase-8 activity, maximum being 3-fold through 18 h, which again was protected by NAC (Fig. 6C). Unlike caspase-3 and -9 the increasing level of this enzyme activity was not attenuated when cells were treated with WA for 18 h. This suggests that turnover number of this enzyme is independent of factors influencing caspase-3 and -9 activities. The increasing caspase-8 activity exhibited strong correlation with time-related enhanced over expression of TNFR-1 in WA treated HL-60 cells analyzed by immunobloting (Fig. 6D).

Activation of caspase-3 by WA is influenced by activation of both capase-9 and -8 signaling

Caspase-3 activity of HL-60 cells treated with WA for 9 h was measured in the presence and absence of caspase-8 inhibitor AC-IETD-CHO and caspase-9 inhibitor z-LEHD-FMK (Fig. 7) in order to evaluate their relative contribution in the activation of caspase-3. The inhibitors were added 3 h before the treatment with WA. The withanolide induced more than 3-fold activation of caspase-3; the stimulated activity was inhibited >80% by caspase-9 inhibitor. On the contrary, the influence of caspase-8 inhibitor was relatively lower at this period of treatment as it could inhibit the caspase-3 activity by about 40%. This suggested that WA induced apoptosis engages at least both caspase-8 and -9 dependent signaling cascades.

Mitochondrial membrane disruption by WA: Release of Cyt. c, AIF and translocation of Bax across outer mitochondrial membrane without affecting the expression of Bcl-2 (Fig. 8 A–G)

Excessive ROS generation is known to contribute to mitochondrial damage where Bax from cytosol is translocated and integrated into the outer mitochondrial membrane to form pores to allow the release of cytochrome c into the cytosol as a prerequisite for mitochondrial mediated pathway of apoptosis [33]. To address the possibility that the WA-induced apoptosis is related to

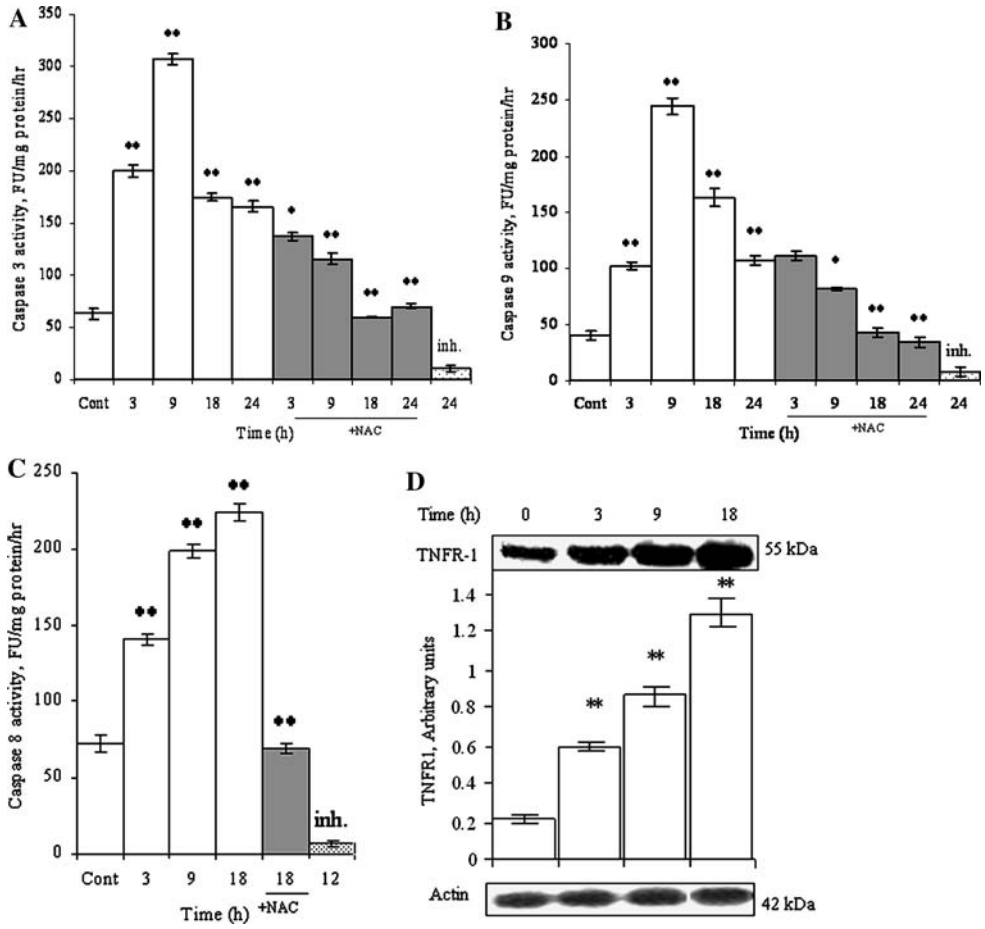


Fig. 6 WA induced activation of caspases and protection by NAC in HL-60 cells. HL-60 cells (3×10^6/2 ml) were exposed to WA 4 μM for indicated time periods for caspase-3 (**A**) caspase-9 (**B**) and caspase-8 (**C**) activities. Wherever indicated, cells were pretreated with 5 mM NAC 1 h before exposure to WA. The caspase activities were determined fluorometrically in the cell lysates of HL-60 cells using BD Apoalert caspase fluorescent assay kits. Specific peptide based inhibitors (inh.) provided along with the assay kits were used for negative control to determine whether fluorescence intensity changes were specific for the activity of caspases as described in Materials and methods. Data are Mean ± S.D. from three similar experiments. (**D**) Immunoblot analysis of TNF-R1 in HL-60 cells treated with WA (4 μM) for indicated time periods. Total cell lysates were prepared and 50 μg protein samples were loaded on SDS-PAGE gel for western blot analysis as described in Material and methods. Relative density of each band indicates arbitrary units of TNF-R1 expression analyzed by Quantity One software of Bio-RAD gel documentation system. *P*-values: $^*P < 0.05$; $^{**}P < 0.001$; WA treated versus untreated control cells or WA + NAC versus WA-treated cells

contributions from the mitochondrial pathway as evidenced by caspase-9 activation, we followed time dependent influence of WA on cytochrome c release and translocation of Bax into the mitochondria by western blot analysis of proteins of WA treated HL-60 cells. WA induced time-dependent progressive increase of Bax expression in the mitochondrial fraction and the concurrent increase of cytochrome c release from mitochondria to the cytosol (Fig. 8A–D). To further examine whether cytochrome c release and Bax translocation were down stream of ROS generation, we pre-incubated cells with NAC before WA treatment (Fig. 8A–D). It was found that NAC has completely abrogated the WA induced cytochrome c release and Bax translocation between mitochondria and cytosol. Cytochrome c was almost drained out from mitochondria after 18 and 24 h treatment that was completely restricted in NAC treated cells. Similarly, a time-dependent reciprocal relationship in the cyosolic fall of Bax was observed which was rescued by NAC. We further examined the effect of WA on the expression of anti-apoptotic Bcl-2 protein, a member of Bcl-2 family that inhibits the translocation of Bax that stops the release of cytochrome c and hence the onset of apoptosis. An interesting observation was that WA was unable to alter the expression of anti-apoptotic Bcl-2 whose level remained unchanged as that of control cells through out the exposure time (Fig. 8E). These results indicate that WA may disarrange the ratio of Bcl-2 and Bax and, therefore, may lead to apoptosis of HL-60 cells. Another important BH3-only protein, Bid is involved in a cross talk between the intrinsic and extrinsic

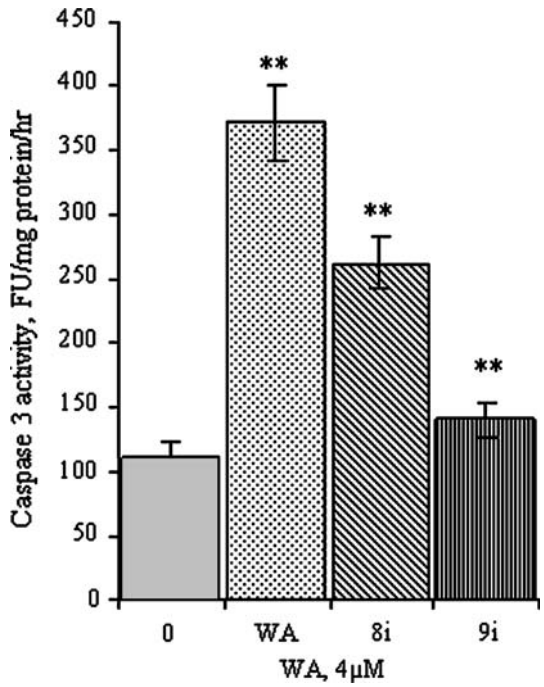


Fig. 7 WA directs caspase-3 activation largely through caspase-9/8 signaling pathways. HL-60 cells were incubated separately with 25 μM of caspase-9 inhibitor (9i, Z-LEHD-FMK) and caspase-8 inhibitor (8i, AC-IETD-CHO) for 3 h. Cells thereafter received treatment with WA (4 μM) for another 9 h and total cell lysates were prepared for the assay of caspase activities as described in Materials and methods. Data are Mean ± SD of three similar experiments.**$P < 0.001$ when compared with untreated control; **<0.001, 9i, vs. WA; *<0.05, 8i, vs. WA

pathways. The death receptor mediated caspase-8 activation leads to truncation of Bid to active pro-apoptotic tBid. The expression of Bid observed a time-dependent decrease in HL-60 cells treated with the withanolide. At 18 and 24 h the expression was almost completely lost possibly because of truncation facilitated translocation to mitochondria. We could not detect tBid with the same antibody used for Bid. However, the degradation was protected by NAC (Fig. 8F). Further we also examined the effect of WA on the relationship between ROS generation with release of apoptosis-inducing factor (AIF) and translocation to nuclear fraction because overwhelming formation of ROS is observed in AIF knock out cells, and that the increase in ROS after AIF depletion has been well established [34]. Early translocation of AIF from mitochondrial intermembrane space to nuclei was observed in less than three hr of WA treatment which increased by at least 4-fold through 24 h (Fig. 8G). Pretreatment of cells with NAC again prevented the AIF translocation corroborating our present and earlier [35] claims of increased ROS generation originating from ETC and its association with AIF release from mitochondria.

WA inhibits NF-kB activation by inducing cleavage in the p65/Rel subunit, and by inhibiting binding to DNA

WithaferinA is reported to inhibit activation of NF-kB in HL-60 cells, and we were interested to find if this inhibition is because of oxidative stress. This was examined by EMSA using ^{32}P-labeled oligonucleotide that contains NF-kB binding sites. HL60 and HUT-78 cells were treated with WA (4 μM) for different time periods, and then nuclear extracts were prepared and assayed for NF-kB binding of DNA. The results indicated that the constitutive NF-kB activity is very high in the untreated cells (9A-I), which was almost completely blocked in WA treated cells in less than 2 h. The down regulation or degradation of NF-kB activity continued through 8 h of treatment. WA strongly inhibited NF-kB activation from an early period of 2 h treatment while free probe with out nuclear extract was run as an assay control. It may be indicated that down regulation of NF-κB appears as a common mechanism of WA action as this effect was also prominent in HUT-78 human lymphoma T cell line (Fig. 9A-II). Further the inhibition was protected by antioxidant NAC returning the activity to the constitutive level of untreated control cells.

We also performed immunoblot analysis of NF-κB expression in cytosolic and nuclear fractions as well as total cell lysate by employing monoclonal antibody which recognizes -COOH terminus epitope of p65/Rel. In total cell lysate, we observed for the first time that WA not only suppressed the expression of NF-κB-p65 but also caused its cleavage during 9–24 h exposure time which was protected completely in cells pre-treated with NAC (Fig. 9B). To understand whether WA induced NF-κB suppression and p65 cleavage occurs in cytosol or after translocation into the nucleus, we observed that NF-kB is cleaved only after its translocation into the nucleus (Fig. 9C) as a result of WA treatment of cells. The cleavage appeared to follow only at a much later stage after it had initially failed to bind DNA. Since NF-kB cleavage is proposed to be mediated by activated caspase-3, we investigated if WA induced caspase-3 is involved in the cleavage (Fig. 9D). For this purpose, HL-60 cells were pre-incubated for 3 h with caspase-3 inhibitor Z-DEVD-CHO before further treating with WA for 18 h. The cleavage of NF-kB was completely protected demonstrating that WA mediated activation of caspase-3 is responsible for NF-KB cleavage.

Discussion

One of the goals of cancer chemotherapy is to explore and develop discovery leads that can selectively induce apoptosis in cancer cells [36]. In this study we report for the first time a novel insight in deciphering the mechanisms

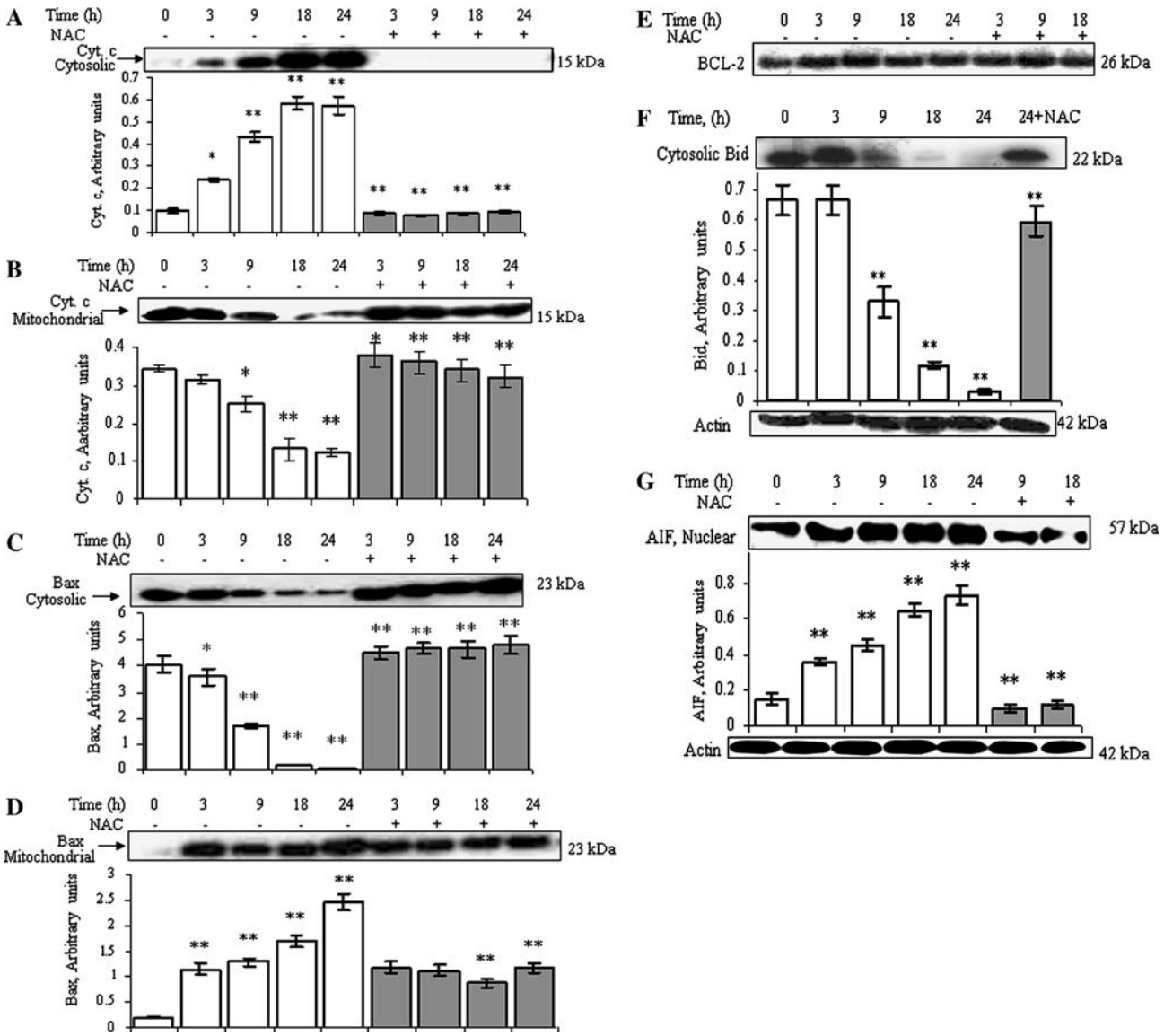


Fig. 8 NAC rescues WA induced altered expression of pro- and anti-apoptotic proteins in HL-60 cells as shown in western blot analysis. HL-60 cells were treated with WA (4 μM) for indicated time periods in the presence and absence of NAC. Immunoblot analysis of cytochrome c, Bax, AIF and BCl-2 was performed in designated sub-cellular lysate. Equal amount of proteins were loaded and resolved on 15% SDS-PAGE, electro transferred to PVDF membrane and probed with specific antibodies. Actin anti-body served as control for the loading protein level. Other conditions are described in Materials and methods. (**A**), (**B**) Cytochrome c; (**C**), (**D**) Bax; E: Bcl-2; (**F**) Bid; (**G**) AIF. Density of each band was calculated using Quantity One software as depicted in bar charts. Data are Mean ± S.D. of three similar experiments. $^{*}P < 0.05$; $^{**}P < 0.001$ for WA versus control, or WA+ NAC treated cells versus WA treated cells

involved in WA induced early events leading to the activation of signaling cascades culminating in apoptotic cancer cell death. Our results demonstrate that exposure of HL-60 cells to WA enabled apoptotic cell death as evidenced by apoptotic bodies formation, increased sub-Go hypo-diploid DNA fraction and enhanced FITC-labeled dUTP incorporation into the 3′-hydroxyl-DNA ends and annexin V binding of cells. However, ROS generation was overwhelmed during the early events of WA exposure before the onset of apoptosis of comparable magnitude suggesting thereby an early pro-oxidative environment triggered by WA. This exquisitely has enabled to induce disruption of mitochondrial function, with concurrent loss of mitochondrial membrane potential ($\Delta\psi_m$). Occurrence of all these events was time dependent and subtle changes appeared within less than 3 h of exposure to WA.

The origin of WA induced ROS formation by WA in cancer cells could not be ascertained in the present studies. The withanolide may be inhibiting respiratory electron transport chain (ETC) delivering electrons to reduce molecular oxygen to form superoxide oxidants as have been observed with mitochondrial ETC inhibitors for

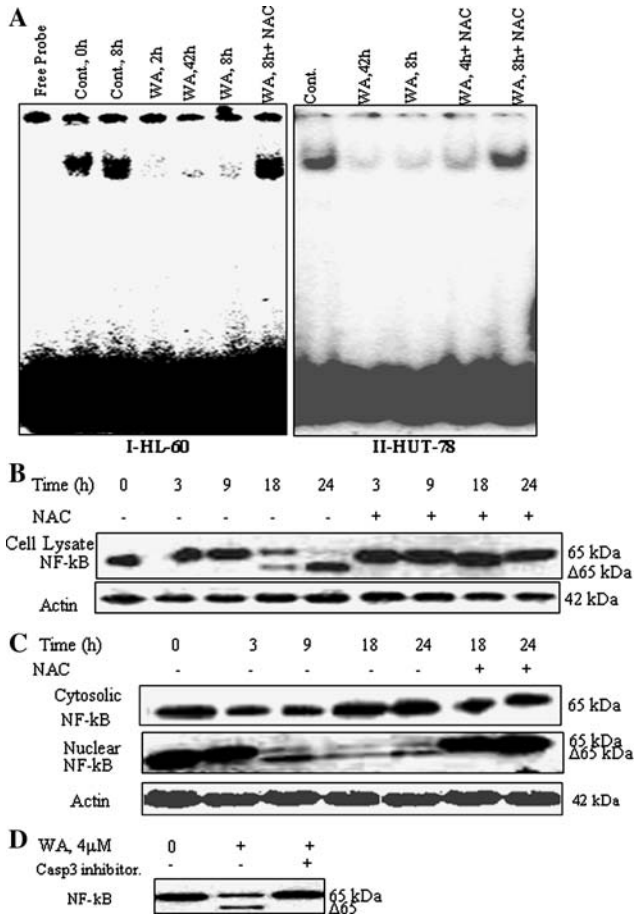


Fig. 9 WA induced inhibition of NF-kB binding of DNA and its nuclear cleavage. (**A**) Electrophoretic mobility shift assay- HL-60 cells were treated with WA (4 μM) for different time periods in the presence and absence of NAC (5 mM) as indicated. Nuclear extract was prepared from HUT-78 and HL-60 cells for the assay of NF-kB binding to DNA by EMSA as described in Material and methods. (**A**-I) shows NFk-B suppression in HL60 cells and (**A**-II) shows the NFk-B suppression in HUT-78 cells. The data are representative of one of the two similar experiments. (**B** and **C**). Immunoblot analysis of WA induced cleavage of NF-kB in HL-60 cells- HL-60 cells were treated with WA (4 μM) in the presence and absence of NAC for indicated time periods. Total cell, nuclear and cytosolic lysates were prepared as described in Materials and methods. The proteins were resolved on SDS-PAGE and probed against p65/rel antibody as described in Materials and methods. Data are representing one of three similar experiments. (**D**) WA induced caspase-3 mediated NF-kB cleavage. HL-60 cells in culture were pre-incubated for 3 h with 25 μM caspase-3 inhibitor Z-DEVD-CHO before treatment with 4 μM of WA for 18 h. Immunoblot analysis of the total cell lysate was performed as described in (**B**)

complex-III and complex-I [37] or by activation of NADPH oxidases. The ETC complexes are the major sites of ROS generation in mitochondria. A blockade of these sites by WA may enable delivery of one electron to molecular oxygen and allow release of superoxide either on the cytoplasmic side or in the mitochondrial matrix resulting in $\Delta\psi_m$ loss. Nevertheless, the ROS formation by WA was strongly blocked by the strong antioxidant NAC.

We expected a significant fall in GSH pool during the early exposure of cells to WA because of the possibility of oxidation of thiol groups by ROS. However, the pool was not affected critically during this period when free radicals generation was at its maximum. The GSH depletion may thus not be the primary cause of cell death because cells exposed to WA are able to maintain the reducing environment due to GSH at least for 3 h contrary to ROS formation. This may be the reason that optimal activation of caspase-3 and caspase-9 was observed through 9 h and thereafter the activity started declining because caspases contain an active site cysteine nucleophile, which is prone to oxidation by ROS in fatally dying cells [38] when GSH levels are not sufficient enough to counter overwhelming ROS accumulated. Caspase-8 however, exhibited a continuing uprising trend with optimal activation at 18 h, which may be viewed as a late complimentary mechanism to support cell death.

The activation of two apoptosis initiators caspases-9 and -8 suggested at least two signaling cascades [36, 39] involved in the apoptotic cell death by WA. Caspase-9 activation by WA suggests engagement of mitochondrial signaling cascade as evidenced by early release of cytochrome c from permeabilized mitochondria to cytosol and simultaneous translocation of Bax to mitochondria. Cytochrome c released is known to bind Apaf-1 (apoptotic protease activating factor 1) in the cytosol to form a complex called apoptosome that recruits and binds procaspase-9 to release active caspase-9, which up-regulates down stream pathways leading to the activation of executioner caspse-3 [39]. Increased level of cytochrome c in the cytosol and its corresponding decrease in mitochondria suggests that release of cytochrome c in fact is a 'point of no return' for cells to enter apoptosis.

WithaferinA also induced early translocation of Bax from cytosol to mitochondria consequent to disruption of mitochondrial membrane functions and $\Delta\psi_{mt}$ loss. The translocation of Bax may also aid and abet the oxidative burst leading to the release of Cyt c from mitochondrial inner membrane [40]. In the cytosol of non-apoptotic cells Bax exists constitutively in inactive form and its activation by ROS mediated cytosolic sensors is often required to oligomerize and stably insert it in the outer mitochondrial membrane. This would enable the onset of MOMP, which is often associated with the loss of $\Delta\psi_{mt}$. Therefore, the translocation of active Bax to mitochondria is a critical event in the discharge of cyt c and other pro-apoptotic molecules from inner mitochondrial space to cytosol. The anti-apoptotic protein Bcl-2 is reported to block the release of cytochrome c and MPT opening [41] by preventing ROS production. We however, could not observe any change in the expression of Bcl-2 though cells were overwhelmed with ROS when exposed to WA suggesting that Bcl-2 may function

differently depending upon various stimuli. For instance, continued Bcl-2 expression was observed in EBV-transformed lymphoblastoid cells undergoing spontaneous apoptosis because of the inhibition of NF-kB in these cells [42].

The loss of $\Delta\psi_m$ by WA is a sign of mitochondrial swelling and disruption of outer mitochondrial membrane [43] with subsequent release of apoptosis inducing factors, i.e., AIF, cytochrome c, from ROS damaged mitochondria of WA treated cells. It is also recognized that translocation of AIF to nucleus is associated with increase in ROS formation and induction of apoptosis [35]; AIF thus released produces peripheral chromatin condensation and high molecular weight DNA fragmentation in the nucleus. WA induced apoptosis consequent to AIF translocation into the nuclei amounts to a similar situation where microinjection of recombinant AIF into the isolated nuclei or cells resulted in apoptotic phenotypes, and mitochondrial membrane potential loss [44].

Another mode of cytotoxicity by WA happened through activation of caspase-8 suggests involvement of extrinsic pathway of apoptosis [39]. Because activation of caspase-8 activity over 18 h period of time corresponded with continuing increased expression of TNFR-1 on cell surface. ROS generation has reportedly been acclaimed to induce over-expression of cell surface death receptors TNFR-1/Fas [45] and WA induced ROS generation increased the activity of caspase-8 with simultaneous expression of TNFR-1. Activation of caspase-8 is also associated with the cleavage of Bid, an important pro-apoptotic member of the Bcl-2 family of proteins. The truncated product tBid translocates to mitochondria and is believed to induce permeabilization of the outer mitochondrial membrane. It is also believed to reorganize the inner mitochondrial membrane leading to rapid release of Cyt c and other molecules involved in apoptotic response. Our studies have shown that WA caused time dependent decrease in the expression of Bid which corresponded with increased enzymatic activity of caspase-8 and over expression of TNFR-1. The decreased expression of Bid may thus be related to its truncation, which forms a central point in the cross talk between caspase-8 and caspase-9 activation. These events clearly demonstrate that WA induced apoptotic cell death is the consequences of involvement of both mitochondrial and non-mitochondrial signaling cascades. The mitochondrial dependent caspase-9 activation by WA however, appeared predominant in regulating cell death during the early hours of exposure. This is evidenced from our studies where caspase-9 inhibitor blocked almost completely the WA activated caspase-3 activity while caspase-8 inhibitor exerted significant but relatively lesser effect after 9 h exposure of HL-60 cells to WA. Thus caspase-8 signaling pathway exerts an important augmenting effect on the major WA activated executioner caspase-9 pathway. Further, the caspases upon activation cleave numerous cellular proteins [46] of which poly (ADP-ribose) polymerase cleavage happened to be an early target of WA induced apoptotic onslaught. All these studies suggested that ROS production functions as a positive regulator of caspases activation. This is a classical example where a dietary product WA is able to mediate ROS production leading to apoptosis, though studies are needed to find precise mechanisms of ROS formation by WA in cancer cells.

Further more, NF-kB family of transcription factor plays a central role in regulation of apoptosis, oncogenesis, inflammatory and immune responses and is activated by a wide range of stimuli. The ability of NF-kB to inhibit apoptosis appears to be stronger than its ability to promote apoptosis [47], and therefore, inhibition of NF-kB is suggested to be a useful strategy for cancer therapy. WA in this regard exhibited its strong ability not only to block completely the binding of the transcription factor to DNA but also caused cleavage of nuclear NF-κB. Such an effect has also been observed earlier with other agents [48]. The failure of NF-kB binding to DNA in the nucleus subjects this protein further to nuclear cleavage by activated caspase-3 observed in our studies thereby abrogating its complete functionality facilitating obliquely the pro-apoptotic machinery of cell death. In other words inhibition of NF-kB may have profound effect on transcription of several anti-apoptotic genes, anti-oxidant enzymes and early formation of ROS. This also suggests that down regulation of the factor can be used as a suitable molecular target for development of anticancer chemotherapeutic agents such as WA and its semi-synthetic analogs.

We further tested the role of oxidant-specific mechanism by pre-exposing HL-60 cells to NAC prior to WA exposure so as to reverse the pro-apoptotic phenotypes. NAC exerted strong protective effect against WA induced ROS mediated apoptosis and the various events involved in intrinsic and extrinsic signaling cascades as represented in Fig. 10. The gradual time dependent protection by NAC appeared to be guided not only by the redox state of the cell, but also on the duration of NAC accessibility to the site(s) of ROS generation. This may be the reason that prolonged incubation of cells with NAC prior to WA treatment offered complete protection to cell viability, annexinV binding and DNA fragmentation. NAC is a scavenger of free radicals as it interacts with ROS through its reactive thiol groups [49] and is increasingly used as chemoprotective agent in clinical trials to ameliorate the toxicity of chemotherapeutics, such as platinum. In case of its chemo protection to WA induced cytotoxicity, NAC may be scavenging ROS by direct interation of its reactive thiol groups with ROS and may also by offering protection against oxidative modifications of critical protein targets affected by WA. In our

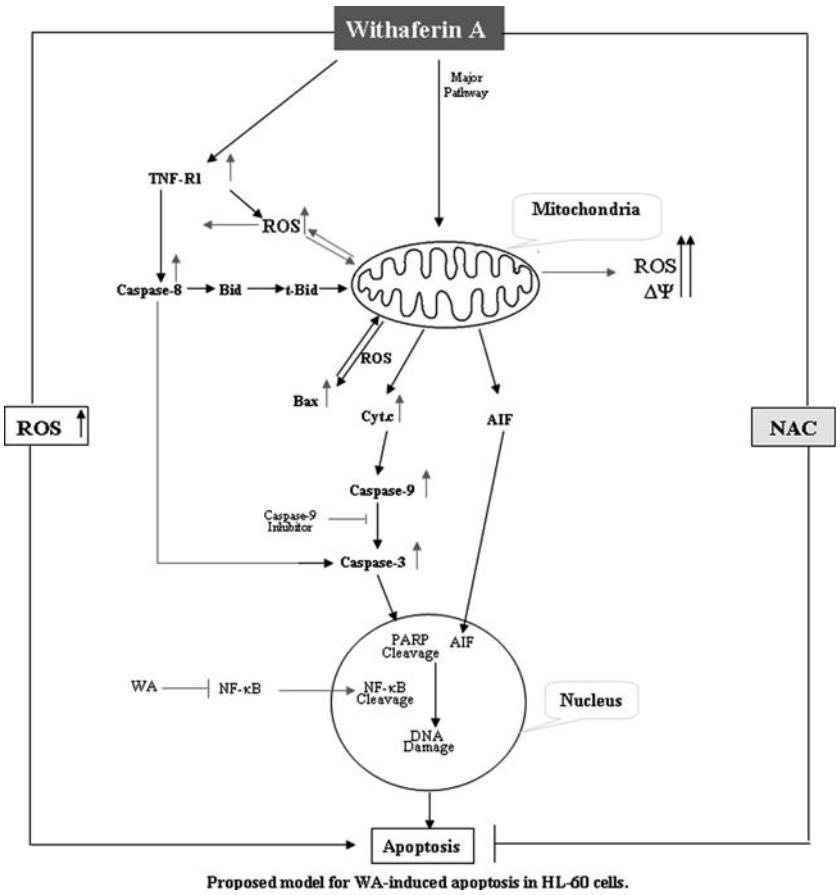


Fig. 10 Schematic representation of various events involved in WA induced apoptosis in HL-60 cells and protection by NAC. Based on the results of our studies on the expression of various apoptosis phenotypes, a scheme is drawn describing the molecular mechanisms of WA action and protection by NAC

studies NAC protected efficiently all pro-apoptotic changes induced by WA in cancer cells. This again demonstrates that though WA may be acting at several targets inside the cell, the hallmark of WA cytotoxicity is through oxidative stress. NAC is also known to prevent apoptosis and promotes cell survival by inhibiting ERK pathways leading to cell survival [50] and it would not be surprising if WA may be activating ERK and MAPK pathways. Our results suggest that WA enters cells rapidly and commits cells to apoptosis which otherwise are rescued by NAC to survival pathways.

Conclusion

In conclusion our studies demonstrate that WA induced early ROS formation and mitochondrial dysfunctions are directly responsible for induction of apoptotic cell death. The withanolide pursued predominantly mitochondrial intrinsic signaling pathway by release of cytochrome c, and AIF from mitochondria and translocation of Bax from cytosol to mitochondria facilitating caspase-9 activation and up regulation of down stream pathways leading to caspase activation and PARP cleavage. WA also increased the activity of caspase-8 with simultaneous expression of TNFR-1 demonstrating activation of extrinsic signal cascade too. However, the intrinsic pathway through caspase-9 signaling exhibited predominance over extrinsic pathway. Further, WA induced decreased expression of Bid may be an important link in the cross talk between caspase-8 and -9 signaling pathways of apoptosis. Another interesting feature was that WA inhibited not only NF-κB binding of DNA but also caused its nuclear cleavage. All these events were reversed by ROS scavenger NAC suggesting that WA alters the redox balance of the cells. The results of these in-depth studies provide putative mechanism of action of WA mediated oxidative stress in cancer cells. The studies therefore raise the potential usefulness of WA as an anti-cancer therapeutic candidate present in the dietary supplement *Withania somnifera*.

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