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Original Article

Withaferin A activates stress signalling proteins in high risk acute lymphoblastic leukemia

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Abstract: Withaferin A, the principal bio-active component isolated from the Withaniasomnifera, has shown promising anti-leukemic activity in addition to anti-invasive and anti-metastatic activity. The present study demonstrates the effect of withaferin A on the cell cycle status and the phosphorylation/activation of proteins involved in signal transduction in t(4;11) and non-t(4;11) acute lymphoblastic leukemia (ALL) cell lines after treatment with withaferin A. The cells after treatment with the vehicle or 25 μM withaferin A for 1, 2, 4 and 8 h were examined using flow cytometric analysis. The results revealed that withaferin A treatment induced cell growth arrest at the S to G2/M phase transition of the cell cycle. Withaferin A treatment also induced the phosphorylation of stress signalling proteins, including the p38 mitogen-activated protein kinase, the c-Jun N-terminal kinase, c-Jun, the heat shock protein 27 and protein kinase B within 0 to 16 h. These results were observed using multiplex technology and Western blotting analysis. Thus withaferin A induces stress response leading to cell death. Therefore, withaferin A can be a potent therapeutic agent for the treatment of high risk ALL with chromosomal translocation t(4;11).

Keywords: Withaniasomnifera, antileukemic activity, signal transduction, cell death, translocation

Introduction

Withaniasomnifera root extract contains 14 withanolides, among which the most abundant is Withaferin A (**Figure 1**) [1, 2]. The extract has a long traditional medicinal importance in East Indian medicine [3]. It is reported that withaferin A induces apoptosis in vimentin expressing tumour cells and inhibits soft tissue sarcoma growth and local recurrence in xenografts [4]. It inhibits vimentin-dependent proteasome activity at micromolar concentration [3, 5]. In breast and prostate cancers withaferin A has shown potent pro-apoptotic and anti-tumour activity [6-8]. It exhibits anti-tumour activity through a variety of targets including NF-Kb, BCL-2, FOXO3A, Hsp90, phosphorylated STAT3 and annexin II27 [9-13]. Withaferin A is known to possess anti-invasive and anti-metastatic activity. It inhibits cancer cell motility and invasion with negligible effects on proliferation in breast cancer at nanomolar concentrations. It leads to vimentin disassembly and hyperphosphorylation of vimentin ser56. Not only the withaferin but its analogs also show vimentin-targeting and anti-invasive activity. Importantly, WFA has potent anti-metastatic efficacy that results in vimentin ser56 phosphorylation, with minimal toxicity to normal tissues [14].

Despite advancement in molecular biology the chromosomal abnormality is associated with poor prognosis [15, 16]. About 60-85% of infants, 2% of children and 3-6% of adults are diagnosed with acute lymphoblastic leukemia (ALL). ALL is resistant to conventional chemotherapeutics; hence the need for a novel effective therapeutic strategy is unmet. Previously, parthenolide has been reported to induced apoptotic cell death in SEM and RS4; 11 cell lines established from patients with ALL carrying the t(4;11)(q21;q23) chromosomal translocation [17]. Parthenolide induces apoptosis in t(4;11) ALL cells at a rapidly compared to the REH leukemia cells which have no translocation. Increased generation of ROS including

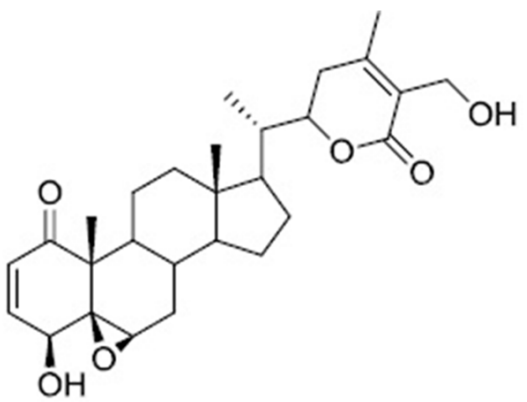

Figure 1. Structure of withaferin A.

nitric oxide, superoxide anion and hypochlorite anion was reported in these cells [17]. The present study was designed to investigate the effects of withaferin A on intracellular protein signalling events leading to apoptosis in leukemia cells. We observed an enhancement of activation (phosphorylation) of several proteins involved in the stress response, including the heat shock protein 27 (HSP27), protein kinase B (Akt), c-Jun N-terminal kinase (JNK), c-Jun and the p38 mitogen-activated protein kinases (p38 MAPK) on withaferin A treatment. Thus withaferin A is a potent therapeutic agent for ALL by activating stress response proteins.

Materials and methods

Cell lines and reagents

The established SEM and RS4;11 cell lines are from patients with high-risk pre-B cell ALL with chromosomal translocation t(4;11)(q21;q23) [14, 15]. The REH cell line was obtained from the American Type Culture Collection (Manassas, VA). The cell lines were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). Withaferin A and other common chemicals were purchased from Sigma (Sigma, St. Louis, MO).

Cell cycle analysis

Cells at a density of 2×10^5/ml were treated with DMSO or 25 μM withaferin A for 1, 2, 4 and 8 h. The cells were then collected, lysed in a hypotonic solution containing 1 mg/ml sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide (PI, Sigma) and examined for cell cycle analysis. FACSCanto fluorescence-activated cell sorter (FACS) with FACSDiva software (Becton-Dickinson, San Jose, CA) was used for analysis of the resulting nuclei. For each sample stained with PI twenty thousand events were collected. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used for the generation of cell cycle profiles.

Multiplex measurements of phosphoproteins

We used the Bio-PlexPhosphoprotein Detection kit (Bio-Rad, Hercules, CA) to examine the phosphorylation of various proteins. The cells after treatment with 25 μM withaferin A or DMSO (control) were lysed at 1, 2, 4, 8 and 16 h. Cell lysates were prepared with lysate buffer supplied with the kit as per the manufacturer's protocol. The concentration of proteins was measured using the Bio-Rad DC Protein Assay and Bovine Á-globulin was used as the standard. The measurements were performed in triplicates using Bio-Plex multiplex flow cytometry instrument (Bio-Rad). All the results were presented as the mean of the median bead intensities normalized to the protein concentration in each well.

Western blot analysis of total and phosphoproteins

The cells after treatment with DMSO or 25 μM withaferin A were lysed, and using dye-binding method (Bio-Rad) protein concentration was determined. The proteins were resolved on 15% SDS-PAGE and transferred to nitrocellulose membranes. Digitonin-based subcellular fractionation technique was used for cytosolic and mitochondrial fractions. Onto DS-PAGE equal volumes of cytosolic and mitochondrial fractions were resolved and transferred to nitrocellulose membranes. The membranes after incubation with primary antibody were washed, and then incubated with horseradish peroxidase anti-mouse or horseradish peroxidase anti-rabbit antibodies. Rabbit polyclonal antibodies against phosphorylated and total Akt, p38 MAPK, JNK, c-Jun, phosphorylated HSP27 and mouse antibody against total HSP27 (Cell Signalling Technology, Danvers, MA) were used. Enhanced chemiluminescence system was used for visualization of immunoreactive bands. The primary antibodies used were: thymidylate synthase (TS), acetyl-H3, poly-(ADP-ribose)-polymerase (PARP), g-tubulin,

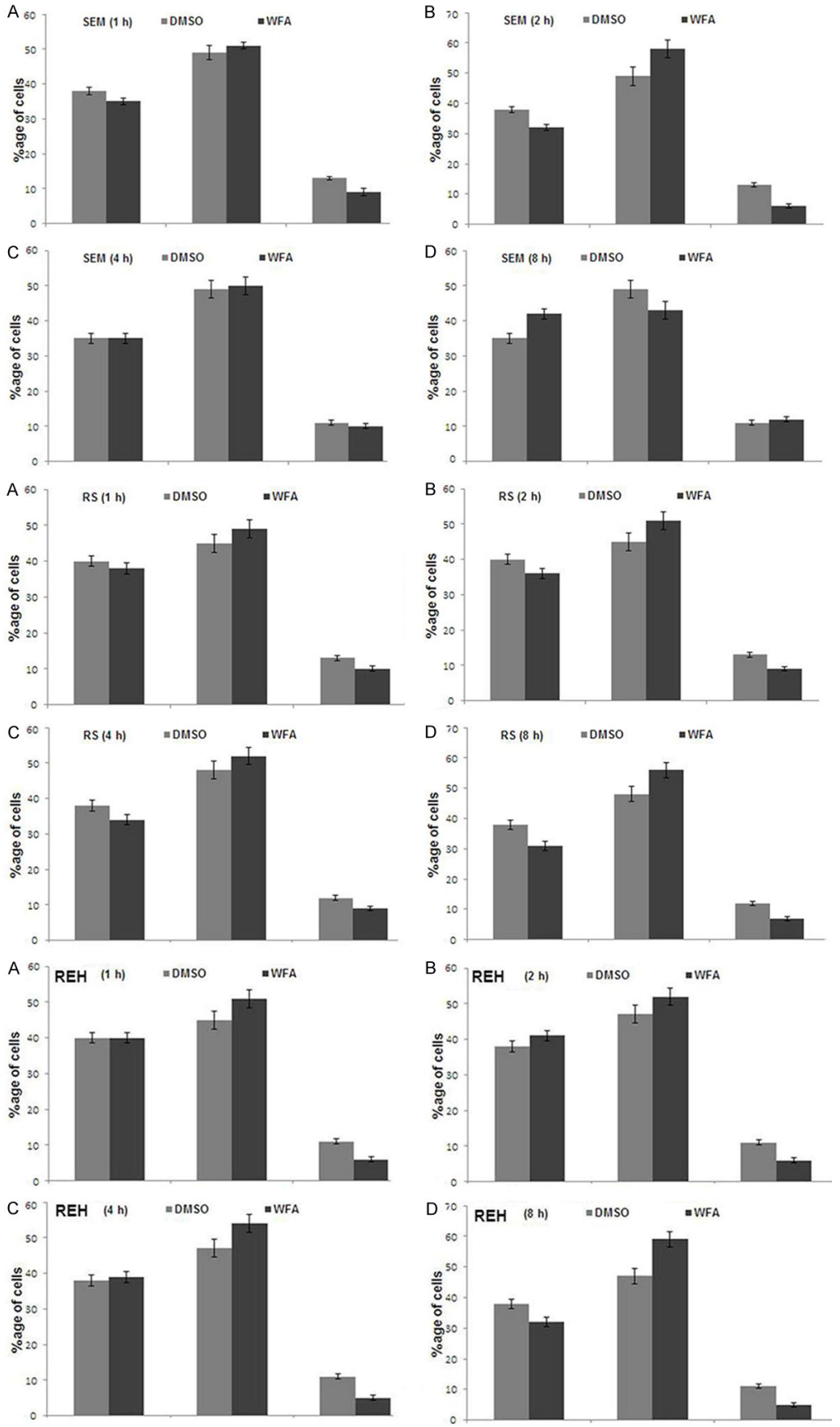
A
SEM (1 h)
DMSO
WFA
%age of cells
B
SEM (2 h)
C
SEM (4 h)
D
SEM (8 h)
A
RS (1 h)
B
RS (2 h)
C
RS (4 h)
D
RS (8 h)
A
REH (1 h)
B
REH (2 h)
C
REH (4 h)
D
REH (8 h)

Figure 2. Effect of withaferin A (WFA) on cell cycle arrest. SEM, REH andRS4 leukemia cell lines were treated with DMSO (control) or 25 μM withaferin A for 1 (A), 2 (B), 4 (C) and 8 h (D), and lysed in hypotonic buffer containing PI. The nuclei were collected on a flow cytometer and the percentage of cells in the G1, S and G2/M phases were calculated using ModFit LT software. The data presented are from 3 separate experiments.

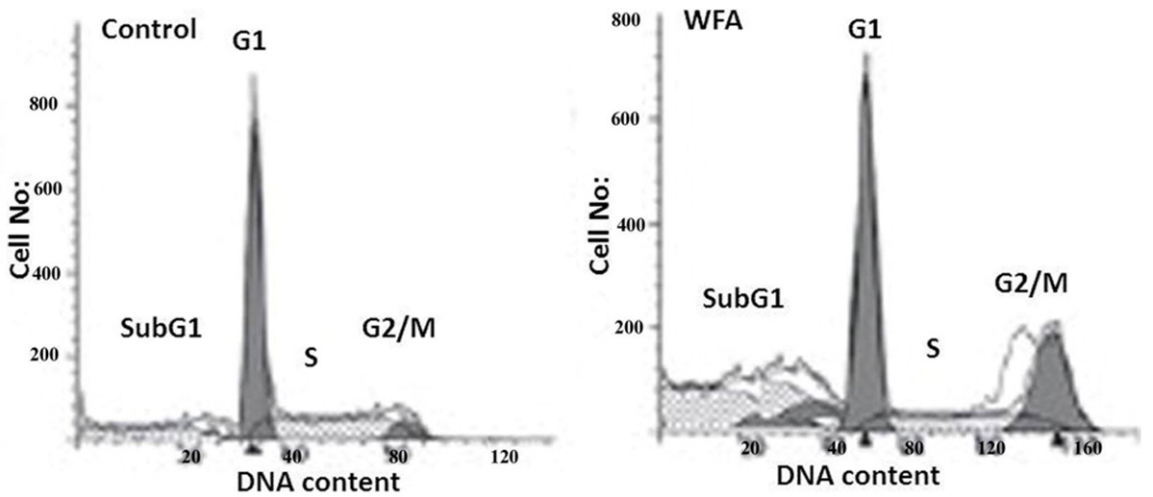

Figure 3. Effect of withaferin A (WFA) on cell cycle in SEM cells after 8 h of treatment. The cells treated with DMSO (control) or 25 μM withaferin A for 8 h were lysed in hypotonic buffer containing PI. Cell cycle histogram profiles were generated with ModFit LT software.

platelet-derived endothelial growth factor (TP) and GAPDH, cleaved caspase 3 and BAX-antibodies from Cell Signalling Technology (Boston, MA, USA).

Statistical analysis

All the results are expressed as the mean of three independent experiments. The statistical significance of differences was determined by two-sided Student's t-test and one-way ANOVA. The differences were considered statistically significant at $P > 0.05$. Sigma Stat software (Systat Software Inc., San Jose, California, USA) was used for all statistical evaluations.

Results

Withaferin A induces cell cycle arrest

The preliminary results revealed that withaferin A induces apoptosis in t(4;11) ALL cell lines at a concentration of 25 μM. Treatment of the cells with DMSO or 25 μM withaferin A was followed by analysis of the cell cycle changes at 1, 2, 4 and 8 h. The results revealed that withaferin A treatment arrests cell cycle at S to G2/M transition in SEM and REH cells after 4 h and in RS4;11 cells after 8 h (**Figure 2**).

In SEM cells treatment with withaferin A lead to a significant increase in the population of cells in subG1 phase after 8 h (**Figure3**).

Withaferin A treatment activates stress response proteins

To determine early signalling events involved in withaferin A induced leukemic cell apoptosis multiplex technology was used. The phosphorylation status of each protein involved in signal transduction at 0, 1, 2, 4, 8 and 16 h after withaferin A treatment was determined. The results showed phosphorylation of several proteins involved in the cellular stress responses leading to the apoptosis. In withaferin A treated cells the levels of phosphorylated p38 MAPK was significantly higher compared to the control cells (**Figure 4A**). In SEM and RS4;11 cells the level of phosphorylated JNK markedly higher than REH cells which in turn expressed higher level compared to untreated cells (**Figure 4B**). Withaferin A treatment also increased the levels of phosphorylated c-Jun compared to the control cells for all three tested cell lines (**Figure 4C**). Interestingly, the enhanced levels of phosphorylated p38MAPK, JNK and c-Jun were maintained in the cells lines for 16 h after withaferin A treatment. This suggests that withaferin A maintained stress on the leukemia cells.

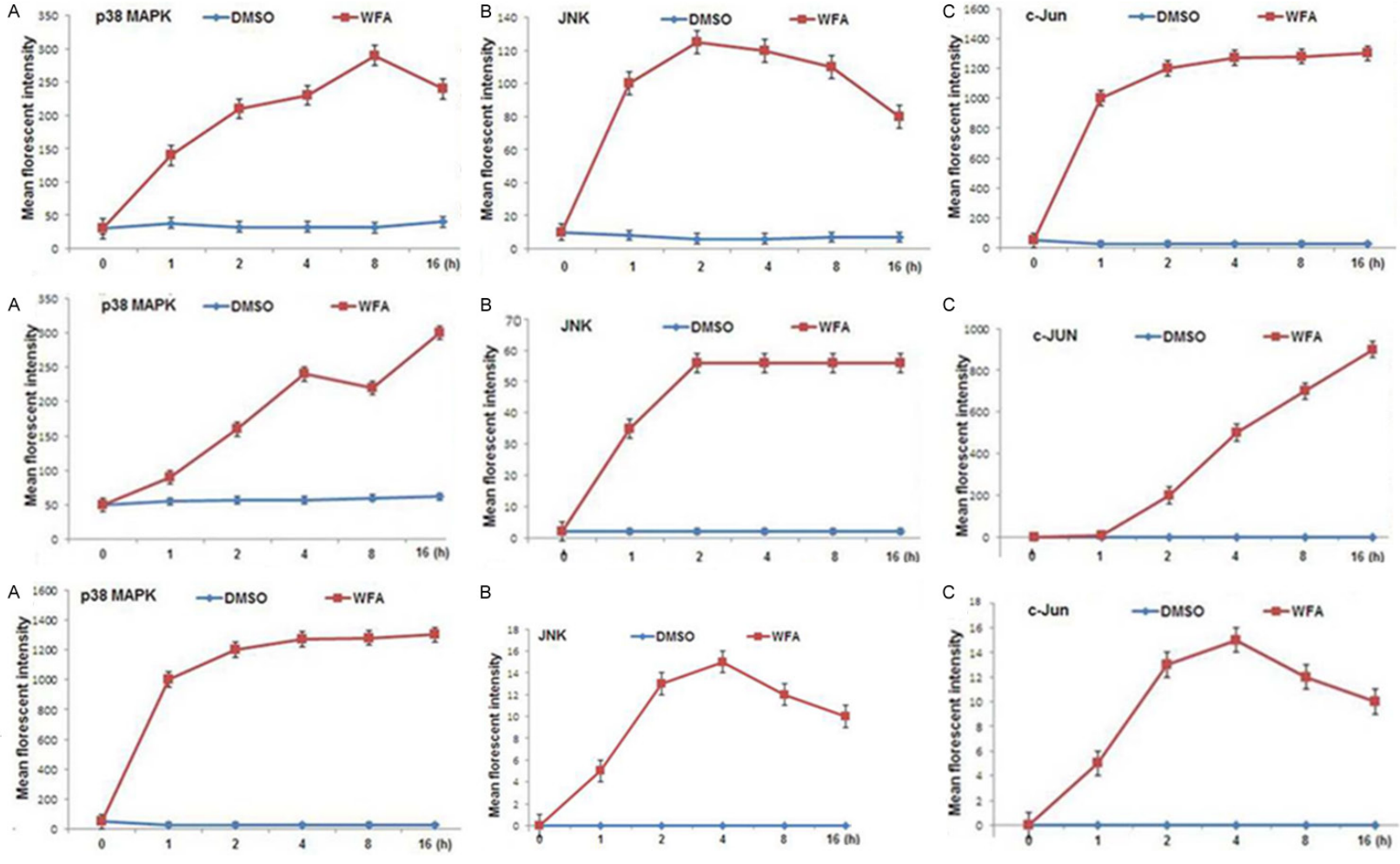

Figure 4. Effect of withaferin A on stress proteins. The SEM, REH andRS4 leukemia cell lines were treated with DMSO (control) or 25 μM withaferin A for 1, 2, 4, 8 and 16 h. Cell lysates were prepared and run in triplicate on a Bio-Plex multiplex instrument. The lysates were analyzed for (A) phosphorylated p38 MAPK, (B) JNK and (C) c-JUN. The data are represented are the mean of the median bead intensities normalized to the protein concentration in each well.

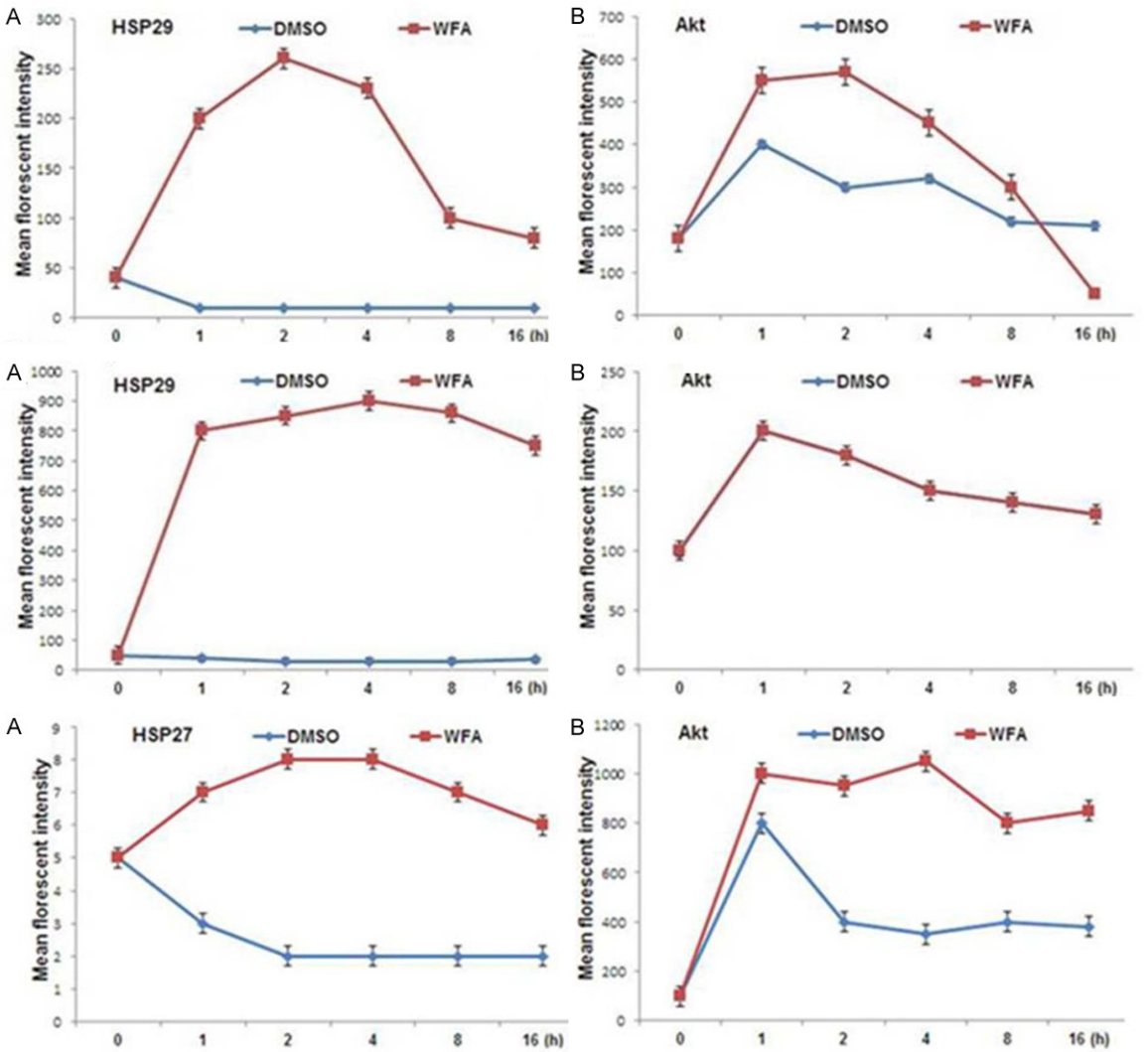

Figure 5. Effect of withaferinA on protective proteins. The SEM, REH andRS4 leukemia cell lines were treated with DMSO (control) or 25 μM withaferin A for 1, 2, 4, 8 and 16 h. Cell lysates were run in triplicate on a Bio-Plex multiplex instrument. The lysates were analyzed for (A) phosphorylatedHSP27 and (B) Akt. The data are represented as the means of the median bead intensities normalized to the protein concentration in each well.

Treatment with withaferin A enhanced the levels of phosphorylated HSP27 for first 8 h which then decreased after 16 h of the treatment (**Figure 5A**). In RS4;11 cells the levels of phosphorylated HSP27 was increased and maintained for all the 16 h after treatment. In REH cells although the levels of phosphorylated HSP27 increased but it was low compared to SEM and RS4;11 cells. The similar results were observed for phosphorylation of Akt in SEM REH and RS4;11 cells (**Figure 5B**). In comparison to untreated cells the level of phosphorylated Akt was significantly higher in withaferin A treated SEM and REH cells.

Confirmation of phosphorylation status by Western blot analysis

The results from western blot analysis of the cell lysates revealed a significant increase in the phosphorylated p38MAPK protein (Thr180/Tyr182) in all the 3 cell lines, with the lowest signal observed in the REH cells (**Figure 6**). In all the tested cell lines increased level of phosphorylated JNK (Thr183/Tyr185) was observed after the withaferin A treatment (25 μM). Similarly increased phosphorylated c-Jun (Ser63) level with two isoforms was observed on withaferin A treatment. Phosphorylated HSP-

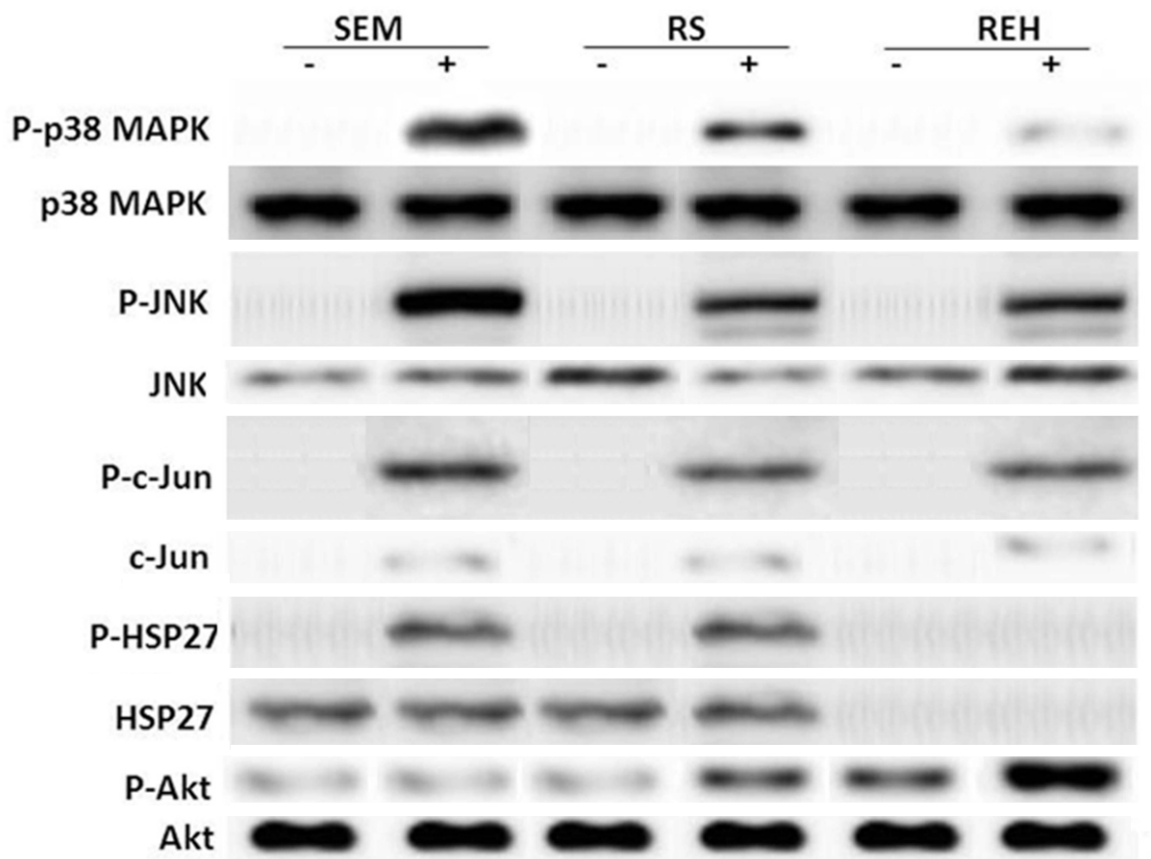

Figure 6. Western blot analysis of the phosphorylated and total proteins. The leukemia cell lines were treated with DMSO (control) or 25 Mm withaferin A for 5 and 16 h.

27 was present in the lysates from the SEM and RS4;11 cells, but not in the REH cells after the treatment with withaferin A. In REH cells the levels of phosphorylated Akt were significantly increased whereas in SEM and RS4;11 lysates cells only slight increase in phosphorylated Akt was observed.

Discussion

The present study demonstrates that withaferin A treatment induces growth arrest and stress signalling responses in leukemia cell lines with or without the chromosomal translocation. Withaferin A induced cell cycle arrest in S to G2/Mtransition phase in all the three tested cell lines after 8 h of treatment. Cell cycle arrest in the G2/M phase due to phosphorylation of p38 MAPK has been shown to be associated with the DNA damage [18-20]. It is reported that ROS generation in leukemia cells [17] can lead to DNA damage and subsequent growth arrest [21]. The activation of p38 MAPK by withaferin A in SEM, RS4;11 and REH cells could be the product of the withaferin A-induced generation of ROS in these cells. Withaferin A treatment of leukemic cells resulted in the phosphorylation of JNK and c-Jun. The JNK signalling pathway plays a major role in apoptosis [22]. JNK has been shown to translocate to the mitochondria and influence mitochondrial-mediated apoptosis [23, 24]. Withaferin A induces mitochondrial-mediated apoptosis in the leukemia cell lines possibly through the action of activated JNK. JNK has also been shown to inactivate the anti-apoptotic proteins, Bcl-2 and Bcl-XL, and to enhance the activation of the pro-apoptotic proteins, Bad, Bim and Bax [24-28]. In the current study, the withaferin A-induced phosphorylation of JNK and c-Jun was reduced in the REH cells compared to that observed in the SEM and RS4;11 cells. These data suggest that the activation of the JNK signalling pathway is an important event for inducing the more rapid

apoptotic death seen in the t(4;11) ALL lines. HSP27 is a member of the small heat shock protein family and has multiple functions [29]. The activation of HSP27 is induced in the cells by many types of environmental stress including heat shock, inflammatory cytokines and oxidants, and plays a protective role [30]. HSP27 has been shown to inhibit stress-induced apoptosis by binding to apoptogenic cytochrome c after release from the mitochondria and the interference with downstream caspase activation [31, 32]. HSP27 inhibits the activation of the pro-apoptotic protein Bax [33]. HSP27 has also been shown to inhibit apoptosis by the direct interaction and activation of Akt [34]. The phosphorylation and activation of HSP27 occurred within the first2 h after the withaferin A treatment of SEM and RS4;11. The SEM cells had elevated levels of both phosphorylated HSP27 and Akt. However, the RS4;11 cells had increased levels of phosphorylated HSP27, but no activation of Akt above the control levels, and the REH cells showed low levels of phosphorylated HSP27, but significant levels of phosphorylated Akt. Furthermore, SEM and RS4;11 were more sensitive to withaferin A-induced apoptosis. Therefore, the importance ofHSP27 and Akt activation as possible protective events in response to withaferin A treatment, is not clear.

The results from our study demonstrate that withaferin A is a novel and potent therapeutic agent to induce apoptosis in ALL cells.

Disclosure of conflict of interest

None.

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