Apoptosis of HL-60 human leukemia cells induced by Asiatic acid through modulation of B-cell lymphoma 2 family proteins and the mitogen-activated protein kinase signaling pathway

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Received June 11, 2014; Accepted February 20, 2015

DOI: 10.3892/mmr.2015.3534

Abstract. The toxicities of conventional chemotherapeutic agents to normal cells restrict their dosage and clinical efficacy in acute leukemia; therefore, it is important to develop novel chemotherapeutics, including natural products, which selectively target cancer-specific pathways. The present study aimed to explore the effect of the chemopreventive agent asiatic acid (AA) on the proliferation and apoptotic rate of the leukemia cell line HL-60 and investigated the mechanisms underlying its anti-tumor activity. The effect of AA on the proliferation of HL-60 cells was evaluated using the MTT assay. Annexin V-fluorescein isothiocyanate/propidium iodide double staining followed by flow cytometric analysis as well as Hoechst 33258 staining were used to analyze the apoptotic rate of the cells. Furthermore, changes of survivin, B-cell lymphoma 2 (Bcl-2), myeloid cell leukemia 1 (Mcl-1), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 expressions were detected by western blot analysis. AA blocked the growth of HL-60 cells in a dose- and time-dependent manner. The IC_{50}-value of AA on HL-60 cells was 46.67±5.08 μmol/l for 24 h. AA induced apoptosis in a dose-dependent manner, which was inhibited in the presence of Z-DEVD-FMK, a specific inhibitor of caspase. The anti-apoptotic proteins Bcl-2, Mcl-1 and survivin were downregulated by AA in a dose-dependent manner. Concurrently, AA inhibited ERK and p38 phosphorylation in a dose-dependent manner, while JNK phosphorylation was not affected. In conclusion, the present study indicated that the p38 and ERK pathways, as well as modulation of Bcl-2 family and survivin proteins were key regulators of apoptosis induced in HL-60 cells in response to AA.

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Key words: asiatic acid, leukemia, apoptosis, b-cell lymphoma 2, mitogen-activated protein kinase

Introduction

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy and primarily treated by chemotherapy. Although various chemotherapeutic agents have been developed for AML treatment, they affect normal cells, which causes unpleasant side effects, including hepatotoxicity, cardiotoxicity, hematotoxicity and infection, and which restricts their dosage and clinical efficacy. Moreover, resistance to conventional chemotherapeutic agents is a common reason for unsuccessful chemotherapy. Recent studies have suggested natural products as potent chemotherapeutic drugs for AML to improve the therapeutic efficacy and lower the side effects (1-3). For instance, β-elemene, an active component of the medicinal herb *Curcuma wenyujin*, induced apoptosis in human leukemia HL-60, NB4, K562 and HP100-1 cells through downregulation of cellular FLICE-like inhibitory protein and generation of reactive oxygen species (4).

In recent years, a series of pentacyclic triterpenoid compounds, including ursolic acid (5), oleanolic acid (6) and betulinic acid (7), were reported to exhibit anti-tumor activity, and therefore, triterpene acids are thought to have great potential as novel drugs for the treatment of malignant tumors. Asiatic acid (AA) is a pentacyclic triterpenoid derived from the medicinal plant *Centella asiatica* (family, *Apiaceae*). A wide range of beneficial effects of AA have been reported in hepatofibrosis (8), diabetes (9), ultraviolet (UV) radiation-induced photo aging and cerebral ischemia (10), and wound healing (11). Furthermore, AA has been reported to be cytotoxic to several solid tumor cell lines, including malignant glioma (12), human hepatoma (13), colon cancer (14), melanoma (15) and gastric cancer (16). Moreover, AA has attracted attention for its multiple protective effects against drug-induced hepatotoxicity, neurotoxicity and ulcers. Due to its potent anti-inflammatory, anti-cancer and chemo-protective activities, AA has been suggested to be a promising chemo-

preventive and therapeutic agent. However, to the best of our knowledge, the effect of AA on hematological malignant cells has not been investigated to date. In the present study, the effect of AA on HL-60 cells was investigated using MTT cell proliferation assays and assessment of the apoptotic rate using flow cytometric analysis and confocal microscopy. Furthermore, the mechanism of the anti-cancer effect of AA was investigated by assessing changes in levels of B-cell lymphoma 2 (Bcl-2) family proteins and proteins involved in the mitogen-activated protein kinase (MAPK) signaling pathway using western blot analysis. The present study provided a molecular basis for the clinical application of AA in patients with acute leukemia.

Materials and methods

Materials. AA ($C_{30}H_{48}O_5$; molecular weight, 488.7 g/mol), dimethyl sulfoxide (DMSO), Hoechst 33258 and MTT were purchased from Sigma-Aldrich (St Louis, MO, USA). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) reagent kit was purchased from Nanjing Key-Gen Biotech Co, Ltd (Nanjing, China). The bicinchoninic acid (BCA) Protein Assay kit, chemiluminescence reagent kit and polyvinylidene difluoride (PVDF) membranes were purchased from Pierce Biotechnology, Inc, USA. The rabbit polyclonal anti-extracellular signal-regulated kinase (ERK)1/2 (cat. no. 9102), rabbit monoclonal anti-phosphorylated (p-)ERK1/2 (cat. no. 4377), rabbit polyclonal anti-c-Jun N-terminal kinase (JNK) (cat. no. 9252), rabbit polyclonal anti-P-JNK (cat. no. 9251), rabbit polyclonal anti-p38 (cat. no. 9212), rabbit monoclonal anti-P-p38 (cat. no. 9215), rabbit polyclonal anti-Bcl-2 (cat. no. 2872), rabbit polyclonal anti-survivin (cat. no. 2803), rabbit polyclonal anti-myeloid cell leukemia 1 (Mcl-1) (cat. no. 4572), and mouse monoclonal anti-β-actin (cat. no. 3700) antibodies were provided by Cell Signaling Technology, Inc, (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 111-035-003) and donkey anti-mouse (cat. no. 715-035-150) immunoglobulin G secondary antibodies were purchased from Jackson Immuno Research Laboratories, Inc (West Grove, PA, USA). Z-DEVD-FMK was purchased from Medical and Biological Laboratories Co, Ltd, (Nagoya, Japan). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co, Ltd (Hangzhou, China).

Cell culture. The leukemia cell line HL-60 was obtained from American Type Culture Collection (Rockville, MD, USA). Human peripheral blood mononuclear cells (PBMCs) from healthy volunteers (five males and five females between 20 and 50 years old) were obtained by Ficoll-Hypaque (Lonza Ltd., Wakersville, MD, USA) density gradient centrifugation at 1,000 x g, according to the manufacturer's instructions. All cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Invitrogen Life Technologies) in a 5% CO_2 humidified atmosphere at 37˚C. Written informed consent for blood utilization was obtained from all volunteers. The procedures were approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology (Hubei, China).

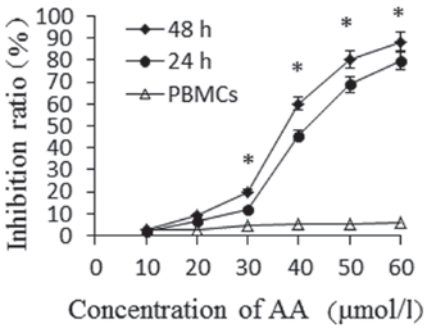

Figure 1. Analysis of the AA-induced proliferation inhibition ratio in HL-60 cells and human PBMCs. The cells were treated with various concentrations of AA for 24 and 48 h. PBMCs were treated with AA for 24 h. An MTT assay was performed to detect the proliferation inhibition ratio. Values are expressed as the mean ± standard deviation of three independent experiments. $^*P<0.05$, as compared with the control cells in both 24 and 48 h treated cells. AA, asiatic acid; PBMCs, peripheral blood monocular cells.

Cell proliferation assay. The effects of AA on the proliferation of HL-60 cells and PBMCs were detected using an MTT assay. Briefly, the cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) until reaching the mid-logarithmic growth phase and then seeded in 96-well plates with or without AA at various concentrations (10, 20, 30, 40, 50 and 60 μmol/l) at a density of $2x10^5$ cells per well. Following incubation for a set period of time, 20 μl MTT (5 mg/ml) was added and the cells were incubated for another 3 h at 37˚C. The supernatant was discarded of and 150 μl DMSO was added. The plate was gently agitated until the blue formazan crystals were fully dissolved. The absorbance (A) was measured at 490 nm using a microplate reader (Infinite F50; Tecan Spectra, Switzerland), and the cell proliferation inhibition ratio (%) was calculated using the following formula: $[1-(A_{experimental\ sample}/A_{control\ sample})]x100$.

Annexin V-FITC/PI double-labeled flow cytometry. The apoptotic rate was measured in HL-60 cells treated with AA at different concentrations alone or in combination with Z-DEVD-FMK. Four-color flow cytometry (FCM) was applied to detect the expression of Annexin V-FITC and the exclusion of PI. The cells positive for Annexin V-FITC and negative for PI represented the early apoptotic cells, whereas the cells positive for both markers represented the late apoptotic cells. The total apoptotic rate was the sum of the number of early and late apoptotic cells. Briefly, HL-60 cells were collected after the treatment using eppendorf tubes, washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich) and resuspended in 500 μl binding buffer. A total of 5 μl Annexin V-FITC was added and the samples were maintained at room temperature for 10 min. Next, 5 μl PI was added and the cells were incubated for another 10 min in the dark. The fluorescence intensity was detected using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Hoechst 33258 staining. HL-60 cells were treated with 40 μmol/l AA for 24 h and the nuclear fragmentation was visualized using Hoechst 33258 staining. Briefly, $1x10^5$ cells were seeded in 12-well plates and incubated with AA. After 24 h, the cells were collected, washed twice with PBS and

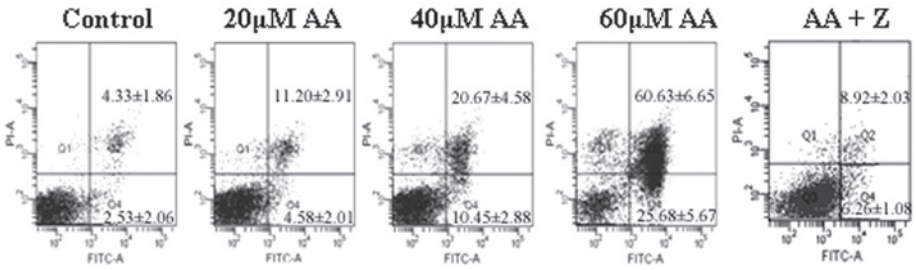

Figure 2. Assessment of the apoptotic rate of HL-60 cells induced by asiatic acid in various concentrations for 24 h. Annexin V-FITC/PI double staining was applied followed by flow cytometric analysis. Q2 quadrant and Q4 quadrant represent late and early apoptotic cells, respectively. The total apoptotic rate was the sum of these two. FITC, fluorescein isothiocyanate; PI, propidium iodide.

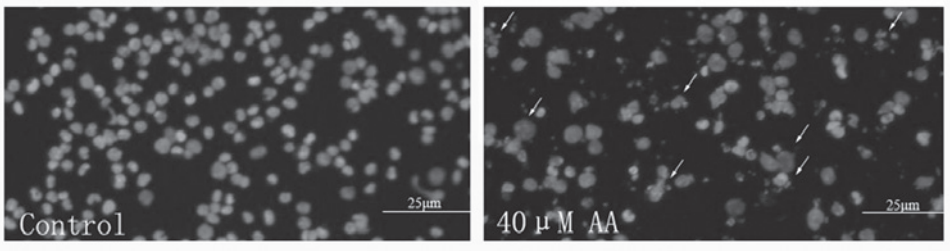

Figure 3. Effect of AA on the apoptosis of HL-60 cells. Following incubation with 40 μmol/l AA for 24 h, the cells were stained with Hoechst 33258 and visualized using a fluorescence microscope. The number of apoptotic bodies exhibiting fragmented or condensed nuclei (indicated by the arrows) were detected in the AA-treated group but not in the control group. AA, asiatic acid.

fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature before being deposited on polylysine-coated slides. After 30 min, the adherent cells were permeabilized by incubation with 0.1% Triton X-100 (Sigma-Aldrich) for 5 min at 4˚C. The cells were then incubated with Hoechst 33258 for 30 min at room temperature, rinsed with PBS and mounted on coverslips using glycerol (Sigma-Aldrich). Finally, cells were visualized using an Olympus BH-2 fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis. All of the HL-60 cells treated with AA at various concentrations for 24 h were collected. The cells were washed twice with PBS and completely lysed in a lysis buffer containing protease inhibitors (Pierce, Thermo Scientific, Waltham, MA, USA). The extracts were centrifuged at 12,000 xg for 15 min at 4˚C, and the clear supernatants containing the total protein were isolated. The protein concentration was quantified using the BCA assay (Pierce, Thermo Scientific). Equal amounts of protein (40 μg per lane) were separated by 10-12% SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk (Sigma-Aldrich) for 2 h at room temperature and then probed with the specific primary antibodies (1:500) at 4˚C overnight, and corresponding secondary antibody (1:1,000) at room temperature for 1 h. The specific protein bands were visualized using an Enhanced Chemiluminescence Detection system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Statistical analysis. Each experiment was repeated three times. Values are expressed as the mean ± standard deviation and analyzed using SPSS 13.0 statistical software for Windows (SPSS, Inc., Chicago, IL, USA). The comparisons between each group were analyzed using a one-way analysis of variance and the Student-Newman-Keuls (SNK) test. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

AA inhibits the proliferation of HL-60 cells. An MTT assay was used to detect the cytotoxicity of different concentrations of AA (0, 10, 20, 30, 40, 50 and 60 μmol/l) on HL-60 cells for 24 and 48 h and PBMCs for 24 h. As shown in Fig. 1, the growth of HL-60 cells was found to be inhibited by AA in a time- and dose-dependent manner. The proliferation inhibition rate of HL-60 cells significantly increased following incubation with AA at various concentrations for 24 h ($P<0.05$), while AA showed low toxicity to PBMCs. When the same concentration of AA was used, the proliferative rate of HL-60 cells was shown to be inhibited in a time-dependent manner ($P<0.05$). The IC_{50}-values of AA for HL-60 cells at 24 and 48 h were 46.67 μmol/l and 36.42 μmol/l, respectively.

AA induces apoptosis in HL-60 cells. Annexin V-FITC/PI double staining and flow cytometric analysis were utilized to assess the apoptotic rate of HL-60 cells treated with AA, as shown in Fig. 2. The early apoptotic rates of HL-60 cells treated with 0, 20, 40 and 60 μmol/l AA for 24 h were 4.58±2.01, 10.45±2.88 and 25.68±5.67%, respectively, which were significantly higher than that of the control group (2.53±2.06%). The late apoptotic rates were 11.20±2.91, 20.67±4.58 and 60.63±6.65%, respectively, while that of the control group was significantly lower (4.33±1.86%). To further identify whether AA-induced apoptosis in HL-60 cells is

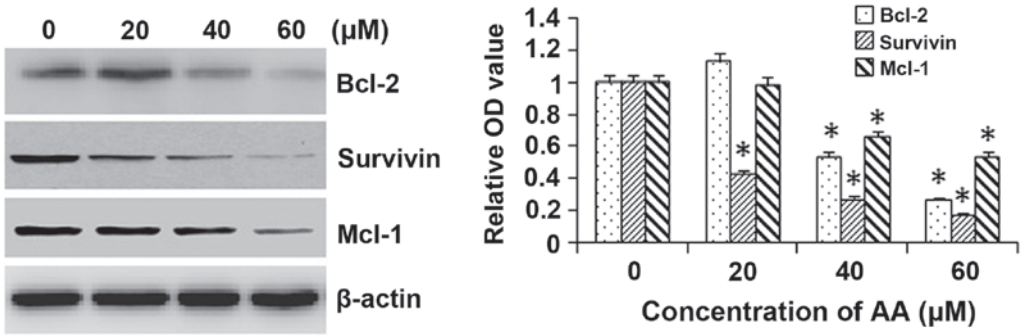

Figure 4. Effect of AA on apoptosis-associated proteins in HL-60 cells. HL-60 cells were treated with 0, 20, 40 or 60 μmol/l AA for 24 h. Cell lysates were prepared and subjected to western blot analysis of Bcl-2, Mcl-1 and survivin. Blots were quantified by densitometric analysis. Values represent the mean ± standard deviation of three determinations. *P<0.05, as compared with the control cells. AA, asiatic acid; Bcl-2, B-cell lymphoma 2; Mcl, myeloid cell leukemia; OD, optical density.

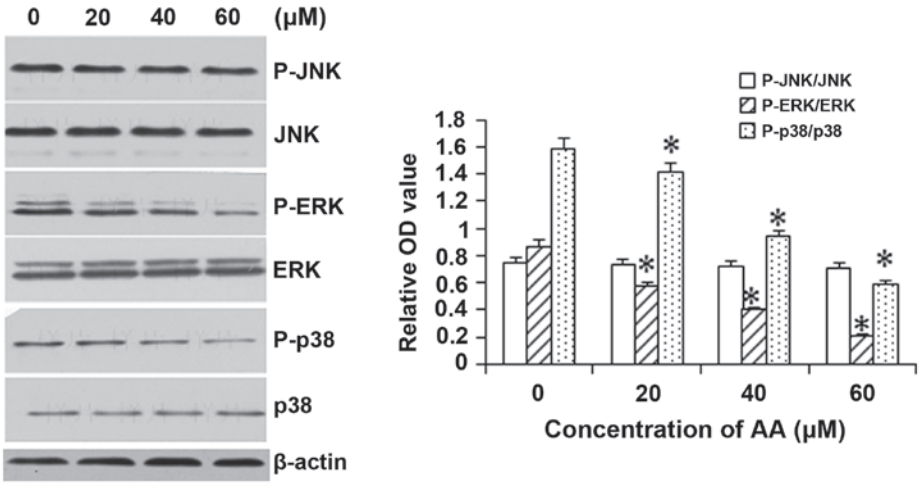

Figure 5. Effect of AA on expression and activation of proteins involved in the MAPK signaling pathway. Cells were treated with 0, 20, 40 or 60 μmol/l AA for 24 h. The cell lysates were prepared, and western blot was performed using antibodies against total or phosphorylated MAPK-signaling proteins. Blots were quantified by densitometric analysis. Values represent the means ± standard deviation of three determinations. *P<0.05, as compared with the control cells. AA, asiatic acid; MAPK, mitogen-activated protein kinase; p-JNK, phosphorylated c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; OD, optical density.

caspase-dependent, caspase inhibitor of Z-VAD-FMK was used in combination with 60 μmol/l AA. Z-DEVD-FMK was found to significantly decrease the apoptotic rate of AA-treated HL-60 cells (15.72±3.82%, P<0.05).

HL-60 cells treated with AA for 24 h were also stained with Hoechst 33258 to visualize the nuclear changes. HL-60 cell nuclei were regular in shape in the control group, whereas apparent apoptotic bodies were observed among HL-60 cells after treatment with 40 μmol/l AA. The nuclei of these apoptotic cells were condensed, and the nuclear envelopes appeared lytic (Fig. 3).

AA decreases the expression of apoptosis-associated proteins in HL-60 cells. To investigate the mitochondrial apoptotic events involved in AA-induced apoptosis, changes in the levels of anti-apoptotic proteins Bcl-2, survivin and Mcl-1 were assessed by western blot analysis. AA treatment attenuated the expression levels of the anti-apoptotic proteins Bcl-2, survivin and Mcl-1 in a concentration-dependent manner in HL-60 cells (Fig. 4). These results suggested that Bcl-2, survivin and Mcl-1 have essential roles in AA-mediated induction of apoptosis.

AA inhibits the activation of ERK and p38 but not JNK. To assess the involvement of the MAPK pathway in the mechanism underlying the cytotoxicity of AA, the levels and activation (phosphorylation) of JNK, p38 and ERK1/2 were assessed in AA-treated HL-60 cells. As shown in Fig. 5, exposure of HL-60 cells to 20, 40 and 60 μM AA resulted in a significant inhibition of phosphorylation of p38 and ERK1/2 in a dose-dependant manner, while basal activation of p38 and ERK was not altered. However, AA treatment did not JNK levels (including the phosphorylated and unphosphorylated forms).

Discussion

In previous studies, numerous natural products, including pentacyclic triterpenoids, have been discovered to be potent anti-leukemic agents for AML therapy (5-7). The present study was the first, to the best of our knowledge, to report the cytotoxic activity of AA on the human acute leukemia cell line HL-60. HL-60 cells treated with AA underwent apoptosis in a dose- and time-dependent manner. It was demonstrated that AA attenuated the expression levels of Bcl-2, survivin

and Mcl-1, and also the phosphorylation of ERK and p38 in a time-dependent manner.

The Bcl-2 family of proteins consists of pro-apoptotic effector proteins, including Bcl-2 associated X protein and Bcl-2 homologous antagonist killer, as well as anti-apoptotic proteins, including Bcl-2, Bcl extra large (Bcl-xL) and Mcl-1. Bcl-2 proteins exert anti-apoptotic effects through regulating the permeabilization of the mitochondrial outer membrane, a key step in apoptosis. Their complex network of interactions in the cytosol and mitochondria determines the fate of the cells (17). Cancer cells often violate key cellular checkpoints that would normally drive the cells to die by programmed cell death. As a result, they require to overcome the apoptotic stress either by reducing the expression of pro-apoptotic factors or, more frequently, by upregulating anti-apoptotic molecules, including Bcl-2, BcL-XL and MCL-1 (18,19). Bcl-2 and MCL-1 are critical for the development and maintenance of hematologic malignancies (20,21). Constitutively high levels of Bcl-2 and Mcl-1 have been associated with a more aggressive malignant phenotype and/or drug resistance to various classes of chemotherapeutic agents in cancer (22,23). This anti-apoptotic subfamily of proteins is currently considered a major target in the development of novel methods to improve treatment outcomes for leukemia patients. In the previous decade, several drugs directed at inhibiting Bcl-2 have been tested in the clinic, with several of them showing promising effects, particularly in lymphoid malignancies (24). Retroviruses encoding BimSL62A/F69A, which selectively bind Mcl-1, were able to selectively diminish survival of cells in two samples of clinical AML (21). In the present study, AA was demonstrated to decrease the anti-apoptotic activity in HL-60 cells through downregulating Bcl-2 and Mcl-1 expression, in consistency with the previously reported effect of AA on Bcl-2 expression in colon cancer, melanoma and breast cancer cells (15,25,26).

Survivin (*BIRC5*) is a member of the family of inhibitors of apoptosis proteins (IAPs) and has been implicated in the control of cell survival as well as regulation of mitosis in cancer, including solid tumors and hematological malignancies (27-29). Upon activation of pro-apoptotic cell signaling, survivin is released from the mitochondria and inhibits caspases-3 and -9. This function requires association with hepatitis B X-interacting protein and/or with X-linked IAP and is inhibited by SMAC-DIABLO (27,28). The regulation of survivin expression and function is complex and occurs at various levels, including transcription, differential splicing, protein degradation and intracellular sequestration via different ligands (28). Overexpression of suvivin is correlated with advanced disease, accelerated time of recurrence, reduced survival and resistance to therapy (30). AML patients with overexpression of survivin showed an unfavorable response to chemotherapy in 81.2% of the patients and shorter median survival time (30 days) compared to that of patients with normal expression (29). Thus, targeting survivin with small-molecule inhibitors by their anti-sense approaches or natural IAP antagonist mimetics may be an attractive strategy of anti-leukemia treatment. Such agents can either directly induce apoptosis of tumor cells or sensitize them to other cytotoxic agents, hence overcoming drug resistance (31,32). Thereofore, the present study evaluated the effect of AA on survivin in HL-60 cells. The results showed that, in addition to bcl-2 protein, survivin is likely to be involved in the mechanism of AA-induced apoptosis.

MAPKs, a family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli. JNK, p38 and ERK1/2 are the three main members of three different MAPK pathways that can be activated by growth factors, DNA damage, cytokines, oxidant stresses, UV light, anti-cancer drugs and osmotic shock (33,34). These signaling pathways regulate a variety of cellular activities, including proliferation, differentiation, survival and death. Deviation from the strict control of MAPK signaling pathways has been implicated in the development of numerous human diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and various types of cancer (35). The present study showed that AA significantly inhibited phosphorylation of p38 and ERK without affecting the JNK pathway. According to previous studies, the role of these three MAPK pathways in cancer has remained controversial, as MAPK pathways have been shown to mediate pro-apoptotic as well as anti-apoptotic signals in different systems, apparently depending on the stimulus and cell type involved. For instance, the apoptosis of K562 cells induced by icaritin was accompanied by the inhibition of activation of p-ERK and p-P38, while activation of p-ERK and p-P38 were shown to be critical mediators in AA-induced cell growth inhibition of human breast cancer cell lines (26,36). JNK and p38 are activated by cellular stress and have been associated with apoptosis (37). However, certain studies have indicated that the JNK is required for interleukin-3-mediated cell survival and that p38 is associated with the development of chemoresistance by activating nuclear factor kappa B (38,39).

In conclusion, the results of the present study suggested that AA induced apoptosis through inhibiting Bcl-2, survivin and MAPK signaling pathways in HL-60 human acute leukemia cells. These results strongly suggested that AA may be a valuable agent for molecular targeted therapy of human acute leukemia.

Acknowledgements

The authors would like to acknowledge Dr Fei Zhao (Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) for analyzing the data.

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