

Pseudolaric acid B induces apoptosis in U937 human leukemia cells via caspase-9-mediated activation of the mitochondrial death pathway

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Abstract. Numerous studies have demonstrated that pseudolaric acid B (PAB) promotes apoptosis in several cancer cell lines. However, thus far, the effect of PAB on human leukemia cells has not been evaluated. In the present study, the anti-tumor activity and molecular mechanisms of PAB in human leukemia U937 cells were investigated. It was demonstrated that PAB induced U937 cell apoptosis, which was confirmed by typical morphological changes and Annexin V-fluorescein isothiocyanate staining. PAB was observed to activate a caspase-dependent apoptotic pathway in U937 cells through the regulation of the Bcl-2 family protein-mediated mitochondrial pathway. Furthermore, the activities of caspase-3 and -9 were increased following treatment with PAB. In conclusion, to the best of our knowledge, this study demonstrated for the first time that PAB was able to enhance the apoptosis of U937 cells, at least in part, through the activation of the mitochondrial death pathway. Moreover, the activation of caspase-3 and -9 mediated the apoptotic induction.

Introduction

Leukemia accounts for the largest number of cases of childhood cancer (1). The predominant therapeutic approaches for human leukemia are radiotherapy, hyperthermia and chemotherapy; however, cure rates remain unsatisfactory (2). Hence, effective drugs, including novel naturally occurring or chemically synthesized compounds, are required to control the malignant progression of leukemia.

Pseudolaric acid B (PAB), a natural diterpene acid present in the traditional Chinese medicinal herb *Tu-Jin-Pi*, has exerted anticancer effects on various types of cancer

cells (3). It was demonstrated that PAB significantly delayed the growth of a taxol-resistant liver cancer without obvious toxicity *in vivo* and may possess a selective anti-proliferative effect in human cancer cells but not in normal cells *in vitro* (1). It was also demonstrated that PAB induced apoptosis via proteasome-mediated Bcl-2 degradation in hormone-refractory DU145 prostate cancer cells (3). Although PAB exhibits cytotoxic activity and induces apoptosis in several cancer cell types, its action in leukemia is unclear.

Apoptosis is a selective process of physiological cell deletion that regulates the balance between cell proliferation and cell death (4). The failure of apoptosis is considered to contribute to the development of human malignancies (5). Therefore, targeting key apoptotic regulators has become a strategy for the development of novel treatments for human cancer (6,7).

Changes in the apoptotic signaling pathway contribute to the development of multiple human diseases, including cancer, autoimmunity, diabetes and neurodegenerative disorders, and may provide rational targets for novel anticancer drugs (8). Apoptosis occurs via the mitochondria- and death receptor-mediated pathways. The two apoptotic pathways converge at caspase-3 and subsequently at other proteases and nucleases that drive the terminal events of apoptosis (9). The mitochondrial pathway is termed the intrinsic pathway of apoptosis (10). Mitochondria are the predominant energy producers in the cell and thus are essential for maintaining cell survival. Moreover, mitochondria are also involved in cell death and contain the majority of the important mediators of apoptosis (11). However, the involvement of the mitochondrial pathway in the anti-leukemia effects of PAB remains to be fully elucidated. Therefore, the principal aim of this study was to investigate the mechanisms underlying PAB-induced apoptosis in U937 human leukemia cells by determining the involvement of the mitochondrial pathway. In the present study, it was demonstrated that PAB activated a caspase-dependent apoptotic pathway in U937 cells through the regulation of the Bcl-2 family protein-mediated mitochondrial pathway. Moreover, the initiation of caspase-3 and -9 activation mediated apoptotic induction. To the best of our knowledge, this is the first study to investigate mitochondrial signaling in U937 cells in response to PAB treatment.

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Materials and methods

Reagents. PAB was purchased from the Liaoning Institute for Food and Drug Control (Shenyang, Liaoning, China). Monoclonal anti- β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cytochrome *c*, apoptosis inducing factor (AIF), anti-Bcl-2, -Mcl-1, -tBid, -Noxa, -Bax and -Bcl-xl were purchased from New England Biolabs (Santa Cruz, CA, USA). RPMI-1640 medium and fetal bovine serum were purchased from Gibco-BRL (Grand Island, NY, USA). An Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from R&D Systems (Abingdon, UK). Cell isolation and tissue culture reagents were obtained from Invitrogen Life Technologies (Lindingö, Sweden). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

U937 cell culture. U937 cells, obtained from Sun Yat-sen University (Guangzhou, China), were used in all experiments. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin, and incubated at 37°C in humidified air containing 5% CO₂. The exponentially growing U937 cells were seeded onto 24-well flat-bottomed plates at a density of 5x10⁵ cells/ml. The cells were collected at 0, 24, 48, 72 and 96 h. Prior to the addition of PAB, the cells were washed once with phosphate-buffered saline (PBS) to remove dead cells, and incubated in tissue-culture plates for 30 min at 37°C. U937 cells in multiwell tissue culture plates were incubated with PAB at different concentrations (0, 1, 2, 4 and 8 μ mol/l) for 48 h or at a concentration of 2 μ mol/l for 0, 24, 48, 72 and 96 h.

Intracellular reactive oxygen species (ROS) detection. Intracellular production of ROS was measured by the Reactive Oxygen Species Assay kit (Enzo Life Sciences, Shanghai, China), which determines the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (12). Following treatment with PAB (2 μ mol/l) for 48 h, the U937 cells were washed three times with serum-free RPMI-1640 and incubated with 10 μ M DCFH-DA in the dark for 20 min at 37°C. Cells were then washed twice with cold PBS. The qualitative analysis of ROS generation was conducted using a fluorescence microscope (Carl Zeiss, Hamburg, Germany). The fluorescence was measured in a Tecan Infinite 200 plate reader (Eastwin Life Sciences, Inc., Haidian, China) with excitation at 485 nm and emission at 520 nm. Values were expressed as the mean absorbance normalized to the percentage of the vehicle control.

Cell viability. To assess the overall viability of the U937 cells following PAB treatment, the cells were treated as described previously. At particular time points (0, 24, 48, 72 and 96 h), the U937 cells were washed twice with PBS and treated with a 0.4% solution of trypan blue. Viable cells were visualized as clear under the microscope; however, U937 cells that were no longer viable exhibited damaged membranes and thus allowed entry of the dye, staining blue. Assays were performed in triplicate and repeated at least three times. The number of intact

viable cells was expressed as a percentage of the total number of cells and was assessed at different time points.

Hoechst 33258 staining. The cells were stained with Hoechst 33258 (Molecular Probes, Inc., Eugene, OR, USA) in a dilution of 1:600 (stock solution, 1 mg/ml) for 5 min in the dark. The samples were observed under a fluorescence microscope. Two-hundred cells were counted from each coverslip and the results were confirmed by visualizing the apoptotic nuclei. There were five coverslips per group, unless otherwise noted in the figure legends.

Flow cytometry analysis. U937 cell apoptosis was quantified by flow cytometry using FITC-conjugated Annexin V and propidium iodide (PI). Specific binding of Annexin V was achieved by incubating 10⁶ cells in 60 μ l binding buffer saturated with Annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with Annexin V and PI prior to analysis. The binding of Annexin V-FITC and PI to the cells was measured by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) using CellQuest software (BD Biosciences). A minimum of 10,000 cells were counted in each sample. Experiments were performed and interpreted as follows: Annexin V/PI⁻ cells (lower left quadrant) were considered to be living cells; Annexin V⁺/PI⁻ cells (lower right quadrant) were considered to be apoptotic cells; Annexin V⁺/PI⁺ cells (upper right quadrant) were considered to be necrotic or advanced apoptotic cells; and Annexin V/PI⁺ staining (upper left quadrant) was considered to indicate bare nuclei, cells in late necrosis or cellular debris.

Measurement of cytochrome *c* release from mitochondria. Cells were treated with 0.1% dimethylsulfoxide (DMSO) for 24 h. The mitochondria and the cytosol were separated using a Cytochrome *c*-Releasing Apoptosis Assay kit (Enzo Life Sciences). Cells were suspended in cytosol extraction buffer and, following 10 min on ice, were homogenized using a Dounce homogenizer (GlobalSpec, East Greenbush, NY, USA) and centrifuged (at 700 x g for 10 min). Subsequent to this, the collected supernatant was re-centrifuged (at 10,000 x g and 4°C for 30 min). The resulting supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were processed for western blot analysis (13).

Western blot analysis. Briefly, following treatment, cells were washed once with ice-cold PBS containing 1 mM Na₂VO₄ and extracted with lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 5 mM EDTA; 5% glycerol; 1% Triton X-100; 25 mM NaF; 2 mM Na₂VO₄; and 10 μ g/ml aprotinin, leupeptin and pepstatin). The preparation of the cytoplasm was conducted using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology, Inc., Rockford, IL, USA). The cell lysates were frozen and thawed three times, and were further centrifuged at 14,000 x g for 10 min at 4°C to pellet insoluble material. The supernatant of the cell extracts was analyzed for the concentration of proteins by a DC Protein Assay kit based on the Lowry method (Bio-Rad, Hercules, CA, USA). Equal quantities of protein (50 μ g) from each sample were separated on 10% sodium dodecyl sulfate-poly-

acrylamide gels and transferred onto polyvinylidene fluoride membranes (Micron Separations, Inc., Westborough, MA, USA). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), and then incubated with rabbit anti-human cytochrome *c*, AIF, anti-Bcl-2, Mcl-1, Bcl-xL, Noxa, Bax and tBid primary antibodies for 60 min. β -actin (dilution, 1:2,000) was used as a control for equal protein loading. The immunoblots were then washed three times with TBST buffer, incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgM; Santa Cruz Biotechnology, Inc.) and developed using a chemiluminescent substrate (Pierce Biotechnology, Inc.). To quantify and compare levels of proteins, the density of each band was measured with a densitometer (GE Healthcare, Little Chalfont, UK).

Measurement of caspase-3 and -9 activity. U937 cells were harvested and centrifuged at 400 \times g for 10 min. Cells were washed twice with PBS (pH 7.4), resuspended with 50 μ l lysis buffer at 4°C and incubated on ice for 10 min. All subsequent steps were performed on ice. Following centrifugation, cell extracts were transferred to fresh tubes, and protein concentrations were measured. Each 50- μ l cell extract containing 100 μ g protein was combined with equal volumes of 2X reaction buffer in a microplate followed by the addition of 5 μ l peptide substrates of caspase-3 and -9. Subsequent to overnight incubation in the dark at 37°C, samples were analyzed in a microplate reader (Tecan Austria, Salzburg, Austria) at 405 nm. Caspase-3 and -9 activity was evaluated by the absorbance ratio of treated to control samples. In certain experiments, the caspase-3 inhibitor (Z-DEVD-FMK) and the caspase-9 inhibitor (Z-LEHD-FMK) were added to the fresh medium of U937 cells 1 h prior to the addition of PAB.

Statistical analysis. Each experiment was conducted in duplicate or triplicate, and three or four independent experiments were performed. Results are presented as the mean \pm standard deviation and analyzed with SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). Results were compared using analysis of variance (ANOVA). When the ANOVA showed a statistically significant difference, a group-by-group comparison was performed using a t-test with Tukey's correction for multiple comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PAB induces apoptosis in U937 cells. The chemical structure of PAB (14) is shown in Fig. 1A. In order to distinguish apoptosis from necrosis in the U937 cells, a trypan blue exclusion assay was conducted. Trypan blue staining demonstrated that $98.12 \pm 1.96\%$ ($n=5$) of the cells incubated with medium alone retained an integrated cell membrane (i.e., resisted trypan blue staining). The percentage of necrotic cells increased with time and with an increase in the concentration of PAB (Fig. 1B).

ROS is involved in PAB-induced apoptosis of U937 cells. Intracellular ROS generation and mitochondrial dysfunction are important in the signal transduction of apoptotic stimuli (15). Therefore, the fraction of cells with high intracellular ROS

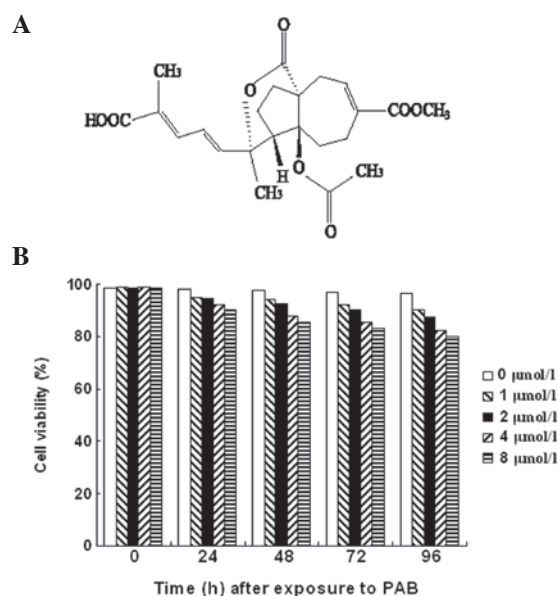


Figure 1. (A) Chemical structure of PAB. (B) Effect of PAB on cell cytotoxicity of U937 cells. U937 cells were incubated with different concentrations of PAB for 48 h or 2 μ mol/l PAB for different times. Viability of U937 cells was determined by a trypan blue assay. The number of dead cells increased with an increasing PAB concentration and an increasing induction time. Data are presented as the mean \pm SD of three determinations. PAB, pseudolaric acid B.

levels was investigated following treatment with PAB. To determine the mechanism involved in PAB-induced apoptosis, ROS production in the U937 cells was investigated. The rate of ROS generation at 0 h was considered to be 100%, and a qualitative and quantitative concentration-dependent increase in the rate of ROS generation was observed as increased levels of fluorescence following treatment with PAB in U937 cells (Fig. 2).

PAB induces apoptosis in human U937 cells. Microscopy of PAB-induced U937 cells revealed morphological changes that were consistent with the induction of apoptosis and necrosis (Fig. 3A). Apoptotic cells were characterized by membrane blebbing and nuclear condensation, while necrotic cells were typically larger and lighter with plasma membrane lesions and mitochondrial abnormalities. The percentage of apoptotic cells was calculated from the observation of 200 cells.

Flow cytometry using FITC-conjugated Annexin V demonstrated that U937 cells exposed to PAB underwent rapid apoptosis. This effect was positively correlated with the exposure time and the concentration of PAB (Fig. 3B). In addition, excessive apoptosis was associated with the loss of membrane integrity in an increased number of U937 cells, which indicated that necrosis or late apoptosis had occurred.

PAB induces apoptosis of U937 cells through the Bax initiated mitochondrial pathway. Members of the Bcl-2 family are divided into pro-apoptotic and anti-apoptotic proteins. The Bcl-2 protein family is responsible for the release of cytochrome *c* from the mitochondria, a pivotal event in the induction of apoptosis. Mcl-1, a member of the anti-apoptotic Bcl-2 protein family, has been demonstrated to be overexpressed in numerous types of cancer (16). Notably, in the present study, PAB significantly increased the expression of Bax and Noxa,

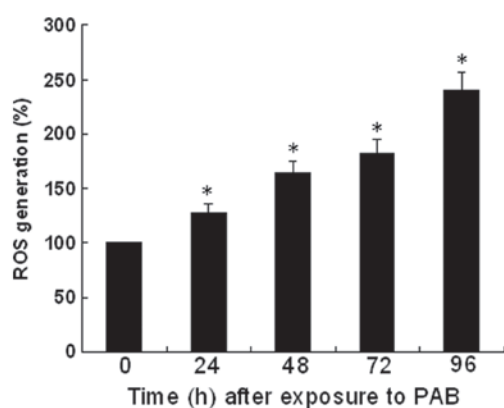


Figure 2. Rate of ROS generation in PAB-induced apoptosis in U937 cells. Data are presented as the mean \pm SD of three independent experiments and analyzed by one-way analysis of variance. * $P < 0.05$ compared with that of PAB treatment at 0 h. ROS, reactive oxygen species; PAB, pseudolaric acid B.

and decreased the expression of Mcl-1, Bcl-xl and Bcl-2 (Fig. 4). However, the expression of tBid was not significantly affected by treatment with PAB. These results suggest that the apoptotic effect may be mediated by raising the ratio of Bax to Bcl-xL, to induce the release of cytochrome *c* into the cytoplasm.

PAB induces translocation of mitochondrial apoptotic factors. The cells were analyzed for alterations in the mitochondria, which have been shown to be important in numerous forms of apoptotic cell death. Mitochondrial damage occurring as a result of drug-induced apoptosis is often accompanied by the release of mitochondrial apoptotic factors into the cytosol (17). Therefore, it was investigated whether AIF was involved in PAB-induced apoptosis. The results of the western blot analysis demonstrated that PAB treatment induced the release of AIF from the mitochondria into the cytosol in a time-dependent manner (Fig. 5A). These characteristics are typically due to apoptosis. In addition, it was determined whether treatment of U937 cells with PAB induced the release of cytochrome *c* (a mitochondrial protein localized in the mitochondrial intermembrane space) into the cytosol. As shown in Fig. 5B, PAB treatment mobilized cytochrome *c* from the mitochondria into the cytosol.

Caspase-3 and -9 activity. The caspase-3 and -9 activity in U937 cells incubated with PAB is shown in Fig. 6. The treatment of U937 cells with PAB for different times at a concentration of 2 mg/ml or for 48 h at concentrations of 0, 1, 2, 4 and 8 μ M demonstrated a significant increase of caspase-3 and -9 activation. The activity of caspase-3 and -9 in U937 cells following PAB treatment was upregulated in a dose- and time-dependent manner. To characterize the pathway of apoptosis execution, experiments were conducted using Z-DEVD-FMK and Z-LEHD-FMK. The rate of apoptosis was significantly reduced by Z-DEVD-FMK and Z-LEHD-FMK (Fig. 7). In conclusion, the data demonstrate that caspase-3 and -9 mediated PAB-induced U937 cell apoptosis.

Discussion

Previous studies have demonstrated that PAB induces growth inhibition, cell cycle arrest and apoptosis in a variety of cancer

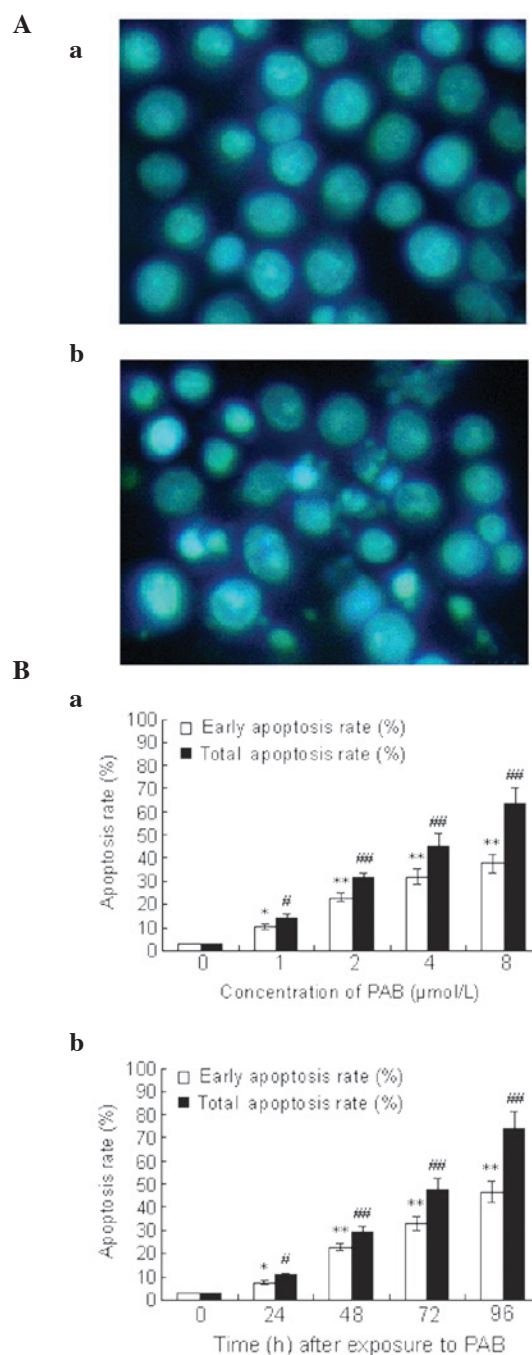


Figure 3. PAB-induced apoptosis in U937 cells. (A) Morphology of U937 cells. U937 cells were cultured (Aa) with or (Ab) without PAB. Control cells and cells treated with PAB were stained with Hoechst 33258 dye (magnification, $\times 400$). (B) Apoptosis rate of U937 cells detected by flow cytometric analysis. U937 cells were harvested (Ba) after 48 h of treatment with different concentrations of PAB or (Bb) following different times of exposure to 2 μ mol/l PAB. The cells were then incubated with FITC-conjugated Annexin V and PI double staining. Flow cytometric analysis was performed. Data shown are representative of three separate experiments. The lower right quadrants represent early apoptotic cells that were stained by Annexin V but not by PI, and upper right quadrants represent late apoptotic cells that were stained by Annexin V and PI. (Ba) * $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$ and **** $P < 0.001$, compared with cells treated with 0 μ mol/l PAB; (Bb) * $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$ and **** $P < 0.001$, compared with cells treated with PAB at 0 h. PAB, pseudolaric acid B; FITC, fluorescein isothiocyanate; PI, propidium iodide.

cell lines, including breast cancer, colon cancer, hepatocellular carcinoma and melanoma cells (18-22). In the present study, the effects of PAB on cell growth and death in U937 cells (a

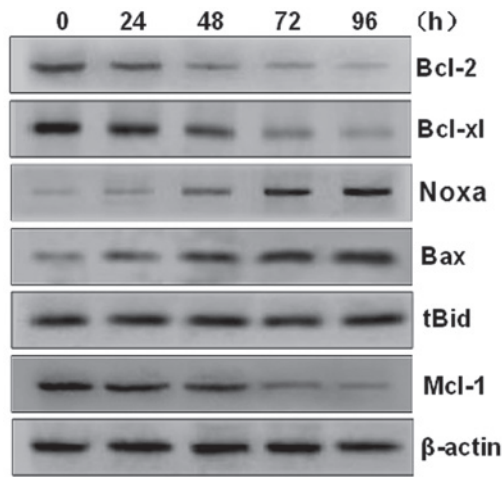


Figure 4. PAB induces the regulation of Bcl-2 family protein expression. The cells were incubated with or without PAB for the indicated time period. Bcl-2, Bcl-xl, Bax, Noxa, tBid and Mcl-1 were detected by western blot analysis using specific antibodies. Triplicate experiments exhibited similar results. PAB, pseudolaric acid B.

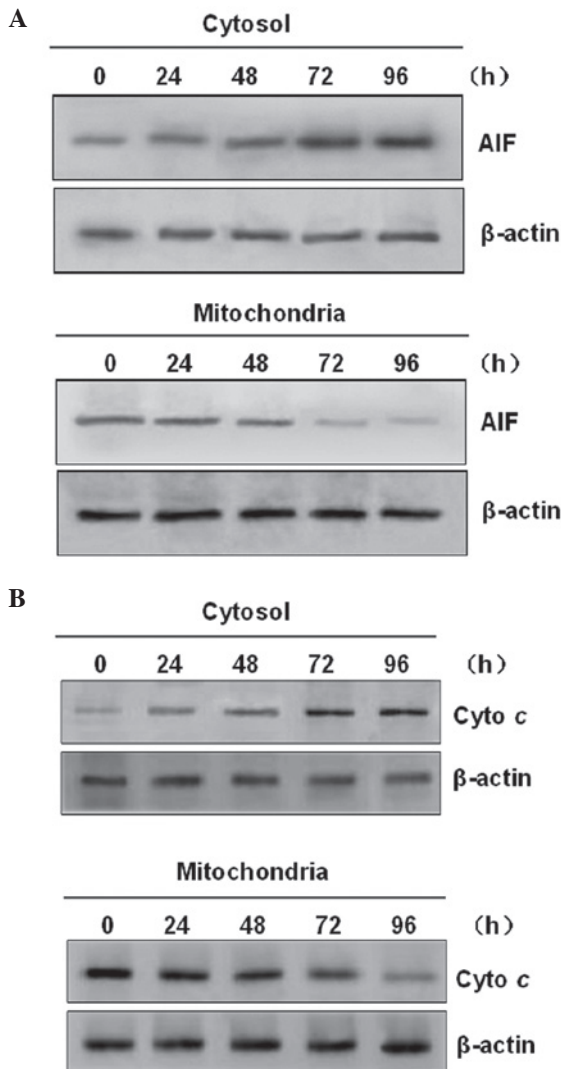


Figure 5. Translocation of mitochondrial apoptotic factors is induced by PAB. Time-dependent release of (A) AIF and (B) Cyto c from the mitochondria into the cytosol was measured by western blot analysis. All cells were treated with PAB. Triplicate experiments exhibited similar results. PAB, pseudolaric acid; AIF, apoptosis inducing factor; Cyto c, cytochrome c.

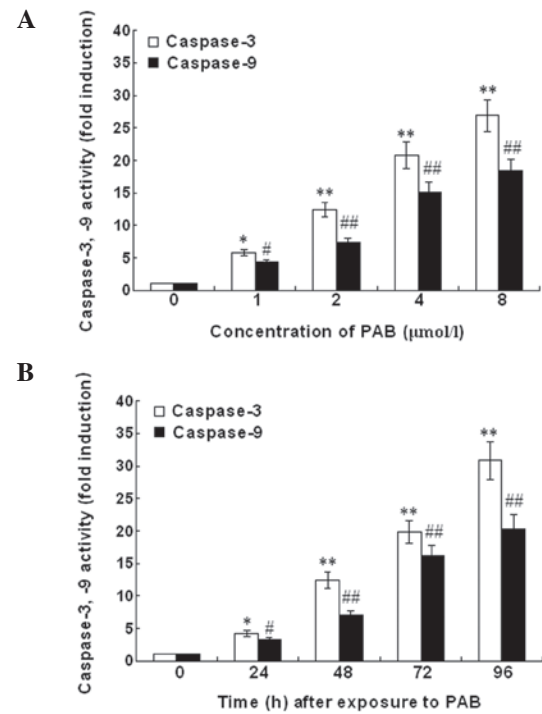


Figure 6. Effect of PAB on the activities of caspase-3 and -9. (A) Dose-dependency of PAB-induced caspase-3 and -9 activity. * $P < 0.01$ and ** $P < 0.001$ compared with cells treated with 0 μmol/l PAB for caspase-3. # $P < 0.05$ and ## $P < 0.001$, compared with cells treated with 0 μmol/l PAB for caspase-9. (B) Time-dependency of PAB-induced caspase-3 and -9 activity. * $P < 0.05$ and ** $P < 0.001$, compared with the control for caspase-3 at 0 h. # $P < 0.05$ and ## $P < 0.001$, compared with the control for caspase-9 at 0 h. PAB, pseudolaric acid B.

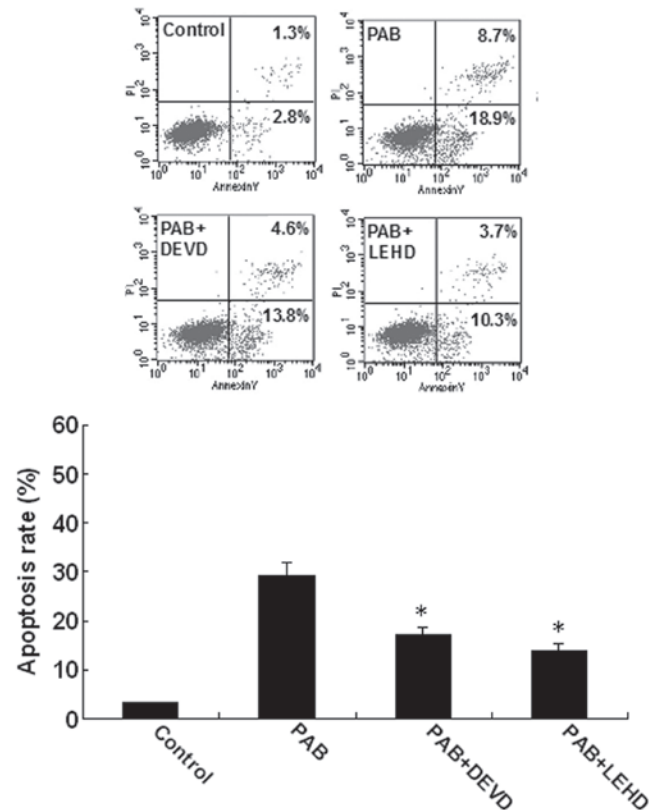


Figure 7. U937 cells were treated with 2 μmol/l PAB and incubated for 48 h with the indicated concentrations of Z-DEVD-FMK (DEVD; a caspase-3 inhibitor) and Z-LEHD-FMK (LEHD; a caspase-9 inhibitor). Values represent the mean ± SD of five experiments performed in duplicate. * $P < 0.05$, compared with that of U937 cells treated with 2 μmol/l PAB. PAB, pseudolaric acid.

human acute monocytic leukemia cell line) and the underlying mechanisms were investigated. It was demonstrated that PAB induced U937 cell apoptosis in a time- and dose-dependent manner.

Apoptosis, or programmed cell death, is important in a variety of physiological processes during fetal development and in adult life (23-25). Cell shrinkage, chromatin condensation and nuclear fragmentation are the morphological hallmarks of apoptosis (26). The inhibition of apoptosis, a universal and efficient cellular suicide pathway, is one of the predominant characteristics of cancer (27). Therefore, the induction of apoptosis is an aim of various antitumor therapies (28). Numerous studies have demonstrated that the majority of anti-cancer agents induce cell death through apoptotic pathways. Previously, several unrelated natural products were observed to elicit their cytotoxic effects by targeting the mitochondria, thereby acting as mitocans (11). The mitochondrial membrane potential and the release of cytochrome *c* are fine-tuned by the balance between Bax and Bcl-xL (13). An increase in the ratio of Bax to Bcl-xL stimulates the release of cytochrome *c* from the mitochondria into the cytosol, where it binds to Apaf-1, leading to the activation of caspase-9. The present study showed that PAB increased the ratio of Bax to Bcl-xL and induced the release of cytochrome *c* into the cytosol in U937 cells. This suggested that the mitochondria may function in the mediation of the apoptotic signal in PAB-induced apoptosis in U937 cells.

The Bcl-2 family of proteins are essential in apoptosis. They are regulators of the mitochondrial membrane permeability and intermembrane space protein efflux, according to the opposing fractions of the anti-apoptotic and pro-apoptotic members (16). The Bcl-2 family of proteins are divided into three subclasses: Bcl-2-like survival members, such as Bcl-xL, Bcl-2 and Mcl-1; Bax-like death members, such as Bax, Bak and Bcl-xs; and BH3-only death members, including Bim, Bid and Bik (16). Noxa upregulation/Mcl-1 downregulation upstream of subsequent mitochondrial outer membrane permeabilization and cytochrome *c* release has recently emerged as a key mechanism underlying potent apoptogenicity of various experimental drugs targeting metastatic melanoma (29). The present study demonstrated that PAB significantly increased the expression of Bax and Noxa, and decreased the expression of Mcl-1, Bcl-xL and Bcl-2. However, the expression of tBid was not significantly affected by treatment with PAB.

As downstream products of cytochrome *c*, caspases are critical mediators of the principle factors in apoptotic cells. Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of numerous important cellular proteins (30). Caspases are classified by their mode of activation as either initiator or effector caspases. Initiators, such as caspase-8 and -9, are referred to as apical caspases, which are activated by a variety of apoptotic signals. In the present study, the activation of caspase-9 was observed to occur in a dose- and time-dependent manner. Previous studies have suggested that caspase-3 is involved in several events in the apoptosis pathway, including cytochrome *c* release, DNA fragmentation and nuclear collapse. Caspase-3 is downstream of caspase-9; thus, it is plausible that applying caspase-3 or -9 inhibitors would reduce PAB-induced apoptosis, as was demonstrated in the present study. These data suggest that apoptosis by PAB

was mediated through the mitochondrial pathway involving caspase-9 and -3.

In conclusion, the results revealed the importance of the mitochondrial signaling pathways in response to PAB, and extended the understanding of the molecular mechanisms mediating these responses. Targeting PAB may be a potential novel therapeutic strategy for leukemia treatment, and these results may provide a foundation for the development of targeted anti-leukemia therapies and drug screening. PAB-induced U937 cell apoptosis is a complex process. Moreover, the *in vivo* relevance of these results obtained from *in vitro* cell culture require verification in animal models.

Acknowledgements

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