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Pseudolaric acid B induces apoptosis via proteasome-mediated Bcl-2 degradation in hormone-refractory prostate cancer DU145 cells

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ABSTRACT

Pseudolaric acid B (PAB), a natural diterpene acid present in the traditional Chinese medicinal herb Tu-Jin-Pi, exerted anticancer effects on various cancer cells. However, the effect of PAB on DU145 cells, an *in vitro* model of hormone-refractory prostate cancer (HRPC), has not been reported previously. In the study, PAB significantly suppressed proliferation of DU145 cells in a dose-dependent manner without obvious cytotoxicity. IC_{50} values of 0.89 ± 0.18 and 0.76 ± 0.15 μM at 48 h was determined by Cell counting kit (CCK-8) assay and clone formation assay, respectively. PAB also induced DU145 cells apoptosis as confirmed by typical morphological changes and Annexin V-FITC staining. Furthermore, we demonstrated that PAB caused a concentration-dependent elevation of reactive oxygen species (ROS) level in DU145 cells, and *N*-acetyl-L-cysteine (NAC, a well-known ROS scavenger) could block PAB-induced ROS generation and apoptosis. Western blotting and/or caspase activity data indicated that PAB downregulated anti-apoptotic Bcl-2 protein and activated caspase-9 and caspase-3, which were largely rescued by NAC or MG-132 (proteasome inhibitor). Taken together, these findings provide the first evidence that PAB may inhibit growth of HRPC DU145 cells and induce apoptosis through ROS generation and Bcl-2 degradation via the activation of the ubiquitin–proteasome pathway.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in men in the USA, a statistic that has remained unchanged for the past 20 years (Jemal et al., 2010). Although androgen ablation therapy is successful in the initial stages, nearly all patients eventually progress to an androgen-independent stage or hormone-refractory prostate cancer (HRPC), unresponsive to further hormone deprivation with a median survival of approximately one year (Moon et al., 2008). The current treatment modality for those patients with HRPC is chemotherapy based on docetaxel (Petrylak, 2000). However, its efficacy is frequently attenuated due to drug resistance, which is considered as the primary cause of high mortality in this cancer (Seruga et al., 2011). Therefore, it is imperative for new chemotherapeutic agents to control drug-resistant HRPC.

Apoptosis is a tightly conserved process of programmed cell death fundamental to development and maintenance in all multicellular organisms. Changes in the apoptotic signaling pathway can contribute to the origin of multiple human diseases, including cancer, autoimmunity, diabetes, and neurodegenerative disorders, as well as provide rational targets for new anticancer drugs (Danial and Korsmeyer, 2004). Commitment of cells to apoptosis is governed largely by protein–protein interactions between members of the Bcl-2 family, including pro- and anti-apoptotic proteins that respond to specific death signals to promote or inhibit cell death. Anti-apoptotic Bcl-2 protein, the cardinal member of this family, protects many cell lines from induced apoptosis (van Delft and Huang, 2006). Several processes, especially Bcl-2 degradation mediated primarily through the ubiquitin–proteasome pathway, tightly regulate the protein levels of Bcl-2 (Breitschopf et al., 2000). Bcl-2, which is localized in the outer mitochondrial membrane, is downregulated under apoptotic stimuli such as

Abbreviations: PAB, pseudolaric acid B; HRPC, hormone-refractory prostate cancer; ROS, reactive oxygen species; CCK-8, Cell Counting Kit-8; NAC, *N*-acetyl-L-cysteine.

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reactive oxygen species (ROS) or diverse chemotherapeutic drugs. This event results in losing mitochondrial membrane potential, releasing several apoptogenic proteins including cytochrome *c*, and triggering the caspase cascade, which can lead to irreversible commitment of cells to apoptosis through the mitochondrial pathway (Green and Reed, 1998; Leber et al., 2010; Simon et al., 2000). More interesting, early studies demonstrated that the Bcl-2 protected prostate cancer cells from apoptotic stimuli *in vitro* and suggested that such protection correlated with the ability to form HRPC *in vivo* (Raffo et al., 1995; Reed, 1995). Recent studies further declared that Bcl-2 plays a key role in the development of drug resistance in many cancer cells including HRPC cells (Bray et al., 2009; Karnak and Xu, 2010; Wang et al., 2008). Targeted therapy against Bcl-2 might enhance the efficacy of chemotherapy in patients with drug-resistant HRPC.

Pseudolaric acid B (PAB) is a novel diterpene acid isolated from the root bark of *Pseudolarix kaempferi* Gordon, known as "Tu-Jin-Pi" in Chinese herb, which has been safely used for centuries in traditional Chinese medicine for treating skin inflammation (Zhou et al., 1983). PAB contains a structural framework that has never been found in any other natural products including a unique polyhydroazulene with a *trans*- substitution pattern at the junction sites (Ma et al., 2010). It was demonstrated that PAB significantly delayed tumor growth of a taxol-resistant liver cancer without showing obvious toxicity to the animals *in vivo*, and might possessed selective anti-proliferative effect in human cancer cells but not in normal cells *in vitro* (Ma et al., 2010; Wong et al., 2005). Several studies also showed that PAB induced apoptosis in uterus, skin, stomach, liver, breast and blood carcinomas in multiple pathways, mainly associated with downregulation of Bcl-2 (Gong et al., 2004; Li et al., 2005; Ma et al., 2010; Meng and Jiang, 2009; Wu et al., 2006; Yu et al., 2007). However, so far, the effect of PAB on human prostate cancer has not been evaluated.

DU145 cell line is an *in vitro* cell model of HRPC, highly tumorigenic and chemotherapy-resistant (Mickey et al., 1977). In the present study, we reported the significant anti-proliferative effects of PAB on DU145 cells. Furthermore, PAB effectively induced DU145 cells to apoptosis through intracellular ROS generation, downregulation of Bcl-2 via the ubiquitin–proteasome pathway, and activation of caspase-3 and caspase-9 in the mitochondrial pathway. Therefore, PAB might be a promising new drug for the treatment of HRPC.

2. Materials and methods

2.1. Chemicals and antibodies

PAB (Fig. 1A) was kindly gifted by Dr. Qinshi Zhao (Kunming Institute of Botany, Chinese Academy of Sciences). It was isolated from the ethanolic extract of the root bark of *P. kaempferi* by column chromatography and recrystallization, with the purity of 99.3% as determined by high-performance liquid chromatography (Yang et al., 2003). A stock solution of 10 mM PAB was prepared in sterilized DMSO and further diluted to appropriate concentrations with cell culture medium immediately before use. DMSO [0.1% (v/v)] was used as a vehicle control throughout the study.

Cell Counting Kit-8 (CCK-8, #CK04) was obtained from Dojindo Laboratories (Kumamoto, Japan). Lactate dehydrogenase (LDH) assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Hoechst Staining Kit (#C0003), Annexin V-FITC Apoptosis Detection Kit (#C1063), Reactive Oxygen Species Assay Kit (#S0033), BCA Protein Assay Kit (#P0012), Caspase-3 Activity Assay Kit (#C1115), Caspase-8 Activity Assay Kit (#C1151), Caspase-9 Activity Assay Kit (#C1157) and BeyoECL Plus kit (#P0018) were obtained from Beyotime Institute of Biotechnology (Haimen, China). RPMI-1640 medium, heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin were acquired from Gibco Co. USA. The antibodies for Bcl-2, Bax, β-actin, and goat anti-rabbit and anti-mouse secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ROS scavenger *N*-acetyl-L-cysteine (NAC), proteasome inhibitor MG-132 and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture

Human prostate cancer DU145 cells were obtained from American Type Culture Collection (ATCC HTB-81, Manassas, VA). Cultures were maintained in 95% air and 5% CO_2 at 37 °C in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin.

To determine the effects of ROS scavenger (NAC) or proteasome inhibitor (MG-132) on PAB-induced apoptosis in DU145 cells, cell culture was pre-incubated for 2 h with NAC (5 mM) or MG-132 (10 μM) before the addition of PAB.

2.3. Cell proliferation assay

2.3.1. Cell viability analysis

Cell viability was measured by using CCK-8 assay (Guan et al., 2009). Briefly, DU145 cells (2×10^5 cells/ml in 96-well culture plates) were treated with DMSO (1:1000) or PAB (0.08, 0.4, 2, 10 and 50 μM) for 48 h. The medium (90 μl) was incubated with 10 μl of CCK-8 solution for 2 h at 37 °C. Absorbance was read at 450 nm on a microplate reader (Bio-Rad Model 550, Hercules, CA). Cell viability was expressed as a percent of the control culture value. IC_{50} value, the concentration of PAB inhibiting 50% of the cell growth at 48 h, was calculated by Reed and Muench's method (Devignat, 1952).

2.3.2. Clone formation assay

DU145 cells seeded at 500 cells/well in six-well culture plates were treated with DMSO (1:1000) or PAB (0.08, 0.4, 2, 10 and 50 μM) for 48 h and then maintained in the routine medium. After 10 days incubation, cell colonies consisting of more than 50 cells stained with crystal violet were counted and pictures were taken by a digital camera (Franken et al., 2006). The ratio of clone formation was calculated following the equation: Rate of clone formation (%) = (clone amount/500) × 100%. IC_{50} value was calculated with the former method.

2.3.3. LDH measurement

Leakage of LDH to the cell culture medium indicates cell membrane damage. After DU145 cells were exposed to DMSO (1:1000) or PAB (0.4, 2 and 10 μM) for 48 h, each culture medium was centrifuged at 250*g* for 10 min. Supernatant was transferred to a 96-well culture plate to determine the amount of LDH according to the manual of the LDH assay kit. LDH activity is reported as percentage relative to control level (Huang et al., 2010). Absorbance of samples was measured at 450 nm. Data were obtained from three independent assays.

2.4. Morphological assessment

2.4.1. Inverted phase-contrast microscope observation

DU145 cells were treated with DMSO (1:1000) or PAB (2 μM) at a fixed incubation time of 48 h (following Sections 2.4.2 & 2.4.3 with the same method). Cells were washed twice with PBS and fixed with 4% paraformaldehyde. Images were captured at 200× magnification with an inverted phase-contrast microscope (Olympus DP10, Japan).

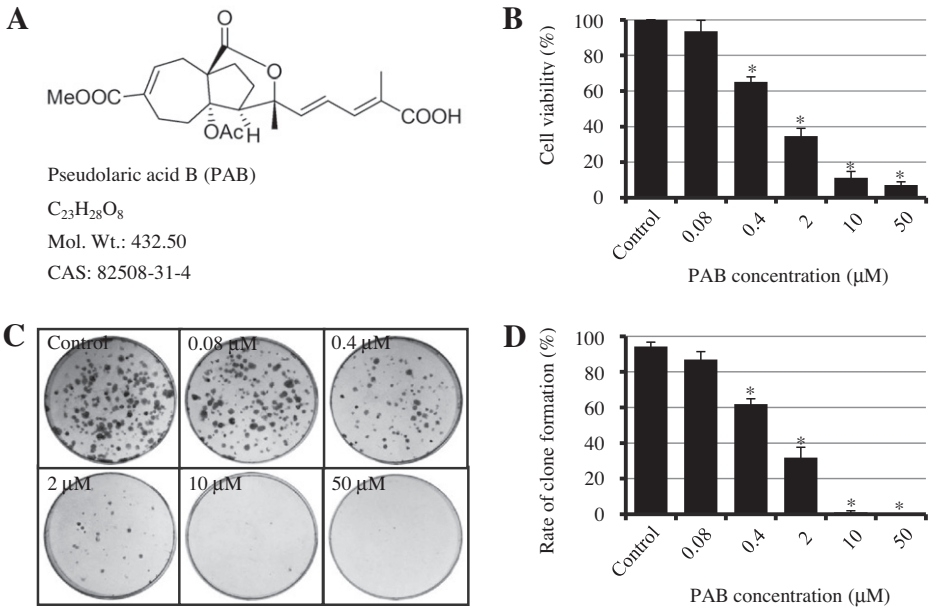

Fig. 1. Effects of PAB on the growth of DU145 cells. (A) Chemical structure of pseudolaric acid B (PAB). (B) Cell viability was determined by CCK-8 assay. Exponentially growing DU145 cells were treated with the indicated concentration of PAB for 48 h. (C) Photographs of colonies of DU145 cells were shown. Pictures were enhances using Adobe Photoshop for brightness, contrast, and sharpening for uniformity of appearance only. (D) Graphs showed the rate of clone formation from (C). Data were presented as means ± SD of three independent experiments and analyzed by one-way ANOVA. $^*p < 0.05$, versus control.

2.4.2. Fluorescence microscope observation

Cells were seeded on sterile cover glasses placed in the 6-well plates the day before treatment. After 48 h, cells were fixed, washed twice with PBS and stained with Hoechst 33258 staining solution from Hoechst Staining Kit according to the manufacturer's instructions (Liu et al., 2007). Stained nuclei were observed under a reflected fluorescence microscope (Nikon MF30 LED, Japan).

2.4.3. Transmission electron microscopy observation

Cells were fixed according to the guidance as described previously (Jiang et al., 2008). The ultrastructure of cells was examined in ultrathin sections by a transmission electron microscope (TEM, Hitachi H-600, Tokyo, Japan).

2.5. Intracellular ROS detection

Intracellular production of ROS was measured by Reactive Oxygen Species Assay Kit with oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to fluorescent 2′,7′-dichlorofluorescin (DCF) (Cathcart et al., 1983). After treatment with PAB (0, 0.4, 2 and 10 μM) for 48 h, DU145 cells were washed with serum-free 1640 for three times and incubated with 10 μM of 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 done using a fluorescence microscope. The fluorescence was measured in a plate reader (TECAN Infinite200, Eastwin Life Science, China) with excitation at 485 nm and emission at 520 nm. The values were expressed as the mean absorbance normalized to the percentage of the vehicle control.

2.6. Annexin V-FITC staining

The apoptotic rate of DU145 cells was detected with Annexin V-FITC Apoptosis Detection Kit (Brumatti et al., 2008). DU145 cells were treated with PAB (2 μM) for 48 h in the presence and absence of NAC (5 mM). The cells were harvested and rinsed twice with cold PBS. A total of 2.5×10^5 cells were stained with Annexin-V FITC/PI (Ji et al., 2011). The stained apoptotic cells were counted by a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA).

2.7. Western blotting analysis

DU145 cells were grown to 85% confluence and synchronized by starvation in serum-free RPMI 1640 for 16 h. In some cases, cells were pretreated for 2 h with NAC (5 mM) or MG-132 (10 μM) before PAB treatment. After being treated with PAB (0.4, 2 and 10 μM) for 48 h, respectively, cells were harvested in cell lysis buffer (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris–HCl, pH 7.0). Protein concentrations were determined using BCA Protein Assay Kit. Western blotting was performed as previously described (Lin et al., 2011). The signal was visualized using a BeyoECL Plus kit. The level of β-actin was used to normalize the amount of the protein of interest. The density of the band was expressed relative to the density in vehicle control cells.

2.8. Caspase-3,-8,-9 activity measurement

The working principles of Caspase-3,-8 or -9 Activity Assay Kit are based on the cleavage of the following substrate: acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA), acetyl-Ile-Glu-Thr-Asp *p*-nitroanilide (Ac-IETD-*p*NA) and acetyl-Leu-Glu-His-Asp *p*-nitroanilide (Ac-LEHD-*p*NA). Lysates of DU145 cells were prepared after treating with PAB (0.4 or 2 μM) for 48 h. In some cases, cells were pretreated for 2 h with the NAC (5 mM) or MG-132 (10 μM) before PAB treatment. Assays were performed on 96-well microplates by incubating 10 μl cell lysate in 80 μl reaction buffer containing 10 μl caspase substrate (2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with a microplate reader at an absorbance of 405 nm (Liu et al., 2007). Caspase activity was expressed as the ratio of treated to vehicle control cells.

2.9. Statistical analysis

All assays were run at least in three independent experiments. Values were expressed as means ± standard deviations (SD). Data

were analyzed by one-way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) version 17.0. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Growth inhibition of PAB-treated DU145 cells

The number of viable cells was assessed by CCK-8 assay. Treatment with PAB (0.08, 0.4, 2, 10 and 50 μM) resulted in a dose-dependent suppression of cell viability (Fig. 1B, $p < 0.05$). IC_{50} value of PAB on DU145 cells was 0.89 ± 0.18 μM at 48 h.

Moreover, clone formation assay was done to determine a long-term effect of PAB on DU145 cell growth. After 10 days incubation of the indicated concentration of PAB, the colony numbers were counted and the colony photos were taken. The results showed that the rate of clone formation was also reduced in a dose-dependent manner (Fig. 1C and D, $p < 0.05$). IC_{50} value was 0.76 ± 0.15 μM with 10 days incubation after PAB treatment for 48 h.

3.2. PAB did not exhibit cytotoxicity against DU145 cells

The LDH assay detects the amount of LDH released by cells with damaged membranes as indicator of necrosis. 48 h treatment with PAB did not affect the concentration of LDH in the supernatant of culture medium (99.8 ± 8.6%, 102.5 ± 6.8% and 103.6 ± 7.8% at 0.4, 2 and 10 μM, respectively, $p > 0.05$). It suggested that the anti-proliferative effect of PAB on DU145 cells was not due to PAB's cytotoxicity at the dose of 0.4, 2 and 10 μM, and these doses were used for treatment of PAB in the following experiments.

3.3. Morphological changes of apoptosis induced by PAB in DU145 cells

To examine whether the anti-proliferative effect of PAB involved apoptosis, cells were incubated with DMSO (1:1000) or PAB (2 μM) for 48 h and then were examined by three different type microscopes. By the inverted phase-contrast microscope, we observed a decrease in the total cell number and an increase in floating cells (data not shown), with cell shrinkage and cytoplasm vacuolization in PAB-treated DU145 cells (Fig. 2A). Nuclear changes were detected by Hoechst staining. Bright green nuclei blebbing and DNA fragmentation were observed by fluorescence microscopy, while the control DU145 cells were intact in shape and stained green homogeneously (Fig. 2B). In addition, photomicrographs exposed to PAB presented typical apoptotic morphology with chromatin condensation and formation of apoptotic bodies by transmission electron microscopy (Fig. 2C). These results showed that PAB-induced apoptosis happened in DU145 cells (Kroemer et al., 1995).

3.4. ROS involved in PAB-induced apoptosis of DU145 cells

To determine the mechanism involved PAB-induced apoptosis, we examined ROS production in DU145 cells. As the % ROS

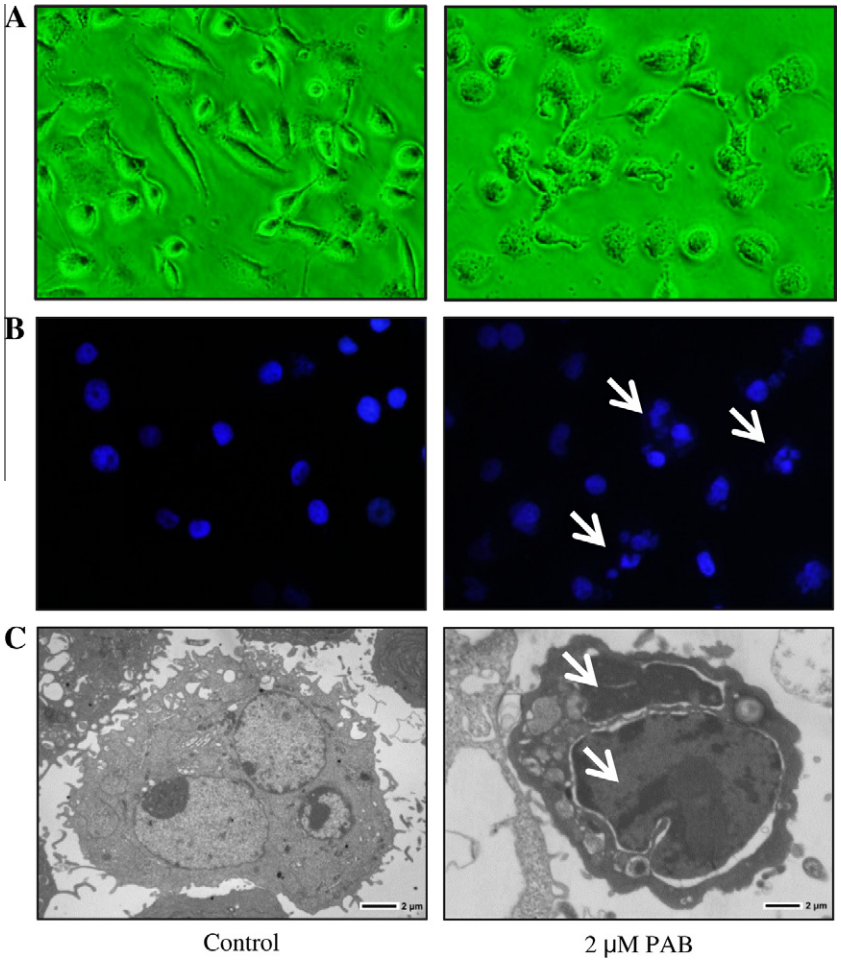

Fig. 2. Morphological changes of apoptosis induced by PAB in DU145 cells. Cells were treated with DMSO (1:1000) or PAB (2 μM) for 48 h and then observed under following microscope. (A) Inverted phase-contrast microscope (200X). (B) Fluorescence microscopy (400×). (C) Transmission electron microscopy (8000×). The white arrows indicate the apoptotic nucleic with chromatin. Results were representative of five independent experiments.

generation of the control cells was considered 100%, a qualitative and quantitative concentration-dependent increase in % ROS generation was observed in the form of fluorescence on treatment with PAB in DU145 cells (116.3 ± 8.3%, 154.3 ± 12.6% and 185.7 ± 10.5% at 0.4, 2 and 10 μM, respectively; Fig. 3A and C, $p < 0.05$).

The percentage of apoptotic DU145 cells with PAB (2 μM) for 48 h, refer to as % Annexin V-FITC cells, was 42.3 ± 4.2%. If cells were pretreated with NAC (5 mM) for 2 h and followed by PAB (2 μM) for an additional 48 h, the percentage of apoptotic cells was reduced to 15.5 ± 3.1%. NAC-treated alone in the absence of PAB showed no significant effect on apoptosis. (Fig. 3B and D, $p < 0.05$).

3.5. Bcl-2 downregulation through the ubiquitin–proteasome pathway in DU145 cells

Western blotting analysis showed PAB (0.4, 2 and 10 μM) for 48 h decreased the anti-apoptotic Bcl-2 protein in a dose-dependent manner ($p < 0.05$), while did not significantly affect the pro-apoptotic Bax protein in DU145 cells (Fig. 4A). In another experiment, some groups were pretreated for 2 h with NAC (5 mM) or MG-132 (10 μM) before PAB treatment. Pretreatment with either NAC or MG-132 inhibited the PAB-induced decline in Bcl-2 (Fig. 4B and C, $p < 0.05$). These results supported that PAB downregulated Bcl-2 protein via ROS generation and Bcl-2 degradation in the ubiquitin–proteasome pathway.

3.6. Caspase activation in response to PAB treatment in DU145 cells

The caspase cascade is crucial for apoptotic signal transduction (Budihardjo et al., 1999). Activities of caspase-3 and caspase-9 were shown to be dose-dependently increased with treatment of PAB for 48 h in DU145 cells ($p < 0.05$), but activities of caspase-8 were not significantly induced. Moreover, effects of NAC (5 mM) or MG-132 (10 μM) on PAB-induced caspase activation were also assessed. The results showed either inhibiting generation of ROS by adding NAC or inhibiting ubiquitin–proteasome by adding MG-132 could significantly blocked caspase-9 and caspase-3 activation in DU145 cells with PAB treatment (Fig. 5, $p < 0.05$). This strongly suggested that both ROS and ubiquitin–proteasome performed a crucial function in PAB-induced apoptosis in DU145 cells, accompanied by the activation of caspases-9 and caspase-3.

4. Discussion

HRPC is the progression of the patients with advanced prostate cancer (Moon et al., 2008). Recently, both experimental and approved chemotherapeutic agents have been developed from natural sources to improve the outcomes of patients with HRPC, but no single agent has so far made a notable impact following docetaxel failure (Cragg et al., 2009; Michael et al., 2009). Thus, it is necessary to look for new drugs to provide therapy for HRPC. Previous studies found that PAB, a novel terpenoid compound, inhibited growth and induced apoptosis towards cancer cell

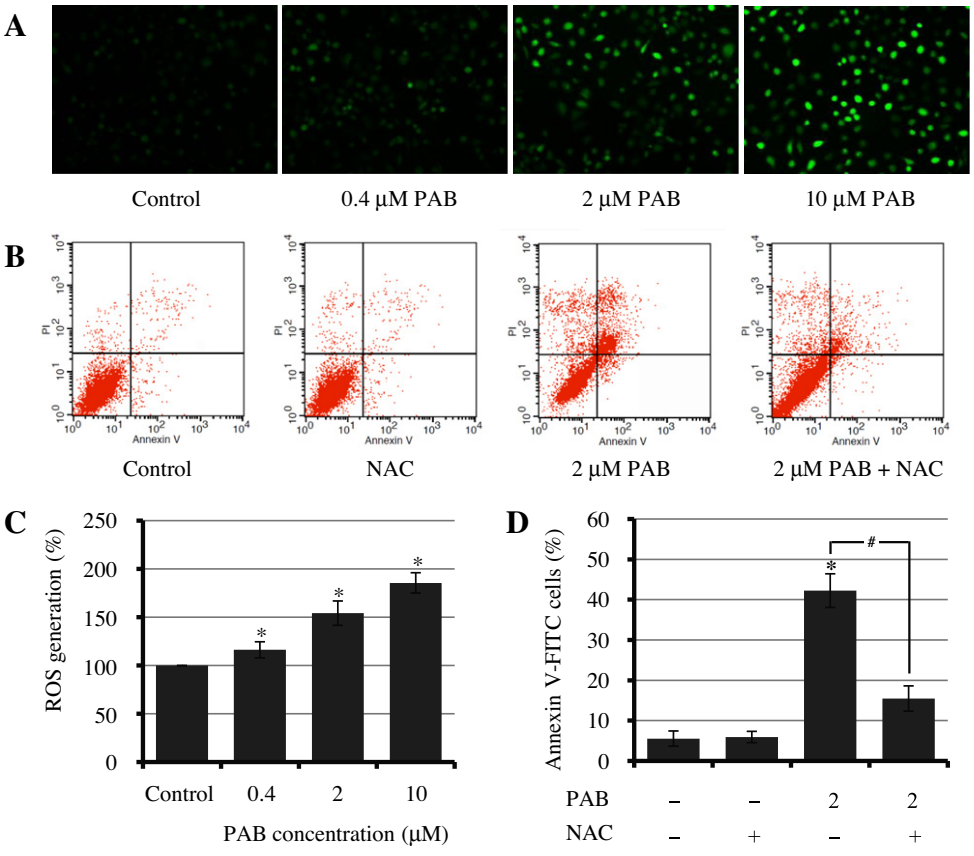

Fig. 3. Effects of ROS on PAB-induced apoptosis in DU145 cells. (A) Photomicrographs of the intracellular ROS in DU145 cells exposed to PAB (0.4, 2 and 10 μM) for 48 h showed increase in fluorescence (100×). (B) Effects of NAC on PAB-induced DU145 apoptosis with Annexin V-FITC Apoptosis Detection Kit by a FACSCalibur Flow Cytometer. Viable cells did not bind annexin V-FITC and PI as reflected in the lower left-hand quadrant of the dot plot. Apoptotic cells are annexin V-FITC positive, with or without PI positivity, as shown in the right-hand quadrant. Graphs showed the % ROS generation from A (C) and annexin positive cells from B (D). Data were presented as means ± SD of three independent experiments and analyzed by one-way ANOVA. $^{*}p < 0.05$, versus control; $^{\#}p < 0.05$, versus PAB-treated alone.

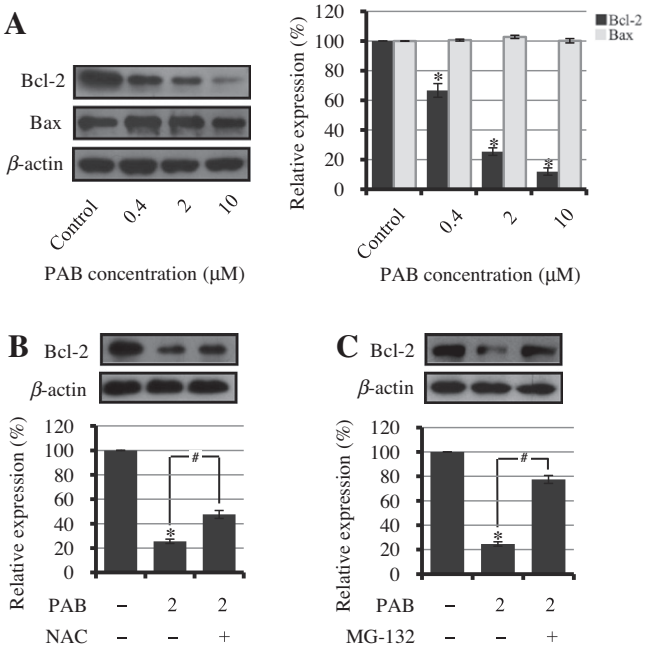

Fig. 4. Effects of PAB on Bcl-2 and Bax expressions in DU145 cells. (A) Bcl-2 and Bax expressions in DU145 cells treated with PAB (0.4, 2 and 10 μM) for 48 h. (B) Effects of NAC on PAB-induced Bcl-2 expressions. (C) Effects of MG-132 on PAB-induced Bcl-2 expressions. In some cases, cells were pretreated for 2 h with NAC (5 mM) or MG-132 (10 μM) before PAB treatment. Data were presented as means ± SD of three independent experiments and analyzed by one-way ANOVA. $^{*}p < 0.05$, versus control; $^{\#}p < 0.05$, versus PAB-treated alone.

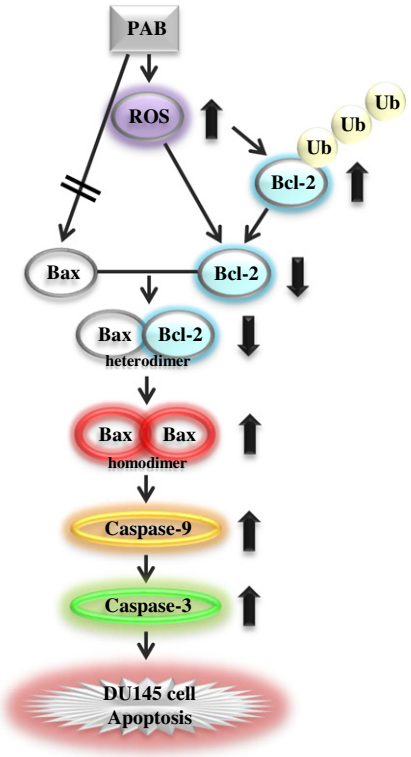

Fig. 6. A proposed schema for PAB-induced apoptosis in DU145 cells.

lines of different origins, including Hela, A375-52, AGS, Bel-7402, MCF-7 and HL-60 (Gong et al., 2004; Li et al., 2005; Ma et al., 2010; Meng and Jiang, 2009; Wu et al., 2006; Yu et al., 2007). In this study, we for the first time evaluated effects of PAB on HRPC DU145 cells *in vitro*.

Consistent with previous studies, our results demonstrated that PAB significantly inhibited the growth of DU145 cells in a concentration-dependent manner by both CCK-8 assay (Fig. 1B) and clone formation assay (Fig. 1C and D). LDH assay indicated 0.4, 2 and 10 μM of PAB could achieve the desired growth inhibition without any obvious necrosis. However, there was a difference in IC_{50} values determined by the two assays. Due to different principles, different methods often yield considerably different values of anti-proliferative activity (Ng et al., 2011). CCK-8 assay is a very convenient and highly sensitive assay for the determination of the number of viable cells, which only reveals changes of cell viability during exposure to the drug. By contrast, clone formation assay is a long-term cell clonogenic ability assay, which demonstrates the metabolic or proliferative capacity of cells after exposure to the drug (Franken et al., 2006). The study exhibited that the IC_{50} value (0.76 ± 0.15 μM) as obtained by clone formation assay was lower than CCK-8 assay (0.89 ± 0.18 μM). We inferred that PAB-treated DU145 cells might perform an irreversible loss of long-term growth ability.

Apoptosis is characterized by a series of typical morphological events (Kroemer et al., 1995). Previous studies demonstrated that PAB could lead to apoptosis. In this work, after 48 h with PAB (2 μM), DU145 cells presented typical apoptotic morphological changes with cell shrinkage, cytoplasm vacuolization, nuclei blebbing, chromatin condensation, and formation of apoptotic bodies (Fig. 2). Because the appearance of apoptotic bodies is regarded as the apoptotic hallmarks, the results suggested that PAB induced the death of DU145 cells mainly through apoptosis. In addition, PAB-induced apoptosis was further demonstrated by Annexin V-FITC apoptosis detection (Fig. 3B and D).

To gain insight into the molecular mechanism involved PAB-induced apoptosis in DU145 cells, production of the intracellular ROS

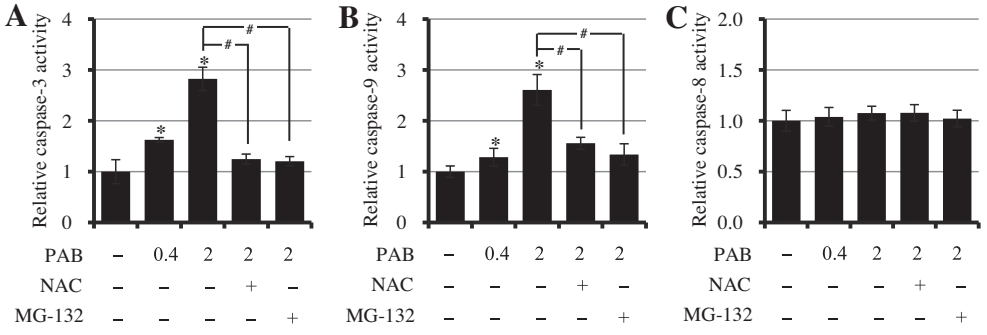

Fig. 5. Effects of PAB on caspase-3, -8, -9 activation in DU145 cells. Graphs of (A), (B) and (C) were drawn from the results using Caspase-3, Caspase-8 and Caspase-9 Activity Assay Kit, respectively. After various treatments as described in "Section 2", caspase activities were measured with a microplate reader at an absorbance of 405 nm. Data were presented as means ± SD of three independent experiments and analyzed by one-way ANOVA. $^{*}p < 0.05$, versus control; $^{\#}p < 0.05$, versus 2 μM PAB-treated alone.

were assessed. ROS generation plays an important role in cellular apoptosis (Simon et al., 2000). Extensive evidence supports involvement of ROS in the mechanism of many anticancer drugs (Kovacic and Somanathan, 2011). Here we hypothesized that PAB causes formation of intracellular ROS, which contribute to apoptosis. We found that treatment with PAB greatly increased ROS production and apoptosis in DU145 cells, which were blocked by NAC, a ROS scavenger (Fig. 3). These findings therefore suggest that PAB-induced apoptosis in DU145 cells is mediated at least partly via ROS production.

Accumulating evidence also indicated that mechanism by which PAB induced apoptosis associated with expression of the Bcl-2 family proteins (Gong et al., 2004; Li et al., 2005). We focused our efforts on two key members of this family that are known to be important drug-resistant HRPC, anti-apoptotic Bcl-2 and pro-apoptotic Bax. Bcl-2 is frequently overexpressed in HRPC and increased following standard hormonal therapy and chemotherapy. High levels of Bcl-2 concurrent with low levels of Bax have been correlated with poor therapeutic response of HRPC (Bray et al., 2009; Fuzio et al., 2011). Bcl-2 protects many cell lines from induced apoptosis by forming heterodimer with Bax (van Delft and Huang, 2006). Present results demonstrated that PAB treatment of DU145 cells dose-dependently downregulated expression level of Bcl-2 without significantly affecting Bax, and ROS could be responsible for the downregulation of Bcl-2 (Fig. 4A and B). Under PAB stimuli, downregulation of Bcl-2 released Bax from the heterodimer and thus permitted Bax to form homodimer leading to apoptosis. Caspase-8 and caspase-9 are the key initiator caspases of the death receptor pathway and the mitochondrial pathway, respectively, that cleave and activate downstream effector caspases such as caspase-3 inducing apoptosis (Green and Reed, 1998). Our results showed that PAB dose-dependently triggered activities of caspase-3 and caspase-9 without significantly inducing activities of caspase-8, which leaded to apoptosis in DU145 cells through the mitochondrial pathway (Fig. 5).

Furthermore, several processes regulate the expression level of Bcl-2 protein, including transcription, posttranscriptional modifications and degradation. Protein degradation is a crucial post-translation modification that plays important roles in the regulation of Bcl-2 protein levels. Among different protein degradation systems, the ubiquitin-dependent proteasomal degradation pathway is the best studied (Breitschopf et al., 2000). However, whether this mechanism is operative in PAB-induced apoptosis is not known. MG-132, which inhibits the proteinase activities of the proteasome, inhibited the PAB-induced decrease expression of Bcl-2 protein and activities of caspase-3 and caspase-9 (Fig. 4C, Fig. 5A and B). Although our present data do not confirm that the ubiquitin–proteasome system is a general regulator of Bcl-2 degradation or whether another pathway performs this function, our findings here indicated that in response to PAB, the ubiquitin–proteasome dependent degradation of Bcl-2 might give rise to the apoptosis of DU145 cells.

In summary, these studies reveal that PAB significantly inhibited growth and stimulated apoptosis in HRPC DU145 cells. We demonstrated a novel finding on the regulatory mechanism of PAB-induced apoptosis, which were associated, at least in part, with the increased intracellular ROS and Bcl-2 degradation through the ubiquitin–proteasome pathway (Fig. 6). Since increased expression of Bcl-2 has been related to drug resistance in many cancer cell lines, the results of this study indicated that PAB may serve as an alternative agent for the treatment of drug-resistant HRPC through regulation of Bcl-2 expression.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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