Pseudolaric acid B induces caspase-dependent cell death in human ovarian cancer cells

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Abstract. Pseudolaric acid B (PAB) is a diterpene acid isolated from the root and trunk bark of Pseudolarix kaempferi Gordon (Pinaceae). Recent studies have reported that PAB exhibits cytotoxic effects in several cancer cell lines. In the present study, we assessed its antitumor activity and molecular mechanisms in HO-8910 and A2780 ovarian cancer cells in vitro. We found that PAB reduced cell viability and induced apoptosis in a dose- and time-dependent manner in HO-8910 and A2780 human ovarian cancer cells. The induction of apoptosis was also accompanied by the regulation of Bcl-2 and XIAP family proteins, cytochrome c and Apaf-1. Moreover, we observed that PAB treatment resulted in the activation of caspase-3 and -9, which may partly explain the anticancer activity of PAB. Collectively, the present study for the first time suggests that PAB enhances apoptosis of HO-8910 and A2780 cells through regulation of Bcl-2 and IAP family proteins. Moreover, the triggering of caspase-3 and -9 activation mediated apoptotic induction. Our results suggest that PAB may be a new therapeutic option for the treatment of ovarian cancers.

Introduction

Ovarian cancer is a fatal gynecological cancer and a major cause of cancer-related mortality worldwide (1). Regrettably, current chemotherapeutics (platinum/taxane-based drugs) have not markedly prolonged recurrence-free survival of this deadly disease. Thus, there is an urgent need for the development of novel treatment strategies (2).

Recently, there has been a growing interest in the use of herbs as a source of new drugs for cancer (3). Pseudolaric acid B (PAB) is a diterpene acid isolated from the root and trunk bark of Pseudolarix kaempferi Gordon (Pinaceae), known as ‘Tu-Jin-Pi’ (4). PAB contains a structural frame-work that has never been found in any other natural products including a unique poly-hydroazulene with a trans-substitution pattern at the junction sites (5). It has been demonstrated that PAB significantly delayed tumor growth of a taxol-resistant liver cancer without showing obvious toxicity to the animals in vivo, and possessed selective anti-proliferative effects in human cancer cells but not in normal cells in vitro (5).

Apoptosis, a process of programmed cell death (PCD), is crucial during development and to maintain homeostasis. However, dysregulation of this process is implicated in various diseases including cancer (6,7). Cell death inhibition is a very successful strategy that cancer cells employ to combat the immune system and various anticancer therapies (8). Recent evidence has shown that PAB treatment leads to apoptosis in many cancer cells (9-11). However, such an effect of PAB on human ovarian cancer cells has not been reported, and the molecular mechanisms are still not fully understood.

Several genes critical in the regulation of apoptosis have been identified, including XIAP, a member of the IAP family. X-linked inhibitor of apoptosis resistance by effectively inhibiting caspase-3, -7 and -9 (12), IAP gene amplification and increased protein expression occur in many types of cancers, and is an important pathway by which cancer cells acquire resistance to chemotherapy and radiation therapy (13).

In the present study, we analyzed the effect of PAB on cell death and apoptosis in ovarian cancer HO-8910 and A2780 cells. The contribution of caspase-3 and -9, XIAP and Bcl-2 family members, and cytochrome c (cyto c) and apoptotic protease activating factor-1 (Apaf-1) in PAB-induced cell death was also investigated.

Materials and methods

Reagents. Monoclonal anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-p53, anti-Bax, anti-Bcl-xl, anti-Bcl-2, anti-Bid, anti-XIAP, anti-cIAP1, anti-cIAP2, anti-Smac, anti-survivin, anti-cyto c and anti-Apaf-1 were obtained from New England Biolabs (Beverly, MA, USA). Stock of the selective XIAP inhibitor Embelin were obtained from Calbiochem Behring (La Jolla, CA, USA). RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). An Annexin V apoptosis detection kit was purchased from R&D Systems (Abingdon, UK). Cell isolation and...
tissue culture reagents were obtained from Invitrogen-Life Technologies (Lidingö, Sweden). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture.** Ovarian cancer cell lines HO-8910 and A2780 were obtained from China Medical University. The cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS; Gibco), 50 µg/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml neomycin. The cells were incubated at 37°C in a humidified CO2 (5%) incubator. HO-8910 and A2780 cells in 24-well flat-bottomed plates were incubated with PAB at different concentrations (0, 2, 4 and 8 µmol/l) for 48 h or at a concentration of 4 µmol/l for 0, 24, 48, 72 and 96 h, respectively. In some experiments, Embelin (XIAP inhibitor) was used 30 min prior to PAB induction.

**Cell viability.** To assess the overall viability of HO-8910 and A2780 cells following PAB treatment, the cells were treated as described above. At particular time-points, the HO-8910 or A2780 cells were washed two times with PBS and treated with a 0.4% solution of trypan blue and visualized as clear cells under a microscope. HO-8910 and A2780 cells that were no longer viable, which had damaged membranes that allowed entry of the dye, were stained blue. Assays were performed in triplicate and repeated at least three times. The number of intact viable cells was expressed as a percentage of total cells and was assessed at different time-points. The percentage of viable cells was calculated as follows: Viable cells (%) = (total number of viable cells per ml of aliquot/total number of cells per ml of aliquot) x 100.

**Acridine orange staining.** Twenty-five microliters of cell suspension (0.5x10⁶ to 2.0x10⁶ cells/ml) was incubated with 1 µl of Acridine orange (AO) solution, and mixed gently. Each sample was mixed just prior to microscopy and quantification. The cell suspension (10 µl) was placed onto a microscopic slide, covered with a glass coverslip and at least 500 cells were examined by fluorescence microscopy using a fluorescein filter.

**Hoechst 33258 staining.** The cells were stained with Hoechst 33258 (Molecular Probes Inc., Eugene, OR, USA) at a dilution of 1:600 (stock solution, 1 mg/ml) for 5 min in the dark. The samples were observed under a fluorescence microscope. Five hundred cells were counted from each coverslip in turn, and the results were confirmed by visualization of the apoptotic nuclei. There were five coverslips in each group.

**Transmission electron microscopy.** The cells treated with 0.1 µmol/l paclitaxel were trypsinized and harvested after 24 h. Subsequently the cells were fixed in 4% glutaral and immersed in Epon 821, embedded in capsules and converged for 72 h at 60°C. The cells were then prepared and placed onto an ultrathin section (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was examined by transmission electron microscopy.

**Flow cytometric analysis.** The apoptosis rates of HO-8910 and A2780 cells were quantified by flow cytometry using FITC-conjugated Annexin V and PI. Specific binding of Annexin V was achieved by incubating 10⁶ cells in 60 µl of 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) using CellQuest software. At least 10,000 cells were counted in each sample. Experiments were performed and interpreted as follows: cells that were Annexin V(-)/PI(-) (lower left quadrant) were considered as living cells, Annexin V(+)/PI(-) cells (lower right quadrant) as apoptotic cells, Annexin V(+)/PI(+), upper right quadrant cells) as necrotic or advanced apoptotic cells, and Annexin V(-)/PI(+) (upper left quadrant) cells may be bare nuclei, were considered as cells in late necrosis or cellular debris.

**Measurement of cyto c and Apaf-1 release from mitochondria.** Cells were treated with 0.1% DMSO or different concentrations of PAB (0, 2, 4 and 8 µmol/l) for 48 h. Mitochondria and the cytosol were separated using a cyto c-releasing apoptosis assay kit. Cells were suspended in cytosol extraction buffer. The cell suspension in extraction buffer was homogenized using a Dounce homogenizer and then centrifuged (700 x g, 10 min) after 10 min on ice. Then, the collected supernatant was re-centrifuged (10,000 x g, 30 min, 4°C). The resulting supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were processed for western blot analysis.

**Western blot analysis.** Western blot analysis using rabbit polyclonal antibody for p53 (1:2,000 dilution), Bcl-2 (1:2,000 dilution), Bcl-xl (1:2,000 dilution), Bax (1:2,000 dilution), XIAP (1:2,000 dilution), cIAP1 (1:2,000 dilution), cIAP2 (1:2,000 dilution), Smac (1:2,000 dilution), Survivin (1:2,000 dilution), cyto c (1:2,000 dilution) and Apaf-1 (1:2,000 dilution) was performed according to standard protocols. β-actin (1:2,000) was used to control for equal protein loading. The immunoblots were then washed three times with TBS-T buffer, incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgM; Santa Cruz Biotechnology), and developed using chemiluminescent substrate (Pierce, Rockford, IL, USA).

**Measurement of caspase-3 and -9 activity.** HO-8910 and A2780 apoptotic cells were harvested and centrifuged at 1,500 rpm for 10 min. Cells were washed two times with PBS (pH 7.4) and then resuspended with 50 µl lysis buffer at 4°C and incubated on ice for 10 min. All subsequent steps were performed on ice. After centrifugation, cell extracts were transferred to fresh tubes, and protein concentrations were measured. Each 50 µl of cell extract containing 100 µg of protein was combined with equal volumes of 2X reaction buffer in a microplate followed by the addition of 5 µl of the peptide substrates of caspase-3 and -9. After overnight incubation in the dark at 37°C, samples were read in a microplate reader at 405 nm. Caspase-3 and -9 activity was evaluated by the absorbance ratio of treated/control samples. In some experiments, inhibitors for caspase-3 (Z-DEVD-FMK) or caspase-9 (Z-LEHD-FMK) were added into fresh medium of HO-8910 or A2780 cells at 1 h before PAB was added.
Statistical analysis. Each experiment was carried out in duplicate or triplicate, and three or four independent experiments were performed. Results are expressed as means ± standard deviation (SD) and analyzed with SPSS 11.5 software. Results were compared using analysis of variance (ANOVA). When ANOVA showed a statistically significant difference, a group-by-group comparison was performed using a t-test with Tukey’s correction for multiple comparisons. Statistical significance was set at P<0.05.

Results

Morphologic analysis of apoptotic HO-8910 cells under light microscopy and transmission electron microscopy. In order to detect HO-8910 or A2780 viability, we performed a trypan blue exclusion assay. Trypan blue staining showed that the percentage of cell viability was decreased with increasing time and concentrations of PAB (Fig. 1).

To investigate whether the growth inhibitory effect was mediated through the induction of apoptosis, we examined the apoptotic morphology of control and PAB-treated HO-8910 cells by Acridine orange staining and transmission electron microscopy. Microscopy of PAB-treated HO-8910 cells revealed morphological changes compared to the control, and the apoptosis rate of HO-8910 increased in a time- and dose-dependent manner. Apoptotic cells were characterized by membrane blebbing and nuclear condensation, while necrotic cells were typically larger and lighter with plasma membrane lesions (Fig. 2). The percentage of apoptotic cells was calculated by observing 500 cells. Transmission electron microscope imaging is considered the gold standard in identifying cellular apoptosis due to its standard and reliable method (14) (Fig. 3).

PAB induces apoptosis in human HO-8910 and A2780 cells. Flow cytometry using FITC-conjugated Annexin V revealed that HO-8910 and A2780 cells exposed to PAB underwent rapid apoptosis (Fig. 4). This effect was positively correlated with the exposure time in the HO-8910 and A2780 cells, and excessive apoptosis was associated with loss of membrane integrity in an increased portion of HO-8910 and A2780 cells, which indicated necrosis or late apoptosis.

PAB treatment modulates the Bax/Bcl-2 ratio in HO-8910 and A2780 cells. Western blot analysis was carried out to verify the involvement of Bax and Bcl-2 proteins during...
PAB-induced apoptosis of HO-8910 and A2780 cells. The expression of Bcl-2 and Bcl-xL proteins was downregulated and the expression of p53 and Bax proteins was upregulated in the PAB-treated cells (Fig. 5). However, the expression of Bid was not altered in the two types of cells. These results suggest that PAB induced apoptosis via alteration of the Bax/Bcl-2 ratio in HO-8910 and A2780 cells.

PAB induces HO-8910 and A2780 apoptotic cell death via modulation of XIAP family proteins. In order to ascertain the apoptotic mechanism in HO-8910 cells induced by PAB, we examined anti-apoptotic protein expression. Members of the mammalian IAP family mainly include XIAP, cIAP-1 and cIAP-2. The results showed that the transcription and expressions of cIAP1/2, XIAP and survivin were decreased in a
dose-dependent manner after challenge with PAB in HO-8910 and A2780 cells. However, the transcription and expression of Smac (second mitochondria-derived activator of caspase), which is an intrinsic antagonist of XIAP, were increased in a time-dependent manner (Fig. 6). These results suggest that changes in expression of cIAP1/2, survivin, Smac and XIAP may contribute to PAB-induced apoptogenesis in HO-8910 and A2780 cells.

PAB induces cyto c and Apaf-1 release from the mitochondria. Mitochondria play an essential role in the apoptosis triggered by chemical (anticancer) agents. The mitochondrial response includes the release of cyto c and Apaf-1 into the cytosol. Therefore, we tested the effect of PAB on cyto c and Apaf-1 release. To analyze the involvement of mitochondria in HO-8910 and A2780 cells, proteins from the cytosolic fraction were prepared and analyzed using western blot analysis. Treatment of HO-8910 and A2780 cells with 0, 2, 4 and 8 µmol/l PAB, respectively, for 48 h resulted in an increase in cyto c and Apaf-1 levels in a dose-dependent manner. These results indicate that PAB promotes cyto c and Apaf-1 release from mitochondria into the cytosol (Fig. 7).

Effects of an inhibitor of XIAP on PAB-induced HO-8910 and A2780 cell apoptosis. To identify the relevance of the XIAP signaling pathway in controlling the apoptotic cell death by PAB, inhibition assays were performed with Embelin (a specific inhibitor of XIAP). The percentage of apoptosis was determined by flow cytometry. HO-8910 and A2780 cells were pretreated with 20 µM Embelin for 30 min, and then cultured with 4 µmol/l PAB for 48 h. The cells were stained with Hoechst 33258 and the samples

Figure 5. p53 and Bcl-2 family proteins are involved in HO-8910 and A2780 cell apoptosis induced by PAB. The expression of p53 and Bcl-2 family proteins in (A) HO-8910 and (B) A2780 cells challenged with 0, 2, 4 and 8 µmol/l PAB, respectively, for 48 h. β-actin was used as a loading control. The data are from one representative experiment.

Figure 6. XIAP family proteins are involved in HO-8910 and A2780 cell apoptosis induced by PAB. The expression of XIAP, cIAP1, cIAP2, survivin and Smac in (A) HO-8910 and (B) A2780 cells challenged with 0, 2, 4 and 8 µmol/l PAB, respectively, for 48 h. β-actin was used as a loading control. The data are from one representative experiment.
were observed under a fluorescence microscope. The results showed that Embelin significantly increased the apoptosis rate (Fig. 8). The same results were also showed by flow cytometric analysis (Fig. 9).

Figure 7. Cytochrome c and Apaf-1 proteins are involved in HO-8910 and A2780 cell apoptosis induced by PAB. The expression of cytochrome c and Apaf-1 proteins in (A) HO-8910 and (B) A2780 cells challenged with 0, 2, 4 and 8 µmol/l PAB, respectively, for 48 h. The data are from one representative experiment.

Figure 8. Morphology of HO-8910 cells following treatment with 20 µM Embelin, 4 µmol/l PAB or 20 µM Embelin plus 4 µmol/l PAB for 48 h. HO-8910 control and treated cells were stained with Hoechst 33258 dye.

Expression of caspase-3 and -9 activity. The expression of caspase-3 and -9 activity in HO-8910 and A2780 cells incubated in the presence of PAB is presented in Fig. 10. Treatment of HO-8910 and A2780 cells with PAB for 48 h at concentrations...
of 0, 2, 4 and 8 µmol/l, respectively, or for different times at a concentration of 4 µmol/l showed marked increase in caspase-3 and -9 activation. Activity of caspase-3 and -9 in HO-8910 and A2780 cells following PAB treatment showed dose- and time-

Figure 9. Effect of inhibitors of XIAP (Embelin), caspase-3 (DEVD), or caspase-9 (LEHD) on PAB-induced HO-8910 and A2780 cell apoptosis. (A and B) HO-8910 and A2780 cells were treated with 4 µmol/l PAB and incubated for 48 h with or without 20 µM Embelin for 30 min. (C and D) HO-8910 and A2780 cells were treated with 4 µmol/l PAB and incubated with DEVD or LEHD for 30 min. After treatment, cells were incubated with FITC-conjugated Annexin V (AV) and PI double staining. Flow cytometric analysis was performed. Values represent means ± SD of five experiments performed in duplicate. (A and B) *P<0.05, **P<0.001 compared with that of the control. (C and D) *P<0.05 compared with that of PAB alone.

Figure 10. Effect of PAB on the activity of caspase-3 and -9 in HO-8910 and A2780 cells. The cells were treated with 0, 2, 4 and 8 µmol/l PAB, respectively, for 48 h or with 4 µmol/l PAB for 0, 24, 48, 72 and 96 h, respectively. (A) Dose-dependent effect of PAB-induced caspase-3 and -9 activity in HO-8910 cells. (B) Time-dependent effect of PAB-induced caspase-3 and -9 activity in HO-8910 cells. (C) Dose-dependent effect of PAB-induced caspase-3 and -9 activity in A2780 cells. (D) Time-dependent effect of PAB-induced caspase-3 and -9 activity in A2780 cells. Values represent means ± SD of five experiments performed in duplicate. *P<0.05, **P<0.001 compared with that of control.
dependent upregulation. Inhibition of XIAP with Embelin potentiated the PAB-induced caspase-3 and -9 activity (data not shown). In order to assess whether PAB-induced cell death occurred due to caspase activation, we used the caspase inhibitor Z-DEVD-FMK (specific for caspase-3) or Z-LEHD-FMK (specific for caspase-9) (Fig. 9). PAB induced cell death in HO-8910 and A2780 cells. The pretreatment of HO-8910 and A2780 cells with Z-DEVD-FMK or Z-LEHD-FMK inhibited PAB-induced apoptosis, suggesting the involvement of caspase(s) in PAB-induced cell death.

Discussion

Several trials are currently being performed to investigate the effect of pseudolactic acid B on various types of solid tumors, including ovarian cancer (4,15). However, knowledge concerning the mechanism by which this compound induces cell death is still limited. Thus, our research was designed to determine whether PAB induces apoptosis in ovarian cancer cells. To the best of our knowledge, the present study for the first time demonstrated that PAB induced HO-8910 and A2780 cell apoptosis in a time- and dose-dependent manner.

A characteristic feature of human cancers is the inability to mount a proper apoptotic response during tumor progression or upon treatment with cytotoxic therapies (16). Therefore, evasion of apoptosis constitutes a critical cause of primary or acquired treatment resistance that frequently occurs in various types of human cancers (17). The molecular pathways leading to apoptosis are evolutionarily conserved and controlled (18). Apoptotic signaling pathways are the most promising therapeutic targets for cancer treatment (19,20).

The Bcl-2 family proteins play an essential role in the apoptotic process. They are regulators of mitochondrial membrane permeability and intermembrane space protein efflux according to the opposing fractions of anti-apoptosis members and pro-apoptosis members (21). The ratio of anti- and pro-apoptotic protein expression, such as Bcl-2/Bax, is crucial for the induction of apoptosis, and it decides the susceptibility of cells to undergo apoptosis (22). Bcl-2 and Bcl-XL act as anti-apoptotic factors, and Bax acts as a pro-apoptotic factor. In the present study, treatment of HO-8910 and A2780 cells with PAB markedly downregulated Bcl-2 and Bcl-XL expression, upregulated Bax and P53, whereas the expression of Bid did not change.

The mitochondrion is generally believed to be the key regulatory element of cell death and the target of many pro-apoptotic signaling pathways (23). Smac/DIABLO (second mitochondria-derived activator of caspses or direct IAP binding protein with low pl), a mitochondrial protein that is released together with cyto c from the mitochondria in response to apoptotic stimuli, was found to promote caspase activation by binding to and neutralizing the IAPs via its N-terminal (24,25). Smac release from mitochondria (26) is a general feature of apoptosis. In the present study, we showed that PAB upregulated the expression of Smac in HO-8910 and A2780 cells. Our results also indicate that PAB promotes cyto c and Apaf-1 release from mitochondria into the cytosol.

Bax/Bak mediate mitochondrial outer membrane permeabilization (MOMP), with consequent release of apoptogenic factors from mitochondria into the cytosol (cyto c, Smac/DIABLO). These apoptogenic factors precipitate activation of the caspase cascade and cell-killing via Apaf-1-mediated activation of the initiator caspase, caspase-9, as well as by derepression of effector caspases by blocking their antagonist XIAP (27,28). Inhibitor of apoptosis (IAP) proteins are a family of endogenous anti-apoptotic proteins (29). Elevated expression of IAP proteins combined with their well-established functional importance for survival of tumor tissues and resistance to anticancer therapies makes IAP proteins attractive targets for therapeutic intervention (8). Among the IAPs, cellular IAP1 (cIAP1) and cIAP2 play a key role in the regulation of death receptor-mediated apoptosis, whereas X-linked IAP (XIAP) inhibits both death receptor-mediated and mitochondrial-mediated apoptosis by binding to and inhibiting caspase-3/7 and caspase-9; three cysteine proteases critical for execution of apoptosis (30). In the present study, we showed that PAB downregulated the expression of XIAP, survivin, cIAP-2 and Bcl-2 and upregulated the expression of Bax. It was also shown that PAB promoted cyto c and Apaf-1 release from mitochondria into the cytosol.

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone; C24H26O3; molecular weight, 294.39) is a type of extract from Japanese Ardisia Herb, and its traditional use in Chinese herbal medicine is to dispel intestinal parasites. It was later identified as a novel cell permeable inhibitor of XIAP (31). To more directly link the XIAP signaling pathway with caspase activation, we examined PAB-mediated caspase-3 and -9. In our study, inhibition of XIAP significantly increased caspase-3 and -9 activity. Moreover, Embelin significantly increased the apoptosis rate.

In conclusion, we found that PAB inhibited cell growth and induced the apoptosis of HO-8910 and A2780 cells. We also studied the underlying mechanisms involved in PAB-induced apoptosis. In the present study, there was a tendency of alterations showing a decreased expression level of Bcl-2, cIAP1/2, survivin and XIAP, and also with an increased expression level of Smac and activation of caspase-3 and -9. Moreover, PAB induced cyto c and Apaf-1 release from the mitochondria.

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