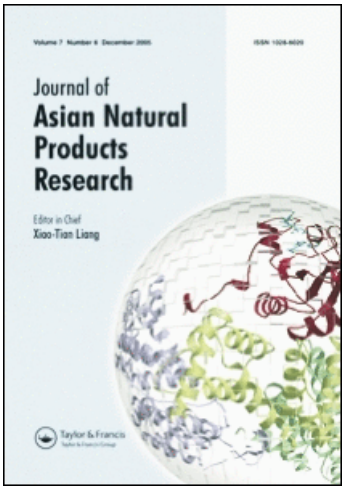

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Studies on anti-tumour activities of pseudolaric acid-B (PLAB) and its mechanism of action

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Studies on anti-tumour activities of pseudolaric acid-B (PLAB) and its mechanism of action

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Pseudolaric acid-B (PLAB), a diterpene acid, was isolated from the root and trunk barks of *Pseudolarix kaempferi*. It showed antifungal and anti-fertility effects as well as cytotoxic activities in previous studies. The present study investigates cytotoxic activity on cultured human cancer cells, inhibition on the growth of transplantable tumours in mice and the mechanism of these actions. The experimental results showed that PLAB had potent cytotoxic effects on cancer cells derived from different tissues. MTT assay showed that its IC_{50} towards these tumour cells was 0.17 to 5.20 μmol/L, and towards one normal human kidney proximal tubular epithelial cell (HKC) was 5.77 μmol/L. Furthermore, the results of cell growth curve and colony formation of cancer cells matched the above results. The results *in vivo* demonstrated that PLAB significantly inhibited the growth of transplantable tumours, such as Lewis lung cancer and hepatocarcinoma 22 (H22) in mice. The inhibitory rate to H22 was 14.4% and 40.1%, and to Lewis lung cancer reached 39.1% and 47.0%, when PLAB was given by intraperitoneal injection (i.p.) at a dose of 30 mg/kg/day and 60 mg/kg/day for 10 days, respectively. It is suggested that PLAB also showed obvious anticancer activity *in vivo*. Inducing apoptosis by PLAB in HeLa cells was assessed by various morphological and biochemical characteristics, including cell shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies, and internucleosomal DNA fragmentation. A typical 'sub-G_1 peak' was also checked through flow cytometry. These results were accompanied by up-regulating P53, down-regulating Bcl-2 and activating Caspase-3, which was revealed by Western blotting. PLAB also caused cell cycle arrest to G_2/M phase in a dose-dependent manner. The experiments suggest that PLAB is a new potent anti-tumour agent.

Keywords: Pseudolaric acid-B; Natural product; Anti-tumour activity; Apoptosis

1. Introduction

During the last decade more attention has been given to uncovering the benefits of natural products in relation to cancers. Pseudolaric acid-B (figure 1), a novel diterpene acid, was isolated and identified as the main antifungal constituent from *Pseudolarix kaempferi Gorden* [1,2]. It also showed an anti-fertility effect [3,4] and was established as a general cytotoxic agent against P-388 lymphocytic leukaemia, KB carcinoma of the nasopharynx,

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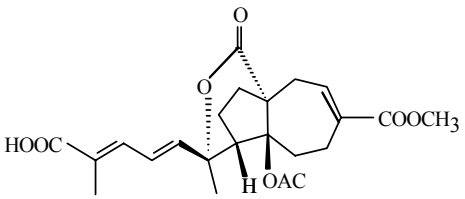

Figure 1. Chemical structure of pseudolaric acid-B.

HT-1080 fibrosarcoma, human breast cancer, human melanoma, human lung cancer and human colon cancer cell [5,6] *in vitro*. However, the mechanisms underlying the tumour cytotoxicity of PLAB and its inhibition effect on transplanted mice tumour cell growth *in vivo* were poorly reported.

The cytotoxicity of most classical anti-tumour drugs is thought to be mediated by their ability to induce apoptosis, which plays an essential role in controlling cell number in many developmental and physiological settings and in chemotherapy-induced tumour cell killing. Apoptosis is a genetically regulated biological process, guided by the ratio of pro-apoptotic and anti-apoptotic proteins. Recently, inducers of apoptosis have been used in cancer therapy. Several studies have attempted to induce apoptosis by triggering the Bcl2 family of proteins and other proteins that inhibit apoptosis; others have targeted the caspases. Some of these therapies have shown promising results [7].

The present work focused on the effects of PLAB on cell proliferation, the anti-tumour activities of PLAB *in vivo* using two transplantable tumour models in mice, and the mechanism of PLAB involving cell cycle distribution and apoptosis.

2. Results and discussion

2.1 Cytotoxic activity of PLAB

In a series of experiments, 11 tumourigenic human cells, including nasopharyngeal carcinoma cell (KB), human colorectal carcinoma cell (HCT-8), human colorectal adenocarcinoma cell (HT-29), human lung adenocarcinoma cell (A549), human kidney

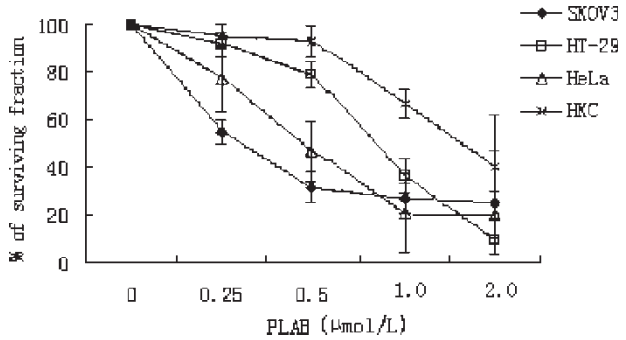

Figure 2. Effect of PLAB cytotoxicity. Human colorectal adenocarcinoma cell (HT-29), human cervical cancer cell (HeLa), human ovarian carcinoma cell (SKOV3) and human kidney proximal tubular epithelial cell (HKC) were exposed to increasing concentrations of PLAB (0.25 to 2.0 μmol/L) for 96 h. Surviving fraction was determined by MTT assay. Data are given as mean of triplicate values ± SD.

carcinoma cell (Ketr-3), human cervical cancer cell (HeLa), human breast adenocarcinoma cell (MCF-7), human bladder carcinoma cell (EJ), human ovarian carcinoma cell (SKOV3, A2780), human stomach carcinoma cell (BGC-823) and one normal human kidney proximal tubular epithelial cell (HKC) were chosen to determine the cytotoxic activity of PLAB. A typical dose-dependent inhibition of cell survival was shown by documenting the surviving cell fraction plotted versus PLAB concentration (figure 2). After incubating for 96 h, increasing concentrations of PLAB (0.125 – 1.0 μmol/L) led to a gradual decrease of viable cells fraction. Because of these variations, the cytotoxic activity of PLAB had been determined against a panel of human tumour cells of different origins. Table 1 shows IC_{50} values varying from 0.17 μmol/L (KB) to 5.20 μmol/L (A-549) with respect to the cells.

HeLa cells were chosen to evaluate the effect of PLAB on growth of cancer cells. The number of control cells increased over fivefold after 6 days, whereas the number of HeLa cells treated with 0.25 μmol/L PLAB increased about threefold. Furthermore, the growth of the group treated with 0.5 μmol/L PLAB was inhibited on the fifth day. From the beginning, tumour cell growth of the group treated with 1.0 μmol/L PLAB was inhibited completely (figure 3A). Inhibition of HeLa cell growth was also assessed using a 10-day clonogenic assay. Tumour cells were plated in a limiting dilution clonogenic assay. Surviving clonogenic units were enumerated by measuring colony formation after 10 days of incubation. Treatment with increasing concentrations of PLAB produced progressively greater inhibition of clonogenic growth of HeLa cells (figure 3B).

2.2 Tumour growth inhibition in vivo

The effect of PLAB on tumour growth was studied in Kunming and C57BL/6 mice using transplanted models with Lewis lung cancer carcinoma and hepatocarcinoma 22 (H22), respectively. Intraperitoneal injection of PLAB (30 mg/kg and 60 mg/kg) was performed every day, followed with tumour cell inoculation. This resulted in an inhibition of tumour growth quantified by means of weighing the excised tumours at the end of the experiment. Compared with the untreated group, inhibition rates were 39.1% and 47.0% of Lewis lung cancer-implanted mice (figure 4A) and 14.4% and 40.1% of H22-implanted mice (figure 4B). Neither death nor altered weight modification of the host occurred, and PLAB seemed to have little toxicity in our experimental conditions.

Table 1. Cytotoxic activity of PLAB on different human cancer cells (IC_{50}).

Cancer cells	*IC_{50} (μmol/L)*
BGC-823	1.13 ± 0.24
HeLa	0.50 ± 0.19
A549	5.20 ± 0.26
HCT-8	1.20 ± 0.04
HT-29	0.68 ± 0.03
KB	0.17 ± 0.035
Ketr3	3.58 ± 2.42
A-2780	0.87 ± 0.08
Skov3	0.74 ± 0.28
Mcf-7	4.25 ± 1.37
EJ	1.04 ± 0.50
HKC	5.77 ± 0.65

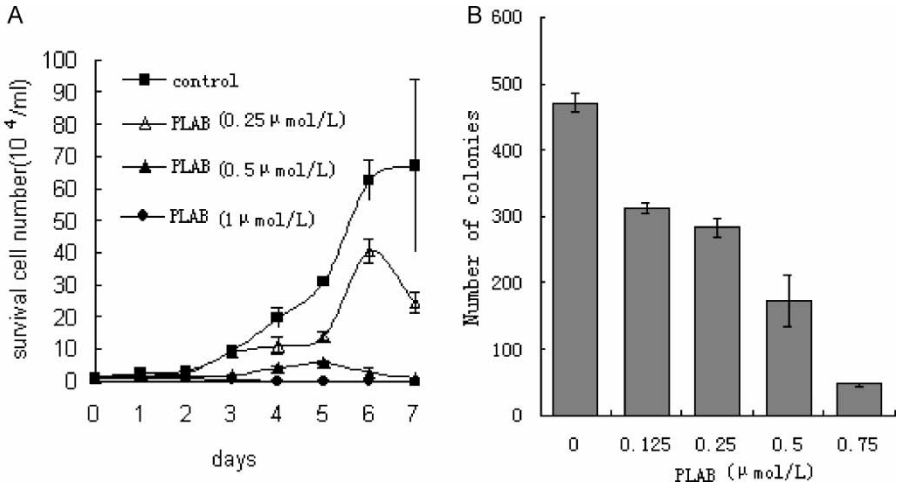

Figure 3. (A) Effect of PLAB on growth in HeLa cells, HeLa cells were treated with PLAB (0–1 μmol/L). Values reflect the mean number of cells ± SE for three replicate wells at different intervals. (B) Clonogenic formation growth of HeLa cells treated with PLAB. Colonies were counted after 10 days of incubation. Values reflect the mean number of colonies ± SE for triplicate experiments.

2.3 PLAB causes cell cycle arrest

To evaluate the possible role of cell cycle arrest in PLAB-caused growth inhibition, HeLa cells were treated with PLAB. Cell cycle distribution was evaluated by flow cytometric analysis after staining of cellular DNA with propidium iodide at the different concentrations. This indicated that PLAB induced an accumulation in G2/M phase of cell cycle. After treatment with 0.25–4 μmol/L of PLAB for 24 h, the number of cells in G2/M phase was higher than that in untreated cells (figure 5A). The percentage of cells arrested in G2/M phase increased with the increase of concentration. The dose-dependent curve of G2/M arrest

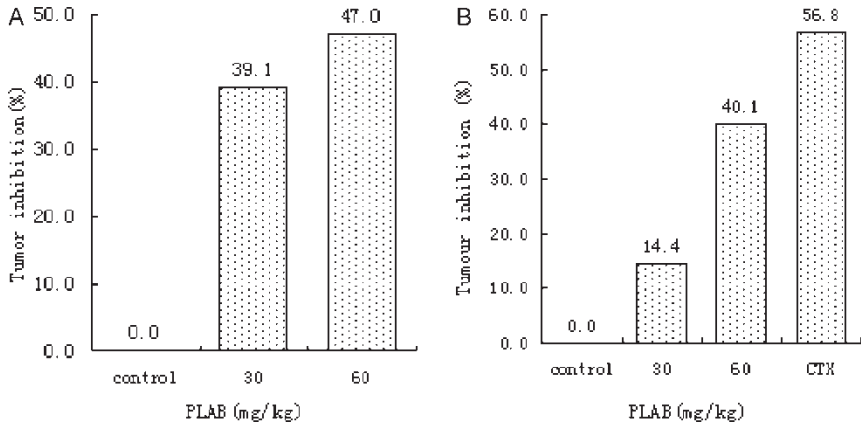

Figure 4. Effect of PLAB on transplanted tumour growth. Thee groups of eight C57BL/6 mice for Lewis lung cancer (A) and four groups of ten Kunming mice for H22 (B) were implanted with tumour cells as detailed in Materials and methods. One day later, PLAB was administered every day by i.p. injection of two doses of 30 mg/kg and 60 mg/kg. As a positive control, CTX (60 mg/kg) was administered by i.p. once in the experimental groups implanted by H22. The negative control group received the solvent in the same conditions. On the 12th day, the mice were killed and tumours were excised then weighed, and the inhibition rate of PLAB was calculated in per cent.

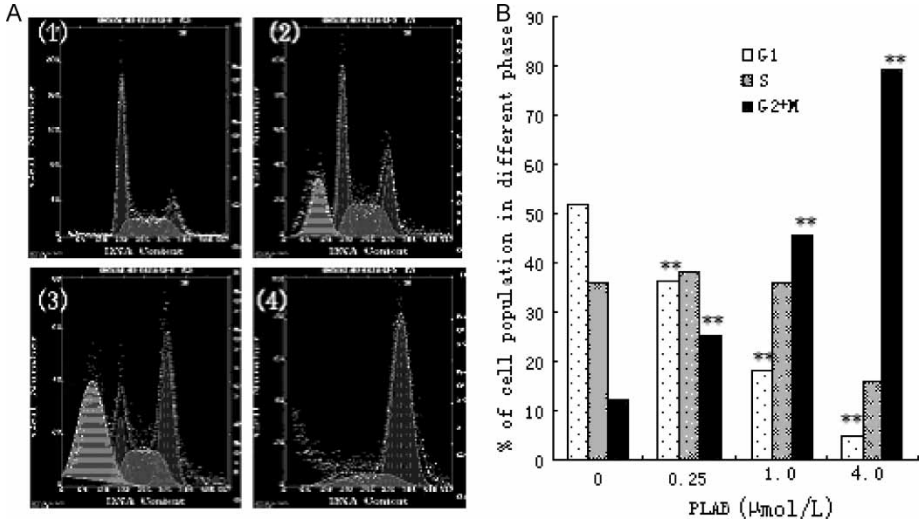

Figure 5. Effect of PLAB on cell cycle distribution. (A) Cell cycle fraction of HeLa cells grown in medium or exposed to 0.1% DMSO (1) and 0.25, 1.0, 4.0 μmol/L of PLAB (2, 3, 4) for 24 h. (B) Dose-effect results of HeLa cells exposed for 24 h to concentrations of PLAB varying from 0.25 to 4.0 μmol/L.

following treatment for 24 h with increasing doses of PLAB is shown in figure 5B. The percentage of cells in G2/M peaked when cells were exposed to 4 μmol/L PLAB.

2.4 PLAB induces apoptosis

PLAB induced morphological changes, which were characteristics of apoptosis in HeLa cells (figure 6). Contrast cells displayed excellent growth characteristics—polygonal shape with round large nucleus featuring prominent multiple nucleoli, and well spread on the growth surface. PLAB evoked typical apoptotic features such as membrane blebbing, cell shrinkage and detachment, and nuclear condensation and fragmentation (arrows in figure 6).

Flow cytometric analysis of HeLa cells exposed to PLAB confirmed the morphological observations above. The DNA fluorescence histograms of PI-stained cells showed low DNA stainability of the PLAB-treated, apoptotic cells, which resulted in a distinct, quantifiable region below the G_1 peak. In contrast, the G_1 peak predominated in control cells (figure 7A). Quantification of dose dependency was done by monitoring the amount of nuclei with sub-diploid DNA content with flow cytometry (figure 7B). The apoptotic cells increased up to 34.3% (1.0 μmol/L) after incubation in PLAB of concentrations higher than 0.25 μmol/L for

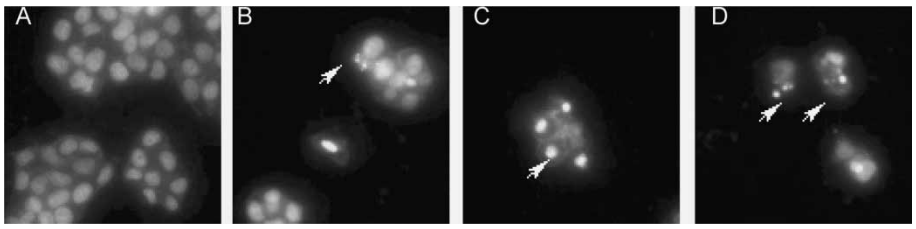

Figure 6. Morphological appearance of cells by fluorescence detection. (A) HeLa cells treated with 0.1% DMSO. (B) HeLa cells treated with 0.25 μmol/L PLAB. (C) HeLa cells treated with 1.0 μmol/L PLAB. (D) HeLa cells treated with 4.0 μmol/L PLAB (× 400).

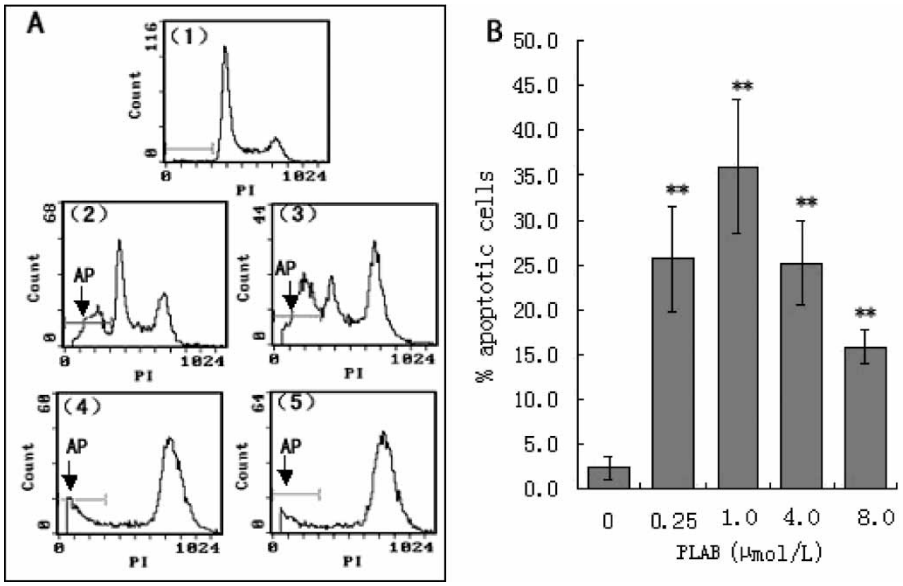

Figure 7. (A) Demonstration of apoptosis by flow cytometric analysis. (1) Untreated HeLa cells. (2)–(5) Appearance of cells with sub-diploid DNA content (Ap) after exposed to increasing concentrations of PLAB (0.25, 1.0, 4.0, 8.0 μmol/L) for 24 h. Ap, apoptotic cells. (B) Dose-dependent induction of apoptosis by PLAB. Cells were incubated with increasing concentrations of PLAB for 24 h. % apoptotic cells, percentage of cells with sub-diploid DNA content as described in Materials and methods. Bars indicate, mean ± standard deviation of three experiments performed in triplicate. $^{**}p < 0.01$ (*t*-test).

24 h. When the cells were treated with PLAB concentrations higher than 1.0 μmol/L, the dead cells increased, so the apoptotic cells decreased relatively.

Finally, agarose gel electrophoresis showed a typical DNA fragmentation pattern and confirmed the apoptosis induced by PLAB (figure 8). DNA fragmentation caused by PLAB was dose-dependent; the intensity of DNA fragments increased as an increasing amount of PLAB (0.25–1.0 μmol/L) was added to the cells. As a positive control, cells were treated with camptothecin (CAM) (1 μmol/L, 24 h).

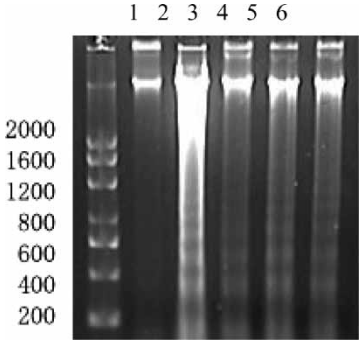

Figure 8. PLAB-induced DNA fragmentation in HeLa cells. DNA was separated and analysed on agarose gel as described in Materials and methods. Lane 1, DNA size marker; HeLa cells untreated (lane 2) and treated with 0.25, 0.5, 1.0 μmol/L (lanes 2, 3, 4) PLAB for 24 h. Demonstration of apoptosis by gel electrophoresis. DNA extracted from HeLa cells untreated (control) or exposed to increasing concentrations of PLAB (0. 25–1.0 μmol/L) for 24 h was separated by agarose gel (1.8%) and stained with ethidium bromide. As a positive control, cells were exposed to Camptothecin (CAM) (1.0 μmol/L, 24 h).

2.5 PLAB regulates expression of P53 and Bcl-2

Expression of P53 and Bcl-2 in HeLa cells exposed to PLAB was investigated by Western blot. Data showed that P53 protein levels increased for 24 h in culture in the presence of PLAB (figure 9A,C), while a significant reduction of Bcl-2 expression in the HeLa cells was observed (figure 9B,D).

2.6 PLAB activates Caspase-3

Figure 10 showed that apoptosis induced by PLAB was associated with activation of caspase-3. Procaspase-3 levels increased significantly when HeLa cells were treated with 0.125–1.0 μmol/L PLAB for 24 h. P17 protein was observable at 0.5 and 1.0 μmol/L.

In conclusion, MTT assay indicated that PLAB exhibits marked anti-tumour activity (table 1), but PLAB also had lower cytotoxic effects on a human kidney proximal tubular epithelial cell (HKC). These results showed that PLAB did not show obvious selectivity. Furthermore, the cell growth curve and colony formation of cancer cells matched with MTT. Its concentration and time-effect relationships were also significant. Previous studies had demonstrated that PLAB could be a cytotoxic against other cancer cells [5,6]. This suggests that the growth inhibition effect of PLAB had no specificity. Effects of PLAB *in vivo* show that daily injection of PLAB at less than toxic doses reduced the tumour weights (figure 4). Of course, these preliminary results demonstrated the inhibition of tumour growth by PLAB, which should be further demonstrated in the nude mice model.

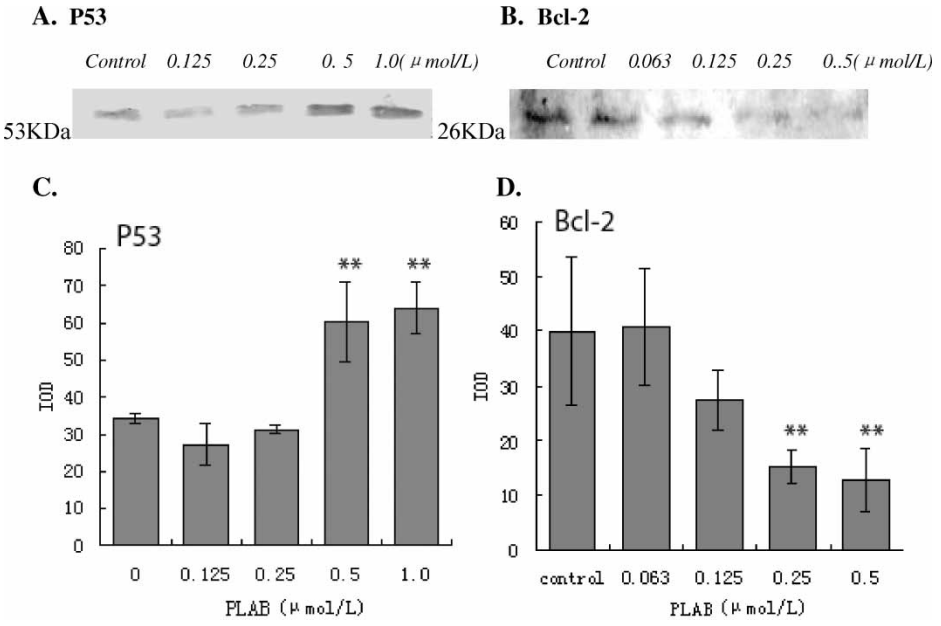

Figure 9. Effect of PLAB on P53 and Bcl-2 protein expression in HeLa cells continuously exposed to PLAB (0.125–1.0 μmol/L); protein expression determined by Western blot analysis as described in Materials and methods. (A, B) Western blot analysis of P53 and Bcl-2. Blots were hybridised with antibodies recognising P53 and Bcl-2. (C, D) Densitometric intensity of absorbance (IOD) of two proteins. Data are expressed mean ± S.D. The data were selected from three similar experiments. $^{**}p < 0.01$.

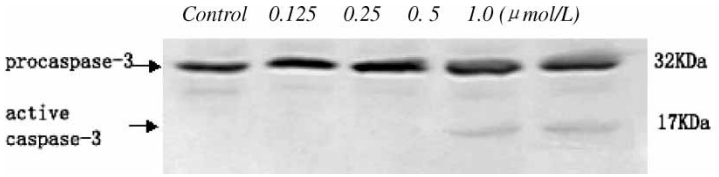

Figure 10. PLAB-activated caspase-3 in HeLa cells. Immunoblotting with antibody recognising the large subunit of procaspase 3; the appearance of the active caspase-3 in 0.5 μmol/L and 1.0 μmol/L PLAB-treated cells was apparent.

Increasing evidence showed that anti-proliferative compounds blocked the cell cycle progression by stimulating a series of checkpoints what control cell cycle transition [8]. Perturbations in cell cycle progression may account for the anti-carcinogenic effects. It has been proposed that G_2-M-phase arrest caused by DNA-damaging agents or stress stimuli could provide an opportunity for DNA repair. In the absence of such a mechanism, the mitotic cells will be premature and will result in a lethal outcome [9]. Exposure of HeLa cells to PLAB at low concentrations resulted in cell cycle arrest at G_2-M phase on concentration dependence and inducing apoptosis. The molecular mechanism related to cell cycle checkpoint, including the cytoskeleton, will be one of our research focuses in the near future.

Apoptosis is a form of physiological cell death, characterised by chromatin condensation, cytoplasmatic blebbing and DNA fragmentation [10]. Apoptosis is controlled by a complex interplay between regulatory proteins, such as P53 and Bcl-2 proteins [11]. The p53 gene is one of the most extensively studied genes. Originally identified as an oncogene, subsequent studies confirmed it to be an important tumour suppressor gene that is highly mutated in a variety of tumours. The p53 gene acts as a transcription factor and mediates its effect by modulating the expression of its downstream target genes [12]. It plays an important role in cell cycle regulation and apoptosis [13]. P53 protein is potentially functional in cervical cancer cells [14]. HeLa cells contain inactive p53 via complexation and degradation with human papillomavirus (HPV) 16/18 E6 protein [15], so blocking E6-mediated p53 inactivation is a main therapeutic goal. It was reported that selective rescue of the p53 pathway may sensitise HPV-positive transformed cells to undergo growth arrest or apoptosis, and inhibition of E6-mediated degradation of p53 in cervical cancer frequently results in increased levels of p53 expression [16].

Bcl-2, an integral membrane oncoprotein, was the first anti-apoptosis gene product discovered [17]. Bcl-2 and related proteins form a growing family of death agonist and antagonist proteins that control apoptosis through regulating a critical step in the mammalian-programmed cell death pathway in order to modulate mitochondrial permeability and function [18]. Previous studies showed Bcl-2 protein expression to block apoptosis in HeLa cells, and bcl-2 expression occurred in the absence of chromosomal translocation or rearrangement of the bcl-2 gene [19], so decreasing Bcl-2 protein levels in HeLa cells exposed to PLAB could induce apoptosis.

Apoptosis is characterised by the activation of caspases. Among the critical cysteine protease family members, caspase-3 plays an essential role in the cleavage of diverse cellular proteins. Caspase-3 exists as a proenzyme in the cell. Upon activation by upstream stimuli, the precursor molecule is cleaved resulting in the removal of the prodomain and the reassociation of the large and small subunits to form a heterodimer [20].

Jaradat et al. reported that PLAB was equally active as an agonist for transcriptional activation of the PPAR α, γ and δisoforms [21]. Recent data showed that ligands for PPARγ exhibit growth inhibitory effects on various types of cancers by affecting differentiation and/or inducing apoptosis both *in vitro* and *in vivo*. So PLAB induced HeLa cell apoptosis by increasing the expression of P53 and decreasing Bcl-2, taking effect on poly-(ADP-ribose) polymerase (PARP), activating caspase-3, etc. The apoptosis also might be triggered by either premature or delayed cell cycle progression. However, there was a controversy over the relationship between cell cycle arrest and apoptosis caused by some chemotherapeutic compounds. It was also reported that PLAB has anti-angiogenic effects [22]. Perhaps this is also one important cause of tumour inhibition *in vivo.*

3. Materials and methods

3.1 General experimental procedures

Pseudolaric acid-B, provide by Professor Hong Chen, was a white solid with mp 145–146°C and was more >98% pure [23]. It was dissolved in dimethylsulfoxide and further diluted in cell culture medium before use. The final concentration of DMSO in the solution was <0.1%. It was stored at 4°C before use. All cells were gifts from professor Xiao Guang Chen. MTT, Proteinase K, RNase A, SDS Na, acrylamide and Hoechst 33342 were purchased from Sigma Chemical Co. (St Louis, MO, USA). RMPI 1640, trypsin, propidium iodide and Tris were products of Gibco Laboratories (Grand Island, NY, USA). Mouse anti-p53, anti-Bcl-2 monoclonal antibodies, rabbit anti-caspase-3 polyclonal antibody, alkaline phosphatase (AP)-conjugated anti-mouse and anti-rabbit antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Kunming and C57BL/6 mice were provided by the Experimental Animal Centre of the Chinese Academy Of Medical Sciences. All other chemicals and reagents were of reagent grade or better and used without further purification.

3.2 3.2 Cell culture

All human tumour cells used were grown in RPMI 1640, and the normal human kidney proximal tubular epithelial cells (HKC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and penicillin/streptomycin. A humidified incubator was set at 37°C; the air contained 5% CO_2.

3.3 MTT assay

Cells were detached by trypsinisation, seeded at $1.0–2.0 \times 10^3$ cells/well in a 96-well microtitre plate overnight, and treated with different concentrations of PLAB in RPMI 1640 with 10% foetal bovine serum. The effects on cell growth were examined by MTT assay. Finally, 50 μL of MTT solution (1 mg/mL in RPMI 1640) was added to each well and incubated at 37°C for 4 h. The MTT-formazan formed by metabolically viable cells was dissolved in 150 μL of DMSO, and monitored by a microplate reader at dual-wavelength of 570 nm, 450 nm; IC_{50} was defined as the drug concentrations that inhibited the cell number to

50% after 96 h. Each experiment was repeated at least three times and the combined data were compared using Student's paired *t*-test.

3.4 Clonogenic survival assay

Cells were seeded into a 6-well plate at a density to produce approximately 500 colonies per dish in the controls and were incubated for 10 days. Colonies were fixed with 75% ethanol and 25% acetic acid, and stained with trypan blue. The number of colonies consisting of more than 50 cells was counted.

3.5 Doubling time assay

Control HeLa Cells and cells treated with PLAB were seeded into each well of a 6-well plate (2.5×10^3 cells/well) and incubated until the control cell population began to decrease. Cells were harvested and counted on a Coulter counter. The doubling time was calculated from the total incubation time divided by the number of times that the cells replicated.

3.6 Assessment of apoptosis by Hoechst staining

To analyse chromatin condensation, which is a sign of apoptosis, 1×10^4 cells were added to each well of the 24-well plates. Cells were exposed to PLAB (0.25–4 mol/L) for 24 h, and Hoechst staining was performed. Hoechst 33342 (Sigma Chemical Co.) was diluted in distilled water (10 mg/mL) and stored as a stock solution at 4°C before use; for use, the stock solution was diluted in PBS to a final concentration of 5–10 μg/mL. At first 4% formaldehyde solution was added to each well in which cells had been exposed to PLAB. The cells were then incubated with the dilute Hoechst 33342 solution for 10 min at room temperature. Finally, each well was examined under a fluorescence microscope equipped with a DM455 filter for chromatin condensation.

3.7 Assessment of apoptosis by flow cytometry

Trypsinised cells were washed with PBS and then fixed with 70% ethanol. The fixed cells were kept at least overnight at − 20°C. The cells were washed with PBS before analysis, then the fluorochrome solution (50 μg/mL propidium iodide in PBS, plus RNase, 50 μg/mL) was added. Distribution of the cell cycle phases was determined by analytical flow cytometry using a Coulter Epics XL (Coultronics, France SA) with an excitation/emission of 488/525 nm. All experiments were performed three times.

3.8 Assessment of apoptosis by DNA fragmentation

Cells incubated with PLAB (0.25–1.0 μmol/L) for 24 h were collected and washed two times by PBS, then resuspended in 0.5 mL of extraction buffer (100 mmol/L NaCl, 10 mmol/L Tris–HCl pH 8.0, 25 mmol/L EDTA pH 8.0, 0.1 mg/mL Proteinase K) at 50°C for 12 h. DNA was extracted with an equal volume of phenol saturated/chloroform/isoamyl alcohol (25:24:1) and extracted again with a combination of chloroform/isoamyl alcohol (24:1), and centrifuged at 12,000 × *g* for 30 min. Precipitated DNA was analysed on a 2.0% agarose gel.

3.9 Western blot analysis

Protein levels of Bcl-2, P53 and caspase-3 were determined by Western blotting using standard protocols. In brief, cells were washed with cold PBS, harvested using a cell scraper, and lysed in buffer (0.15 mmol/L NaCl, 0.5 mmol/L EDTA, 1% Tritonx-100, 10 mmol/L Tris–HCl) for 30 min on ice. Cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C and the protein content was determined by the G250 method. Lysates containing equal protein were added in equal volume of 2 × reducing sample buffer (100 μmol/L Tris–HCl pH 6.8, 200 μmol/L dithiothreitol, 4% SDS, 20% glycerol, 0.2% bromophenol blue). Samples were electrophoresed on (10–15%) polyacrylamide gels according to the molecular weight of protein under constant voltage (80–120 V), then electrotransferred to Protan nitrocellulose membrane (Neuro Probe Co.). The membranes were blocked with 5% non-fat milk in TTBS (100 mmol/L Tris–HCl pH 7.5/0.9% NaCl/0.1% Tween-20) at 4°C overnight, incubated with primary antibody (anti-Bcl-2 antibody or anti-P53 antibody; anti-caspase-3 antibody), 1:500 dilution, at room temperature for 2–3 h, washed three times with TTBS and incubated (1–2 h) with appropriate secondary antibody alkaline phosphatase (AP)-conjugated anti-mouse or anti-rabbit antibody (1:500 dilution). The membranes were washed three times with TTBS. The band intensity was determined from scanned images using Gel-Pro Analyser 3.1 Automated Digitising System software. Each experiment was repeated at least three times and the combined data were compared by Student's paired *t*-test.

3.10 Assessment of PLAB anti-tumour activity in vivo

Both male and female mice were used, weighing between 18 and 22 g. Mouse Lewis lung cancer cells and mouse hepatocarcinoma 22 cells (H22), 1×10^6 cells in 0.2 mL of PBS, were then injected under aseptic conditions into C57BL/6 mice and Kunming, respectively. After 1 day, tumour-bearing mice were randomly divided into groups for treatment with PLAB: groups of untreated controls; groups of mice given i.p. injections of PLAB (30 mg/kg or 60 mg/kg in a total volume of 0.4 mL/injection) every day; and positive control Kunming mice given i.p. injections of cyclophosphamide (60 mg/kg in a total volume of 0.4 mL/injection) once. The resulting tumour weight was measured. Results shown are the mean ± S.E.M., and each group contained 8–10 animals.

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