

Full Paper

Bcl-2 Family Proteins Were Involved in Pseudolaric Acid B–Induced Autophagy in Murine Fibrosarcoma L929 CellsJinghua Yu¹, Xiangru Li¹, Shin-ichi Tashiro², Satoshi Onodera², and Takashi Ikejima^{1,*}¹China-Japan Research Institute of Medical Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, P.R. China²Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194-8543, Japan

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Abstract. Pseudolaric acid B (PAB) exerted cytostatic activity on murine fibrosarcoma L929 cells. The cytostatic mechanism of PAB on L929 cells was investigated in this paper. At 36 h, after 80 μ M PAB treatment, the inhibitory ratio was $65.37 \pm 4.12\%$, and the MDC staining ratio was strongest in L929 cells. 3-Methyladenine (3-MA), an inhibitor of autophagy, inhibited the generation of autolysosomes induced by PAB. The expression of autophagy-associated Beclin 1 protein was up-regulated, and microtubule-associated protein light chain 3 I (LC3 I) was cleaved into LC3 II after 80 μ M PAB treatment from 12 h. Therefore, it was concluded that PAB exerted a cytostatic effect on L929 cells through autophagy. However, 80 μ M PAB treatment did not induce apoptotic body formation, but 3 mM 3-MA promoted apoptosis, so autophagy might inhibit apoptosis. PAB had no effect on mitochondrial membrane potential (MMP), but up-regulated the expressions of Bax and Bcl-2. Immunoprecipitation analysis showed that PAB inhibited Bcl-2 binding with Beclin 1. Additionally, PAB inhibited the localization of Bax in mitochondria, but Bcl-2 still was in the mitochondria to sustain MMP. Meanwhile PAB promoted the phosphorylation of cytoplasmic Bcl-2. Therefore the phosphorylation of Bcl-2 in the cytoplasm and the mitochondrial location of Bcl-2 might be the reasons why PAB inhibited the binding of Bcl-2 and Beclin 1.

Keywords: autophagy, L929 cell, pseudolaric acid B (PAB), Bcl-2 family protein

Introduction

Pseudolaric acid B (PAB) (Fig. 1) is a diterpene acid isolated from the root and trunk bark of *Pseudolarix kaempferi* GORDON (*Pinaceae*), known as “*Tu-Jin-Pi*” in Chinese, which has been used to treat dermatological fungi infections (1). PAB exerts potent cytotoxic activity in vitro (2, 3) and has a cell cycle arrest effect (4–6). However, its effect in murine fibrosarcoma L929 cells is still not clear.

Apoptosis and autophagy are two types of programmed cell deaths: apoptosis is called type I programmed cell death and autophagy is type II (7). Apoptosis is characterized by a series of typical morphologic events such as cell shrinkage, DNA fragmentation,

finally fragmentation into membrane-bound apoptotic bodies, and rapid phagocytosis by neighboring cells (8). Differing from apoptosis, autophagic cell death exhibits the appearance of vacuoles that engulf bulk cytoplasm and cytosolic organelles such as mitochondria and endoplasmic reticulum. Then lysosomal system of the

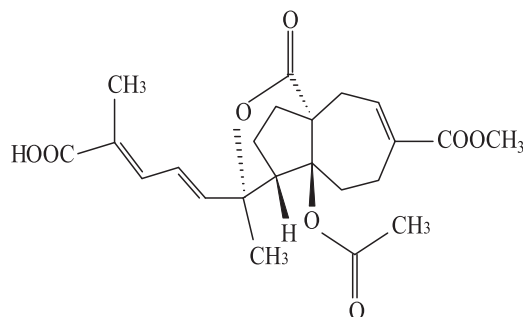


Fig. 1. Chemical structure of PAB.

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same cell dissolves the autophagic vesicles and their contents (9–13). The relationship of autophagy and apoptosis is complex and varies with cell and stress distinction. In a cell, sometimes autophagy and apoptosis happens instantaneously after stress stimuli or sometimes there is only autophagy or apoptosis (14). Class III phosphatidylinositol 3-kinase (PI3K) is important in autophagy, and it was reported that 3-methyladenine (3-MA), an inhibitor of Class III PI3K, inhibited autophagy (15). Beclin 1 functions in autophagy as part of a complex with hVps34/Class III PI3K. Generation of phosphoinositide-3-phosphate (PI3P) by the Beclin 1/hVps34 complex is thought to be important in mediating the localization of other autophagy proteins to preautophagosomal membranes (16). Bcl-2 is an anti-apoptotic protein, and it interacts with Beclin 1 and down-regulates Beclin 1-dependent autophagy through preventing Beclin 1/hVps34 complex formation (10). The Bcl-2 family includes proapoptotic proteins (e.g., Bax, Bad, and Bak) and antiapoptotic proteins (e.g., Bcl-2, Bcl-xl, and Mcl-1), and the balance between these two groups determines the fate of cells. Bax acts as a homologous (combined with Bax) or heterogenous dimer (such as Bcl-2) on the mitochondria to induce the mitochondrial permeability transition (MPT) (17–20). In addition, if Bcl-2 is phosphorylated at the mitotic phase or after microtubule disruption, they do not exert the function of combining with Bax, but phosphorylation of Bcl-2 has not been certain to induce cell death (21, 22). Therefore, the effect of Bcl-2 family proteins on autophagy was investigated in this study. Microtubule-associated protein light chain 3 (LC3), a homolog of Apg8p in yeast and a homolog of mAp8p in mouse, is essential for autophagy and associated to the autophagosome membranes after processing. Two forms of LC3, the cytosolic LC3 I and the membrane-bound LC3 II, are produced post-translationally. LC3 I is formed by the removal of the C-terminal 22 amino acids from newly synthesized LC3, followed by the conversion of a fraction of LC3 I into LC3 II (23, 24). When autophagy is induced, some LC3 I is converted into LC3 II. The amount of LC3 II or the LC3 II/LC3 I ratio correlates with the number of autophagosomes.

Therefore, in this study, the function of Bcl-2 family proteins was investigated on the PAB-induced autophagy.

Materials and Methods

Materials

PAB, which was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), was dissolved in dimethyl

sulfoxide (DMSO) to make a stock solution. DMSO concentration was kept below 0.01% in all the cell cultures and did not exert any detectable effect on cell growth or cell death. Monodansylcadaverine (MDC), rhodamine 123, 3-MA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Rabbit or mouse polyclonal antibodies against Beclin 1, Bcl-2, Bax, LC3 (LC3, which can detect the mAp8 of mouse cell), β -actin, and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit or goat-anti-mice) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A Sepharose CL-4B was purchased from Amersham Biosciences (Tokyo).

Cell culture

Murine fibrosarcoma L929 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM glutamine (Gibco, Grand Island, NY, USA), penicillin (100 U/ml), and streptomycin (100 μ g/ml) and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cell growth inhibition test

The inhibition of cell growth was determined by the cell counting test. L929 cells (1.0×10^4 cells/well) were seeded into 96-well culture plates (Nunc, Roskilde, Denmark). After 24-h incubation, various concentrations of PAB were added to the plates for 36 h. The L929 cells were trypsinized, and counted in a Blood Counting Chamber (Yancheng Cordial, Jiangsu, China).

Cell inhibitory ratio (%)

$$= [N(\text{control}) - N(\text{sample})] / N(\text{control}) \times 100$$

Flowcytometric analysis of the MDC staining

A fluorescent compound, MDC, has been proposed as a tracer for autophagic vacuoles (25). L929 cells (1×10^6 cells/bottle) were cultured in a 25-ml culture bottle overnight, then harvested with different doses of PAB for 36 h, and rinsed with PBS. Then the cells were stained with 0.05 mM MDC at 37°C for 1 h. After incubation, the cells were washed once with PBS. The samples were analyzed by a FACScan flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Lactate dehydrogenase (LDH) activity-based cytotoxicity assays

3-MA is the most commonly used inhibitor of auto-

phagic sequestration (26). L929 cells (1×10^4 cells/well) were cultured in a 96-well plate overnight and then harvested with $80 \mu\text{M}$ PAB and/or 3 mM 3-MA for 36 h. Floating dead cells were collected from culture medium by centrifugation ($240 \times g$ for 10 min at 4°C), and the LDH content from the pellets lysed in 0.1% NP-40 for 15 min was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic cell death. The adherent and viable cells were lysed in 0.1% NP-40 for 15 min to release LDH (intracellular LDH or LDHi). The substrate reaction buffer of LDH [0.5 mM L(+)-lactic acid, 0.66 mM indonitro-tetrazolium (INT), 0.28 mM phenazine methosulfate (PMS), 1.3 mM nicotinamide adenine dinucleotide (NAD^+) in pH 8.2 Tris-HCl] was added. The A value at 490 nm of reaction for 1 and 5 min were assayed.

$$\text{LDH activity} = (A_{5\text{min}} - A_{1\text{min}}) / 4$$

The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\text{apoptosis\%} = \text{LDHp} / (\text{LDHp} + \text{LDHe} + \text{LDHi}) \times 100$$

$$\text{necrosis\%} = \text{LDHe} / (\text{LDHp} + \text{LDHe} + \text{LDHi}) \times 100$$

The analysis of MDC staining

L929 cells (5×10^5 cells/well) were cultured in a 6-well plate overnight, and L929 cells were treated with $80 \mu\text{M}$ PAB and/or 3 mM 3-MA for 36 h and then incubated with 0.05 mM MDC at 37°C for 1 h. After incubation, the cells were washed once with PBS. Intracellular MDC was measured by fluorescence microscopy at excitation wave length of 380 nm with emission filter of 525 nm (Leica, Nusslich, Germany).

Rhodamine 123 analysis of the mitochondrial membrane potential (MMP) changes by fluorescence microscopy

The rhodamine 123 staining intensity is intensified with the increased MMP. L929 cells (5×10^5 cells/well) were cultured in a 6-well plate overnight, L929 cells were treated with $80 \mu\text{M}$ PAB for 0, 12, 36, and 48 h and then incubated with 5 mg/l rhodamine 123 at 37°C for 30 min. After incubation, the cells were washed once with PBS. The intensity of rhodamine 123 staining was measured by fluorescence microscopy at an excitation wave length of 505 nm with emission filter of 534 nm (Leica).

Flowcytometric analysis by the rhodamine 123 staining

L929 cells (1×10^6 cells/bottle) were overnight cultured in 25-ml culture bottles. L929 cells were harvested and rinsed with PBS. Then the cells were stained with 5 mg/l rhodamine 123 at 37°C for 30 min. After incubation, the cells were washed once with PBS. The samples were analyzed by flowcytometry.

Immunoprecipitation analysis of Bcl-2 protein complexes

Pretreatment of all the materials were performed at 4°C and all IP steps were done in Ependorf tubes on ice unless noted otherwise. After L929 cells (1×10^6 cells/bottle) were cultured in 25-ml culture bottle overnight, PAB treatment was performed for 36 h. The cells were washed twice with PBS in a culture dish and dried on filter paper. A 0.5-ml aliquot of RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM sodium fluoride (NaF), $1 \mu\text{g/ml}$ aprotinin, $1 \mu\text{g/ml}$ leupeptin, and $1 \mu\text{g/ml}$ pepstatin) was added to the culture dish, and then the cells were scraped and put together in an Ependorf tube. After lysis for 30 min and centrifugation at $14,000 \times g$ for 15 min, the supernants were collected. A $25\text{-}\mu\text{l}$ aliquot of Protein A Sepharose beads was added (50% in RIPA buffer) to immunoprecipitate the non-special antibody. The total proteins were quantitated by Bradford assay. A $3\text{-}\mu\text{l}$ aliquot of Bcl-2 antibody was added and incubated overnight; then another $25\text{-}\mu\text{l}$ Protein A Sepharose beads was added, followed by incubation for 2 h. The samples were centrifuged at $14,000 \times g$ at 4°C for 3 min and the precipitates were collected. The precipitates were washed three times with RIPA buffer, $2 \times$ SDS buffer was added, the samples were boiled for 5 min, and then the supernants were collected. The expression of Beclin1 was detected by Western blotting.

Western blot analysis of protein expression

L929 cells (1×10^6 cells/bottle) were cultured in a 25-ml culture bottle overnight, and the cells were treated with $80 \mu\text{M}$ PAB for the indicated time. Both adherent and floating cells were collected and frozen at -80°C . Western blot analysis was performed for total proteins as follows: Briefly, the cell pellets were resuspended in lysis buffer containing 50 mM Hepes (pH 7.4), 1% Triton-X 100, 2 mM Na_3VO_4 , 100 mM NaF, 1 mM edetic acid, 1 mM egtazic acid (EGTA), 1 mM PMSF, 0.1 g/l aprotinin, 0.01 g/l leupeptin, and then lysed in 4°C for 1 h. After $13,000 \times g$ centrifugation for 10 min, the protein content of the supernatant was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Protein was loaded in each lane, separated by 12% SDS polyacrylamide gel electrophoresis, and then blotted onto a nitrocellulose membrane. Protein expression was detected using primary polyclonal antibody (1:400) and secondary polyclonal antibody (1:500) conjugated with peroxidase. The extraction of cytoplasmic and mitochondrial proteins are described as follows: the cell pellets were resuspended in $100 \mu\text{l}$

lysis buffer (pH 7.5) containing 250 nM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin and then lysed at 4°C on ice for 1 h. After 12,000 × *g* centrifugation for 30 min, the supernatant containing the cytoplasm proteins was collected. The pellet was resuspended in 30 µl lysis buffer that contained 50 mM Hepes (pH 7.4), 1% Triton-X100, 100 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM PMSF, 10 mg/l leupeptin, 10 mg/l aprotinin, 10 mg/l pepstatin A, and lysed at 4°C on ice for 1 h. After 12,000 × *g* centrifugation for 30 min, the supernatant containing the mitochondrial proteins was collected. Protein levels were evaluated by densitometry (Fluochim v2.0 Alpha; Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± S.D. *P*-values of less than 0.05 were considered to represent a statistically significant difference.

Results

PAB inhibited L929 cell growth and increased the positive ratio of MDC staining

To detect the growth inhibition of PAB-exposed L929 cells, the cells were treated with various doses of PAB ranging from 0 to 160 µM for 36 h. At 36 h, the inhibitory ratio of 80 µM PAB was 65.37 ± 4.12%, and the inhibitory effect was increased in a dose-dependent manner (Fig. 2A).

Flow-cytometric analysis indicated that MDC-labeled cells increased in a dose-dependent manner at concentrations up to 80 µM at 36 h. When the PAB concentration was 80 µM, the MDC staining positive ratio was the highest, and 160 µM PAB induced similar but slightly reduced staining (Fig. 2B). Therefore, 80 µM PAB was used to explore the mechanism of autophagy induced by PAB in L929 cells.

PAB did not induce apoptosis in L929 cells, but 3-MA promoted apoptosis

The amount of LDH released from viable cells and floating dead cells and in the culture medium were used to determine the proportion of apoptotic and necrotic cells (27). At 36 h, compared with the control, there were no obvious necrotic and apoptotic cells by 80 µM PAB treatment, but with addition of 3-MA, autophagy inhibitor, PAB increased the apoptotic ratio from 6.13 ± 3.67% to 36.69 ± 6.12% compared to treatment with PAB alone (Fig. 3).

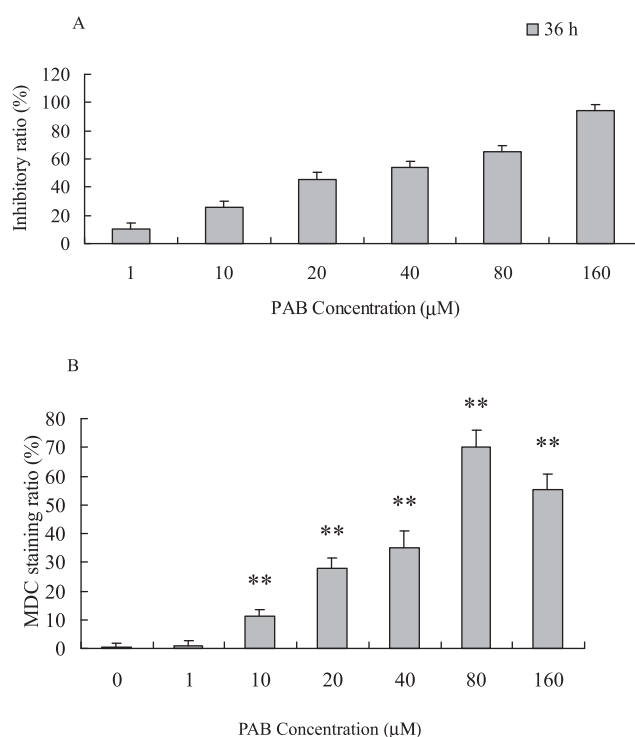


Fig. 2. PAB inhibited cell growth and increased the positive ratio of MDC staining in L929 cells. L929 cells (1.0×10^4 cells/well) were seeded into 96-well culture plates overnight. A: The cells were incubated with various concentrations of PAB for 36 h. PAB inhibited the cell growth of L929 cells in a dose-dependent manner. Growth inhibition was evaluated by the cell counting test. Mean ± S.D., *n* = 3. B: At 36 h, 80 µM PAB displayed the most potent positive ratio of MDC staining in L929 cells. Mean ± S.D., *n* = 5, ***P* < 0.01 vs control.

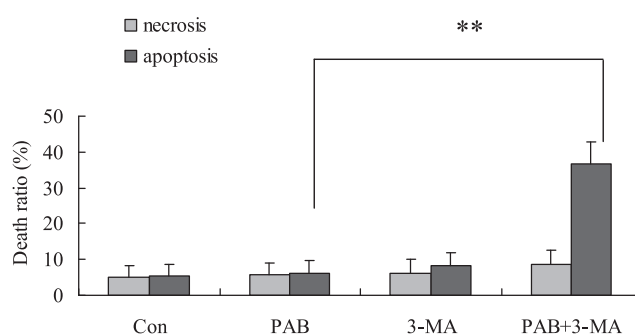


Fig. 3. PAB was not involved in apoptosis, but in the presence of 3-MA, PAB promoted apoptosis. L929 cells (1.0×10^4 cells/well) were seeded into 96-well culture plates overnight. After 80 µM PAB and/or 3 mM 3-MA treatment, LDH analysis was done to determine the percentage of apoptosis and necrosis. Mean ± S.D., *n* = 5, ***P* < 0.01 vs PAB group.

PAB enhanced the MDC staining ratio in L929 cells, and 3-MA inhibited the enhancement

In this study, at 36 h compared with the control (Fig. 4Aa), the bright green dots indicating auto-

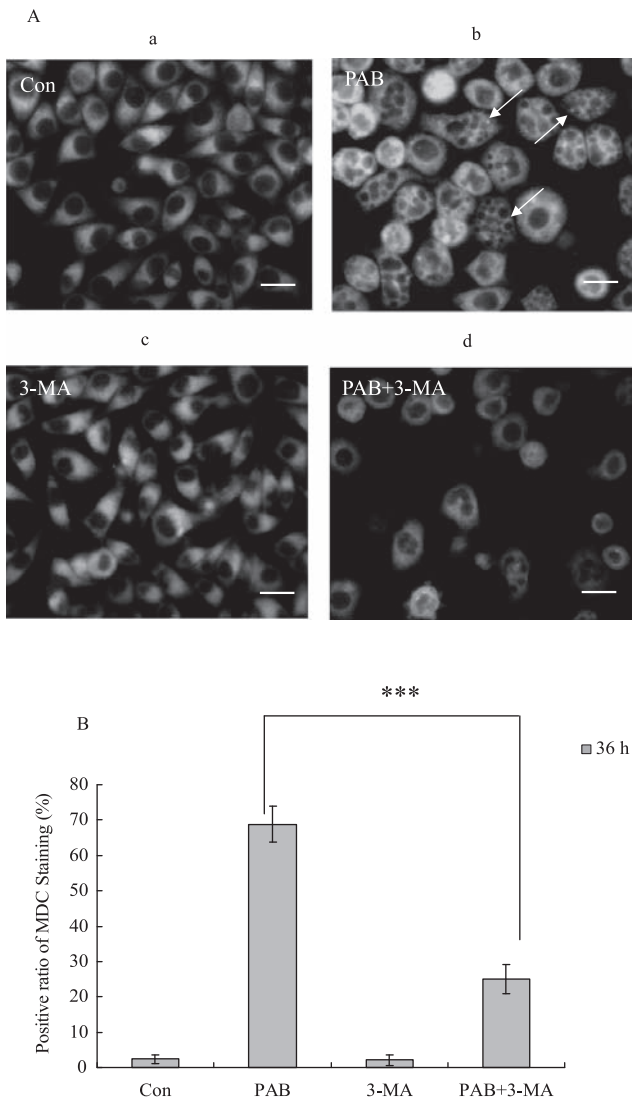


Fig. 4. Upon addition of 3-MA, PAB did not increase the positive ratio of MDC staining. MDC staining of autophagic vacuoles in L929 cells treated with 80 μ M PAB or 3 mM 3-MA for 36 h. A: Fluorescence microscopy showed that PAB increased the number of dots, showing the position of autophasosomes, in the cytoplasm of the L929 cells. However, 3 mM 3-MA inhibited the appearance of these dots and promoted apoptosis of L929 cells. Arrows indicate the positive MDC staining. Bar = 30 μ m. Triplicate experiments were done. B: Quantitative analysis detected a positive ratio of MDC staining by flowcytometric analysis. Mean \pm S.D., n = 5, ***P < 0.001 vs PAB group.

phagosomes were increased in the cytoplasm, and the larger L929 cells appeared after 80 μ M PAB treatment (Fig. 4Ab). Cells treated with 3 mM 3-MA by itself were similar to the control cells (Fig. 4Ac), but in the presence of 3-MA, PAB decreased the number of these dots and promoted apoptosis (Fig. 3Ad). Consistent with the results of Fig. 3Ab, flowcytometric analysis showed that 3-MA decreased the MDC staining ratio induced by PAB from $68.83 \pm 5.11\%$ to $25.12 \pm 4.13\%$ (Fig. 4B).

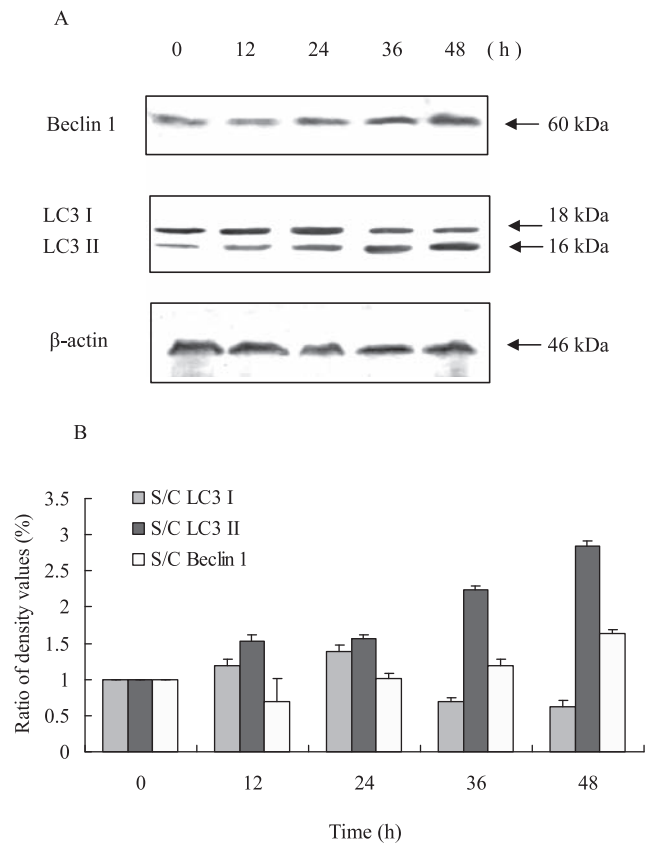


Fig. 5. The expression of Beclin 1 and LC3 in 80 μ M PAB-treated L929 cells at different time points. A: By Western blot analysis. B: The densitometry was applied to quality the protein density of the Western blot. S/C: (band density of the sample after PAB treatment for different time) / (band density of control). Mean \pm S.D., n = 3.

The effect of PAB on the expression of Beclin 1 and LC3

Beclin 1 complemented the defect in autophagy in *apg6*-defective yeast strains and induced autophagy when over-expressed in mammalian cells (28). After 80 μ M PAB treatment for 12 h, the expression of autophagy-associated Beclin 1 protein was up-regulated and then sustained at a stable level.

During the formation of mammalian autophagosomes, two ubiquitylation-like modifications are required, Atg12-conjugation and LC3-modification. LC3 is an autophagosomal ortholog of yeast Atg8. A lipidated form of LC3 II has been shown to be an autophagosomal marker in mammals, and LC3 I was cleaved into LC3 II from 12 h, which was the marker of autophagy (Fig. 5). Considering the results from Fig. 3, it was concluded that PAB induced autophagy in L929 cells.

PAB had no effect on MMP

Rhodamine 123 staining indicates the intensity of MMP. The stable intensity of rhodamine 123 staining was observed by fluorescence microscopy in the 80 μ M

PAB-treated L929 at 0, 12, 36, and 48 h. Like Fig. 4Ab, L929 cells became large after 80 μ M PAB treatment (Fig. 6A). In Consistent with the observations by fluorescence microscopy, flowcytometric analysis indicated that PAB had no effect on MMP (Fig. 6B).

Up-regulated Bcl-2 and Bax expression induced by PAB

Anti-apoptotic protein Bcl-2 sustains stable MMP (17–20). After 12 h of PAB treatment, the expression of Bcl-2 was up-regulated (Fig. 7). Therefore, Bcl-2 might stabilize MMP after PAB treatment in L929 cells. Pro-apoptotic protein, Bax, is supposed to decrease the MMP. However, unexpectedly Bax expression was also up-regulated after 12-h PAB treatment, and it was probable that Bcl-2 might bind with Bax or Bax existed outside of mitochondria (Fig. 7).

PAB inhibited the binding of Bcl-2 with Beclin 1

At 36 h, Bcl-2 was precipitated to identify the protein binding to Bcl-2. From immunoprecipitation analysis, PAB inhibited the binding of Beclin 1 protein with Bcl-2 (Fig. 8).

PAB promoted the recruitment of Bcl-2 on mitochondria and inhibited Bax on mitochondria

To detect the distribution of Bax and Bcl-2 on mitochondria, the proteins of mitochondria and cytoplasm were extracted. Bcl-2 was found to be mainly in mitochondria and Bax was in the cytoplasm, which accounted for the sustained MMP. However, cytoplasmic Bcl-2 was phosphorylated, and the Bcl-2 phosphorylation in the cytoplasm time-dependently increased (Fig. 9).

Discussion

PAB obviously inhibited the L929 cell growth in a dose-dependent manner at 36 h, and the inhibitory ratio of 80 μ M PAB was $65.37 \pm 4.12\%$. PAB at 80 μ M had the strongest positive ratio of MDC staining, which is one indicator of autophagy; consequently, 80 μ M PAB was applied to explore the mechanism of autophagy. Surprisingly 80 μ M PAB did not promote apoptosis in L929 cells and exerted only a cytostatic effect, suggesting that autophagy inhibited apoptosis and might inhibit the L929 cell growth. MDC staining is one of the autophagic markers, so the other indicators were used to further prove actual PAB-induced autophagy. 3-MA, a specific inhibitor of autophagy, decreased the positive ratio of MDC staining from $68.83 \pm 5.11\%$ to $25.12 \pm 4.13\%$ in cells treated with PAB. Meanwhile PAB up-regulated the expression of the autophagy-relative protein Beclin 1 and promoted the conversion

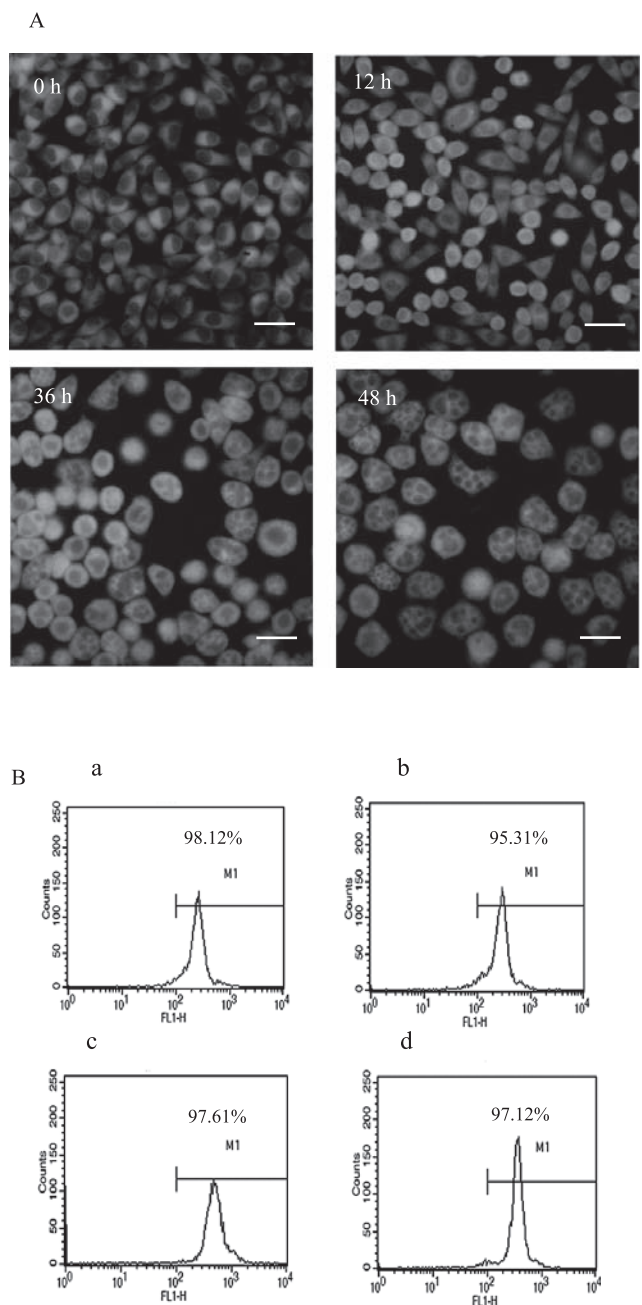


Fig. 6. Changes of mitochondrial membrane potential (MMP) at different time points (0, 12, 36, and 48 h) in L929 cells treated with 80 μ M PAB. A: under fluorescence microscopy, the intensity of Rodamine staining remained at a stable level at different time points. Bar = 30 μ m. Triplicate experiments were done. B: Flowcytometric analysis of MMP of L929 cells treated with 80 μ M PAB at different time points, a: 0 h, b: 12 h, c: 36 h, d: 48 h.

of LC3 from LC3 I to LC3 II. Therefore, it was concluded that PAB induced autophagy in L929 cells. In addition, it was curious that 3-MA promoted the apoptosis of PAB-treated L929 cells. Autophagy inhibited apoptosis and 3-MA abolished autophagy in L929 cells induced by PAB, thereby promoting

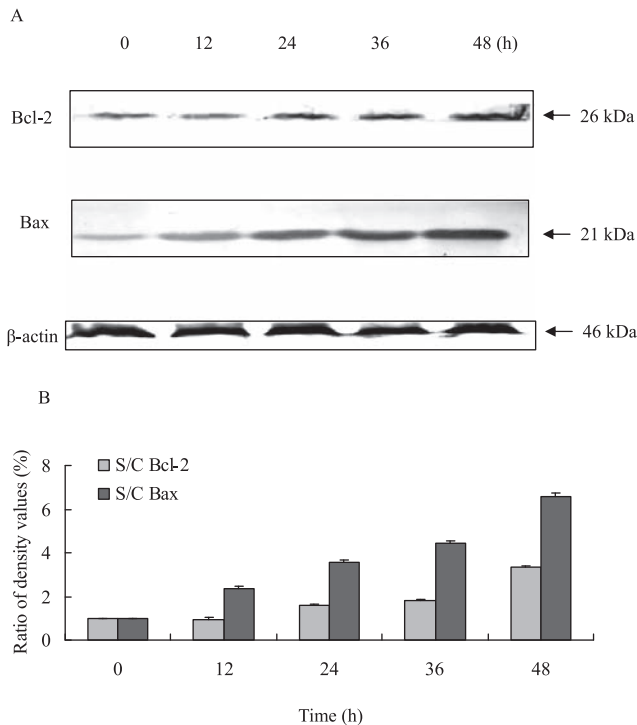


Fig. 7. PAB up-regulated the expression of Bcl-2 and Bax. A: Western blot analysis of the expression of Bcl-2 and Bax in 80 μ M PAB-treated L929 cells at 0, 12, 24, 36, and 48 h by Western blot analysis. B: The densitometry was applied to quantify the protein density of the Western blot. S/C: (band density of the sample after PAB treatment for different time)/(band density of control). Mean \pm S.D., $n = 3$.

apoptosis; and 3-MA together with PAB activated apoptotic pathways. The detailed mechanism for the conversion from autophagy to apoptosis by 3-MA in PAB-treated L929 cells will be investigated in the future.

In this study, the MMP was stable, but the expression of Bax was up-regulated, indicating that there might be some substances that combined with Bax to inhibit the decrease of MMP. After PAB treatment, the expression of Bcl-2 was also up-regulated; therefore, it was possible that Bcl-2 was combined with Bax to keep the MMP stable. It was interesting that most of the Bcl-2 was found in the mitochondrial, but the cytoplasm contained most of the Bax. Therefore the stable MMP was due to the transportational inhibition of Bax from the cytoplasm to mitochondria, and mitochondrial Bcl-2 was bound to mitochondrial Bax. PAB up-regulated the expression of Bcl-2 in mitochondria, and cytoplasmic Bcl-2 was not obviously up-regulated, but they were markedly phosphorylated by PAB. When cells enter the mitotic phase, Bcl-2 is phosphorylated, and if microtubule disruption occurs, Bcl-2 may be also phosphorylated in malignant cells (21, 22). Therefore, PAB might induce

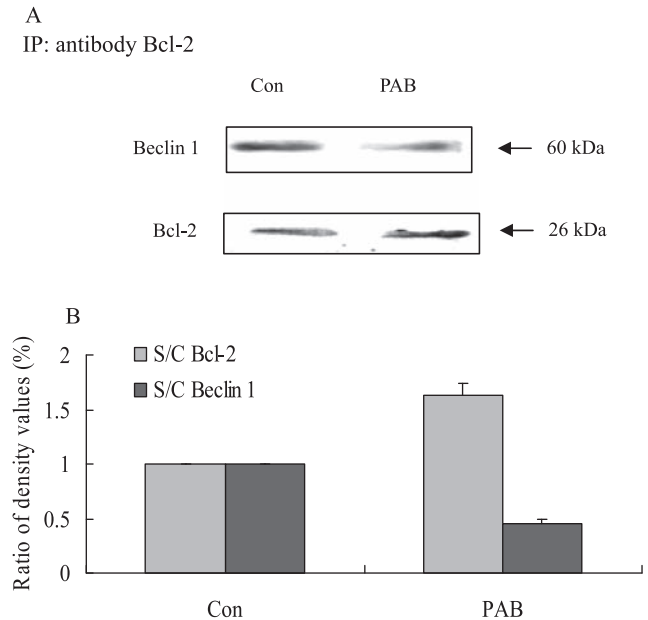


Fig. 8. PAB inhibited the binding of Beclin 1 with Bcl-2. A: The complex of Beclin 1 and Bcl-2 was coprecipitated with Bcl-2 antibody at 36 h in 80 μ M PAB-treated L929 cells by immunoprecipitation analysis. B: Densitometry was applied to quantify the protein density of the Western blot. S/C: (band density of the sample after PAB treatment for different time)/(band density of control). Mean \pm S.D., $n = 3$.

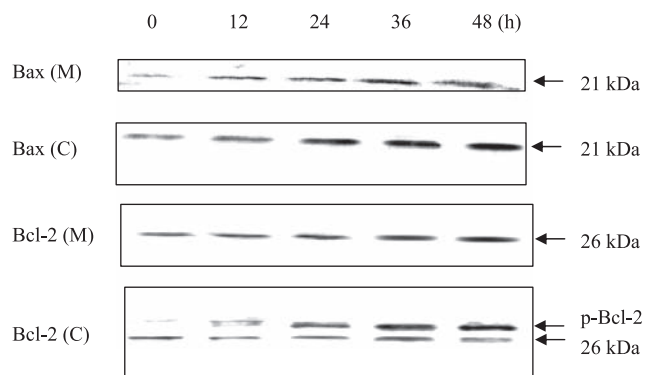


Fig. 9. The distribution of Bcl-2 and Bax on mitochondria. PAB promoted Bcl-2 in mitochondria, but inhibited Bax in mitochondria. PAB promoted the phosphorylation of Bcl-2 in mitochondria. The cell lysates were separated by 12% SDS-PAGE, and the protein expression was detected by Western blot analysis. Triplicate experiments were done.

mitotic arrest and microtubule disruption in L929 cells. Actually PAB induced mitotic arrest in human breast cancer MCF-7 cells (3) and microtubule inhibition in other cells (6). Meanwhile, it was observed that L929 cells became larger after PAB treatment than in the medium control, which might be related to mitotic arrest and microtubule inhibition. Whether mitotic arrest and

microtubule inhibition are induced by PAB in L929 cells need to be proved in the future.

Beclin 1 functions in autophagy as part of a complex with hVps34/Class III PI3K. Generation of PI3P by the Beclin 1/hVps34 complex is thought to be important in mediating the localization of other autophagy proteins to preautophagosomal membranes (16). Bcl-2 is an anti-apoptotic protein, and it interacts with Beclin 1 and down-regulates Beclin 1-dependent autophagy through preventing Beclin 1/hVps34 complex formation (10). To further observe the effect of Bcl-2 on autophagy, immunoprecipitation analysis was performed to observe the binding of Bcl-2 with Beclin 1. PAB reduced the binding of Beclin 1 with Bcl-2, which enabled Beclin 1 to free itself from Bcl-2. The attenuated binding of Bcl-2 with Beclin 1 was due to the location of Bcl-2 on mitochondria, and the phosphorylation of Bcl-2 in cytoplasm might be another factor. Therefore, Beclin 1, which was up-regulated and not inhibited by Bcl-2, induced the autophagy. L929 cells treated by PAB possibly switched apoptosis to autophagy through mitochondria.

Taken together, PAB induced autophagy in L929 cells, which might be attributed to reduction in the binding of Bcl-2 to Beclin 1.

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