

Original Research Article

Pumilaside A from Litchi semen induces apoptosis in human gastric cancer BGC823 cells via activation of death receptor- and mitochondria-mediated apoptotic pathways

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Sent for review: 10 February 2018

Revised accepted: 19 November 2018

Abstract

Purpose: To investigate the cytotoxic effect of pumilaside A from Litchi semen against human gastric cancer BGC823 cells, and unravel its possible mechanism(s) of action.

Methods: The cytotoxic activity of pumilaside A (5 - 40 µg/mL) against BGC823 cells was assessed by thiazolyl blue tetrazolium bromide assay. The pro-apoptotic effect of PA (10, 20 or 40 µg/mL) on BGC823 cells was monitored by flow cytometry, while the mechanisms involved were investigated using western blot.

Results: Pumilaside A significantly produced cytotoxic activity against BGC823 cells ($IC_{50} = 25.43$ µg/mL) and induced apoptosis in BGC823 cells ($p < 0.01$). Treatment with pumilaside A led to significant upregulation of pro-apoptotic factors (Fas, FasL, FADD, Bax, Apaf-1, and c-caspase -8, 9 and 10), and downregulation of anti-apoptotic factors (survivin and Bcl-2, $p < 0.05, 0.01$). In addition, pumilaside A increased the cytoplasmic levels of Smac and cytochrome c in BGC823 cells by enhancing their mitochondrial release, and significantly upregulated the levels of executioner c-caspases-3, 6 and 7 ($p < 0.05, 0.01$).

Conclusion: Pumilaside A shows good cytotoxic activity against BGC823 cells via a mechanism related to activation of death receptor- and mitochondria-mediated apoptotic pathways. Thus, pumilaside A has a potential for use as an anti-gastric cancer agent.

Keywords: Litchi semen, Pumilaside A, BGC823 cells, Cytotoxicity, Apoptosis

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INTRODUCTION

At present, gastric cancer is the second leading cause of cancer death, and ranks fourth in global cancer prevalence [1,2]. Surgery, interventional therapy, radiotherapy, chemotherapy and biotherapy constitute the main treatment strategies for gastric carcinoma [3-6]. It has been suggested that chemotherapy is a vital aspect of

this treatment [7]. Prolonged use of chemotherapy drugs by gastric cancer patients is necessary to enhance therapeutic outcomes. However, prolonged use of these drugs is associated with development of drug resistance by the cancer cells, leading to reduced effectiveness of chemotherapy [8]. Thus, it is crucial to evolve newer and more effective chemotherapy drugs for treating gastric cancer.

Anticancer drugs from Chinese medicine have attracted the interest of researchers in the past decades [9].

It has been reported that litchi semen, the seeds of *Litchi chinensis* Sonn. (Sapindaceae family), exerts anticancer, radical-scavenging and antioxidant activities [10-12]. Pumilaside A (PA), a sesquiterpene glucoside from litchi semen, has been shown to exert cytotoxic activity against some cancer cells, but the mechanisms of action are not yet understood [13].

The aim of the present study was to investigate the cytotoxic activity of PA against human gastric cancer BGC823 cells using thiazolyl blue tetrazolium bromide (MTT) assay. The cytotoxic mechanisms of PA against BGC823 cells were also studied by monitoring apoptosis and apoptosis-related proteins using flow cytometric and western blot procedures.

EXPERIMENTAL

Plant material

Litchi semen was obtained from Anguo Chinese herbal medicine shop in 2015 and authenticated by Jun Liu, a taxonomist in the Department of General Surgery, Taian City Central Hospital. A voucher specimen (voucher no. TACCH DP20150481) was kept in the Department of Pharmacy, Taian City Central Hospital for future reference.

Chemicals and reagents

All solvents used in this study were of analytical grade, and were purchased from Haiyang Chemical Co. (Qingdao, China). Diaion HP-20 macroporous resin was product of Mitsubishi Chemical Co., Japan. Fetal bovine serum (FBS) and RPMI-1640 medium were products of Gibco Life Technologies (Grand Island, NY, USA), while MTT and dimethyl sulfoxide (DMSO) were products of Sigma (St. Louis, MO, USA). Kits for enhanced BCA protein assay and Annexin V-FITC/PI apoptosis assay were supplied by Beyotime Biotechnology (Haimen, China) and JRDUN Biotech (Shanghai, China), respectively. Total, cytoplasmic and mitochondrial protein extraction kits were purchased from BioDee (Beijing, China), Nanjing Biobox Biotech (Nanjing, China) and Bangyi-bio (Shanghai, China), respectively. Polyvinylidene fluoride (PVDF) membrane was obtained from Roche Diagnostics (Mannheim, Germany). Primary antibodies against Fas, FasL, FADD, Bax, Bcl-2, Survivin, Apaf-1, cytochrome c, Smac, cleaved (c)-caspases-8, 10, 9, 3, 6 and 7, as well as β -

actin and COX IV were supplied by cell Signaling Technology (Beverly, MA, USA), Abcam (Cambridge, UK) or Sigma (St. Louis, MO, USA). HRP-conjugated antibody was product of Jackson Immuno Research Laboratories (West Grove, Pennsylvania, USA).

Extraction and isolation of PA

Litchi semen (10 kg) was finely ground and extracted thrice with 15 L 95 % ethanol at 30 °C for 4 days. The extract was dried using low-pressure evaporation to yield 1735 g of material, which was subsequently dissolved in water and then successfully subjected to partitioning in a solvent mixture of petroleum ether, ethyl acetate and n-butyl alcohol. The fraction from n-butyl alcohol (208 g) was applied to a column of Diaion HP-20 resin eluted with water and methanol to afford a methanol eluate (113 g). This was purified further using silica gel column chromatography eluted with chloroform: methanol at volume ratios in the range 90:10 - 60:40. Eluates from chloroform: methanol (85:15 and 80:20) were pooled and subjected to repetitive silica gel column chromatography to obtain 35 mg of PA. Thereafter, mass spectrometry, NMR and HPLC were used for structural elucidation and purity assessment of the PA.

Cell culture

BGC823 cell line was obtained from ATCC (Manassas, VA, USA). The cells were maintained at 37 °C on RPMI-1640 medium containing 100 U/mL penicillin, 100 U/mL streptomycin and 10 % FBS in a humidified incubator with 5 % CO₂.

Cell viability assay

MTT assay was carried out to assess the cytotoxic activity of PA against BGC823 cells. In this procedure, the BGC823 cells (5×10^3 cells/well) were seeded in 96-well plates for 24 h. Thereafter, the cells were exposed to varying doses of PA (0, 5, 10, 15, 20, 25, 30, 35 or 40 μ g/mL) for 48 h, following which 50 μ L of 2 mg/mL solution of MTT was introduced into each well. The plates were subjected to incubation for 4 h at 37°C, at the end of which the RPMI-1640 medium was removed, and the cells were incubated with 200 μ L DMSO in the dark for 30 min to dissolve the resultant blue-violet formazan crystals. The absorbance values of the formazan solutions were read in a Bio Rad microplate reader (Model 680, USA) at 570 nm against a blank of DMSO [14,15].

Cell apoptosis assay

The BGC823 cells (5×10^5 cells/well) were seeded in 12-well plates for 24 h and treated with PA (0, 10, 20 or 40 $\mu\text{g/mL}$) for 48 h, followed by harvesting and washing (thrice) in chilled PBS, and staining with Annexin V-FITC/PI. The stained cells were then subjected to flow cytometric analysis using FACSCalibur from BD Biosciences (San Jose, CA, USA) [16].

Western blot assay

Following treatment with PA (0, 10, 20 or 40 $\mu\text{g/mL}$) for 48 h, BGC823 cells were harvested, and their total, cytoplasmic and mitochondrial protein contents were separately extracted with the appropriate kits. The total protein was used to investigate the expressions of Fas, FasL, FADD, Bax, Bcl-2, survivin, Apaf-1, and c-caspases-8, 10, 9, 3, 6 and 7 proteins. The cytoplasmic and mitochondrial proteins were used to investigate the release of mitochondrial cytochrome c and Smac proteins into the cytoplasm compartment.

Protein concentration was determined using enhanced BCA kits in line with the kit protocol [17]. The protein (40 μg) was subjected to SDS-polyacrylamide gel electrophoresis, and the resultant bands were transferred onto PVDF membrane. Non-specific binding was blocked using 5 % non-fat milk, prior to incubation of the membrane overnight at 4 °C with the appropriate primary antibodies, and subsequently with HRP-conjugated antibody for 2 h at room temperature. Finally, the protein bands were subjected to chemiluminescence analysis. In this assay, β -actin served as endogenous control for total protein and cytoplasmic protein [18], while COX IV served as endogenous control for mitochondrial protein.

Statistical analysis

The results obtained are presented as mean \pm standard deviation (SD). Statistical differences among groups were determined by one-way analysis of variance (ANOVA). All statistical analyses were carried out using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were assumed to indicate statistical significance.

RESULTS

Purity and identification of PA

Results from mass spectrometry indicated that the molecular formula of the target analyte, an

amorphous powder, was $\text{C}_{21}\text{H}_{39}\text{O}_8$. Comparing NMR data of the target analyte with existing literature [19], it was identified as PA (Figure 1). Results from HPLC area normalization method indicated that the level of purity of PA was 98.3 %.

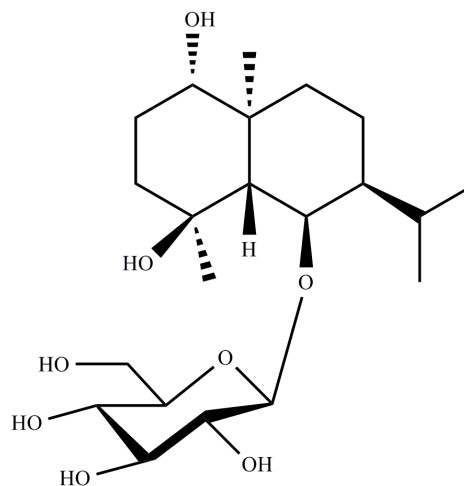


Figure 1: Chemical structure of PA

Cytotoxicity of PA against BGC823 cells

Results from MTT assay (Figure 2) indicated that PA (5, 10, 15, 20, 25, 30, 35 or 40 $\mu\text{g/mL}$) significantly reduced the viability of BGC823 cells, relative to the untreated control ($p < 0.01$). The IC_{50} value of PA was 25.43 $\mu\text{g/mL}$.

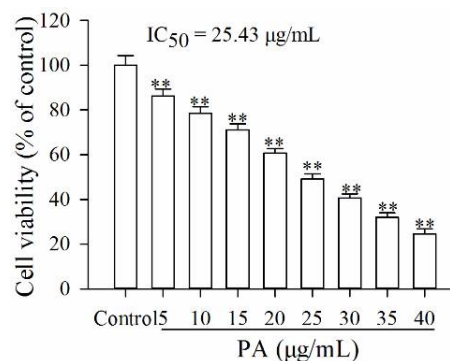


Figure 2: Cytotoxic activity of PA against BGC823 cells; ** $p < 0.01$, relative to the control

PA induced apoptosis in BGC823 cells

Results from MTT assay indicated that PA exerted good cytotoxic activity against BGC823 cells. Therefore, flow cytometry analysis was carried out to see if the cytotoxicity was related to apoptosis. The results showed that PA (10, 20 or 40 $\mu\text{g/mL}$) induced significant apoptosis in BGC823 cells, relative to the control ($p < 0.01$; Figure 3). These results revealed that the cytotoxicity of PA was due to apoptosis.

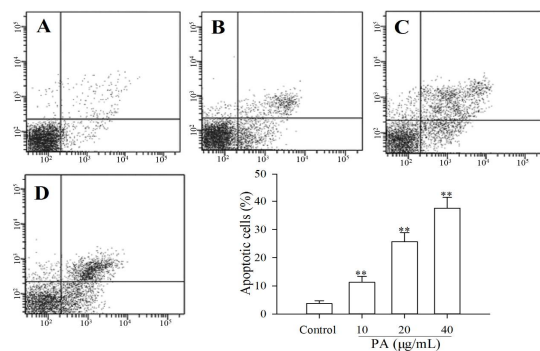


Figure 3: Apoptosis-inducing effect of PA in BGC823 cells; A is control, while B, C and D represent 10, 20 and 40 µg/mL PA, respectively; ** $p < 0.01$, relative to the control

PA upregulated expressions of pro-apoptotic proteins in the death receptor-mediated apoptotic pathway

The expressions of the pro-apoptotic proteins (Fas, FasL, FADD, c-caspase-10 and c-caspase-8) were significantly increased by PA (10, 20 or 40 µg/mL), relative to the control ($p < 0.05, 0.01$; Figure 4).

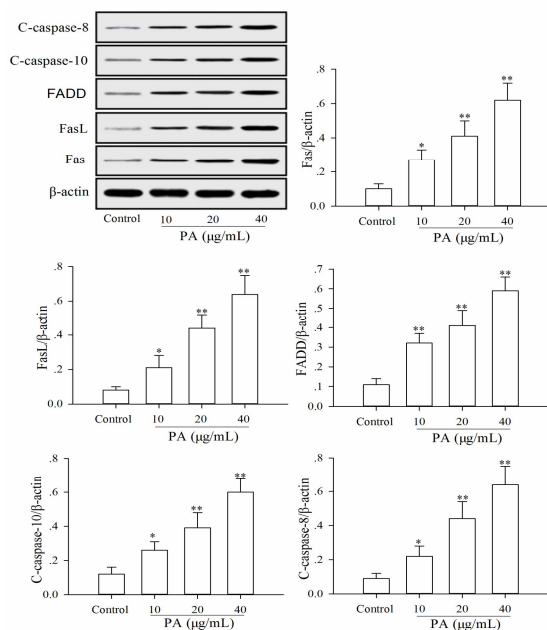


Figure 4: Up-regulatory effect of PA on Fas, FasL, FADD, c-caspase-10 and c-caspase-8; * $p < 0.05$, ** $p < 0.01$, relative to the control

PA regulated expressions of apoptosis-related proteins in the mitochondria-mediated apoptotic pathway

As shown in Figure 5 and Figure 6, relative to the unexposed control, PA (10, 20 or 40 µg/mL) significantly downregulated the anti-apoptotic

proteins (survivin and Bcl-2), and upregulated the pro-apoptotic proteins (Bax, Apaf-1 and c-caspase-9) ($p < 0.05, 0.01$). Moreover, PA significantly increased the release of pro-apoptotic proteins (Smac and cytochrome c) from mitochondria to cytoplasm ($p < 0.05, 0.01$).

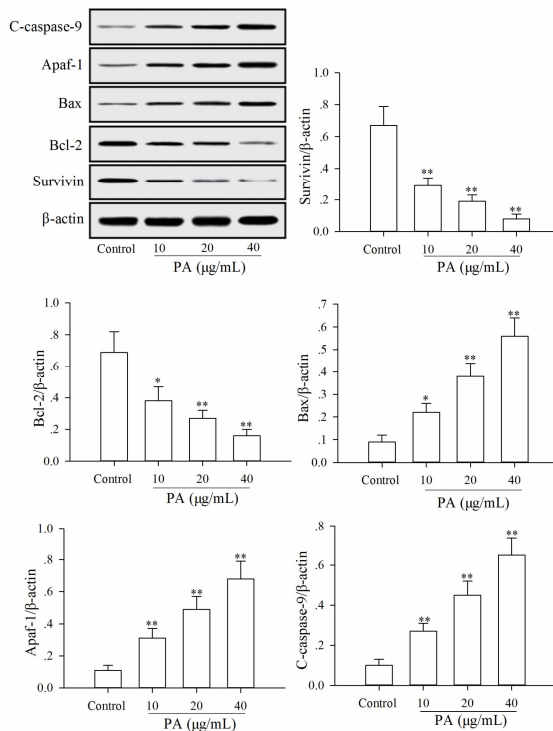


Figure 5: Regulatory effect of PA on survivin, Bcl-2, Bax, Apaf-1 and c-caspase-9; * $p < 0.05$, ** $p < 0.01$, relative to the control

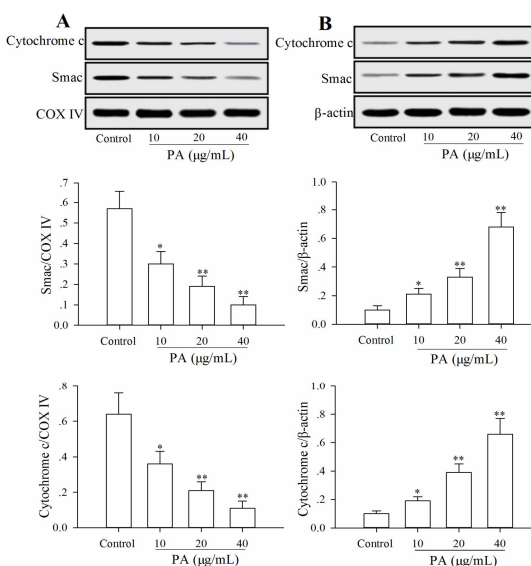


Figure 6: Positive effect of PA on release of Smac and cytochrome c from mitochondria to cytoplasm; A and B represent amounts of Smac and cytochrome c in mitochondria and cytoplasm, respectively; * $p < 0.05$, ** $p < 0.01$, relative to the control

PA increased expressions of executioner caspases

The executioner c-caspases-3, 6 and 7 were significantly upregulated in BGC823 cells treated with PA (10, 20 or 40 $\mu\text{g/mL}$), relative to the control ($p < 0.05, 0.01$; Figure 7).

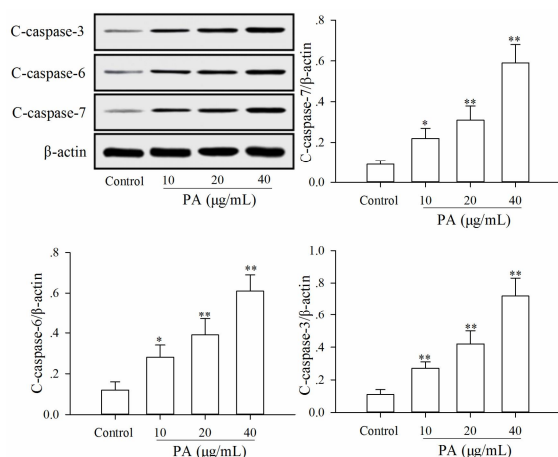


Figure 7: Up-regulatory effect of PA on executioner c-caspases-3, 6 and 7; * $p < 0.05$, ** $p < 0.01$, relative to the control

DISCUSSION

In the present study, the cytotoxic activity of PA against BGC823 cells was related to apoptosis, and the mechanisms of action were linked to activation of death receptor- and mitochondria-mediated apoptotic pathways. The MTT assay is a standard procedure for assessing the viability of cancer cells [20], while flow cytometry is used for investigating apoptosis in cancer cells [21]. The results obtained from these assay methods indicated that PA exerted good apoptosis-related cytotoxic activity against BGC823 cells.

Death receptor- and mitochondria-mediated apoptotic pathways are crucial in induction of apoptosis. Studies have suggested that c-caspases -10 and 8, as well as Fas, FasL and FADD are essential pro-apoptotic mediators in the death receptor-mediated apoptotic pathway [22,23]. Following combination of death receptors such as Fas and TNFR1 with corresponding ligands such as FasL, TNF and DR4/DR5, multimerization and conformational changes in receptors are induced [24]. The interaction of death domain in death receptor of multimerization recruits FADD, and then the interaction of death effector domain in FADD promotes the combination of FADD with caspase-8 or caspase-10 [25].

Subsequently, caspase-8 or caspase-10 as a component of the death-inducing signaling

complex (comprising death receptor, death ligand, FADD and caspase-8 or caspase-10) becomes activated to generate c-caspase-8 or c-caspase-10 [26]. Then, the executioner pro-caspases-3, 6 and 7 are activated by c-caspase-8 or c-caspase-10, resulting in executioner c-caspases-3, 6 and 7, which immediately induce cell apoptosis by acting on apoptosis-related substrates [27]. In the present investigation, PA significantly upregulated the expressions of FasL, Fas, FADD, as well as c-caspases-10, 8, 3, 6 and 7 in BGC823 cells, suggesting that the PA-associated apoptosis in BGC823 cells occurred through activation of the death receptor-mediated apoptotic pathway.

It has been established that the key proteins in mitochondria-mediated apoptotic pathway are Bcl-2, survivin, Bax, Apaf-1, cytochrome c, Smac and c-caspase-9 [28]. Cytochrome c forms apoptosome with Apaf-1, procaspase-9 and dATP, and then procaspase-9 is activated to generate c-caspase-9 [29]. Finally, the executioner pro-caspases-3, 6 and 7 undergo activation by c-caspase-9 to generate executioner c-caspases-3, 6 and 7, which immediately induce cell apoptosis by acting on apoptosis-related substrates [30].

The activation of executioner pro-caspases is suppressed by survivin, but the function of survivin is inhibited by Smac released from mitochondria [31]. However, Bcl-2 suppresses the release of cytochrome c and Smac from the mitochondria, and the function of Bcl-2 is inhibited by Bax [32]. The results obtained in the present study showed that PA significantly upregulated the expressions of Bax and Apaf-1, as well as c-caspase-9, 3, 6 and 7. PA increased the cytoplasmic levels of cytochrome c and Smac, and downregulated the expressions of survivin and Bcl-2 in BGC823 cells. These results suggest that the PA-associated apoptosis in BGC823 cells occurred through activation of the mitochondria-mediated apoptotic pathway.

CONCLUSION

The findings of this work indicate that PA exhibits good cytotoxic activity against BGC823 cells, and the mechanisms of action are related to activation of death receptor- and mitochondria-mediated apoptosis. Thus, PA has a potential for development into an anti-gastric cancer agent.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Jun Liu and Xiao Zheng conceived and designed the study, while Xiao Zheng, Jian-Wei Liang, Gang Cui and Lei Zhang performed the experiments, and collected and analyzed the data. All authors read and approved the manuscript for publication.

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