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Physalin B induces cell cycle arrest and triggers apoptosis in breast cancer cells through modulating p53-dependent apoptotic pathway

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ABSTRACT

Physalin B (PB), one of the major active steroidal constituents of Cape gooseberry (*Physalis alkekengi* L.), possesses a wide spectrum of biological activities. Although the anticancer activity of PB was reported in previous studies, the underlying mechanisms are still not well stated. In this study, the anticancer effect and the underlying mechanisms of PB were investigated in breast cancer cells. PB significantly reduced the viability of three human breast cancer cell lines, MCF-7, MDA-MB-231 and T-47D, in a concentration- and time-dependent manner. PB induced cell cycle arrest at G2/M phase and promoted cleavage of PARP (poly (ADP-ribose) polymerase), caspases 3, caspase 7 and caspase 9 to stimulate cell apoptosis. Further studies showed that PB induced breast cancer cells apoptosis in a p53-dependent manner in MCF-7 cells. PB also suppressed the phosphorylation of Akt (protein kinase B) and PI3K (phosphoinositide 3-kinase), and increased the phosphorylation of GSK-3β (glycogen synthase kinase 3β). Taken together, our results indicated that PB might serve as a potential therapeutic agent for breast cancer.

1. Introduction

Breast cancer is the most common malignancy in women around the world and is the second leading cause of cancer-related deaths among women [1]. Breast cancer diagnosis and treatment is associated with increases in anxiety, depression and distress, which significantly reduces the quality of life of cancer patients [2,3]. Therefore, there is a high demand for new drugs that are potent toward breast cancer. Due to complexity of constituents, poor quality control and lack of dosage instructions, herbal extracts show many disadvantages, including unexpected side effect and unstable pharmacological activity, which limit their clinical application as anti-cancer agents. Thus, there is a growing interest in identifying compounds with anticancer potential from herbs, and many natural products are clinically used for chemotherapies [4].

Physalins, a type of steroids with 13,14-seco-16,24-cycloergostane skeleton, are the characteristic constituents of Chinese lantern (*Physalis alkekengi* L.) [5]. Some of the plants from the genus *Physalis* are traditionally used in China for the treatment and prevention of tumors, leishmaniasis, sore throat, cough, eczema, hepatitis and urinary problems [6–8]. Previous studies investigated the anti-proliferative effect of physalin B (PB, Fig. 1A) on human colon cancer cells [9], human melanoma A375 cells [10], prostate cancer cells [11] and leukemia cells [12]. PB has been identified to target different pathways to exert its activities, including inhibition of the ubiquitin-proteasome pathway and induction of incomplete autophagic response [9], triggering NOXA-related apoptosis pathway [10], activation of cell apoptosis and downregulation of androgen receptor expression [11]. Although the anticancer activities of physalins have been widely investigated, the anti-proliferative effects of physalins on breast cancer and underlying mechanisms were not detailed explained. In the current study, we investigated the efficacy of PB on three human breast cancer cell lines, and the potential mechanisms were further investigated.

2. Materials and methods

2.1. Materials and reagents

Physalin B with a purity ≥98% by high performance liquid

Abbreviatrions: Akt, protein kinase B; GSK-3β, glycogen synthase kinase 3β; PARP, poly (ADP-ribose) polymerase; PB, physalin B; PI3K, phosphoinositide 3-kinase

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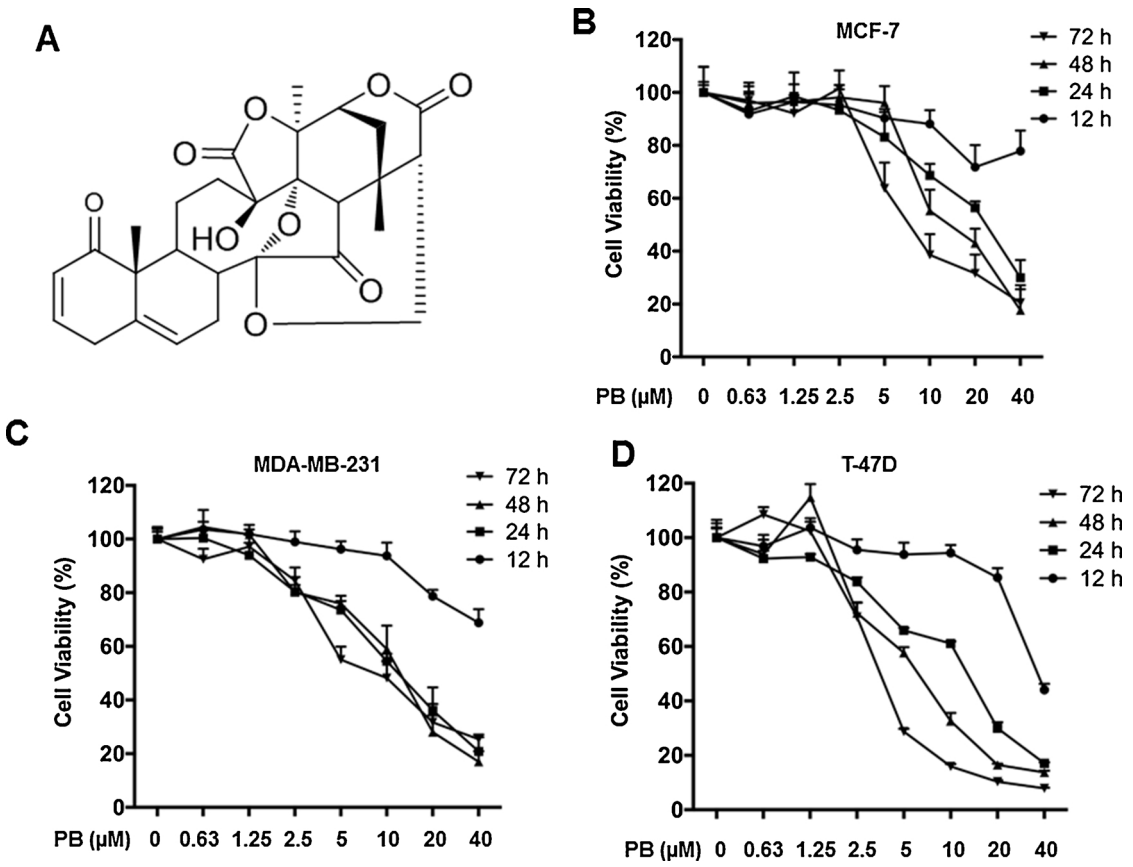

Fig. 1. Anti-proliferative effect of PB in human breast cancer cells. (A) The chemical structure of PB. Cell viabilities of MCF-7 (B), MDA-MB-231 (C), and T-47D (D) cells when treated with different concentrations of PB for different time. Data represented means ± SEM. $n = 9$.

chromatography analysis was isolated and identified by our group [13]. PB was well dissolved in DMSO (dimethyl sulfoxide, cell culture grade) at a concentration of 40 mM as stock solution and the final DMSO concentration in cell media was less than 0.1%. Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640, penicillin-streptomycin (P/S), phosphate-buffered saline (PBS) powder, 0.25% (w/v) trypsin/1 mM EDTA and fetal bovine serum (FBS) were purchased from Gibco™ (Waltham, MA, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2-H-tetrazolium bromide (MTT), DMSO, phenylmethanesulfonyl fluoride (PMSF), Hoechst 33342 and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V/propidium iodide (PI) assay kit was purchased from BD Biosciences (Qume Drive San Jose, CA, USA). p53 siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RIPA lysis buffer for protein extraction was purchased from Beyotime Biotechnology (Beyotime, Shanghai, China). All primary and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). And the detailed information about these antibodies are list as following: p53 (Cat. NO, 9282; Rabbit), $p21^{Waf1/Cip1}$ (Cat. NO, 2947; Rabbit), cdc25c (Cat. NO, 4688; Rabbit), p-cdc2 (Cat. NO, 9111; Rabbit), cdc2 (Cat. NO, 28439; Rabbit), β-actin (Cat. NO, 3700; Mouse), caspase-9 (Cat. NO, 9502; Rabbit), cleaved-caspase 9 (Cat. NO, 9505; Rabbit), caspase-7 (Cat. NO, 9494; Mouse), cleaved-caspase 7 (Cat. NO, 8438; Rabbit), caspase-3 (Cat. NO, 9662; Rabbit), cleaved-caspase 3 (Cat. NO, 9664; Rabbit), PARP (Cat. NO, 9532; Rabbit), cleaved PARP (Cat. NO, 5625; Rabbit), Akt (Cat. NO, 4685; Rabbit), p-Akt (Cat. NO, 13038; Rabbit), PI3K (Cat. NO, 4292; Rabbit), p-PI3K (Cat. NO, 4228; Rabbit), GSK-3β (Cat. NO, 12456; Rabbit), p-GSK-3β (Cat. NO, 5558; Rabbit), anti-mouse IgG (Cat. NO, 7076) and anti-rabbit IgG (Cat. NO, 7074) HRP-linked secondary antibody. All the primary antibodies were diluted 1:1000 (v/v) in TBST, and the secondary antibodies were diluted 1:2000 (v/v) in TBST.

2.2. Cell culture

Human breast cancer MCF-7, MDA-MB-231 and T-47D cells were purchased from American Type Culture Collection (Rockville, MD, USA). MCF-7 and T-47D cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) P/S, and MDA-MB-231 cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS and 1% (v/v) P/S. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell viability assay

Cell viability was assayed as described previously [14–16]. In brief, MCF-7, MDA-MB-231 and T-47D cells were seeded in 96-well plates at a density of 1×10^4 cells/well. When 70–80% confluence, cells were treated with or without different concentrations of compounds at indicted time. Subsequently, the culture medium was discarded and cells were incubated with fresh culture medium containing MTT (1 mg/mL) for 4 h, followed by dissolving the formazan crystals with 100 μL DMSO. The absorbance at 570 nm was measured by a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and presented as relative cell viability. The results were analyzed based on at least three independent experiments.

2.4. Cell cycle assay

Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and cultured for 24 h. To analyze the intracellular DNA content, cells were treated with serum-free culture medium for 24 h and then with

different concentrations of PB for another 48 h. Cells were harvested and fixed in 70% ethanol at −20 °C overnight. Cells were washed with PBS twice, and suspended in PBS containing 50 μg/mL PI and RNase A, incubated at 37 °C for 30 min and analyzed with a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), 1×10^4 events per run were recorded for each sample. Data output was analyzed using Modifit LT software (Verity Software House, Topsham, ME, USA). All experiments were conducted in triplicate.

2.5. Apoptosis assay

Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and cultured for 24 h. Cells were treated with or without different concentrations of PB for 48 h, and then culture medium in each well were collected and centrifuged (300 g, 5 min) to get the floating cells. For the attached cells, 100 μL/well trypsine was added and incubated at 37 °C for 5 min, followed by adding 1 mL culture medium and centrifuging to get the cells. Subsequently, the trypsinized and floated cells in each well were respectively mixed together and washed with PBS twice, and diluted to 1×10^6 cells/mL followed by 15 min incubation with Annexin V and PI staining in the dark at room temperature. The cell suspensions were adjusted to 500 μL with binding buffer and counted using a flow cytometer (Becton Dickinson), 1×10^4 events per run were recorded for each sample. The results were analyzed based on at least three independent experiments.

2.6. Nucleus morphology observation

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h. Cells were treated with or without different concentrations of PB for 48 h. After washed with PBS twice, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with Hoechst 33342 (1 μg/mL) for 10 min. The morphology of cell nuclei was monitored using an In Cell Analyzer 2000 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.7. Western blotting analysis

Cells were seeded in 100 mm dishes at a density of 1×10^6 cells/dish and cultured for 24 h. Cells were treated with or without various concentrations of PB for 48 h. After washed with PBS twice, cells were harvested by treating with ice-cold RIPA lysis buffer containing PMSF and protease inhibitor cocktail (100:1:1, v/v/v). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes, and then blocked with 5% nonfat milk in TBST (100 mM NaCl, 10 mM Tris–HCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature and incubated with the respective primary antibodies in TBST overnight at 4 °C with shaking. After washed with TBST three times, secondary HRP-linked antibody was added and incubated at room temperature for 4 h, followed by washing in TBST three times. Then membranes were incubated with West Femto Sensitivity Substrate and protein bands were detected using ChemiDoc XRS Molecular Imager system (BIO-RAD, Hercules, CA, USA). Intensity of individual bands in Western blots was quantified using Image J densitometry software, and expressed relative to reference protein signal, as a measure of protein relative abundance in the different samples.

2.8. siRNA transfection

MCF-7 cells were seeded in 6-well plates and allowed to grow overnight. Transient transfections were performed using Lipofectamine 2000 (Life technologies, CA, USA) according to the manufacturer's guide. Briefly, siRNA and transfection reagent were diluted with Opti-MEM medium and mixed for 20 min. After transfection for 4 h, the medium was changed with fresh complete medium. The cells were treated with PB for cell viability and Western blotting assay.

2.9. Statistical analysis

Data were expressed as mean ± SEM based on at least three independent experiments and analyzed by Graphpad Prism 6 (GraphPad Software, San Diego, CA, USA). The significance of differences between groups was assessed by one-way analyses of variance (ANOVA) test using SPSS software 16.0 (Chicago, IL, USA). $P < 0.05$ indicated the presence of a statistically significant difference, and $P < 0.01$ was considered significantly different.

3. Results

3.1. PB suppresses the viability of breast cancer cells

To assess the effect of PB on cell viability, we examined the anti-proliferative effect of PB in MCF-7, MDA-MB-231 and T-47D cells using MTT assay. At concentrations below 2.5 μM, PB only caused less than 20% cell death in three cell lines, even treated up to 72 h (Fig. 1B–D). At concentrations from 2.5 to 40 μM, PB decreased the cell viabilities of three cell lines in a dose-dependent manner (Fig. 1B–D). Meanwhile, PB also decreased the cell viability in a time-dependent manner. When treated with 20 μM PB, the cell viabilities were reduced to 71.8%, 52.0%, 43.0% and 31.8% for MCF-7 cells, 78.7%, 36.2%, 28.0% and 33.2% for MDA-MB-231 cells, and 85.3%, 29.9%, 16.5% and 10.3% for T-47D cells, respectively, after 12, 24, 48 and 72 h (Fig. 1B–D). All three kinds of breast cancer cells were almost dead when treated with 40 μM PB for 24 h or longer. These results demonstrated that PB decreased the viability of breast cancer cells in a concentration- and time-dependent manner. And, MCF-7 cells, one of the most commonly used breast cancer cell line, were chosen in the following studies.

3.2. PB induces G2/M cell cycle arrest in MCF-7 cells

To address the underlying mechanism responsible for PB-mediated anti-proliferative effect, the cell cycle distribution profile was further examined using flow cytometry. As shown in Fig. 2A, the percentage of MCF-7 cell population at G2/M was significantly increased after treatment with PB at concentrations ranging from 2.5 to 10 μM for 24 h. Quantitative analysis indicated treatment with PB at 2.5, 5, and 10 μM increased the distribution of MCF-7 cells at G2/M phase from 3.1% to 22.6%, 25.6%, and 30.0%, respectively, showing a concentration-dependent increase (Fig. 2B). Furthermore, the expression of cell cycle-related proteins was detected by Western blotting analysis. The results showed that PB upregulated the expressions of p53 and $p21^{Waf1/Cip1}$, while downregulated the expressions of cdc2 and p-cdc2 in MCF-7 cells (Fig. 2C). Since the mutant p53 protein in MDA-MB-231 and T-47D cells, the expression of this protein was detected to confirm whether this protein was envolved in regulation of the proliferation of cells [17,18]. Interestingly, PB decreased the expressions of mutant p53 and increased the expressions of $p21^{Waf1/Cip1}$ in MDA-MB-231 and T-47D cells in a dose-dependent manner (Fig. 2D). Taken together, these data showed that PB triggers G2/M cell cycle arrest by altering the key molecules of G2/M cell cycle transition-phase.

3.3. PB induces apoptosis in MCF-7 cells

As induction of apoptosis is one of the major contents that mediate cell proliferative inhibition, apoptosis was further investigated. The nuclei morphology was observed by Hoechst 33342 staining to characterize the apoptotic cells. Upon challenged with PB for 48 h, the cells produced condensed chromatin in the periphery of the nuclei and fragmented nuclei (indicated by yellow arrows in bottom panel in Fig. 3A), which were morphological hallmarks of apoptotic cell death. At the highest concentration of PB (10 μM), severe fragmentation of

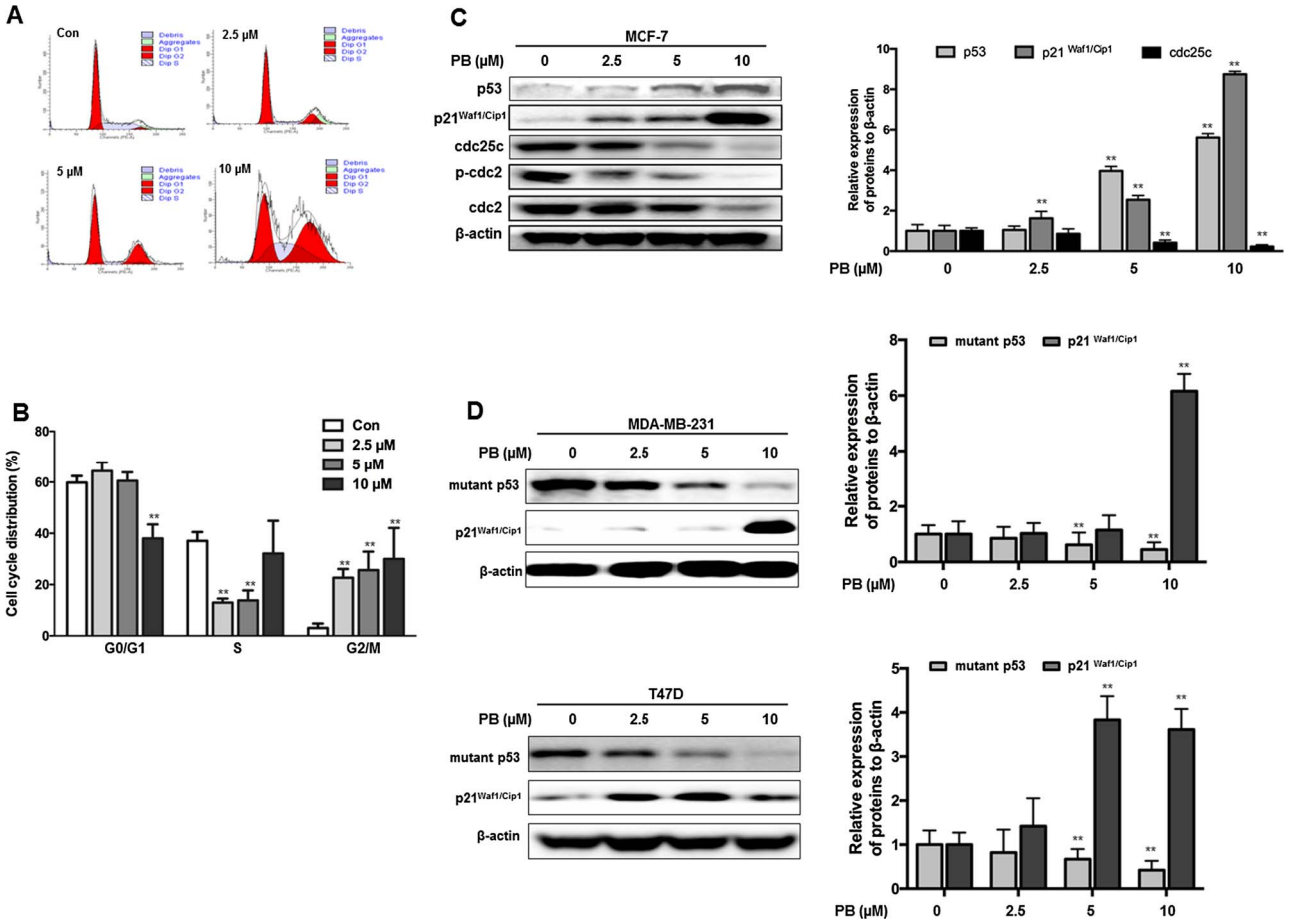

Fig. 2. The cell cycle arrest effect of PB in human breast cancer cells. MCF-7 cells were treated with 10 μM PB for 24 h and the various phases of the cell cycle were evaluated by flow cytometry qualitively (A) and quantitatively (B). (C) Expressions of cell cycle related proteins in MCF-7 cells. (D) Expressions of mutant p53 and $p21^{Waf1/Cip1}$ in MDA-MB-231 and T-47D cells. Data represented means ± SEM. $^{**}P < 0.01$ *vs* untreated control group.

nuclei was detected, accompanied with the formation of apoptotic bodies. To quantify the apoptosis, Annexin V/PI double staining was performed. As shown in Fig. 3B, quantitative assay indicated that the apoptotic rate of MCF-7 cells was increased to 16.4%, 21.6%, and 35.4% after treated with 2.5, 5, and 10 μM PB for 48 h, respectively. The expressions of important signaling proteins involved in cell apoptosis were investigated by Western blotting. During apoptosis, caspases (caspases-3, -7 and -9) are cleaved and activated, causing poly (ADP-ribose) polymerase (PARP) cleavage and production of an 89 kDa C-terminal fragment [19]. As shown in Fig. 3C, the protein expression of cleaved PARP, cleaved caspase 3, cleaved caspase 7, and cleaved caspase 9 were all remarkably increased after treatment with PB in MCF-7 cells. All the results indicate that PB provokes MCF-7 cell apoptosis by activating the caspase cascade.

3.4. PB induces MCF-7 cell apoptosis in a p53-dependent manner

The p53 tumor suppressor protein plays a key role in cellular response to DNA damage and other genomic aberrations [20]. The relationship between p53 and PB-induced MCF-7 cell apoptosis was investigated. As shown in Fig. 4A, silencing of p53 gene using specific siRNA resulted in a substantial reduction of p53 protein level in MCF-7 cells. Remarkably, the cytotoxic effect of PB was significantly decreased in the p53 silenced cells, when compared with the normal control cells (Fig. 4B). Similarly, the Western blot analysis of apoptotic related proteins demonstrated p53 silence blocked PB-induced cleavage of PARP, caspase 3 and caspase 7 (Fig. 4C). Taken together, these results suggest that PB induces cell apoptosis in a p53-dependent manner.

3.5. Decreased PI3K/Akt activation is involved in PB-induced cytotoxicity

Akt plays a critical role in controlling cell survival and apoptosis [21]. Akt promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets [22,23]. As shown in Fig. 5A, the phosphorylation forms of Akt and PI3K were significantly decreased by PB, while the expression of total Akt and PI3K were not changed. Furthermore, PB treatment did not change the total GSK-3β expression, but increased the phosphorylation of GSK-3β in a dose dependent manner in MCF-7 cells (Fig. 5A). Similarly, PB decreased the phosphorylation level of Akt and increased the phosphorylation level of GSK-3β in MDA-MB-231 and T47D cells in a dose-dependent manner (Fig. 5B). These data indicated that the cell cycle arrest and apoptosis induced by PB was partially mediated by Akt pathway.

4. Discussion

In this study, the anti-proliferative effect of PB in human breast cancer cells was investigated. Our results demonstrated that PB decreased the cell viability of three breast cancer cell lines in both a concentration- and time-dependent manner. PB induced cell cycle arrest at G2/M phase and promoted cleavage of PARP, caspases 3, caspase 7 and caspase 9 to stimulate cell apoptosis. Further studies showed that the effect of PB was associated with the activation of p53 and inhibition of Akt in MCF-7 cells; while inactivation of mutant p53 and Akt in MDA-MB-231 and T47D cells. However, these observations require further investigations.

Breast cancer encompasses a group of hetero-geneous diseases,

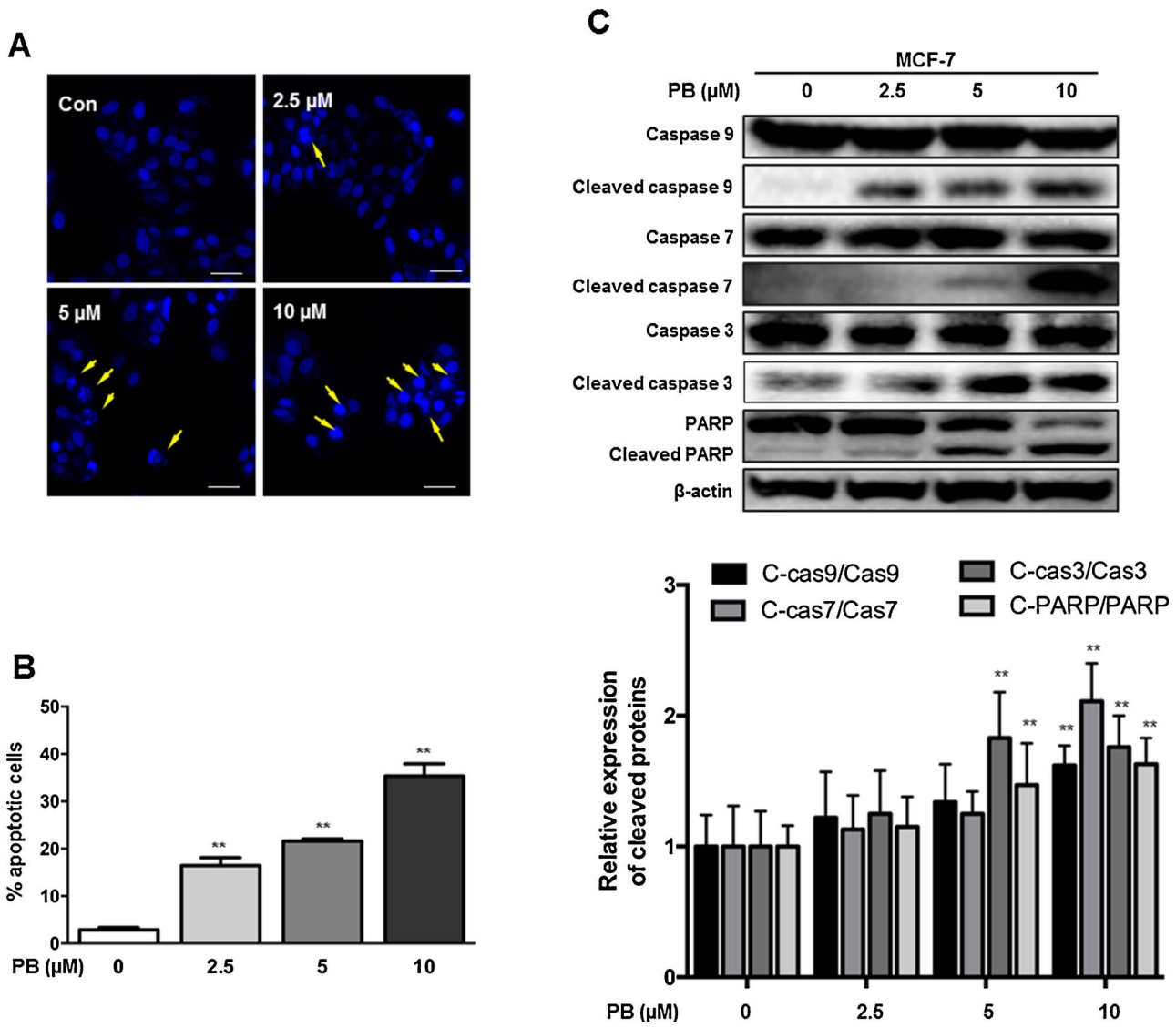

Fig. 3. PB induces apoptosis in human breast cancer MCF-7 cells. (A) The effect of PB on the nuclei morphology change was observed by Hoechst 33342 fluorescent staining. (B) Flow cytometry analysis of the apoptotic cells treated by different concentrations of PB. (C) The expression of apoptosis-related proteins was determined by Western blotting. $^{**}P < 0.01$ *vs* untreated control group. Scale bar, 25 μm.

which can be classified by the molecular biomarkers, histopathologic and clinical levels [24]. The heterogeneity among different breast cancer cells has been well proved, and at least five subtypes of breast cancer could be classified: luminal A, luminal B, HER2, basal and normal according to the gene expression profiling and the immunohistochemical expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 [24,25]. In this article, three kinds of breast cancer cells, including MCF-7, MDA-MB-231 and T47D, were chosen to study the anti-tumourigensis effect of PB. Among the breast cancer cells, MCF-7 and T47D are classified as luminal A type which have the ER^+, $PR^{+/-}$and $HER2^-$ immunological profile and with characteristic of low Ki67, endocrine responsive and often chemotherapy responsive [26–29]. While, MDA-MB-231 breast cancer cell belongs to normal type which is triple-negative in ER, PR and HER2 and potent with characteristic of low Ki67, E-cadherin, claudin-3, claudinin-4 and claudinin-7 and intermediate response to chemotherapy [26,30,31]. Although these selected cell lines fail to reflect all the subtypes of breast cancer, the current achievements demonstrated that PB triggered breast cancer cells apoptosis through an ER, PR and HER2 independent manner.

Among the selected three breast cancer cell liness, MCF-7 expresses widetype p53 with function of suppressing tumour proliferation, and the other two cell lines carry with mutant p53 which promotes tumorigenesis and metastasis [18,32]. The mutant p53 is highly expressed in MDA-MB-231 breast cancer cells, which is stabilized by high phospholipase D (PLD) activity [17]. It is reported that mutant p53 recruits p300 to induce histone acetylation, which permits tumour progression proliferation in MDA-MB-231 breast cancer cells [18]. Mutant p53 was also reported to interact with a wide variety of other proteins, resulting in interference in a multitude of cellular pathways, including inhibition of p63 [33], the MRE11-Rad51-NSB complex, p73, and SP-1 to induce genomic instability, chemoresistance, or proliferation [34–36], promoting the function of proteins including SREBP, VDR, ETS2, or NRF2, resulting in increased proliferation, cholesterol synthesis, accumulation of reactive oxygen species, and enhanced cell survival [37–39]. In this article, we observed that PB significantly upregulated p53, which contributed as a tumour suppressor in MCF-7 cells. On the other hand, the expression of mutant p53 in MDA-MB-231 and T47D breast caner cells was down-regulated by PB. However, the underlying mechanisms for the observations in MDA-MB-231 and T47D is still deserved deep exploration. Nevertheless, it is obvious that PB regulates p53 expression, no matter widetype or mutant, which plays a key role in anti-proliferation in breast cancer cells.

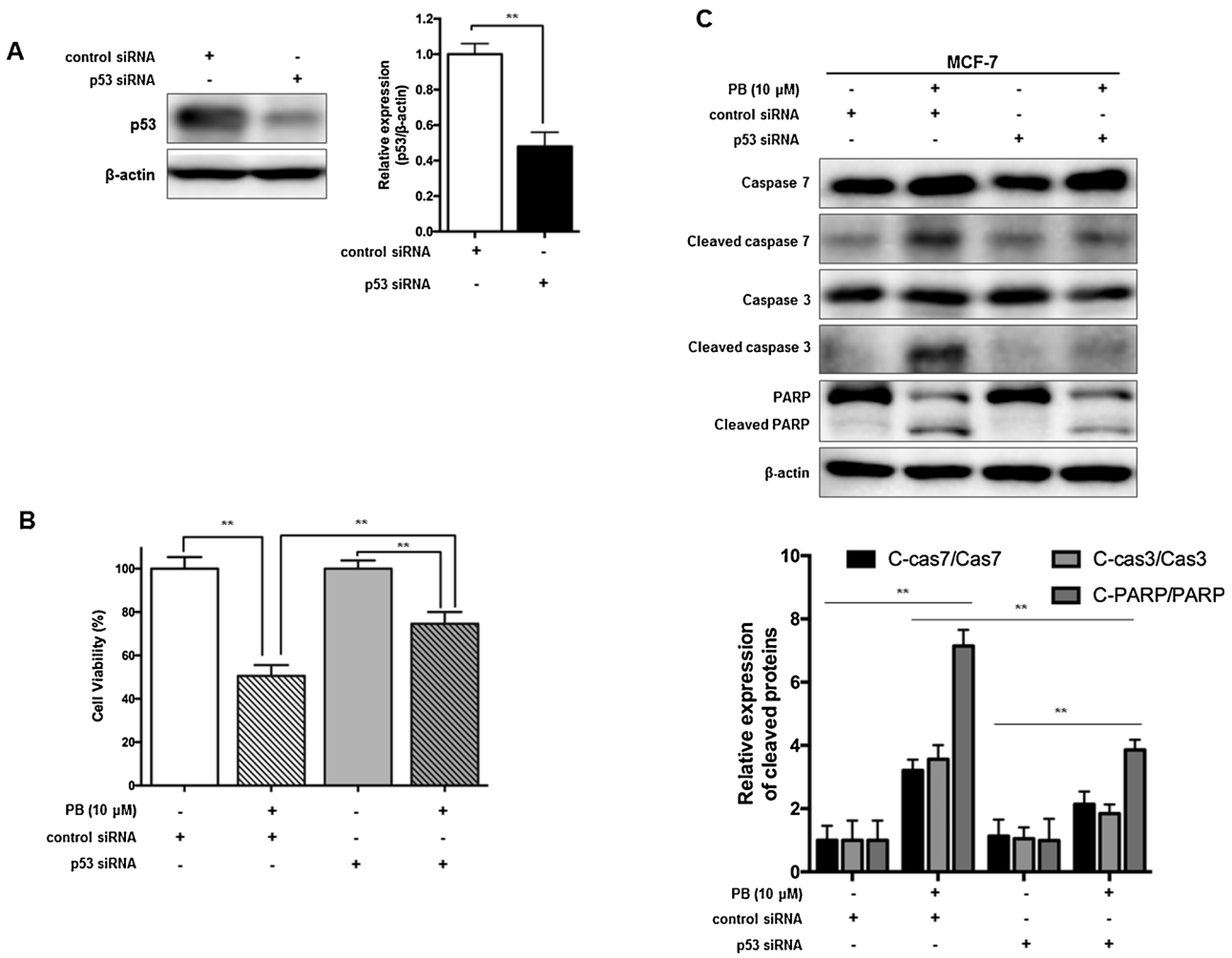

Fig. 4. Effect of p53 silencing on PB-induced cytotoxicity and apoptosis. Cells were transfected with p53 siRNA or control siRNA (A), and then treated with 10 μM of PB for 48 h, the cell viability and expression of apoptosis-related proteins were assessed by MTT assay (B) and Western blotting (C), respectively. $^{**}P < 0.01$.

The cell cycle progression is controlled by a series of signaling cascades by which cell replicates its DNA, divide and proliferate [1]. Anti-cancer drugs can activate p53-dependent and p53-independent pathways to arrest cancer cells at the G2/M checkpoint and form entry into mitosis [40–42]. The activation of p53 can transcriptionally increase the expression levels of its target genes, especially p21 [43]. In this study, PB treatment significantly increased the G2/M phase cycle arrest accompanied by the significantly increased expressions of p53 and $p21^{Waf1/Cip1}$ protein. Cdc2 activity has been shown to be essential for progression into mitosis and the progression into S phase [44]. We also found that PB suppressed the phosphorylation and expression of cdc2, which might also contribute to its effect in inducing G2/M phase cycle arrest.

Apoptosis is an energy-dependent genetically programmed cell death that is of importance in cancer research since abnormal cells that do not undergo apoptosis may become malignant, and reduced apoptosis has been associated with resistance to chemotherapy and progression of cancer, and most of the current anticancer drugs induce apoptosis as their mechanism of action [45]. One of the main biochemical hallmarks is the activation of the caspase cascades, in which a series of cysteinyl aspartic acid-specific proteases are activated by cleavage of the zymogen form to activate the two main pathways of apoptosis: the extrinsic death receptor-mediated and intrinsic mitochondrial-mediated pathways [46]. In the current study, it was found that PB induced apoptosis by cleavage of caspase-9 which further processes other caspase members, including caspase-3 and caspase-7, to initiate a caspase cascade [47]. It further induced the cleaved PARP to facilitate cellular disassembly and serves as a marker of cells undergoing apoptosis [48,49].

As one of key factors controlling cell survival and apoptosis [21], Akt is activated and phosphorylated at Ser473 by PDK2 [50]. Another essential Akt function is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β [51,52]. Akt is also involved in cell cycle regulation by preventing GSK-3β-mediated phosphorylation and degradation of cyclin D1 [53] and by negatively regulating the cyclin dependent kinase inhibitors $p27^{Kip1}$ [54] and $p21^{Waf1/Cip1}$ [55]. This phenomenon could be explained by that decreased recruitment of Akt by appropriate survival signals lead to inactivation of Mdm2, activation of p53, and eventually promotion of p53-dependent apoptosis [56–58]. The current results showed PB supreesed the phosphorylation of AKT, PI3K and GSK-3β, which indicated that Akt singaling might be partially involved in PB-induced apoptosis.

5. Conclusions

In conclusion, our results revealed the anti-proliferative effect and potential mechanisms of PB in human breast cancer cells. This study provides evidence that PB has potential for further development as a novel antitumor drug candidate for breast cancer.

Conflict of interests

The authors declare no competing financial interest.

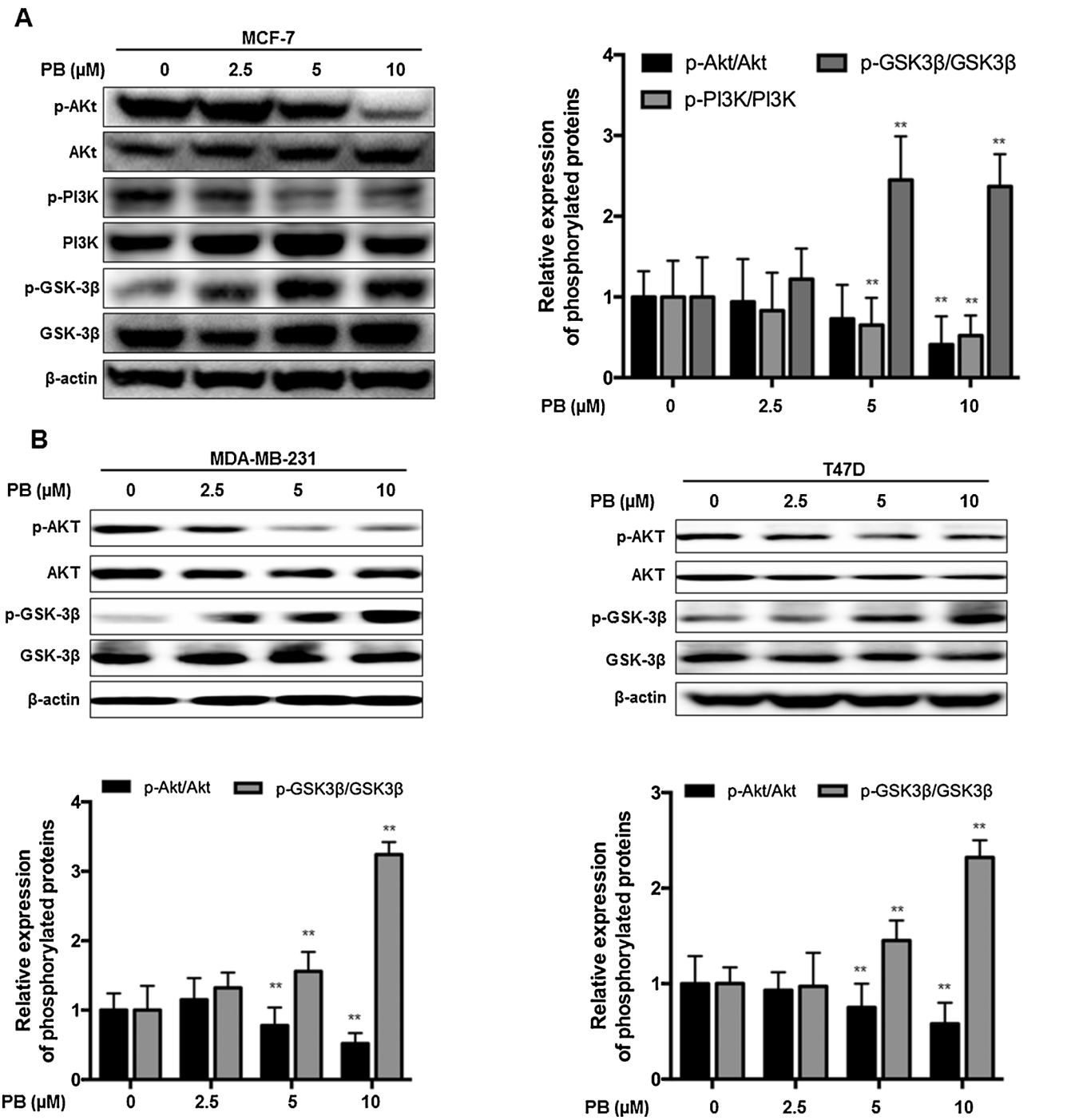

Fig. 5. Akt is partially responsible for PB-induced cytotoxicity. (A) The effect of PB on the expressions of Akt and related proteins in MCF-7 cells. (B) The effect of PB on the expressions of Akt and related proteins in MDA-MD-231 and T-47D cells. $^{**}P < 0.01$.

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