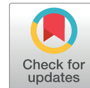

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Panduratin A Inhibits Cell Proliferation by Inducing G0/G1 Phase Cell Cycle Arrest and Induces Apoptosis in Breast Cancer Cells

Qiuming Liu, Yali Cao*, Ping Zhou, Shimin Gui, Xiaobo Wu, Yong Xia and Jianhong Tu

Department of Breast Surgery, Breast Cancer Institute, The Third Hospital of Nanchang, Nanchang 330009, China

Abstract

Because of the unsatisfactory treatment options for breast cancer (BC), there is a need to develop novel therapeutic approaches for this malignancy. One such strategy is chemotherapy using non-toxic dietary substances and botanical products. Studies have shown that Panduratin A (PA) possesses many health benefits, including anti-inflammatory, anti-bacterial, anti-oxidant and anti-cancer activities. In the present study, we provide evidence that PA treatment of MCF-7 BC cells resulted in a time- and dose-dependent inhibition of cell growth with an IC_{50} of 15 μM and no to little effect on normal human MCF-10A breast cells. To define the mechanism of these anti-proliferative effects of PA, we determined its effect critical molecular events known to regulate the cell cycle and apoptotic machinery. Immunofluorescence and flow cytometric analysis of Annexin V-FITC staining provided evidence for the induction of apoptosis. PA treatment of BC cells resulted in increased activity/expression of mitochondrial cytochrome C, caspases 7, 8 and 9 with a significant increase in the Bax:Bcl-2 ratio, suggesting the involvement of a mitochondrial-dependent apoptotic pathway. Furthermore, cell cycle analysis using flow cytometry showed that PA treatment of cells resulted in G0/G1 arrest in a dose-dependent manner. Immunoblot analysis data revealed that, in MCF-7 cell lines, PA treatment resulted in the dose-dependent (i) induction of $p21^{WAF1/Cip1}$ and p27Kip1, (ii) downregulation of Cyclin dependent kinase (CDK) 4 and (iii) decrease in cyclin D1. These findings suggest that PA may be an effective therapeutic agent against BC.

Key Words: Breast cancer, Panduratin A, Apoptosis, Cell cycle arrest, Cyclin D1

INTRODUCTION

Breast cancer is the most common malignancy in women throughout the world, accounting for 18% of all female cancers, and there are approximately 600,000 annual deaths worldwide (Kumar *et al*., 2013). Approximately 76% of all breast tumors have been categorized as invasive breast cancers (Sathya *et al*., 2010). Surgery, radiotherapy and chemotherapy remain the primary option for the treatment of BC. The chemotherapy of BC leads to an emerging drug resistance and tumor relapse, and post-treatment toxicity limits their use in the clinic; these hurdles facilitate BC as the number one killer in women (Sathya *et al*., 2010; Crown *et al*., 2012). Due to high mortality and the associated side effects of chemotherapy and/or radiotherapy, cancer patients often seek alternative forms of therapies such as natural or herbal medicines (Sadagopan *et al*., 2015; Zheng *et al*., 2016). This has led to an increased interest and active search for novel anticancer agents from natural sources. Historically, many potent anticancer agents such as vincristine, vinblastine, paclitaxel, etoposide, camptothecin, topotecan, and doxorubicin were derived from plants (Bhanot *et al*., 2011; Yao *et al*., 2016). Vinblastine, vincristine, vinorelbine, vindesine, Taxol, etoposide, topotecan and irinotecan are well used in clinical practice as combinational therapy for many malignancies (Mann, 2002; Newman and Cragg, 2012).

Apoptosis is a form of cell death, a goal of cancer treatment, and is characterized by cell shrinkage, plasma membrane blebbing, and chromatin condensation, which are associated with cleavage of DNA into ladders (Matsuura *et al*., 2016). However, in response to some effective therapeutic treatments, a decreased ability to undergo apoptosis occurred in human malignant tumor cells (Dabrowska *et al*., 2016). Therefore, the further development of agents that can induce or enhance the extent of apoptosis seems to be a promising strategy in the treatment of cancer. On the other hand, many agents target the cell cycle and its signaling proteins to inhibit the proliferation of cancer cells (El-Naa *et al*., 2016; Zhu *et al*.,

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***Corresponding Author**
E-mail: caoyali@medmail.com.cn; leecao@yahoo.com
Tel: +079186615534, Fax: +079186615534

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2016). Hence, we analyzed the effect on apoptosis and cell cycle arrest in MCF-7 cells.

Panduratin A (PA) is a cyclohexanyl chalcone isolated from *Boesenbergia pandurata* (*Roxb*.) *Schltr*., commonly called finger root, that has demonstrated anticancer, antioxidant, anti-inflammatory, antibacterial, and anti-biofilm activities (Yun *et al*., 2003; Sohn *et al*., 2005; Yun *et al*., 2006; Yanti *et al*., 2009; Rukayadi *et al*., 2010). PA was reported to inhibit cell proliferation and induce apoptosis in colon cancer cells. Additionally, it was found to decrease the incidence of aberrant crypt foci (ACF) (pre-neoplastic lesions) in rats with colon cancer (Kirana *et al*., 2007). PA has benefits as an NF-κB inhibitor to inhibit the proliferation of cancer cells by blocking cells in the G2/M phase and by inducing apoptosis in A549 non-small cell lung cancer cells (Cheah *et al*., 2011). PA is also known to inhibit the cell proliferation of CaP cells by apoptosis, which was associated with the upregulation of the Fas death receptor and G2/M phase arrest (Yun *et al*., 2006).

This study was aimed to determine the cytotoxic effect of PA on MCF-7 human breast cancer cells *in vitro*. In addition, the mechanism of action of PA related to its cytotoxicity was elucidated through the detection of cell cycle-related proteins and the apoptotic pathway.

MATERIALS AND METHODS

Cell lines and cell culture

MCF-7, MCF-10A and T47D breast cancer cells were obtained from the American Type of Cell culture (ATCC, VA, USA), and the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) and 3.7 mg/mL of $NaHCO_3$ at 37°C in 5% CO_2.

Cytotoxicity assay

The cytotoxicity of PA was procured from Hangzhou Dayangchem Co. Ltd (Zhejiang, China) and was analyzed by the MTT assay. Briefly, 1×10^4 cells/well were seeded in a 96-well plate and were incubated at 37°C in 5% CO_2. After 24 h, the cells were treated with different concentrations (2.5, 5, 7.5, 10, 15, 20 and 30 μM) of PA. PA was dissolved in 0.01% DMSO, and it was used as the vehicle control. The cells were incubated for 24 and 48 h at 37°C. Subsequently, 50 μl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/ml) was added and incubated for 2 h. After the incubation, the media was discarded, and 100 μl of DMSO was replaced into each well to dissolve the formazan crystal. The colorimetric assay was quantified at a wavelength of 570 nm using a Chameleon V microplate reader (Hidex, Turku, Finland). The anti-proliferation activity of PA was expressed as an IC_{50} value. The percentage of cell viability was calculated.

Cell cycle analysis

The effect of PA on cell cycle analysis was performed using propidium iodide (PI). Briefly, cells were seeded in a 25-cm^2 flask, and then 60% confluent cells were treated with PA (10, 20 and 20 μM) or without PA for 24 h. Following incubation, the cells were harvested and spun down for 5 min at 2,000 rpm. The supernatant was removed, and the cells were then fixed in 1 ml of ice-cold 70% ethanol overnight at −80°C. Fixed cells were washed with 1 ml of 1X PBS and were stained in 500 μl of PI containing 5 μg/ml DNase-free RNase for 30 min at room temperature in total darkness. The DNA content of the cells was analyzed by flow cytometry. The percentages of G0-G1, S and G2-M cells were then calculated using fluorescence-activated cell sorting (FACS) software (BD Biosciences, CA, USA).

Analysis of apoptosis by annexin V-FITC

Briefly, MCF-7 cells were plated in chamber slides at the concentration of 1×10^5 and were allowed to attach overnight. Next, the cells were treated with PA at three concentrations for 24 h. After the incubation, the slides were washed with PBS and then were incubated with ice-cold methanol-acetone (1:1) at −20°C for 10 min. After air drying, the cells were washed two times with cold PBS and then were incubated with Annexin V-FITC conjugate (Santa Cruz Biotechnology Inc., TX, USA) for 30 min in the dark. After washing with PBS, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:5000 v/v; Invitrogen, Carlsbad, CA, USA) to stain the nucleus for 10 min. After incubation, the cells were washed with PBS, and the slides were mounted, coverslipped and visualized under a FSX100 fluorescence microscope (Olympus, Tokyo, Japan). Similarly, a portion of the cells will be analyzed for annexin V-FITC expression using a flow cytometer (BD FACS Count™ Flow Cytometer, CA, USA).

Measurement of caspase- 7, 8 and 9 activities

The activities of caspases-8 and -9 in MCF-7 cells was assessed using a colorimetric assay kit (Sigma Aldrich, St. Louis, MO, USA) based on the spectrophotometric detection of the caspase enzymes after cleavage from the labeled substrate, and caspase 7 was assayed using a fluorometric-based assay according to the manufacturer's instructions (Abcam, Cambridge, UK). The absorbance was read using the Universal Microplate Reader (Bio-TEK. Instrument. Inc., Winooski, VT, USA).

Isolation of mitochondria for cytochrome c determination

To study the cytochrome c protein level of mitochondria, the instructions issued by Mitochondria Isolation Kit for Cultured Cells were followed (Thermo Scientific, Rockford, IL, USA). The protein content was measured from the supernatants that contain the mitochondrial fraction. All the wells were loaded with 25 μg of protein.

Western blot analysis

Briefly, 1×10^5 cells/flask were seeded and treated with PA in a dose-dependent manner for 24 h. Later, the cells were collected and lysed in RIPA buffer supplemented with a 10-μl protease inhibitor cocktail, sodium orthovanadate, and PMSF (Santa Cruz, USA). The lysate was stored at −80°C until further use. A 40-μg protein sample was resolved on 10% SDS-PAGE and was transferred to an Immun®-Blot polyvinylidene difluoride (PVDF) membrane (BIO-RAD, CA, USA). The membrane was blocked in 5% BSA for 1 h at room temperature following an overnight incubation at 4°C with the following primary antibodies: anti-Cyclin D1, anti-CDK4, anti-cytochrome C, anti-Bax, anti-Bcl-2, anti-p21, anti-p27 and mouse anti-β-actin (Cell Signaling Technology, USA) antibodies. Membranes were washed with 1× TBS-T prior to incubating with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies for 2 h at room temperature. The membranes were washed 3

times with 1× TBS-T to remove excess antibodies before the protein-antibody complex was detected with Amersham ECL prime western blotting detection reagent (GE Healthcare, IL, USA).

Statistical analysis

Statistical analysis was processed according to conventional procedures using the Statistical Program for Social Sciences (SPSS) software for Windows (NY, USA), Version 20.0 (Post hoc, Turkey's test). A *p*-value <0.05 was considered to be statistically significant.

RESULTS

PA inhibits the cell proliferation of MCF-7 and T47D breast cancer cells

The viability of MCF-7 and T47D breast cancer cells treated with various concentrations of PA was measured using the MTT assay. Additionally, to check whether PA has any adverse effects on non-tumorigenic cells, we tested using MCF-10A breast cells. As shown in Fig. 1, PA could cause cytotoxicity towards the cells in a dose-dependent manner at 24 and 48 h. The data suggest concentration-dependent inhibition in MCF-7 cells with an IC_{50} value of 15 µM at 24 h and 11.5 µM at the 48 h time point. In the case of T47D cells, the IC_{50} was 17.5 µM at 24 h and 14.5 µM at 48 h. Notably, PA treatment had no effect on the proliferation of the non-tumorigenic MCF-10A cells.

PA induces cell cycle arrest in the G0/G1 phase

Flow cytometric analysis of the cell cycle distribution of MCF-7 breast cancer cells treated with PA for 24 h was performed. Fig. 2 shows the relative percentages of MCF-7 cells in each phase of the cell cycle following treatment. We noticed an increase in the mean percentage of cells in the G0/G1 phase of the cell cycle from 53.4 ± 2.3% (control) to 67.2 ± 3.5% (20 µM). G0/G1 phase cell cycle arrest was accompanied by a decrease in the percentage of cells in the S and G2/M phases of the cell cycle. The FACS results showed that PA inhibited the proliferation of MCF-7 cells during the G0/G1 phase.

PA deregulates the expression of cell cycle-related proteins

To understand the mechanism by which PA induces cell cycle arrest in MCF-7 cells, the expression of cell cycle-related proteins were examined using western blotting (Fig. 3A). We observed a dose-dependent increase in the expression of cell cycle-inhibitory proteins (p21 and p27) and decrease in cell cycle-promoting proteins (CDK4 and Cyclin D1), respectively, after 24 h of treatment. The quantification of the respective blots was represented in Fig. 3B and 3C.

PA induces apoptosis in MCF-7 breast cancer cells

As shown in Fig. 4A, treatment with PA resulted in an increase in the cell population that was positive for annexin V-FITC staining. Starting from 15 to 20 µM at the 24 h time point, MCF-7 cells undergo apoptosis as detected by the green fluorescence of annexin V-FITC when compared with that of the untreated control cells. Hence, we confirmed that PA induced apoptosis as evidenced by the increased phosphatidyl serine (PS) exposure. To further confirm apoptosis, we analyzed annexin V-FITC/PI expression by flow cytometry

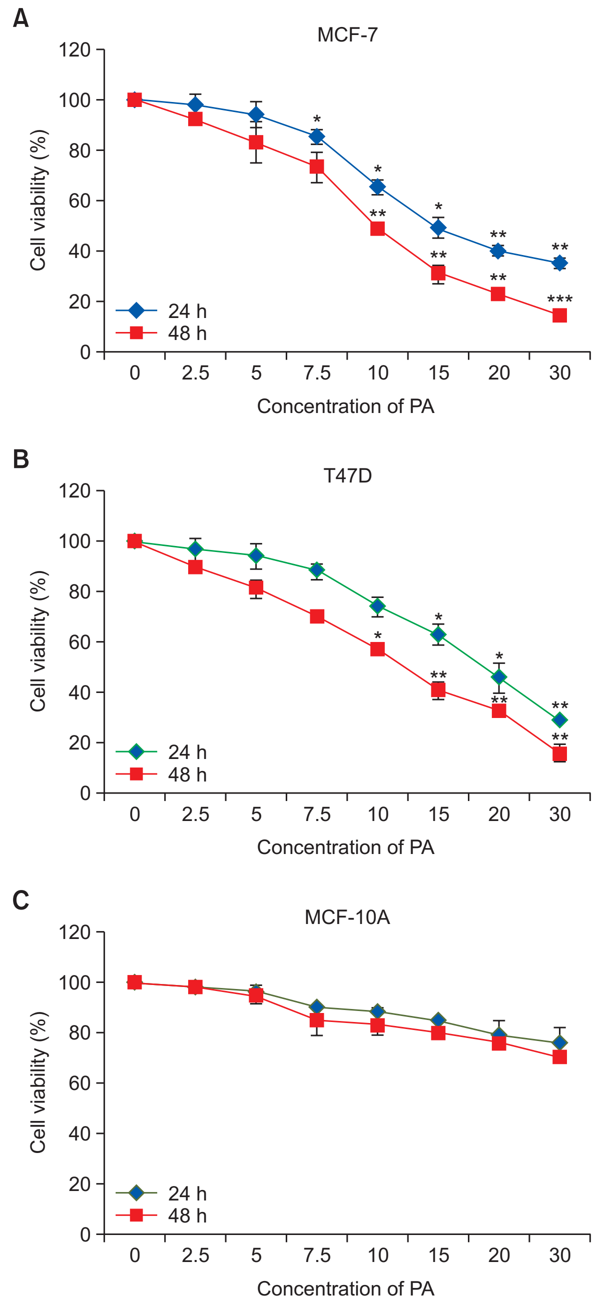

Fig. 1. Effect of panduratin A on the viability of MCF-7, T47D and MCF10A. (A-C) MCF-7 T47D and MCF10A cells were treated with the specified concentrations of PA for 24 and 48 h, and cell viability was determined by the MTT assay as described in the Materials and methods section. The values are represented as the percentage cell inhibition where vehicle-treated cells were regarded as 100%. The data represent the means ± SD of three independent experiments each conducted in triplicate. Treated groups compared with untreated control, *p<0.05 and **p<0.01.

(Fig. 4B). The MCF-7 cells were treated with PA at the doses of 10, 15 and 20 µM. The control cells exhibited a lower percentage of cells in early and late apoptosis (3.1% and 9.4%, respectively), and PA dose dependently increased annexin V expression. The percentages of early and late apoptosis for 10

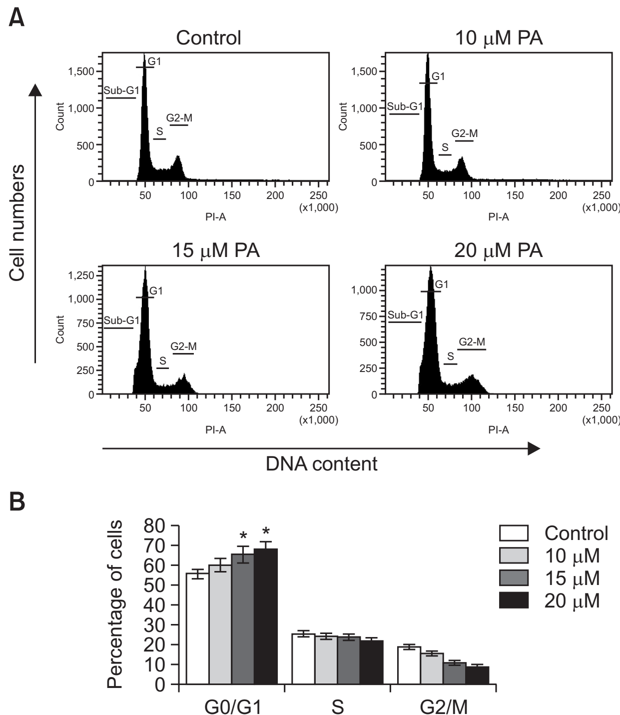

Fig. 2. PA induces G0/G1 phase arrest in MCF-7 cells. (A) MCF-7 cells were treated with the indicated concentrations of PA (10, 15 and 20 μM) for 24 h. Cells were permeabilized by ethanol and stained with PI. Cell cycle progression was assessed by flow cytometry. Representative figures of the cell cycle distribution (G0-G1, S, and G2-M) show accumulation of PA-treated cells in G0/G1phase. MCF-7 cells were arrested in G0/G1 phase 24 h after PA treatment. (B) The average percentage of the cell cycle phases is represented. The results are from 3 independent experiments and are presented as the means ± SD. Treated groups compared with the untreated control, *p<0.05.

μM were 3.4 and 13.2%, respectively, those for 15 μM were 4.1% and 17.4%, respectively, and those for 20 μM were 9.3% and 32.1%, respectively. We also analyzed the expression of the mitochondrial cytochrome c, pro-apoptotic Bax and anti-apoptotic Bcl-2 expression by western blotting (Fig. 4C-4E). Incubation of MCF-7 cells with PA showed decreased expression of Bcl-2 and increased expression of mitochondrial cytochrome c and Bax.

Caspases are the key enzymes in the cell that can be activated either by auto-catalytic processing or by another caspase (Bell and Megeney, 2017). Generally, these proteases can be classified into two groups: ‘initiator’ caspases, e.g., caspase-8 and -9 that have long pro-domains at the NH2-termini and can be self-cleaved on oligomerization, and ‘effector’ caspases, e.g., caspase-3, -6 and -7 that have short pro-domains and can be activated by initiator caspases or by activated effector caspases. Once activated by apoptotic stimuli, caspases contribute to the morphological and biochemical changes of apoptosis (Dabrowska *et al*., 2016; Duclos *et al*., 2017). Initially, we checked the activity of caspase-3 in MCF-7 cells and noticed no activity of caspase 3 (data not shown). We observed a significant (p<0.05) increase in the activities of caspase-7, 8 and -9 following the incremental dose of PA, demonstrating a dose-dependent manner (Fig. 5). We assumed that PA-induced apoptosis is mediated by the activation of caspases 7, 8 and 9.

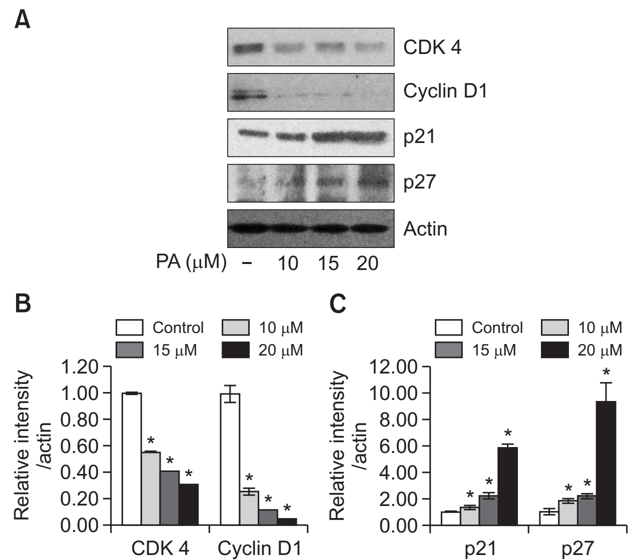

Fig. 3. PA modulates cell cycle signaling proteins in MCF-7 cells. (A) Western blot analysis shows a dose-dependent increase and decrease in the expression of cell cycle-inhibitory genes (p21 and p27) and cell cycle-promoting genes (CDK4 and Cyclin D1), respectively, after 24 h of treatment. (B, C) Quantification of the respective blots was represented in the graphs. Treated groups compared with the untreated control, *p<0.05 and **p<0.01.

DISCUSSION

For a therapeutic agent to be truly effective, it should be toxic to tumor cells without affecting normal cells. From the literature, it appears that natural products fulfill this criterion. Many natural products are reported to have anti-tumor effects by multiple mechanisms. We checked the cytotoxic effect of PA on MCF-7, T47D (human breast cancer) and MCF-10A (breast non-tumor) cells. The IC_{50} of PA in MCF-7 cells displayed an IC_{50} value of 15 μM at 24 h and 11.5 μM at 48 h. In the case of T47D cells, the IC_{50} was 17.5 μM at 24 h and 14.5 at 48 h. It is noteworthy that PA treatment does not affect the proliferation of MCF-10A cells. We further analyzed the effect of PA on the cell cycle arrest and apoptosis-inducing property in MCF-7 human breast cancer cells.

Cell cycle progression is orchestrated by a complex network of interactions among proteins, including cyclins, CDKs, E3 ubiquitin ligase complexes, CDK activating kinase, CDC25 phosphatases and CDK inhibitors (Diaz-Moralli *et al*., 2013). To explore the mechanisms by which PA induces cell cycle arrest in MCF-7 cells at the G0/G1 phase, western blot analysis was used to assess the modulation of the cell cycle regulatory proteins. The results showed that PA treatment resulted in decreased expression of cyclin D1 and CDK4 and increased expression of p21Cip1 and p27, explaining the G0/G1-phase arrest observed. Cyclin D1 and CDK4 are specific G0/G1-phase regulatory proteins (Diaz-Moralli *et al*., 2013). Previously, Yun *et al*. (2006) reported that PA isolated from *Kaempferia pandurata* induces cell cycle arrest in androgen-independent PC3

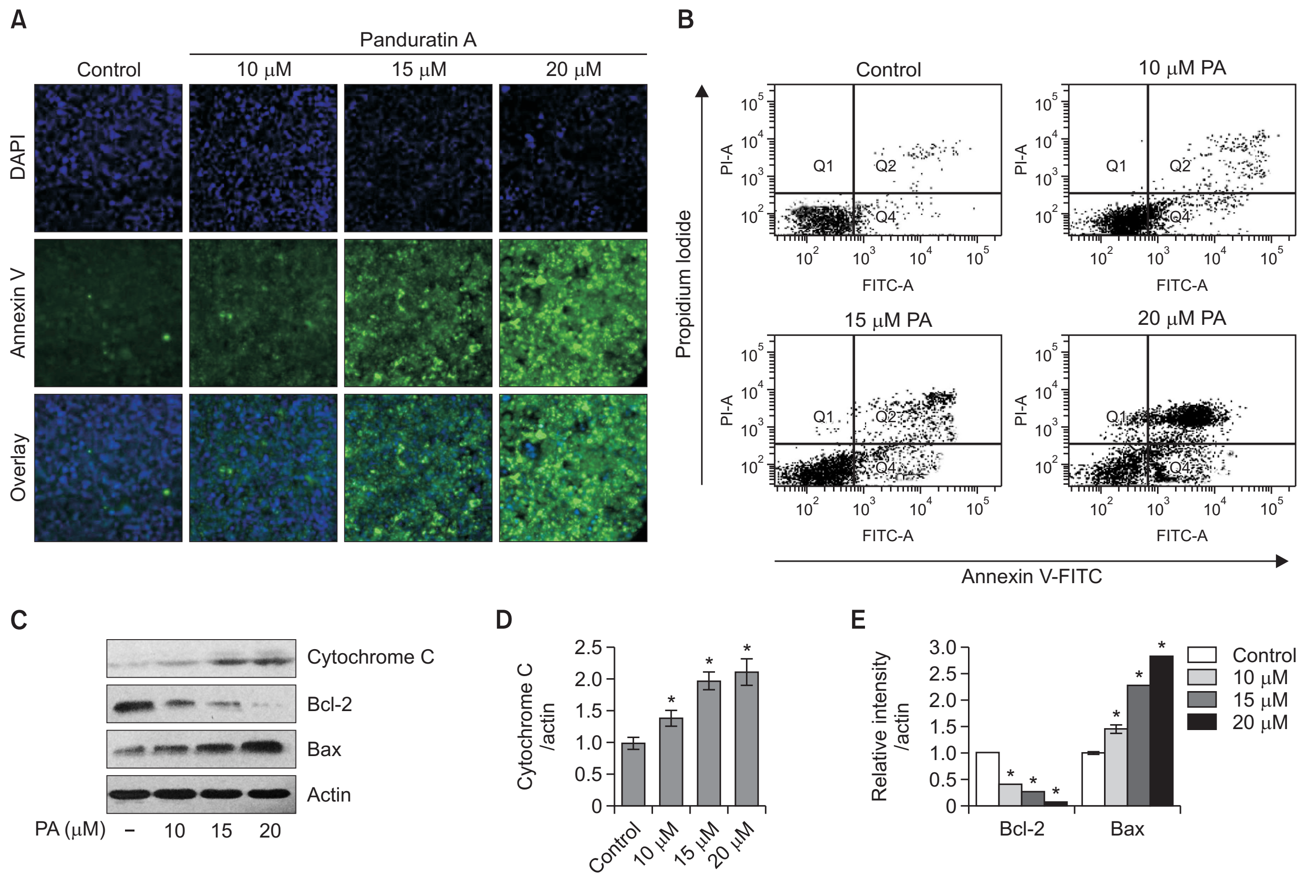

Fig. 4. PA induces apoptosis in MCF-7 cells. (A) MCF-7 cells were grown in coverslips and were treated with PA with three different concentrations (10, 15 and 20 μM) for 24 h. Next, the medium was removed and washed with PBS. Additionally, the cells were fixed with ice-cold acetone:methanol for 20 min. After air drying, the cells were incubated with Annexin V (FITC conjugated) for 30 min in the dark. After counter staining, the coverslips were placed in a slide with anti-fading agent. PA dose-dependently increased the expression of Annexin V-FITC at 24 h. (B) Flow cytometric analysis of Annexin V-FITC/PI was represented. PA administration, dose-dependently increased the apoptosis (both early and late). (C) Western blot analysis of mitochondrial cytochrome C, Bax and Bcl-2. Treatment with PA for 24 h increased the expression of mitochondrial cytochrome C, Bax and decreased the expression of Bcl-2. (D, E) Quantification of the respective western blots (Cytochrome C, Bax and Bcl-2) was represented in graphs. The results are from 3 independent experiments and are presented as the means ± SD. *Indicates a significant difference by Tukey's test ($p<0.05$) relative to their respective control.

and DU145 human prostate cancer cells. Additionally, it (i) induces p21WAF1/Cip1 and p27Kip1, (ii) downregulates cdks 2, 4 and 6 and (iii) decreases cyclins D1 and E (Yun *et al.*, 2006). Thus, we confirmed that PA-induced G0/G1 phase arrest was mediated through the modulation of p21 and p27.

Inter-nucleosomal DNA fragmentation is one of the hallmarks of apoptosis. Because the low-molecular-weight DNA fragments are extracted during cell staining in aqueous solutions, apoptotic cells can be identified on DNA content frequency histograms as cells with fractional ("sub-G1") DNA content (Kajstura *et al.*, 2007). We analyzed the population of cells of the sub-G1 phase in untreated and dose-dependent PA-treated MCF-7 cells. We found that the sub-G1 content in the untreated control population was 1.17 ± 0.11 and those of PA-treated (10, 15 and 20 μM) cells were 1.84 ± 0.18, 2.62 ± 0.21 and 4.52 ± 0.28, respectively. We assumed that the increase in PA treatment leads to the DNA fragmentation in MCF-7 cells that was confirmed as the cell population in the sub-G1 phase.

Activation of the intrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins. The Bcl-2 family proteins play vital roles in the regulation of cell death mechanisms (Adams and Cory, 1998; Cory *et al.*, 2003). Anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL) can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members (Bax, Bak, Bad) can trigger these changes. It is well known that caspases play central roles in the terminal execution of apoptosis induced by various stimulations (Salvesen and Riedl, 2008). To understand the induction mechanism of apoptosis by PA, we examined the expression levels of Bcl-2 and Bax by immunoblot analysis. The treatment of MCF-7 cells with PA resulted in a marked decrease in Bcl-2 protein levels in a dose-dependent manner (Fig. 4C). By contrast, the mitochondrial cytochrome c and Bax protein expression levels were increased compared with that in untreated control cells.

Components of the apoptosis signaling cascade, including caspases (Boatright and Salvesen, 2003; Philchenkov *et al.*, 2004) and triggers and regulators such as Fas ligand 45 and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death (Bouillet and

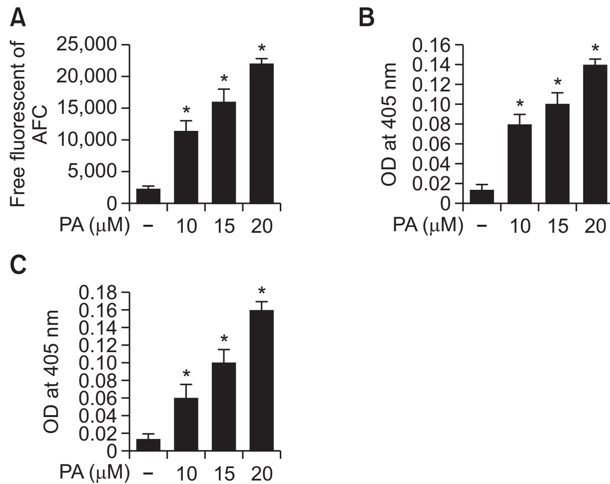

Fig. 5. PA increases the activities of caspase 7, 8 and 9 in MCF-7 cells. (A) PA increased the protein expression of caspase-7 in a dose-dependent manner (10, 15 and 20 μM of PA). The results were expressed as free fluorescent of DEVD-AFC. (B) PA increased the activity of caspase-8 at the highest dose of PA (20 μM). (C) PA increased the activity of caspase-9 at the highest dose of PA (20 μM). The caspase-7, 8 and -9 activities were determined by incubation with the specific substrates Ac-DEVD-AFC (abbreviated as N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Trifluoromethylcoumarin) (for caspase 7), Ac-IETD-pNA (abbreviated as Ac-Ile-Glu-Thr-Asp-pNA (pNA=p-Nitroaniline)) (for caspase 8) and Ac-LEHD-pNA (abbreviated as Ac-Leu-Glu-His-Asp-pNA (pNA=p-Nitroaniline)) (for caspase 9). The optical density was measured at 405 nm as described in the Materials and Methods section. The results are from 3 independent experiments and are presented as means ± SD. *Indicates a significant difference by Tukey's test (p<0.05) relative to their respective control.

Strasser, 2002; Liu *et al*., 2005). Caspases can be regarded as the central executors of the apoptotic pathway. Among the family of caspases, caspase-3 has been reported to be the most frequently activated caspase protease in apoptotic cells, indicating its crucial role in the cell death process (Cohen, 1997). In our study, we analyzed the activity of caspase 3 in MCF-7 cells after treatment with PA but found no activity of caspase 3 in MCF-7 cells (data not shown). It was reported that MCF-7 cells do not express caspase 3. In the absence caspase 3, cells proceed via the sequential activation of caspases 9 and 7 (Ye *et al*., 2001). Furthermore, the activities of caspases 7, 8 and 9 were dose dependently increased by the administration of PA to MCF-7 cells (Fig. 5). Many natural agents target the apoptotic process to kill cancer cells. It was known that PA induces apoptosis by modulating the Bax:Bcl-2 ratio and induction of caspases in many types of cancers (Yun *et al*., 2006; Cheah *et al*., 2011).

The *in vitro* anti-cancer activity of PA in MCF-7 breast cancer cells is highly positive. The results of our study indicate that PA has strong anti-proliferative effects by inducing apoptotic cell death, causing G0/G1 phase arrest of MCF-7 breast cancer cells. Because the modes of action of these bioactive compounds are unclear, a greater understanding of their mechanisms of action will help in providing useful information for their possible application in cancer prevention and perhaps also in cancer therapy and various ailments. In the future, we would like to continue our studies in understanding the efficacy of PA in triple-negative breast cancer *in vitro* and *in vivo*.

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