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Physalin B from *Physalis angulata* triggers the NOXA-related apoptosis pathway of human melanoma A375 cells

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ABSTRACT

Melanoma is a lethal form of skin cancer that can metastasize rapidly. While surgery and radiation therapy provide palliative therapy for local tumor growth, systemic therapy is the mainstay of treatment for metastatic melanoma. However, limited chemotherapeutic agents are available for melanoma treatment. In this study, we investigated the anti-melanoma effect of physalin B, the major active compound from a widely used herb medicine, *Physalis angulata* L.

This study demonstrated that physalin B exhibits cytotoxicity towards *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF)-mutated melanoma A375 and A2058 cells (the IC_{50} values are lower than 4.6 μg/ml). Cytotoxicity is likely resulted from apoptosis since the apoptotic marker phosphatidylserine are detected immediately under physalin B treatment and apoptotic cells formation. Further examination revealed that physalin B induces expression of the proapoptotic protein NOXA within 2 h and later triggers the expression of Bax and caspase-3 in A375 cells. These results indicate that physalin B can induce apoptosis of melanoma cancer cells via the NOXA, caspase-3, and mitochondria-mediated pathways, but not of human skin fibroblast cells and myoblastic cells. Thus, physalin B has the potential to be developed as an effective chemotherapeutic lead compound for the treatment of malignant melanoma.

1. Introduction

Numerous natural products reported for pharmacological application exhibit anti-tumor effects by inducing cancer cell death. Several studies examined the appealing theory that tumor cells could be eliminated by artificially triggering cell death through apoptosis (Kerr et al., 1972; Saraste and Pulkki, 2000; Thompson, 1995). Therefore, apoptosis may be a clinically relevant mode of tumor cell death triggered by anti-cancer agents.

Physalis angulata L. is a herbaceous annual plant widely distributed in tropical and subtropical regions of the world, and has long been used for the treatment of malaria (Ankrah et al., 2003), tuberculosis and inflammation (Choi and Hwang, 2003; Januario et al., 2002). Extracts of this plant have been used in herbal medicine to treat lung, cervical, and hepatic cancers (Makino et al., 2003; Wu et al., 2004). The crude extract of *P. angulata* has been proven capable of inhibiting cell proliferation and inducing G2/M arrest and apoptosis in human breast cancer cell lines (Hsieh et al., 2006). Withangulatin A, a compound isolated from *P. angulata*, can promote type II DNA topoisomerase-mediated DNA damage (Juang et al., 1989). Previous studies demonstrated that physalins purified from *P. angulata* had suppressive effects on human leukemia K562 and APM1840 cell lines, and sarcoma cell lines (Chiang et al., 1992; Magalhaes et al., 2006), despite insufficient knowledge of its effect on melanoma. Additionally, physalins, including physalin B and F isolated from the plant *Witheringia solanacea* can induce apoptosis in the Jurkat leukemia cell line (Jacobo-Herrera et al., 2006). Physalin B was also reported to contribute to apoptosis and induce the proapoptotic protein NOXA in the DLD-1 4Ub-Luc colon cancer cell line (Vandenberghe et al., 2008).

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In numerous clinical cases, the melanoma cells had invaded deep into the dermis at the time of diagnosis. The prevalence of these patients is increasing rapidly and their survival rate is poor. Systemic therapy is the mainstay of therapy for most patients with stage IV melanoma. Chemotherapeutic agents with modest antitumor efficacy in metastatic melanoma include doxorubicin and dacarbazine. These agents have been used alone or in combination. Despite its modest efficacy and lack of data for survival benefit, dacarbazine continues to be the "standard treatment" for metastatic melanoma.

Ours preliminary studies have discovered that crude extract of *P. angulata* had an inhibitory effect on the *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF)-mutated melanoma A375 cell line at the concentration of 20 mg/ml, and with the bioactivity-guided fractionation approach, physalin B has been determined to be the main active compound (data not show). In attempt to evaluate the chemopreventive role of physalin B on cancer progress *in vitro*, we purified and identified physalin B, and assayed its activities on cellular toxicity and apoptosis against several cell lines. Our results revealed that physalin B can induce NOXA expression and then apoptosis in melanoma cells but not in nontumor cells. This study is critical in developing new therapeutic strategies for patients with melanoma.

2. Materials and methods

2.1. Compound preparation

The dried whole plants of *P. angulata* were provided from Tainan District Agricultural Research & Extension Station, COA, Tainan County, Taiwan, in June, 2007. The plant materials (3.5 kg) were extracted with methanol (MeOH) at room temperature and concentrated under reduced pressure. The MeOH extract (297 g) was partitioned between ethyl acetate (EtOAc) and H_2O to yield EtOAc and H_2O extracts. These extracts were evaporated to give dark-green viscous residues. The EtOAc extract was separated by silica gel column chromatography using a gradient of *n*-hexane–EtOAc–MeOH to yield 19 fractions. Fractions 8 (203.4 mg) and 9 (154.8 mg) were combined and then chromatographed on a silica gel column using *n*-hexane–EtOAc (2:1) as eluting solvent to obtain three fractions. Fraction (8 + 9)-2 was purified on preparative TLC using *n*-hexane–EtOAc (1:1) for elution and recrystallized from MeOH to yield physalin B (25.2 mg). The chemical structure of physalin B and its purity (>98%) was identified using mass spectrometry (MS), and nuclear magnetic resonance (NMR).

2.2. Cell line and cell culture

The human malignant melanoma cell lines (A375 and A2058) and rat cardiac myoblast cell line (H9c2) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, USA).

CCD-966SK cells, a human skin fibroblast, were maintained in minimum essential medium (Eagle) in Earle's BSS with 10% FBS, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate.

T/G HA-VSMC cells, a human normal aorta smooth muscles, were maintained in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate supplemented with 10 mM HEPES, and 0.03 mg/ml endothelial cell growth supplement (ECGS) with 10% FBS. The cells were placed in 10 cm dishes and grown at 37 °C in a humidified atmosphere comprised of 95% air and 5% CO_2. All cell lines used in this study are obtained from the Bioresources Collection and Research Center (BCRC, Taiwan).

2.3. Mitochondrial MTT reduction assay

The effect of physalin B on cytotoxicity was measured with a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco USA) assay according to standard procedure (Mosmann, 1983). Compounds were assayed for cytotoxicity against A375, A2058, H9c2, CCD-966SK and T/G HA-VSMC cells. The IC_{50} is the concentration of agent that reduces cell growth by 50% under experimental conditions. Physalin B was dissolved in DMSO to achieve a concentration of 0.1% for the final reaction volume.

2.4. Annexin V and PI analysis

Cells were incubated for 24 h with either DMSO (control) or physalin B (3 μg/ml). Upon washing with ice-cold PBS, the cells were collected and incubated in the dark with annexin V-FITC and propidium iodide (PI) for 15 min at room temperature, according to instructions from the manufacturer (BD Pharmingen). Late apoptotic or necrotic (annexin V and PI double positive) cells were examined using a flow cytometer (Becton Dickinson, San Jose, CA, USA). The physalin B induced apoptosis in A375 cells was morphologically identified using an inverted microscope (Axiovert 40; Carl Zeiss).

2.5. DNA relaxation experiments

To test whether cellular toxicity were related to topoisomerases inhibition, the effect physalin B on topoisomerase DNA relaxation was performed. Negatively supercoiled plasmid DNA (pUC119) is prepared using the Qiagen plasmid purification kit (Qiagen Corp.). DNA topoisomerase I was purchased from TaKaRa Biotechnology Co., Ltd. (TaKaRa, Japan), and bacteriophage T2 topoisomerase II is kindly provided by Nei-Li Chan, Ph.D. (National Taiwan University, Taipei, Taiwan). DNA relaxation assays were performed according to procedures described in previous studies (Osheroff et al., 1983). The DNA product was analyzed via electrophoresis. Electrophoresis was carried out in a 1% agarose gel at 50 V for 1 h. The agarose gel was stained with ethidium bromide and photographed under UV light. Camptothecin and 4-(9-acridinylamino) methanesulfonm-anisidide (*m*-AMSA) were used as positive controls for inhibiting topoisomerase I and II activity, respectively.

2.6. Western blot analysis

A375 and CCD-966SK cells in a 10 cm Petri dish were treated with physalin B. The cells pellets were harvested in 30 μl of lysis buffer containing 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40. The entire cell extracts (~30 μg) were resolved under denaturing conditions by SDS–polyacrylamide gel electrophoresis in 10–15% gradient gels. The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes by electroblotting and incubated overnight with anti-Apaf-1 (Becton Dickinson), anti-NOXA (Calbiochem), anti-procaspase 3 (Becton Dickinson), and anti-Bax (Becton Dickinson) monoclonal antibodies. A secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG) from Millipore was diluted at 1:2000 and incubated with the membrane for 2 h. Proteins were detected using an ECL Western blotting kit according to the manufacturer's instructions (Invitrogen). Bands corresponding to the protein levels were exposed to X-ray films and quantified using Quantity one (Bio-Rad, USA).

3. Results

3.1. Physalin B induced cell death in melanoma cancer cell lines but not in nontumor cells

Stage IV melanoma has limited chemotherapeutic agents choice and all the conventional chemotherapy is restricted by the toxicity of the drugs to normal tissues. To test whether physalin B could differentiate melanoma from nontumor cells, we selected the nontumor cell lines CCD-966SK, T/G HA-VSMC and H9c2 cells along with melanoma A375 and A2058 for cytotoxicity test. We isolated and purified physalins B from dried plant *P. angulata* L., and determined its chemical structure as shown in Fig. 1A. Its molecular formula was established as $C_{28}H_{30}O_9$ by electrospray ionization mass spectrometry (m/z 533 $[M+Na]^+$) and NMR spectra (in Supplementary Fig. 1 and legend). The most significant signals of its ^1H NMR spectrum were those corresponding to three tertiary methyl groups [δ 1.22 (3H, s, H-19), 1.27 (3H, s, H-28), 1.97 (3H, s, H-21)], a oxymethylene group [δ 3.77 (1H, d, J = 13.8 Hz, H-27), 4.53 (1H, dd, J = 13.8, 4.8 Hz, H-27)] and three olefinic protons [δ 5.58 (1H, br d, J = 5.6 Hz, H-6), 5.92 (1H, dd, J = 10.0, 2.0 Hz, H-2), 6.79 (1H, m, H-3)]. Results demonstrated that physalin B induced the cell death of A375 and A2058 cells in a time- and dose-dependent manner. The IC_{50} values for 24 h treatment are 3.8 and 4.6 μg/ml for the melanoma A375 and A2058, respectively. When incubated with 10 μg/ml of physalin B, the viability of the above two melanoma cells dropped to 10% for 24 h treatment and essentially no viable cell remained after 48 h. In contrast, the viability of human skin fibroblast cells CCD-966SK and T/G HA-VSMC were both approximately 90% and 80% after 24 and 48 h of treatment,

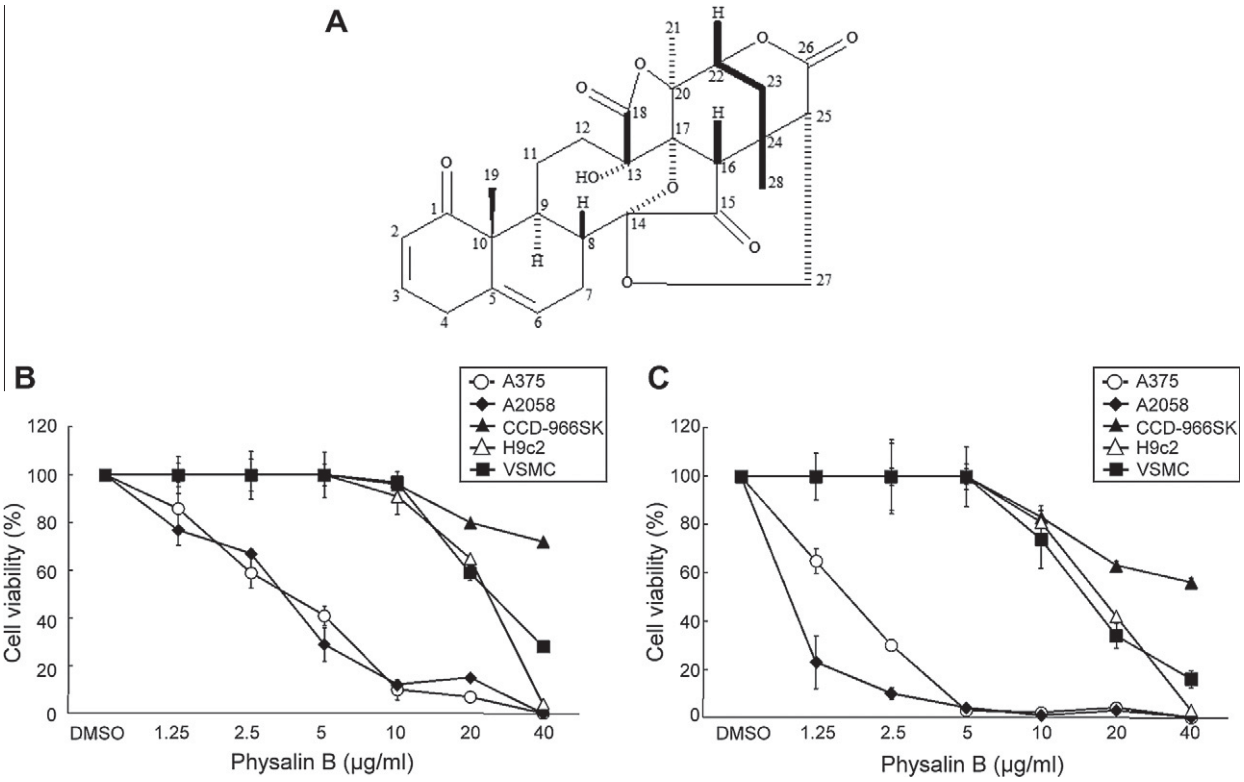

Fig. 1. The dose- and time-dependent cytotoxic effects of physalin B on melanoma and nontumor control cells using the MTT viability assay. (A) Chemical structure of physalin B. (B) A375 (○), A2058 (♦), CCD-966SK (▲), H9c2 (△) and T/G HA-VSMC (■) cells were treated with physalin B at indicated concentrations for 24 h. The final concentration of DMSO in the test medium was less than 0.1%. Tests for each concentration were performed three times. The values are expressed as a mean percentage of the control activity, and the error bar represents the standard deviation. (C) A375 (○), A2058 (♦), CCD-966SK (▲), H9c2 (△) and T/G HA-VSMC (■) cells were treated with physalin B at indicated concentrations for 48 h.

respectively. Conventional chemotherapeutic agent for melanoma such as doxorubicin was reported to be cardiotoxic (Shan et al., 1996), we therefore specifically test the effect of physalin B on a cardiomyoblast H9c2. When the embryonic rat heart-derived H9c2 cells were treated with physalin B, the IC_{50} values were 13 and 9 μg/ml for 24 and 48 h, respectively, approximately 4.5 times higher than melanoma cells (Fig. 1B and C). These results indicated that the cytotoxicity effect of physalin B was selective for melanoma cells.

3.2. Physalin B induced apoptosis of A375 cells

In attempt to evaluate the cause of cytotoxicity, we examined the appearance of apoptotic A375 cancer cells following treatment with 3 μg/ml of physalin B using flow cytometry. In early stage of apoptosis, the plasma membrane keeps its integrity and thus excludes viability dyes such as propidium iodide (PI), but the externalization of phosphatidylserine in the outer cell membrane enable the binding of annexin V. However, in late stage of apoptosis, the disintegration of cell membrane makes both annexin-V and PI stain positive. The total percentage of apoptotic cells (upper right panel for late stage plus lower right panel for early stage of apoptosis in Fig. 2A) increased from 3.28% in pre-treated A375 cells to 56.4% after physalin B treatment of 24 h (Fig. 2A). During the early process of apoptosis, cell shrinkage and chromatin condensation are visible by microscopy. Morphological change in physalin B treated A375 cells were also detected during apoptotic cells formation under a microscope. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed (Kerr et al., 1972). In untreated A375 and CCD-966SK cells, no change of morphology was detected after equivalent DMSO exposure (Fig. 2B). These results demonstrated that physalin B can induce apoptosis, and cellular morphological changes were observed during apoptotic cells formation.

3.3. The effect of physalin B on NOXA in A375 and CCD-966SK cells

When the melanoma A375 cells were treated with 3 μg/ml of physalin B for a 24 h duration, Western blot results showed that NOXA accumulation could be detected past 2 h, and reached a maximal level after 12 h. Physalin B slight increased the procaspase-3 protein expression after 24 h. NOXA was consistently induced in A375 cells significantly, in contrast to the absence of NOXA expression in the CCD-966SK human skin fibroblast cell line (Fig. 3A). A slight increase of procaspase-3 and Bax was observed after 24 h, however, no change of apoptotic protease-activating factor-1 (Apaf-1) level was detected before and after physalin B treatment (Fig. 3B).

3.4. The interaction of physalin B with DNA topoisomerases

The chemical structure of physalin B is similar to that of withangulatin A, another compound extracted from *P. angulata*. Previous studies showed that withangulatin A had an inhibitory effect for topoisomerase II (Juang et al., 1989). Inhibition of either type I or type II topoisomerases will induce DNA damage and thereafter trigger apoptosis, and topoisomerase inhibitors have long been used as anti-cancer drugs (Denny, 1989; Liu, 1989). Bacteriophage T2 and mammalian type II topoisomerases share conserved amino acid sequences and display similar enzymatic properties (Wyckoff et al., 1989). Our unpublished data demonstrated that crude extracts of *P. angulata* had a moderate inhibitory effect on T2

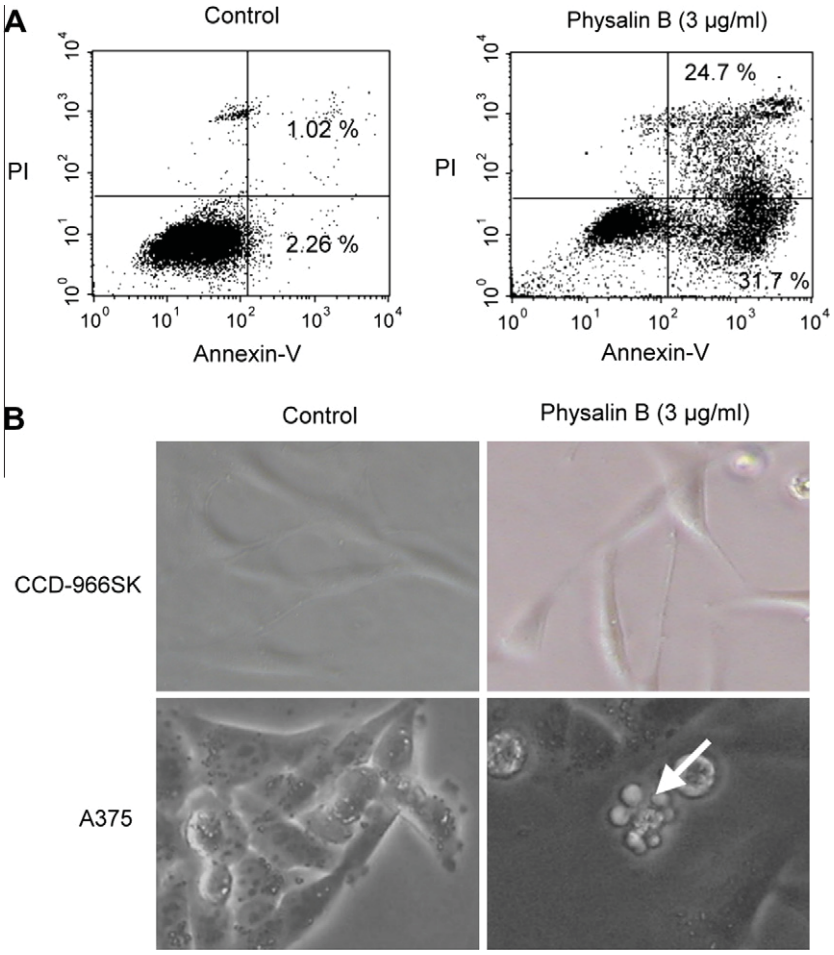

Fig. 2. Physalin B induces human melanoma A375 cells apoptosis. (A) Apoptosis was evaluated after treating A375 cells with 3 μg/ml of physalin B, and stained with annexin-V and PI at 24 h. Control represents the mock treatment with DMSO. The flow cytometry profile represents annexin-V-FITC staining in *x* axis and PI in *y* axis. (B) A375 and CCD-966SK cells were treated with either DMSO at 0.1% or physalin B at 3 μg/ml for 24 h and examined under a microscope. Arrow indicates the formation of an apoptotic cell.

topoisomerase at a concentration of 20 mg/ml (data not shown). We therefore examined the effect of purified physalin B on both type I and type II DNA topoisomerases. Our data showed that physalin B failed to affect the relaxation activity of either type I or II topoisomerase at various concentrations (Fig. 4). These results suggested that the apoptotic effect of physalin B was not through the effect of topoisomerases.

4. Discussion

The cancer chemopreventive agent is intended to eliminate malignant cells via cell cycle inhibition or induction of apoptosis, but with less or no toxicity to nontumor cells (Srivastava and Gupta, 2006; Stolarska et al., 2006). Doxorubicin is classified as a topoisomerase II poison and is one of the effective anti-tumor drugs including melanoma. However, its clinical use is limited by its cumulative dose-related cardiotoxicity, which can lead to severe and irreversible cardiomyopathy (Shan et al., 1996). This study demonstrated that physalin B could potently decrease the viability of *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF) mutated melanoma A375 cells, and this effect is potentially caused by the induction of apoptosis. In A375 cells, physalin B induced the activation of NOXA, caspase-3, and Bax, suggesting mitochondria-mediated apoptosis was involved in the physalin B-induced cytotoxicity. In addition, physalin B was also shown to be less cytotoxic for not only the CCD-966SK and T/G HA-VSMC cells, but also the cardiac myoblastic H9c2 cells.

Malignant melanoma is a serious skin cancer and the five-year survival rate is less than 10%. To date, there is still no effective therapeutic treatment for malignant melanoma. The current therapies for malignant melanoma are locoregional treatment modalities, such as surgery or radiation. However, the cornerstone of melanoma treatment is systemic chemotherapy. Approximately 65–70% of melanomas harbor a mutation in kinase BRAF. Previous studies have shown that PLX4032 (RO5185426; Plexxikon/Roche, Berkeley, CA) is a potential drug for melanoma treatment and could effectively inhibit the oncogenic BRAF gene mutating at the residue 600. An early phase 1 trial of this compound demonstrated that PLX4032 could result in complete or partial tumor regression in the majority of patients (Flaherty et al., 2010). Though the early response to PLX4032 appears to occur reliably, responsive tumors can develop resistance to the treatment. The mechanism of secondary tumor resistance is not yet known. Therefore, an effective drug for the treatment of malignant melanoma is absent from clinical practice. Our study demonstrated that physalin B significantly suppressed the survival of A375 and A2058 cells, but was less cytotoxic for CCD-966SK, T/G HA-VSMC and H9c2 cells. These suggested that physalin B has the advantage of preserving nontumor cells while confers the anti-cancer effect in BRAF-mutated melanoma cells.

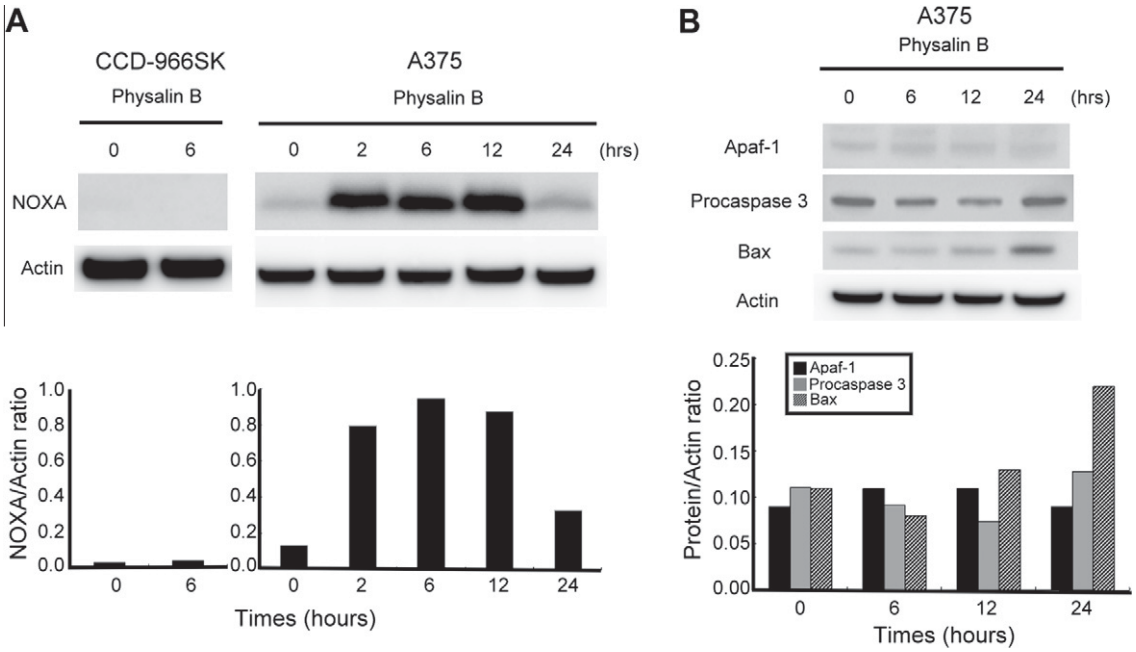

Fig. 3. The induction of apoptotic proteins by physalin B in A375 cells. (A) The control skin CCD-966SK cell line and melanoma A375 cells were exposed to physalin B (3 μg/ml) for 6 and 24 h, respectively. (B) The A375 cells were treated with 3 μg/ml of physalin B for 24 h. Whole cell lysate were extracted and immunoblotted for NOXA, Apaf-1, procaspase-3, and Bax as described in Section 2. The expression level of apoptotic proteins was the mean value of two independent experiments and normalized according to the actin protein.

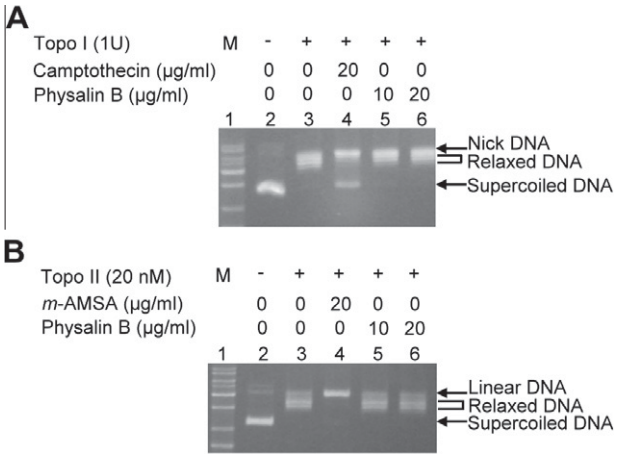

Fig. 4. Physalin B has no effect on DNA topoisomerases. (A) Supercoiled DNA was incubated with topoisomerase I (1 U) in the absence or presence of camptothecin and physalin B. (B) Supercoiled DNA was incubated with T2 topoisomerase (20 nM) in the absence or presence of *m*-AMSA and physalin B. The concentration of camptothecin and *m*-AMSA used here can strongly inhibit the relegation activity of topoisomerases, therefore generate nicked or linear DNA product as indicated by arrow heads. The DNA relaxation assay was performed as described in Section 2.

Previous studies determined numerous anti-cancer drugs which can suppress tumor progression through apoptotic pathways. The death receptor (extrinsic) and mitochondrial (intrinsic) pathways are two major apoptotic pathways that cause caspases activation. In the mitochondrial pathway, the BH3-only pro-apoptotic proteins known as NOXA act upstream, while the Bax family proteins act downstream in mitochondrial disruption. According to a published report (Nickoloff et al., 2005), gamma-secretase inhibitors (GSI) treatment induced NOXA protein in melanoma cells. Application of PLX4032 in malignant melanoma cell lines also suggests that increased NOXA protein facilitates the apoptosis (Landi et al., 2006). Moreover, physalin B can provoke the accumulation of NOXA protein in DLD-1 4Ub-Luc colon cancer cell line (Vandenberghe et al., 2008). However, no published results regarding the mechanism of physalin B on human melanoma cell line was reported yet. Previous studies also lacked information concerning the pharmacogenetic mechanism and selectivity of physalin B in melanoma and nontumor cells. We used physalin B to investigate its anticancer potential against human A375 and A2058 melanoma cells. A375 is a BRAF-mutated human malignant melanoma cells commonly used for studying the genomic effects of anti-cancer compounds (Tap et al., 2010). This study analyzed NOXA protein expression following physalin B treatment in A375 melanoma and human skin fibroblast cells, and the results demonstrated that upregulation of NOXA occurred in the initial stages following exposure of A375 cells to physalin B, but not in the CCD-966SK cells. These results suggest the apoptotic effect of physalin B is through NOXA expression and is selective for melanoma cells.

The induction of apoptosis by physalin B was demonstrated by using two independent methods, detections of cell apoptosis/necrosis (annexin V/PI double staining method), and detection of cellular apoptosis proteins (Western blot assay). Exposing the A375 cells with physalin B increased the early apoptotic cell population in a flow cytometric assay. Physalin B is a proteasome inhibitor in the ubiquitin/proteasome pathway (Vandenberghe et al., 2008). Recent evidence suggests that ubiquitin/proteasome inhibition triggers the mitochondrial signaling pathways, and plays an important role in apoptosis (Zhang et al., 2004). Apoptotic molecules, including pro-survival proteins Bcl-2 and Bcl-x, are regulated by the ubiquitin/proteasome pathway, which can therefore facilitate interaction with the pro-apoptotic proteins, Bax and Bak, located in the mitochondrial membrane. Therefore, this study implied that physalin B could inhibit the ubiquitin/proteasome pathway, and trigger mitochondrial signaling to cause apoptosis. A375 has a defect in tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) gene induction (Chawla-Sarkar et al., 2003); therefore, additive cellular responses from endogenous Apo2L/TRAIL could be avoided. Recent reports demonstrate that NOXA pathways converge on the intrinsic mitochondrial

pathway through direct or indirect interactions with Bax and/or Bak (Letai et al., 2002; Willis and Adams, 2005). The intrinsic pathway of apoptosis is triggered following mitochondrial damage, which leads to cytochrome *c* release, apoptosome formation and caspase activation (Hajra and Liu, 2004). Our data indicated that the mitochondrial pathway was involved in physalin B driven apoptosis.

Apoptotic process is mediated through the formation of apoptosome, a large, multimeric, caspase-9 activating complex containing seven molecules of Apaf-1, a cytochrome *c*, and ATP (Zou et al., 1999). Our study observed the formation of apoptotic cells, but not upregulation of Apaf-1 after physalin B treatment. Previous studies indicate that Apaf-1 is barely detectable in selected melanoma cell lines, suggesting that even extremely low Apaf-1 levels is sufficient for proteasome inhibitors to induce apoptosis (Nickoloff et al., 2005). Our findings are consistent with their results.

In conclusion, physalin B from *P. angulata* has selective cytotoxicity for human melanoma cells. Physalin B can induce apoptosis of melanoma cells through the NOXA, caspase-3, and mitochondria-mediated pathways. Therefore, physalin B could be developed as an effective chemotherapeutic lead compound for the treatment of malignant melanoma.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2011.12.017.

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