

# Crude saponins from *Platycodon grandiflorum* induce apoptotic cell death in RC-58T/h/SA#4 prostate cancer cells through the activation of caspase cascades and apoptosis-inducing factor

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Received October 16, 2012; Accepted December 13, 2012

DOI: 10.3892/or.2013.2256

**Abstract.** Saponins are a major active component of *Platycodon grandiflorum* (*P. grandiflorum*) and are known to induce apoptosis in metastatic prostate cancer cell lines. However, thus far, no research has been conducted on the anticancer activity of saponins in RC-58T/h/SA#4 primary prostate cancer cells. In this study, we show that the treatment of prostate cancer cells with saponins extracted from *P. grandiflorum* (SPG) inhibits cell proliferation in a dose-dependent manner. SPG significantly induced apoptotic cell death, resulting in an increase in the sub-G1 apoptotic cell population, apoptotic DNA fragmentation and morphological changes. Pre-treatment with a caspase inhibitor modestly attenuated the SPG-induced increase in the sub-G1 cell population, suggesting that caspases play a role in SPG-induced apoptosis. Moreover, SPG-induced apoptosis was associated with changes in caspase activity, the upregulation of the apoptotic protein, Bax and the downregulation of the anti-apoptotic protein, Bcl-2. Furthermore, the caspase-independent mitochondrial apoptosis factor, apoptosis-inducing factor (AIF) was upregulated following SPG treatment. These findings indicate that SPG exerts its anticancer effects on RC-58T/h/SA#4 primary prostate cancer cells through mitochondrial caspase-dependent and -independent apoptotic pathways.

## Introduction

Prostate cancer is the most commonly diagnosed cancer in males worldwide (1). Unlike other malignant tumors, prostate cancer is a very slow growing tumor and may not cause

pathognomonic signs or problems for years (2,3). For this reason, prostate cancer patients are diagnosed after symptoms of illness worsen substantially. The late detection of prostate cancer can lead to its wide spread to delicate organs such as the epithelium, spinal cord and brain; therefore, early suppression of prostate cancer progression is mandatory to prevent the development of degenerative symptoms (3).

To date, human prostate cell lines, such as PC-3, DU-145, and LNCaP, derived from bone, brain and lymph node metastases, are the most commonly used cell culture models of prostate cancer and have been used in almost all prostate cancer trials (4-7). However, it is particularly difficult to duplicate the genetic makeup or stimulate the bioactive behavior of primary tumors within these cell lines (8). Therefore, primary prostate tumor cell lines are urgently required to examine pre-clinical and early molecular prostate cancer lesions.

RC-58T/hTER is a well-characterized human cancer cell line derived from a primary tumor of a prostate cancer patient (9). Early passage RC-58T cells were transduced through infection with a retrovirus vector expressing human telomerase reverse transcriptase (hTER), and the original phenotypes of the primary cells were maintained with prostate-specific markers. Among these cell lines, the RC-58T/h/SA#4 cell line derived from soft agar was isolated and further characterized phenotypically and genetically (9-11).

The roots of *Platycodon grandiflorum* A. De Candolle (*P. grandiflorum*) have been used both as a food material and a traditional oriental medicine in Eastern Asia (12). *P. grandiflorum* contains various chemical compounds, including lignoceric acid, cerotic acid, n-octacosanoic acid and  $\alpha$ -monopalmitin, and triterpenoid saponins constitute its main bioactive component (13). These saponins are present in *P. grandiflorum* at approximately 1-4% and have a variety of medical applications (14-18). The extraction of crude saponins from *P. grandiflorum* generally involves the use of butanol as a solvent. However, since they are severely toxic to humans, crude saponins obtained by butanol extraction are not suitable for medical purposes or as health supplements (19). In this study, we aimed to separate the non-toxic and edible saponins by using a previously reported method that involves the use of Diaion HP-20 resin (20).

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**Key words:** *Platycodon grandiflorum*, apoptosis, primary prostate cancer cells, caspase, apoptosis-inducing factor

Saponins extracted from *P. grandiflorum* (SPG) have pharmacological activities, including anti-oxidative (21) and immunomodulatory activities (22). They can also ameliorate lipid metabolism of the liver in rats with hypercholesterolemia (23) and increase hemolytic activity and humoral immune responses in ICR mice (24). Previous studies have reported that saponins inhibit HT-1080 human fibrosarcoma cell invasion and matrix metalloproteinase (MMP) activity (25), induce apoptosis in MCF-7 human breast cancer cells (26), and suppress acrolein-induced MUC5AC expression by inhibiting the activation of NF- $\kappa$ B in A549 lung cancer cells (27). Although there are several reports on the chemical and biological characteristics of SPG, there have been no significant findings regarding its effect on primary prostate cancer cells.

Therefore, in the present study, we aimed to investigate the cytotoxicity and potential mechanisms of SPG obtained by using Diaion HP-20 resin in RC-58T/h/SA#4 primary human prostate cancer cells.

## Materials and methods

**Preparation of SPG.** The dried roots of *P. grandiflorum* (500 g) were pulverized and extracted with 80% EtOH 5 times. After the powder particles had settled down, the clear yellow supernatant was filtered with a 0.22- $\mu$ m pore size polytetrafluoroethylene (PTFE) filter, and concentrated by vacuum evaporation. The 80% EtOH extract was concentrated in a vacuum at 40°C to produce a residue (101.6 g), which was fractionated on Diaion HP-20 and eluted with H<sub>2</sub>O, 20% EtOH, and 100% EtOH, yielding 3 fractions.

**High performance thin layer chromatography (HPTLC) and liquid chromatography-mass spectrometry (LC-MS)/MS analysis.** Chromatography was performed on silica gel 60F254 HPTLC plates (20x10 cm; 0.25-mm layer thickness). The chromatograms were evaluated by Camag densitometry (Camag model-3 TLC scanner equipped with Camag CATS 4 software). The slit was set 15x8 mm and data acquisition and processing were performed using CAT windows software. Samples (10  $\mu$ l) were applied to layers at 8-mm wide bands, positioned 10 mm from the bottom of the plate, using a Camag Linomat IV automated TLC applicator with nitrogen flow providing delivery from the syringe at a speed of 10  $\mu$ l/sec, maintained for all analyses. TLC plate development was performed using a Camag Automated Multiple Development system. The solvent front was allowed to rise to a height of 14 cm. TLC analyses were performed at room temperature. A mixture of butanol:ethyl acetate:distilled water (50:10:40) was used as the mobile phase; after development, the layers were dried and the components were visualized under UV light.

**Cell culture and proliferation.** The RC-58T/h/SA#4 (primary prostate cancer cells; androgen-positive cells) cells and RWPE-1 (human prostate epithelial cells) cells were obtained from the Center for Prostate Disease Research (CPDR). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml) (Gibco BRL, Life Technologies, Grand Island, NY, USA) in an incubator containing a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

Cell proliferation was determined by sulforhodamine B (SRB; Sigma, St. Louis, MO, USA) assay. The cancer cells were seeded at a concentration of 1x10<sup>5</sup> cells/well in 24-well tissue culture plates and incubated with various concentrations of SPG for 24 h. Following treatment, the medium was aspirated and 10% trichloro-acetic acid was added. After a 1-h incubation at 4°C, the plate was washed 5 times with DW-R10 dry wash resin and air-dried. The cells were stained with 0.4% (w/v) SRB at room temperature for 1 h and then washed 5 times using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris, and the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA).

**Cell cycle analysis for sub-G1 population.** Cells were seeded at a density of 1x10<sup>6</sup> cells per well in 6-well plates and cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of SPG for 24 h. The cells were then collected and fixed in ice-cold 70% ethanol in medium and stored at 4°C overnight. After resuspension, the cells were washed and incubated with 1  $\mu$ l of RNase (1 mg/ml) (Sigma), 20  $\mu$ l of propidium iodide (PI; 1 mg/ml) (Sigma), and 500  $\mu$ l of PBS at 37°C for 30 min. After staining, flow cytometry was used to analyze the sub-G1 DNA content.

**Detection of morphological apoptosis.** Morphological changes characteristic of apoptosis were assessed by fluorescence microscopy using bis-benzimide (Hoechst 33258) staining. Briefly, the cells were seeded in 6-well plates at a density of 1x10<sup>6</sup> cells per well, followed by treatment with SPG for 24 h. After harvesting, the cells were washed twice with PBS and then stained with 200  $\mu$ l of bis-benzimide (5  $\mu$ g/ml) for 10 min at room temperature. Subsequently, 10  $\mu$ l of this suspension were placed on a glass slide and covered with a cover slip. The cells were examined under a fluorescence microscope (Olympus Optical Co. Ltd., Japan) to determine nuclei fragmentation and chromatin condensation.

**Analysis of DNA fragmentation.** The cells were seeded at a density of 1x10<sup>7</sup> cells in a 100-mm dish and cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of SPG for 24 h, followed by centrifugation. The pellets were lysed by lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0, 0.5% Triton X-100, 20% SDS, and 10 mg/ml of proteinase K) and then centrifuged. After extraction with phenol:chloroform:isoamyl alcohol (25:24:1), DNA was precipitated with 2 vol of cold absolute ethanol. The resulting pellets were incubated with TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) and RNase (2 mg/ml) for 1 h at 37°C. Separation by electrophoresis was then performed on 2% agarose containing ethidium bromide. The resulting DNA bands were examined using a UV Transilluminator Imaging System.

**Assay for caspase activity.** Cells were seeded at a density of 5x10<sup>5</sup> cells/well and then cultured for 24 h in DMEM. The cells were then pre-incubated with z-VAD-fmk for 2 h, followed by treatment with the indicated concentrations of SPG for 24 h. For growth inhibition analysis and measurement of sub-G1 DNA content, the cells were collected and fixed in ice-cold 70%

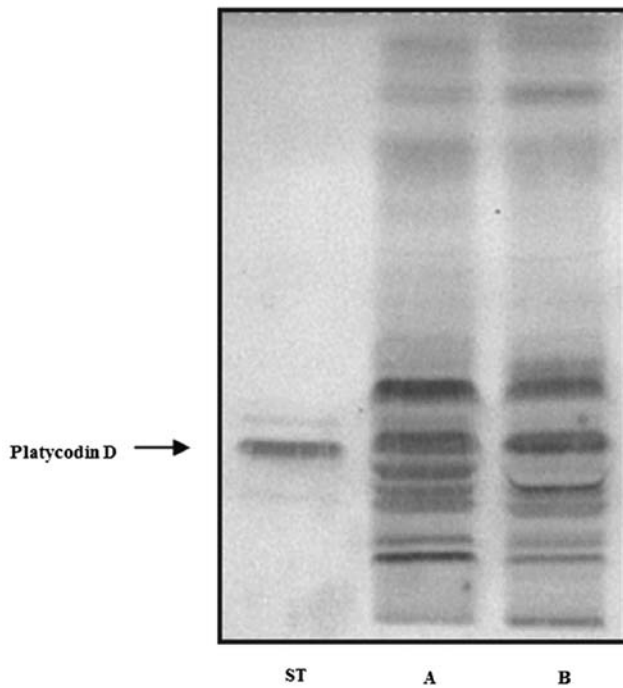


Figure 1. TLC pattern of platycodon saponins prepared by different methods. ST, standard; lane A, Diaion HP-20 adsorption; lane B, butanol extraction. Developing solvent, BuOH:EtOAc:H<sub>2</sub>O [50:10:40 (v/v)]. Spray reagent, 10% H<sub>2</sub>SO<sub>4</sub>.

ethanol in medium, followed by storage at 4°C overnight. After resuspension, the cells were washed and incubated with 1 µl of RNase (1 mg/ml) (Sigma), 20 µl of PI (1 mg/ml), and 500 µl of PBS at 37°C for 30 min. After staining, flow cytometry was carried out to analyze the sub-G1 DNA content.

**Assay for caspase inhibitor activity.** The cells were seeded at a densities of 1x10<sup>5</sup> cells per well in a 24-well plate and 1x10<sup>6</sup> cells per well in a 6-well plate, then cultured for 24 h in DMEM. The cells were pre-incubated with z-VAD-fmk for 2 h and then treated with the indicated concentrations of SPG for 24 h. The cells were collected and counted using trypan blue dye (Gibco BRL), and were then fixed in ice-cold 70% ethanol in medium, and then stored at 4°C overnight. After resuspension, the cells were washed and incubated with 1 µl of RNase (1 mg/ml) (Sigma), 20 µl of PI (1 mg/ml) (Sigma), and 500 µl of PBS at 37°C for 30 min. After staining, flow cytometry was used to analyze the sub-G1 DNA content.

**Western blot analysis.** The cells were seeded at a density of 1x10<sup>7</sup> cells in a 100-mm dish, and then cultured for 24 h in DMEM. After culturing, the cells were treated with the indicated concentrations of SPG for 24 h, followed by centrifugation. The resulting pellets were lysed by lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM PMSF and 2 µg/ml of aprotinin) for 30 min on ice. The protein content of the supernatant was measured using the BCA protein kit (Pierce, Rockford, IL, USA). The protein samples were then loaded at 10 µg of protein/lane and then separated by 12% SDS-PAGE at 100 V of constant voltage/slab for 1.5 h. Following electrophoresis, the proteins were

Table I. Platycodin D contents in crude saponins prepared by LC/MS/MS.

Method	Platycodin D (mg/g)
Diaion HP-20 method	25.1±1.54
Butanol method	14.1±1.78

transferred onto nitrocellulose membranes. After blocking with 2.5 and 5% bovine serum albumin (BSA) for 1 h at 37°C, the membranes were incubated with primary antibody [anti-Bid, anti-Bax, anti-Bcl-2, anti-poly(ADP-ribose) polymerase (PARP) and anti-apoptosis-inducing factor (AIF)] at 4°C overnight. Finally, the membranes were treated with horseradish peroxidase-coupled secondary antibodies for 1 h at 4°C. The membranes were then washed with T-TBS after each antibody binding reaction. Detection of each protein was performed using an ECL kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

**AIF translocation.** RC-58T/h/SA#4 cells were seeded in 6-well plates at seeding densities of 1x10<sup>6</sup> cells per well, followed by treatment with SPG for 24 h. After harvesting, the cells were washed twice with PBS and then blocked with blocking buffer (2% BSA in T-TBS) for 1 h. The cells were incubated with AIF primary antibody overnight at 4°C, followed by anti-rabbit secondary antibody for 1 h. AIF translocation was analyzed under a fluorescence microscope (Olympus Optical Co., Ltd.).

**Statistical analysis.** The data were analyzed using the Student's t-test to evaluate significant differences. Levels of \*p<0.05 and \*\*p<0.01 were considered to indicate statistically significant differences.

## Results

**Contents of platycodin D were determined by HPTLC and LC-MS/MS analysis.** Platycodin D is a major constituent of triterpene saponin in platycodon saponins (28). We initially confirmed the platycodin D contents of SPG using HPTLC and LC-MS/MS. TLC comparison of saponin fractions prepared by Diaion HP-20 and the butanol method showed similar patterns, and they contained platycodin D (Fig. 1). In addition, saponin fractions prepared by the Diaion HP-20 adsorption method showed a higher content of platycodin D (25.1 mg/g) compared to those prepared by the butanol method (14.1 mg/g) (Table I). These results suggested that SPG prepared by the Diaion HP-20 adsorption method was purer compared to the saponins prepared by the butanol method.

**SPG inhibits RC-58T/h/SA#4 human prostate cancer cell growth.** We investigated the IC<sub>50</sub> value of SPG against human prostate cancer cells (RC-58T/h/SA#4, PC-3 and LNCap.FGC) and normal cells (RWPE-1). These cells were treated with 10, 30, 50 and 80 µg/ml SPG for 24, 48 and 72 h. As shown in Table II, SPG treatment decreased the IC<sub>50</sub> value in RC-58T/h/SA#4 cells in a time-dependent manner. Although the IC<sub>50</sub> value of SPG in the RC-58T/h/SA#4 cells was higher than

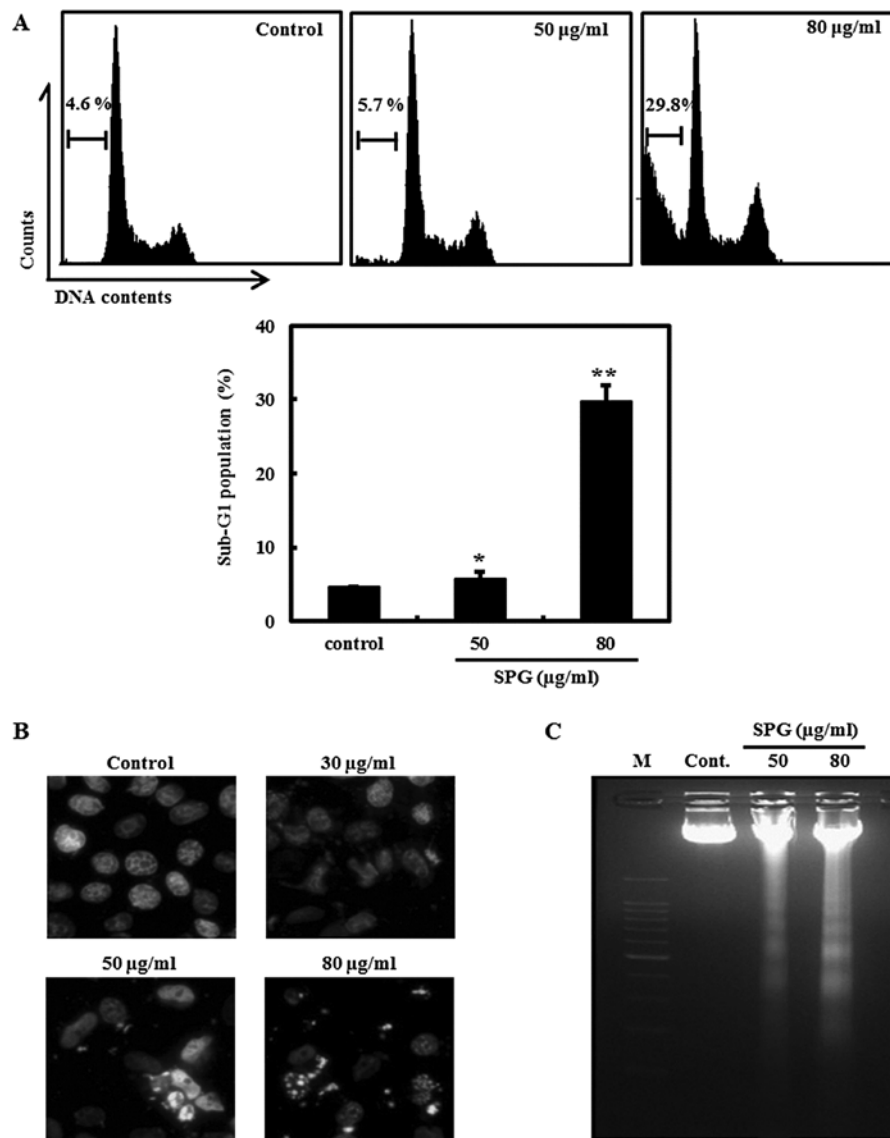


Figure 2. Induction of apoptotic cell death in RC-58T/h/SA#4 human prostate cancer cells by SPG. Cells were treated with or without SPG for 24 h. (A) Quantitative flow cytometric analysis of apoptotic cell death induced by SPG. Significant differences were compared with the control at  $^{**}p < 0.01$  using the Student's t-test. (B) Representative images of nuclear condensation in response to SPG treatment as detected by Hoechst staining assay. (C) DNA fragmentation was observed by 2% agarose gel electrophoresis. M, 100 bp DNA marker.

Table II.  $IC_{50}$  values of saponins isolated from *Platycodon grandiflorum* A. De Candolle in the 4 cell lines.

Cell line	$IC_{50}$ ( $\mu\text{g/ml}$ )		
	24 h	48 h	72 h
RWPE-1	62.81 $\pm$ 4.23	58.07 $\pm$ 3.46	45.25 $\pm$ 6.03
RC-58T/h/SA#4	49.86 $\pm$ 3.63	44.61 $\pm$ 1.01	37.04 $\pm$ 0.96
LNCap.FGC	38.33 $\pm$ 1.23	37.56 $\pm$ 0.97	33.58 $\pm$ 2.11
PC-3	48.33 $\pm$ 0.97	37.3 $\pm$ 0.38	28.84 $\pm$ 0.38

Data values are expressed as the means  $\pm$  SD of triplicate determination.

that in the PC-3 and LNCap.FGC cells, the RC-58T/h/SA#4 cells were found to be more sensitive to SPG as compared to RWPE-1 human prostate epithelial cells.

*SPG leads to apoptosis in RC-58T/h/SA#4 cells.* In order to determine whether SPG induces apoptosis in RC-58T/h/SA#4 cells, we performed flow cytometry, Hoechst staining and a DNA fragmentation assay. As shown in Fig. 2A, the proportion of the sub-G1 peak was negligible for the control RC-58T/h/SA#4 cells not treated with SPG, whereas the exposure of RC-58T/h/SA#4 cells for 24 h to 50 and 80  $\mu\text{g/ml}$  SPG resulted in dose-dependent accumulation of cells in the sub-G1 phase.

Induction of apoptosis by SPG was further examined using Hoechst staining and DNA fragmentation assay. After SPG treatment for 24 h, the morphological characteristics of apoptosis were observed in the RC-58T/h/SA#4 cells treated with 50 and 80  $\mu\text{g/ml}$  SPG, while the untreated cells did not show these characteristics (Fig. 2B). DNA fragmentation, another distinct feature of apoptosis, was observed in the RC-58T/h/SA#4 cells after treatment with SPG at concentrations of 50 and 80  $\mu\text{g/ml}$ , while the untreated cells did not show the

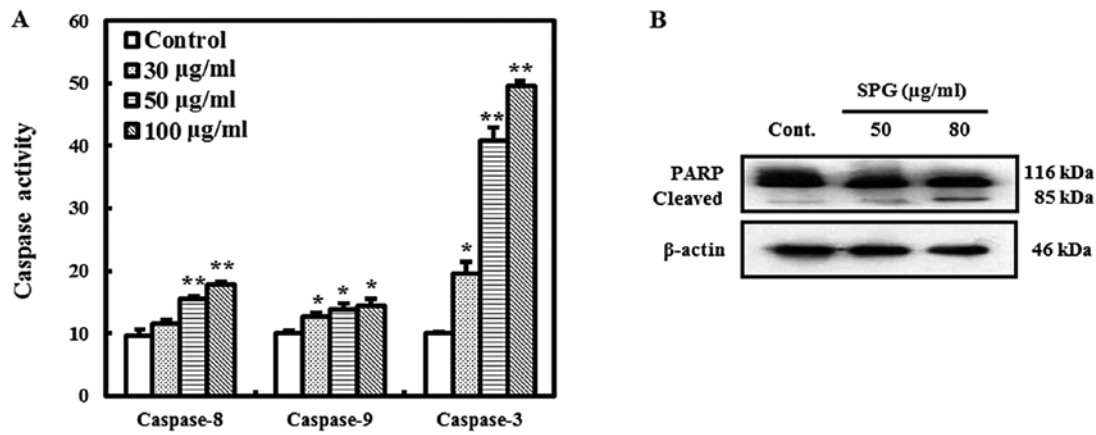


Figure 3. Effect of SPG on activation of caspases and PARP. (A) RC58T/h/SA#4 cells were treated with SPG for 24 h. Data values are expressed as the means  $\pm$  SD of triplicate determinations. Significant differences were compared with the control at \* $p < 0.05$  and \*\* $p < 0.01$  by Student's t-tests. (B) Apoptotic PARP cleavage in RC58T/h/SA#4 cells treated with SPG for 48 h as shown by western blot analysis.

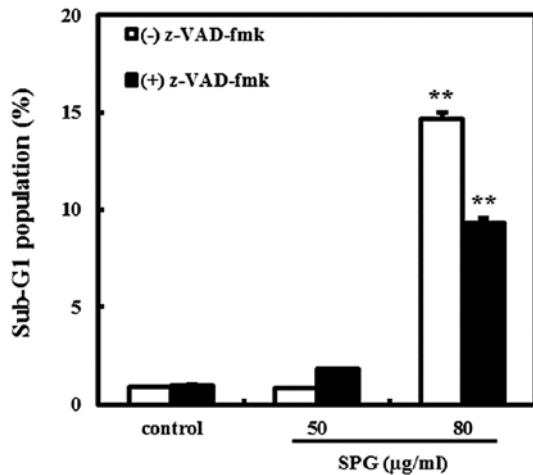


Figure 4. Effect of caspase inhibitor (z-VAD-fmk) on cell death induced by SPG. RC58T/h/SA#4 cells were pre-incubated with 2  $\mu$ M z-VAD-fmk for 2 h, followed by treatment with SPG. Variation in sub-G1 content in cells treated with or without z-VAD-fmk. Data values are expressed as the means  $\pm$  SD of triplicate determinations. Significant differences were compared with cells treated with or without z-VAD-fmk at \* $p < 0.05$  and \*\* $p < 0.01$  by Student's t-tests.

ladder formation (Fig. 2C). These results indicate that SPG induces apoptosis in RC-58T/h/SA#4 cells.

**SPG induces apoptosis through caspase activity.** To confirm the importance of caspase activation in SPG-induced apoptosis, the activities of initiator caspases (caspase-8 and -9) and the effector caspase (caspase-3) were investigated. As shown in Fig. 3A, SPG markedly induced the activities of caspase-8, -9, and -3 at concentrations of 30, 50, and 80  $\mu$ g/ml in the RC-58T/h/SA#4 cells. Furthermore, PARP, a family of proteins involved in DNA repair and apoptosis, was identified by its predicted cleavage product of 89 kDa following SPG treatment in the RC-58T/h/SA#4 cells, but not in the untreated cells (Fig. 3B). These data indicate that SPG-induced apoptosis occurs through caspase activation.

**Caspase inhibitor reduces SPG-induced apoptosis.** In order to elucidate whether the apoptosis induced by SPG is involved

in the activation of caspases, we used a universal caspase inhibitor, z-VAD-fmk. As shown in Fig. 4, the percentage of cells in the sub-G1 phase was almost 15% at an SPG concentration of 80  $\mu$ g/ml in the absence of z-VAD-fmk, whereas approximately 10% of cells were in the sub-G1 phase in the presence of z-VAD-fmk (10  $\mu$ M). These data indicate that the activation of caspases is involved in SPG-induced apoptosis in RC-58T/h/SA#4 cells.

**SPG induces apoptosis through activation of the mitochondrial pathway.** Caspases, particularly caspase-8, are thought to regulate the expression of proteins involved in the mitochondrial pathway, such as Bid, Bcl-2 and Bax. Therefore, we determined whether Bid, a pro-apoptotic Bcl-2 family member, plays a role in mitochondrial damage caused by SPG. As shown in Fig. 5A, full-size Bid (22 kDa) was cleaved to yield a 15-kDa fragment (tBid) following the treatment of cells with SPG. In addition, SPG treatment resulted in the upregulation of Bax and the downregulation of Bcl-2 expression (Fig. 5B). These data indicate that apoptosis induced by SPG is involved in the expression of mitochondrial-related proteins, indicating that SPG induces apoptosis via mitochondrial pathways.

**SPG stimulates release of AIF from the mitochondria.** Mitochondrial pathways and the Bcl-2 family are thought to induce the expression of AIF upon treatment with SPG (29,30). Thus, we examined whether SPG can modulate the level of AIF in RC-58T/h/SA#4 prostate cancer cells. As shown in Fig. 6A, AIF levels gradually increased following SPG treatment. In addition, the transfer of AIF from the mitochondria to the nucleus was detected by immunostaining (Fig. 6B). These results suggest that AIF translocation into the nucleus is required for SPG-induced apoptosis in RC-58T/h/SA#4 cells.

## Discussion

Representative Saponins extracted from *P. grandiflorum* (SPG) have been identified as platycodin D, platycodin D2, platycodin D3, platycoside A, platycoside E, deapioplatycoside D and polygalacin D2 (31). Using these saponins, a number of previous studies have shown apoptosis induction in metastatic

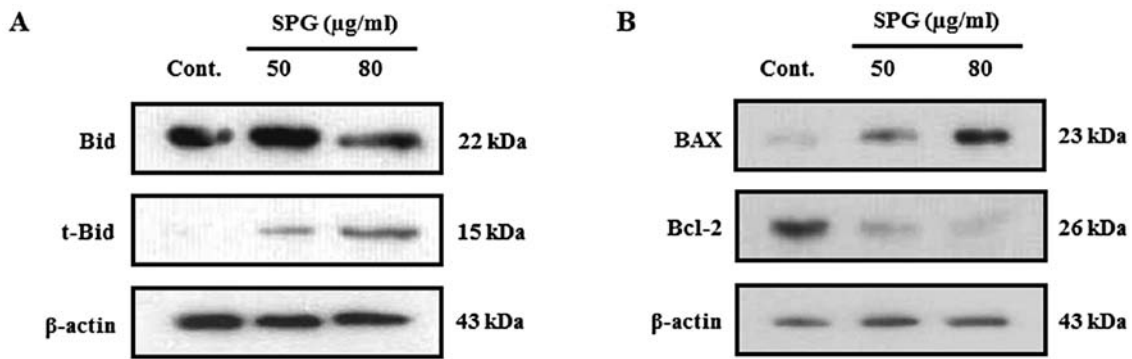


Figure 5. Western blot analysis of the expression of Bid (A) and Bcl-2 (B) family proteins in RC58T/h/SA#4 cells treated with SPG. Cells were treated with or without SPG for 24 h, after which the cells were harvested and lysed. Protein lysates were subjected to 12% SDS-PAGE and then immunoblotted with corresponding antibodies.

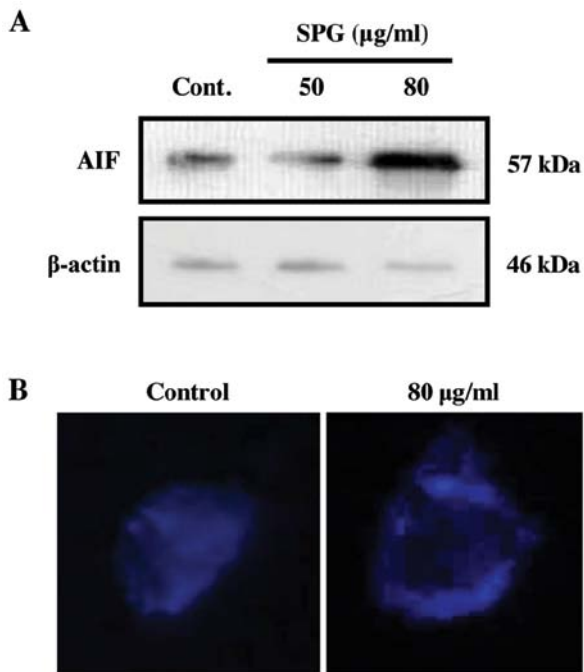


Figure 6. Expression level of AIF protein and representative confocal image of AIF translocation into the nucleus in RC58T/h/SA#4 cells treated with SPG. (A) Expression level of AIF protein in SPG-treated RC58T/h/SA#4 cells after 24 h. An equal quantity of proteins was analyzed by 12% SDS-PAGE. Cell fractions were isolated and analyzed by western blot analysis using anti-AIF antibody. (B) Representative images of the translocation of AIF into the nucleus in SPG-treated RC58T/h/SA#4 cells.

prostate cancer cell lines (32-35). However, the anti-cancer activity in RC-58T/h/SA#4 primary prostate cancer cells has not yet been investigated. In this study, to our knowledge, we are the first to report the growth-inhibitory effect and apoptosis mechanism of SPG in primary human prostate cancer cells (RC-58T/h/SA#4).

Apoptotic cell death is a genetically programmed mechanism that allows cells to commit suicide. Apoptosis is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation. It is a cell-intrinsic, programmed suicide mechanism that results in the controlled breakdown of the cell into apoptotic bodies (36,37). Previous studies have reported that triterpenoid saponins have a cytotoxic effect on

tumor cells, including prostate cancer cells (33). The present study demonstrates that SPG is an efficient growth inhibitor in RC-58T/h/SA#4 cells. Treatment with SPG decreased the proliferation of RC-58T/h/SA#4 cells, which were found to be more sensitive to SPG than RWPE-1 human prostate epithelial cells. RC-58T/h/SA#4 cells treated with SPG had clearly increased sub-G1 populations and DNA fragmentation patterns. The presence of condensed nuclei and apoptotic bodies was confirmed by Hoechst 33258 staining in the SPG-treated RC-58T/h/SA#4 cells. Taken together, these data indicate that SPG inhibits RC-58T/h/SA#4 prostate cancer cell proliferation and induces apoptosis in a dose- and time-dependent manner.

Our results are in agreement with those from previous studies describing the antiproliferative and apoptosis-inducing effects of platycodin D on colon cancer HT-29 cells (30), immortalized keratinocytes (5), and breast cancer MCF-7 cells (26). Platycodin D is the major constituent of SPG. Upon performing TLC for a comparison of the saponins extracted from *P. grandiflorum*, we detected platycodin D, which was quantified by performing LC-MS/MS; we observed 3.01% platycodin D. Our results suggest that platycodin D may be the majorly active saponin in the SPG-induced apoptosis of RC-58T/h/SA#4 cells. However, future studies are required to determine which saponin compounds of SPG are involved in its antiproliferative effect on RC-58T/h/SA#4 cancer cells.

During the process of apoptosis, caspases, a family of cysteine-dependent proteases, are essential for the activation of cell death in response to various stimuli (38). Previous studies on apoptosis signaling mechanisms mainly support the hypothesis of the existence of caspase-dependent pathways leading to cell death: an extrinsic (death receptor) pathway leads to the activation of caspase-8 or -10, which then activates downstream effector caspases capable of PARP cleavage, such as caspase-3 and -7; and an intrinsic (mitochondria) pathway for apoptosis is triggered by various cellular stress stimuli (39,40). In this pathway, mitochondria release apoptotic proteins including cytochrome *c*, which activates caspase-9, and is regulated by the Bcl-2 family (41-44). In this study, we demonstrated that SPG induced PARP cleavage and an increase in the number of apoptotic cells promoted by the activation of caspase-8, -9, and -3. Furthermore, treatment with a pan-caspase inhibitor (*z*-VAD-fmk) markedly blocked the apoptotic cell death in SPG-treated RC-58T/h/SA#4 cells. These results clearly indi-

cate that SPG-induced apoptosis is associated with caspase activation, which involves the degradation of the 116-kDa PARP into 85-kDa fragments.

The mitochondrial pathway for apoptotic cell death is governed by members of the Bcl-2 family. These proteins mediate mitochondrial outer-membrane permeabilization and act as pro-apoptotic (Bid, Bad, Bim and Puma) or anti-apoptotic (Bcl-2, Bcl-xL, Bcl-W and Bcl-B) regulators (45). Bid, a family that links the Bcl-2 family members and caspases is a pro-apoptotic Bcl-2 family containing only the BH3 domain (46). Several studies have demonstrated that Bid is cleaved by caspase-8 following death-receptor stimulation (45). We found that the expression of full-length Bid decreased following SPG treatment, and this led to the cleavage of Bid to tBid. Our results also showed that SPG inhibited Bcl-2 expression and increased Bax expression, leading to the decrease in the Bcl-2/Bax ratio. The Bcl-2/Bax ratio is one of the critical factors determining whether the cell will undergo apoptosis (47). Taken together, these data suggest that SPG-induced apoptosis leads to the downregulation of Bcl-2 expression and the upregulation of Bax expression, which induces outer mitochondrial membrane permeabilization and the loss of mitochondrial potential in the apoptotic death of RC-58T/h/SA#4 cells.

Previous studies have demonstrated that apoptosis occurs through either a caspase-dependent or -independent pathway in prostate cancer cells (48,49). AIF, a hallmark of caspase-independent apoptosis, is translocated from the mitochondrial intermembrane space to the cytosol, as well as to the nucleus during apoptosis (50). Our data show that SPG treatment induces AIF release from the mitochondria, which supports the contention that SPG induces caspase-independent apoptosis. Therefore, the results from the present study suggest that SPG induces at least 2 different signaling pathways in RC-58T/h/SA#4 cell death.

In conclusion, to our knowledge, our study is the first to demonstrate the effectiveness of SPG as an AIF in primary human prostate cancer cells (RC-58T/h/SA#4). We confirmed that the apoptotic mechanisms of SPG are mediated by caspase-dependent and -independent pathways in RC-58T/h/SA#4 cells. These findings uncover a basic mechanism for the anticancer properties of SPG and suggest that SPG can be used in pre-clinical strategies, such as chemoprevention, chemotherapy and cytotoxin therapy for the treatment of early prostate cancer.

### Acknowledgements

The authors wish to thank Dr John S. Rhim of the Center for Prostate Disease Research (CPDR) for providing the RC-58T/h/SA#4 primary human prostate cancer cells and RWPE-1 human prostate epithelial cells.

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