20(S)-Protopanaxadiol Triggers Mitochondrial-Mediated Apoptosis in Human Lung Adenocarcinoma A549 Cells via Inhibiting the PI3K/Akt Signaling Pathway

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Abstract: 20(S)-Protopanaxadiol (PPD), an aglycone saponin ginsenoside isolated from Panax quinquefolium L, has been shown to inhibit the growth and proliferation in several cancer lines. However, the underlying molecular mechanisms remain poorly understood. In this study, we investigated the apoptosis-induced effects and the mechanism of 20(S)-PPD on human lung adenocarcinoma A549 cells. 20(S)-PPD showed a potent antiproliferative activity against A549 cells by triggering apoptosis. 20(S)-PPD-induced apoptosis was characterized by a dose-dependent loss of the mitochondrial membrane, release of cytochrome c, second mitochondria-derived activator of caspase (Smac) and apoptosis-inducing factor (AIF), activation of caspase-9/-3, and cleavage of poly (ADP-ribose) polymerase (PARP). Caspase-dependence was indicated by the ability of the pan-caspase inhibitor z-VAD-fmk to attenuate 20(S)-PPD-induced apoptosis. After treatment with 20(S)-PPD, the proportion of A549 cells at the G0/G1 phase increased, while cells at the S and G2/M phases decreased. Furthermore, 20(S)-PPD also triggered down-regulation of phosphorylated Akt (Ser473/Thr308) and glycogen synthase kinase 3β (GSK 3β). Knockdown of GSK 3β with siRNA promoted the apoptotic effects of 20(S)-PPD. These results revealed an unexpected mechanism of action for this unique ginsenoside: triggering a mitochondrial-mediated, caspase-dependent apoptosis via down-regulation of the PI3K/Akt signaling pathway in...
A549 cells. Our findings encourage further studies of 20(S)-PPD as a promising chemopreventive agent against lung cancer.

Keywords: 20(S)-Protopanaxadiol; Lung Cancer; Apoptosis; Mitochondria; PI3K/Akt Pathway.

Introduction

Lung cancer is the second most common fatal cancer in the United States, with 222,520 new cases and 157,300 deaths reported in 2010 (Jemal et al., 2010). In China, the highest predicted increase of cancer cases is that of lung cancer, with the population of male lung cancer patients increasing from 26,000 in 2000 to 33,000 in 2005. According to recent statistics, approximately 80% lung cancer cases is non-small cell lung cancer (NSCLC). Lung adenocarcinoma is the most common histological type comprising about 60% of NSCLC, and a majority of patients presented in advanced stage (Hoffman et al., 2000). Chemotherapy is an important therapeutic strategy for cancer treatment and remains the mainstay for the management of human malignancies; however, radiotherapy and chemotherapy fails to eliminate all tumor cells because of intrinsic or acquired drug resistance, which is the most common cause of tumor recurrence (Wang et al., 2010).

The PI3K/Akt pathway plays a pivotal role in the initiation and progression of malignancies such as breast, lung, melanoma and leukemia, enhancing cell survival by stimulating cell proliferation and inhibiting apoptosis (Attele et al., 1999; Lin et al., 2001). Akt acts to promote cell proliferation and survival, and regulates multiple signaling pathways such as maintaining cell cycle, proliferation, and resistance to apoptosis. The activation of Akt is highly prevalent and a poor prognostic factor for type of NSCLC, and this signaling pathway may be a potential target for cancer chemotherapy and radiotherapy.

Ginseng, the root and rhizomes of different Panax species (Araliaceae), has been used as a traditional herbal medicine in East Asian countries for thousands of years, and now is famous medicine widely in Europe and North America for therapeutic purposes (Attele et al., 1999; Lee et al., 2011). The major pharmacological active ingredients of ginseng are ginsenosides, saponin-like compounds, and have been responsible for many biological activities including anti-allergic, anti-inflammatory, anti-depressant and anti-tumor, et al. (Xu et al., 2010; Wang et al., 2012). 20(S)-Protopanaxadiol (PPD) is an active ginseng metabolite, which is the final form of protopanaxadiol saponins metabolized by human intestinal microflora (Fig. 1A) (Kitts et al., 2000). We have screened several lung cancer lines in humans, including A549, H441 (K-Ras mutation), PC-9, HCC827 (EGFR mutation), SPC-A1, H292 (non K-Ras and EGFR mutation), Calu-3, H1299, and LETP-a-2. However, not all types of tumor cells were targeted by 20(S)-PPD, excluding the adenocarcinoma A549, Calu-3, and SPC-A1. During evaluation of the three cell lines, the IC₅₀ value of 20(S)-PPD was estimated lower in the adenocarcinoma A549 cells. Based on above results, we have gained invention patent (patent number: ZL02146549.5) about the anti-cancer effect. At present, 20(S)-PPD has been developed into a Chinese medicine,
named “Yijinsheng Capsule”, to assist radiotherapy and chemotherapy, currently in clinical stage 3. However, the detailed molecular mechanisms of apoptosis induced by 20(S)-PPD on human lung adenocarcinoma A549 cells remains largely unknown.

This present study was undertaken to investigate the molecular mechanism of 20(S)-PPD that contributed to an apoptosis via the mitochondrial death pathway and inhibited the phosphorylation of Akt and its substrate GSK3 was probably involved in this effect.

Materials and Methods

Chemicals

20(S)-Protopanaxadiol (PPD) was provided from Hainan Asia Pharmaceutical Co. Ltd., (China). The purity of 20(S)-PPD used in experiments was >95% as determined by HPLC. Antibodies against Akt, phospho-Akt (Ser^{473}/Thr^{308}), pro-caspase-3/9, capase-3/9, poly (ADP-ribose) polymerase (PARP), apoptosis-inducing factor (AIF), Second mitochondria-derived activator of caspase (Smac), cytochrome c, Bcl-2, Bcl-XL, Bax, Bad, glycogen synthase kinase 3β (GSK 3β), Forkhead-related transcription factor (FKHR), LY294002 (a PI3K inhibitor), GSK 3β siRNA, and negative control RNA were purchased from Cell Signal Technology (Beverly, MA, USA). Benzyloxycarbonyl-Val-Ala-Asp-fluromethylketone (z-VAD-fmk) was ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Micro BCA protein assay reagent kit was product of Pierce (Rockford, IL, USA). Hoechst 33342 staining kit and Caspase activity assay kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Annexin V-FITC Apoptosis Detection Kit was obtained from KeyGen Biotech Co. Ltd., (Nanjing, China). Rhodamine123 (fluorescence probes, 2-(6-Amino-3imino-3H-xanthen-9-yl) benzoic acid methyl ester), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide (PI) and all other reagents were purchased from Sigma-Adrich Co. (St. Louis, MO, USA).

Cell Culture and Treatment

Human lung adenocarcinoma A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, and antibiotics (10 U/ml penicillin and 100 µg/ml streptomycin) under standard culture conditions (37°C, 95% humidified air, and 5% CO₂). 20(S)-PPD dissolved in dimethyl sulfoxide (DMSO, less than 1%) and neutralized in medium, was added to the culture media to the final concentrations specified in the text. Control cells were treated with an equivalent amount of the solvent alone.

Cytotoxicity Assay

Cell viability was assessed by MTT metabolism test, as described previous (Wang et al., 2007). Cells were cultured for 12, 24, 48 hours with different concentration of 20(S)-PPD.
at a concentration of $1 \times 10^4$ cells/well in 96-well plates for cell viability assays. Control cells were cultured with the relevant amounts of DMSO. After incubation at 37°C in a humidified incubator, 10 μl MTT (5 mg/ml in PBS) was added to each well and incubated for four hours. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm with a microtiter plate reader (SpectraMax plus384, Molecular Devices, USA). The cell viability-drug dilution was obtained from sigma plots, and drug concentrations that inhibited growth by 50% were calculated from multiple runs (IC$_{50}$).

**Apoptotic Morphology and Cell Cycle Analysis**

Apoptotic morphology was analyzed by the Hoechst 33342 staining assay (Wu et al., 2006). Cells were seeded on coverslips in the indicated concentrations for 24 h. The cover glasses were washed twice with PBS and fixed with 4% formaldehyde for 10 min, and stained by Hoechst 33342 for 5 min. The condensed or fragmented nuclei of apoptotic cells were observed under fluorescence microscopy (excitation, 365 nm; emission, 480 nm; Nikon TE-2000U, Nikon Corporation, Tokyo, Japan).

To quantify 20(S)-PPD-induced apoptotic death of A549 cells, Annexin V- Fluorescein isothiocyanate (FITC) and PI staining was done followed by flow cytometry (Agarwal et al., 2002). After treatment 20 μmol/L 20(S)-PPD treated for 24 h, $5 \times 10^4$ cells were resuspended in 300 μl of binding buffer containing 1 μg/ml PI and 0.05 μg/ml Annexin V-FITC. The samples were incubated in the dark for 15 min at room temperature, and were analyzed by flow cytometry (FACSCalibur, BD, UK).

Cell cycle assay was performed by PI single staining. Cells were first treated with 20(S)-PPD (20 μmol/L) for different times, then trypsinized and resuspended in 70% absolute ethanol. After an incubation at 4°C overnight, the cells were resuspended in the cell cycle assay buffer [PBS (pH 7.4) containing 0.1 mg/ml RNase A and 50 mg/ml propidium iodide (PI)] for 30 min at room temperature in the dark. Finally, the percentage of cells in different phases of cell cycle was determined by flow cytometry.

**Caspase Activity Assay**

Caspase-3/caspase-9 activity was measured by colorimetric protease assay, following manufacturer’s protocol as published (Agarwal et al., 2002). Briefly, cell lysate was prepared in cell lysis buffer (TBS containing detergent) at the end of treatment with different dose or time of 20(S)-PPD, or a pan-caspases inhibitor z-VAD-fmk (20 μmol/L), or both. Protein lysate (100 μg per sample) was mixed with 2× reaction buffer and 200 μmol/L substrate (Ac-DEVD-pNA for caspase-3; Ac-LEHD-pNA for caspase-9) and incubated at 37°C for 2h in the dark. Developed color was measured at 405 nm in microtiter plate reader.

**Mitochondrial Transmembrane Potential ($\Delta \psi_m$) Analysis**

Alterations in $\Delta \psi_m$ were analyzed by flow cytometry using the mitochondrial membrane potential sensitive cationic dye Rhodamine123 and following vendor’s protocol with some
modification (Uddin et al., 2005). Briefly, $5 \times 10^4$ cells/ml was treated with different dose of 20(S)-PPD for 24 h, after that stained with 1 μmol/L Rhodamine123. After incubation up to 4 h at 37°C in the dark, cells were washed twice with ice-cold PBS and then analyzed by flow cytometry, with excitation and emission setting of 507 nm and 529 nm, respectively.

**Preparation of Cytosolic Extract for Cytochrome c Release and Bcl-2 Family Expression**

The cytosolic fraction was prepared from the cells as described previously (Uddin et al., 2005). A549 cells were treated with and without 20(S)-PPD, resuspended in ice-cold lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 10 μ/ml aprotinin and 250 mM sucrose) for 15 min. Cells were homogenized with 20 strokes of prechilled Dounce homogenizer, and the homogenates were centrifuged at 1000 g for 5 min at 4°C and at 12,000 g for 15 min at 4°C. Supernatants were transferred to new tubes and centrifuged again at 12,000 g for 15 min, and the resulting supernatants were separated to perform western blotting with anti-cytochrome c and Bcl-2 family antibody.

**GSK 3β siRNA Transfection Assay**

GSK 3β siRNA transient transfection assay was used to knock down the expression of GSK 3β as we described previously (Pang et al., 2007). A549 cells were transfected with GSK 3β siRNA or negative control RNA at concentration of 50 nM using Lipofectamine2000 according to the manufacturer (Invitrogen, Life Technologies, Inc.). Twenty four hours after transfection, the cells were exposed to 20(S)-PPD (20 μmol/L) for 24 h and then were harvested to perform western blotting and flow cytometry analysis.

**Western Blot Analysis**

Cells lysates were lysed in extraction buffer as previously described (Choi et al., 1997). The protein concentration was determined using the Micro BCA protein assay kit. Cell extracts (60 μg) were resolved by 12% SDS–polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking with TBST [Tris-buffered saline (TBS) containing 0.1% Tween 20] containing 5% (W/V) non-fat dry milk for 1 h. The transferred membrane was incubated with appropriate primary antibodies at 4°C overnight, and then membranes were washed three times with 0.1% Tween 20 in TBS for 10 min and incubated with secondary antibodies at room temperature for 1 h. Antibody binding protein bands were detected by enhanced chemiluminescence reagent.

**Statistical Analysis**

All data from MTT assays, FACS analyses, Western blot analysis, and caspase activity assays derived from at least three independent experiments gave similar patterns. All data was presented as mean±S.D. Statistical differences were evaluated applying Student’s
-test or one-way analysis of variance (ANOVA). A value of $p < 0.05$ was accepted as an indication of statistical significance.

**Results**

20(S)-PPD Suppresses the Proliferation and Induces Apoptosis in A549 Cells

To verify the effect of 20(S)-PPD on the cell viability, we first evaluated the cytotoxic effect of 20(S)-PPD with 0 to 100 $\mu$mol/L on A549 cells by MTT assay. As shown in Fig. 1B, 20(S)-PPD inhibited proliferation in a dose- and time-dependent manner. The IC$_{50}$ value of 20(S)-PPD on growth inhibition was estimated to be 20 $\mu$mol/L in cells with 24 h treatment. Based on these observations, we selected a dose of 10, 20, 40 $\mu$mol/L, and a time period of 24 h post-20(S)-PPD treatment for further mechanistic studies.

![Chemical structure of 20(S)-Protopanaxadiol (PPD)](image)

![Effect of 20(S)-PPD on A549 cells viability, morphology and apoptosis](image)

**Figure 1.** Effect of 20(S)-PPD on A549 cells viability, morphology and apoptosis. (A) Chemical structure of 20(S)-Protopanaxadiol (PPD). (B) A549 cells were treated with different concentration of 20(S)-PPD at indicated incubation times and the cell viability was determined by the MTT assay. (C) A549 cells were treated with 0, 10, 20, 40 $\mu$mol/L 20(S)-PPD for 24 h and the nuclear DNA was monitored with Hoechst 33342 staining. (D) Quantitative representation of 20(S)-PPD-induced apoptosis was measured using the Annexin-FITC/PI staining and FACS cytometry. Data is expressed in values DMSO-treated cells and correspond to mean±S.D. of at least three independent experiments. *$p < 0.05$, **$p < 0.01$ for 20(S)-PPD-treated cells vs. DMSO-treated cells.
To determine whether the growth inhibition induced by 20(S)-PPD in A549 cells was caused by apoptosis. We tested the different apoptotic features using Hochoest 33342 and Annexin V-FITC/PI staining. As shown by representative picture (Fig. 1C), the nuclei of cells were round and homogeneously stained in DMSO-treated cells after 24 h. However, cells treated with 20(S)-PPD (10, 20, 40 \mu mol/L) showed that significant condensation and morphology changes, such as cell shrinkage, cell nuclei condensation and apoptotic body formation. Furthermore, we clearly determined both early and late apoptosis cells increased with the concentration of 20(S)-PPD, shown in Fig. 1D. The percentage of early apoptotic cells treated by 20(S)-PPD was 6.2 ± 1.0%, 34.8 ± 2.8%, (48.1 ± 3.3%, respectively, whereas DMSO-treated cells was 3.2 ± 0.8%. The cells treated by 20(S)-PPD for 24 h were happened a significant sub G1 and G0/G1 phase block (Table 1).

20(S)-PPD Activates the Caspase Cascade and Induces Cleavage of PARP

Further downstream in the apoptotic pathway, we investigated two caspases during 20(S)-PPD-induced apoptosis. As shown in Fig. 2A, caspase-3 and caspase-9 activity is markedly increased after exposure to 20 \mu mol/L 20(S)-PPD within 18 h, but slightly decreased after 24 h. Different concentration of 20(S)-PPD treatment for 24 h resulted in caspase-3 and-9 activity in a dose-dependent manner (Fig. 2B). Additionally, Fig. 2C showed that z-VAD-fmk markedly attenuated 20(S)-PPD-simulated DNA fragmentation,

Table 1. Effects of 20(S)-PPD on the Apoptosis and Cell Cycle

<table>
<thead>
<tr>
<th>20(S)-PPD (\mu mol/L)</th>
<th>n</th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2-M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>58.25 ± 3.54</td>
<td>32.11 ± 1.76</td>
<td>9.65 ± 0.67</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>63.68 ± 2.64</td>
<td>27.43 ± 1.19</td>
<td>8.89 ± 0.63</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>78.85 ± 2.33*</td>
<td>14.30 ± 0.87*</td>
<td>7.05 ± 0.65*</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>85.56 ± 2.63*</td>
<td>10.62 ± 0.74*</td>
<td>3.82 ± 0.31*</td>
</tr>
</tbody>
</table>

Note: *p < 0.05, compared to without 20(S)-PPD group. Data represent mean ± S.D. of at least three independent experiments.
indicating that 20(S)-PPD-induced apoptosis is largely dependent on the activation of caspase-9 and caspase-3. 20(S)-PPD treatment activated caspase-9 and caspase-3 expression as determined by the cleavage of caspase-3 and PARP (Figs. 2D and 2E).

Figure 2. 20(S)-PPD induced apoptosis of A549 cells via caspases-dependent mechanisms. (A) A549 cells were treated with 20 μmol/L 20(S)-PPD for indicated times and caspase-9/-3 activity were assessed by the substrate Ac-LEHD-pNA and Ac-DEVD-pNA, respectively. (B) A549 cells were incubated with different concentration of 20(S)-PPD for 24 h and caspase-9/-3 activities assay as described above. (C) DNA fragmentation induced by 20(S)-PPD was abolished by a pan-caspase inhibitor (z-VAD-fmk) preincubation in A549 cells. (D), (E) Proteins expression of caspases and PARP were determined by Western blotting. All values were expressed as mean ± S.D. (n = 3). Values were presented significant as *p < 0.05, **p < 0.01 vs. DMSO-treated cells.
Figure 3. 20(S)-PPD-induced the mitochondrial dysfunction and the abnormal expression of Bcl-2 family proteins. (A) Treatments of A549 cells with indicated dose of 20(S)-PPD for 24 h and mitochondrial membrane potential was evaluated by flow cytometry. (B) A549 cells were treated with indicated dose of 20(S)-PPD for 24 h and cytochrome c, Smac and AIF release were determined by Western blotting. (C) Protein expression of Bcl-2 family was determined by Western blotting. All values were expressed as mean±S.D. (n=3). *p < 0.05, **p < 0.01 vs. DMSO-treated cells.
20(S)-PPD Induces Mitochondrial Dysfunction and Abnormal Expression of Bcl-2 Family Proteins

Treatment with 20(S)-PPD for 24 h significantly induced the loss of $\Delta \Psi_m$ in a dose-dependent manner (Fig. 3A). There was a marked release in apoptotic induce factors from mitochondrial membrane to cytosolic fractions, such as cytochrome c, Smac, and AIF (Fig. 3B). With 20(S)-PPD treatment, Bcl-2 and Bcl-XL protein levels were down-regulated in cytosol, Bax and Bad expression were accordingly increased (Fig. 3C).

Inhibition of the PI3K/Akt Signaling Pathway Enhances the Sensitivity of Apoptosis Induced by 20(S)-PPD

Treatment with 20(S)-PPD decreased the levels of the phosphorylated form of Akt (Ser$^{473}$) and Akt (Thr$^{308}$) sites at indicated dose (Figs. 4A and 4B) and time (Figs. 4C and 4D), whereas, the level of total Akt and Forkhead-related transcription factors (FKHR) was not affected by the 20(S)-PPD treatment. Meanwhile, we found the constitutive phosphorylation of and GSK 3β, the Akt downstream substrates, was markedly decreased.
Knockdown of GSK 3\(\beta\) with siRNA Promoted 20(S)-PPD-Induced Apoptosis

To further confirm the role GSK 3\(\beta\) in the 20(S)-PPD-mediated apoptosis, A549 cells were transiently transfected with GSK 3\(\beta\) siRNA and subsequently treated with 20 \(\mu\)mol/L 20(S)-PPD (20 and) for 24 h. After GSK 3\(\beta\) siRNA transfection, the expression of GSK 3\(\beta\) was significantly down-regulated (Fig. 5A), and cell viability was decreased.

Figure 4. 20(S)-PPD inhibited the phosphorylation of Akt and GSK 3\(\beta\). (A), (B) A549 cells were incubated with different concentration of 20(S)-PPD for 24 h and protein expression of Akt and its substrate was determined by Western blotting. (C), (D) A549 cells were treated with 20 \(\mu\)mol/L 20(S)-PPD for indicated times and A549 cells were incubated with different concentration of 20(S)-PPD for 24 h and protein expression of Akt and its substrate was determined by Western blotting. All values were expressed as mean±S.D. (n = 3). *\(p<0.05\), **\(p<0.01\) vs. DMSO-treated cells.

**Knockdown of GSK 3\(\beta\) with siRNA Promoted 20(S)-PPD-Induced Apoptosis**

To further confirm the role GSK 3\(\beta\) in the 20(S)-PPD-mediated apoptosis, A549 cells were transiently transfected with GSK 3\(\beta\) siRNA and subsequently treated with 20 \(\mu\)mol/L 20(S)-PPD (20 and) for 24 h. After GSK 3\(\beta\) siRNA transfection, the expression of GSK 3\(\beta\) was significantly down-regulated (Fig. 5A), and cell viability was decreased.
Figure 5. Knockdown of GSK 3β with siRNA promoted 20(S)-PPD-induced apoptosis. (A) GSK 3β expression after siRNA transfection was viewed by Western blotting. (B) A549 cells were treated with 20 µmol/L 20(S)-PPD for 24 h and the cell viability was determined by the MTT assay. (C), (D) A549 cells were treated with 20 µmol/L 20(S)-PPD for 24 h and proteins expression of caspases was determined by Western blotting. (E), (F, G) A549 cells were treated with 20 µmol/L 20(S)-PPD for 24 h and quantitative representation of apoptosis was measured using PI single staining and FACS cytometry. All values were expressed as mean±S.D. (n = 3). **p < 0.01 vs. DMSO-treated cells.
Accordingly, knockdown of GSK 3β combined with 20(S)-PPD treatment promoted the caspase-3 and -9 activation (Figs. 5C and 5D) and the apoptosis effects (Figs. 5E–5G).

Discussion

The ginseng has been used as an herbal medicine for over 2000 years, in the belief that it is a panacea and promotes longevity. As described in Chinese traditional medicine textbooks, its effectiveness reaches mythical proportions (Gillis, 1997). It is an herb with many active components; most pharmacological actions of ginseng are attributed to ginsenosides (Hasegawa et al., 1996; Gillis, 1997; Wang et al., 2011). The ginsenoside, 20(S)-PPD, has been used in both anti-cancer and antioxidant effects in clinic in China, also used in radiotherapy and chemotherapy assistant medicine (Shibata, 2001; Wang et al., 2010). Our previous study demonstrated 20(S)-PPD played effective roles on lung cancer and gastrointestinal tumor in animal model (Zhang et al., 2008a,b). However, the underlying molecular mechanisms remained poorly understood. This study provides novel insight into the mechanism of 20(S)-PPD, showing that treatment with 20(S)-PPD is associated not only with increased apoptosis but also with inhibited PI3K/Akt pathway. 20(S)-PPD has a positive effect on cell proliferation and mitochondrial-mediated apoptosis, lending support to a potential new role for the drug in the treatment or chemoprevention of lung cancer.

Apoptosis is a highly ordered cellular suicidal mechanism that regulates normal physiological processes, and plays a crucial role in maintaining normal homeostasis (Hail et al., 2006). 20(S)-PPD induced morphological changes in A549 cells and G0/G1 phase arrest and apoptosis. Some medicines from natural products, such as berberine (Patil et al., 2010), grape proanthocyanidins (Singh et al., 2011), Angelica sinensis (Cheng et al., 2004), gypenosides (Lu et al., 2008), and ginsenosides CK (Cho et al., 2009), have same characteristics of cell cycle arrest. Therefore, we speculate that 20(S)-PPD might negatively regulate cyclin complexes at the G1-S transition similar to these above medicines.

A mitochondrial-dependent step is associated with the change in the membrane permeability and subsequent loss of membrane potential (∆Ψm). This process is controlled by the release of apoptotic proteins including AIF, Smac, and cytochrome c from the inter-membrane space of mitochondria into the cytosol (Kuo et al., 2005). Once these proteins localized within the inter-membrane space released into the cytosol, cytochrome c, a key mitochondrial apoptotic factor, triggers caspase-9 activation by binding and activating the apoptosis protease activation factor-1 in a multi-protein complex called the apoptosome. Consequently, activated caspase-9 activates effector caspase (caspase-3), which cleave several substrates to induce the apoptosis phenotype. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Hail et al., 2006; Kuo et al., 2005). In agreement of these above observations, we found that 20(S)-PPD-induced loss of mitochondrial membrane potential (∆Ψm) was accompanied by the release of cytochrome c, Smac and AIF, and activation of caspase-3/-9. Moreover, z-VAD-frm, a universal inhibitor of caspase, prevented caspase-3 activation and PARP cleavage and
inhibited 20(S)-PPD-induced cell growth inhibition. Thus, we suggested that mitochondrial dysfunction may be involved in 20(S)-PPD-induced A549 apoptosis.

The mitochondrial pathway of apoptosis is partly dependent on the Bcl-2 family proteins transition and release. These proteins are subdivided into two functional groups, an anti-apoptotic group, including Bcl-2 and Bcl-XL, and a pro-apoptotic group, including Bax and Bad. Among the Bcl-2 family members, Bax and Bad are mainly associated with loss of mitochondrial membrane potential, which activated by activity-related conformational changes following an apoptotic stimulus (Chipuk and Green, 2008). Our results suggest that 20(S)-PPD has a beneficial effect on Bcl-2 family transition, an important efficacy measure of lung cancer treatment that is indicative of a better prognosis.

PI3K/Akt signal plays a crucial role in tumor genesis and tumor progression by promoting cell proliferation and inhibiting apoptosis (Jung et al., 2000; Lin et al., 2001). Increased Akt phosphorylation has been reported in various cancers, which suggested that Akt is a potential target for cancer prevention and therapy. Among the compounds of panax ginseng and its extracts, 20(S)-gensenoside-Rh2 (Rh2) and Compound K (CK) were effective inhibitors of cell growth and proliferation and inducers of apoptosis and cycle arrest via Akt pathway (Park et al., 2010; Tsutsumi et al., 2011). This information illustrated that the structure/function relationship of ginsenoside attributed to biological activity. In this present study, we confirmed that Akt is constitutively phosphorylated at both Ser^{473} and Thr^{308} in A549 cells.

As the downstream substrates of Akt, GSK 3β and FKHR family of transcription factors have been reported to be involved in the cells survival (Jin et al., 2007). Akt promoted cells survival by activating the phosphorylation of GSK 3β and FKHR family protein of transcription factors, which prevented the apoptosis (Brunet et al., 2001). However, there are two opposing views on the role of GSK 3β in the pathogenesis of cancer. On one hand, some believe that inhibition of GSK 3β can promote tumor initiation and progression, and tumor growth will be inhibited if GSK 3β is activated (Takahashi-Yanaga et al., 2009). On the other hand, some other researchers hold the view that inhibition of GSK 3β can prevent tumor development, and initiation and progression of tumor will be promoted if GSK 3β activated (Wang et al., 2008). Here we provide evidence that expression of GSK 3β was down-regulated while expression of FKHR was normal in 20(S)-PPD treatment. We further knock down the expression of GSK 3β by siRNA transfection promoted 20(S)-PPD-induced apoptotic effects.

To the best of our knowledge, this is the first documentation that 20(S)-PPD triggers a mitochondrial-mediated apoptosis in human lung adenocarcinoma A549 cells via inhibition of Akt and down-stream substrate GSK 3β. Therefore, we encourage further studies of 20(S)-PPD as a promising chemopreventive agent against lung cancer.

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