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Research article

Ginsenoside 20(S)-protopanaxadiol induces cell death in human endometrial cancer cells via apoptosis

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ABSTRACT

Background: 20(S)-protopanaxadiol (20(S)-PPD), one of the aglycone derivatives of major ginsenosides, has been shown to have an anticancer activity toward a variety of cancers. This study was initiated with an attempt to evaluate its anti-cancer activity toward human endometrial cancer by cell and xenograft mouse models.

Methods: Human endometrial cancer (HEC)-1A cells were incubated with different 20(S)-PPD concentrations. 20(S)-PPD cytotoxicity was evaluated using MTT assay. Apoptosis was detected using the annexin V binding assay and cell cycle analysis. Cleaved poly (ADP-ribose) polymerase (PARP) and activated caspase-9 were assessed using western blotting. HEC-1A cell tumor xenografts in athymic mice were generated by inoculating HEC-1A cells into the flank of BALB/c female mice and explored to validate 20(S)-PPD anti-endometrial cancer toxicity.

Results: 20(S)-PPD inhibited HEC-1A cell proliferation in a dose-dependent manner with an IC_{50} value of 3.5 μM at 24 h. HEC-1A cells morphologically changed after 20(S)-PPD treatment, bearing resemblance to Taxol-treated cells. Annexin V-positive cell percentages were 0%, 10.8%, and 58.1% in HEC-1A cells when treated with 0, 2.5, and 5 μM of 20(S)-PPD, respectively, for 24 h. 20(S)-PPD subcutaneously injected into the HEC-1A cell xenograft-bearing mice three times a week for 17 days manifested tumor growth inhibition by as much as 18% at a dose of 80 mg/kg, which sharply contrasted to controls that showed an approximately 2.4-fold tumor volume increase. These events paralleled caspase-9 activation and PARP cleavage.

Conclusion: 20(S)-PPD inhibits endometrial cancer cell proliferation by inducing cell death via a caspase-mediated apoptosis pathway. Therefore, the 20(S)-PPD-like ginsenosides are endowed with ample structural information that could be utilized to develop other ginsenoside-based anticancer agents.

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1. Introduction

Endometrial cancer is the seventh most common cancer among females globally, trailing only breast, cervical, colorectal, gastric, lung, and ovarian cancer. The number of endometrial cancer patients continues to increase each year due to advances in cancer diagnosis technology. For instance, 35,000 new patients are added each year in America alone, and it is the most common cancer among female genital cancers. About 50,000 patients, corresponding to 1.7% of all female cancer patients who die, die of endometrial cancer each year [1]. Global prevalence of endometrial cancer is partially attributed to the lack of effective chemotherapy regimens for many [2,3], highlighting the growing need for novel chemotherapeutics against endometrial cancer.

There have been many anticancer treatment regimens against endometrial cancer, including radiation therapy and chemotherapy. However, most of them inevitably cause some toxicities and side effects. Therefore, natural product therapies or derivatives that are minimally toxic in the context of normal human body functions are highly favored [2]. Ginseng is a root component of *Panax ginseng* Meyer, which is widely distributed in Northeast Asia. Ginseng has long been used as a traditional Chinese medicine. The major active ingredients of ginsengs are saponins called ginsenosides. The major saponins found in the *P. ginseng* root include ginsenosides Rb1, Rb2, Rc, Re, and Rg1, which constitute >80-90% of total ginsenosides [2,4–6]. These ginsenosides have diverse bioactivities like anti-aging, anti-diabetic, anti-cancer, immune regulation, neuron regulation, and wound and ulcer healing [7–9]. However, it is noteworthy that many protopanaxadiol-type saponins and protopanaxatriol-type saponins are not easily absorbed through the intestine due to their hydrophobicity, suggesting that they are poorly absorbed through the gastrointestinal tract after oral intake [10]. To tackle or circumvent this problem, many ginsenoside derivatives have been purified or synthesized to enhance its bioavailability without sacrificing bioactivity [7].

20(S)-protopanaxadiol (20(S)-PPD) and 20(S)-protopanaxatriol (PPT), which can be produced by metabolizing ginsenosides via the activity of intestinal microorganisms through deglycosylation or acid hydrolysis (Fig. 1), are formulated to increase its bioavailability [11]. Here, we focused on the effect of 20(S)-PPD on human endometrial cancer. The lack of data on human endometrial cancer prompted us to investigate 20(S)-PPD's cytotoxic effects toward human endometrial cancer both *in vitro* and *in vivo* to elucidate its underlying mechanisms. We hope that this study will broaden the pharmacological scope of the ginsenoside aglycone moiety as an anti-cancer agent.

2. Materials and methods

2.1. Chemicals

20(S)-PPD was prepared by chemically modifying ginseng saponins with periodic acid as described previously [12]. The 20(S)-PPD sample was dissolved in dimethyl sulfoxide (DMSO) at 10 mM as a stock solution and diluted to the indicated concentrations with phosphate-buffered saline (PBS) before use. Paclitaxel (Taxol™) was obtained from Bristol-Myers Squibb Pharmaceutical (Montreal, Quebec), dissolved in DMSO, and stored at −20°C in the dark. Antibodies against cleaved poly (ADP-ribose) polymerase (PARP) and caspase-9 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). The annexin V-FITC/PI apoptosis detection kit was from BD Biosciences (San Diego, CA). The ECL membrane was from GE Healthcare Life Sciences (Pittsburgh, PA). RIPA buffer, the BSA protein assay kit, and the SuperSignal® West Pico Chemiluminescent Substrate kit were purchased from ThermoFisher Scientific (Massachusetts, MA). Fetal bovine serum (FBS) was obtained from Gibco-BRL (Carlsbad, CA). DMEM/F12 medium was from PAN Biotech (Aidenbach, Germany). All other chemicals, otherwise specified, were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human endometrial cancer (HEC)-1A cells were purchased from the American Type Culture Collection (ATCC) (Manassa, VA) and maintained in DMEM/F12 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS under standard culture conditions of 37°C, 95% humidified air and 5% CO_2. Human endometrial fibroblast cells were isolated using surgical curettages of the hysterectomized uteri of patients at the Ajou University Hospital (Suwon, S. Korea) with no other apparent endometrial disease diagnoses. Informed consent was obtained from patients before surgery according to the Ajou University IRB preapproval (Suwon, S. Korea).

2.3. MTT assay

The MTT assay was used to quantify cell cytotoxicity. Briefly, HEC-1A cells and endometrial fibroblast cells were seeded onto 24-well plates and treated with different concentrations of 20(S)-PPD at 0, 2.5, 5 μM, or paclitaxel at 100 nM. After a 24 h incubation at 37°C, 1 ml of MTT solution at 5 mg/ml after dilution in serum-free culture medium was added to each well. The plates were incubated for 4 h at room temperature. 420 μl of DMSO was added to each well, and the plates were shaken for 20 min. The optical absorbance was read at 570 nm with a microplate spectrophotometer (model SpectraMAX 190, Molecular Devices, Sunnyvale, CA).

2.4. Cell cycle analysis

Cell cycle analysis was performed using flow cytometry after propidium iodide (PI) staining as previously described [13]. Briefly, HEC-1A cells were seeded onto 6-well plates and treated with 20(S)-PPD at final concentrations of 1 μM, 2.5 μM, and 5 μM. After incubating for 24 h at 37°C, cells were collected by centrifuging at 300 g, fixed with 70% cold ethanol at 4°C, washed with PBS, stained in PI staining solution (0.1% Triton X-100, 10 μg/ml PI, 100 μg/ml RNase A in PBS) and incubated in the dark at 37°C for 20 min. The numbers of cells at each cell cycle phase were quantified using a flow cytometer (model FACSCaliber™, BD Bioscience, Franklin Lakes, NJ) with excitation at 536 nm and emission at 617 nm.

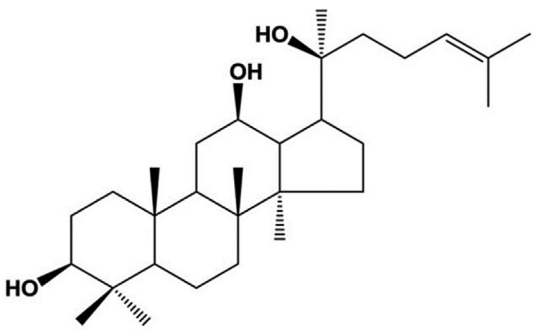

Fig. 1. The chemical structure of 20(S)-PPD.

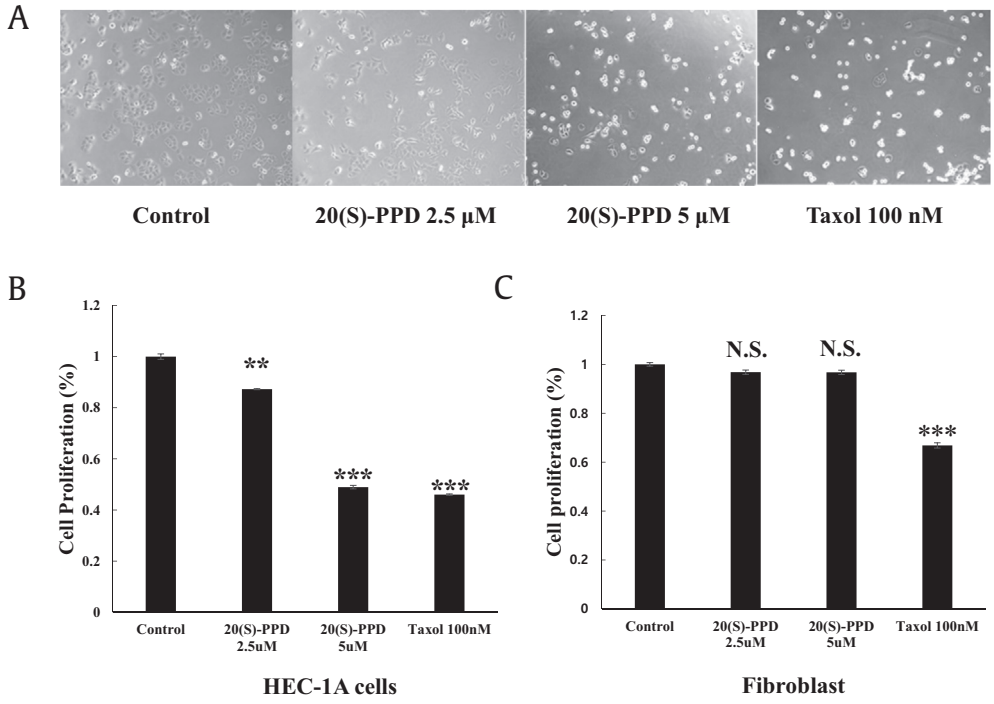

Fig. 2. 20(S)-PPD inhibits HEC-1A cell growth. (A) Phase contrast microscopic images of HEC-1A cells after treating with different concentrations of 20(S)-PPD at 0, 2.5, 5 μM, and paclitaxel at 100 nM for 24 h. The cytotoxicity of 20(S)-PPD was assessed using the MTT assay. Cell growth inhibition was relative to controls without 20(S)-PPD in HEC-1A cells (B) and in endometrial fibroblast cells . (C) Data represent means ± SD of at least triplicate measurements. (**$P < 0.01$, ***$P < 0.001$ *vs.* control).

2.5. Annexin V binding assay

Annexin V-FITC/PI staining was performed to quantify apoptotic cell populations induced by 20(S)-PPD in HEC-1A cells according to the manufacturer's protocol. HEC-1A cells were seeded onto 100∮ cell culture dishes and treated with 20(S)-PPD at final concentrations of 0, 2.5, 5 μM, or paclitaxel at 100 nM. After incubating for 24 h at 37°C after 20(S)-PPD treatments at different concentrations for 24 h, HEC-1A cells were washed twice in PBS and harvested by centrifuging at 300 g. After washing with PBS twice, cells were resuspended in 200 μl of 1X binding buffer before 5 μl of propidium iodide (PI) and anti-annexin V-FITC solution was added. The mixtures were incubated for 15 min at room temperature in the dark and analyzed using flow cytometry [14].

2.6. HEC-1A cell tumor xenografts

Female athymic mice, BALB/C nu/nu, aged 5 weeks were obtained from Nara Biotech (Pyeongtaek, S. Korea). The animals were housed in sterile steel cages with controlled temperature, lighting, and humidity under the supervision of a veterinarian working at the Laboratory Animal Research Center at Ajou University Hospital, S. Korea (http://larc.ajoumc.or.kr). Food and water were available *ad libitum*. All animal experimental procedures were pre-reviewed and approved by the Institutional Animal Research Ethics Committee at Ajou University, S. Korea. Subcutaneous tumor xenografts in nude mice were performed according to a previous report [15,16]. In brief, HEC-1A cells were injected at 5×10^6 cells/100 μl PBS subcutaneously into the left flank of a mouse (n = 10 mice/group) and allowed to grow for 1 week to reach the proliferative phase. When the external mean diameter of the tumors reached approximately 0.5 cm after 3 weeks of growth, the mice were randomly divided into following five groups. The mice assigned to group 1 were subcutaneously (*s.c.*) administered 0.1% DMSO (control). The mice in group 2 were administered 20(S)-PPD at 10 mg/kg. The mice in group 3 were administered 20(S)-PPD at 40 mg/kg. The mice in group 4 were administered 20(S)-PPD at 80 mg/kg. The mice in group 5 were treated with paclitaxel at 10 mg/kg. When the tumor size reached approximately 200 mm^3, the mice in each group were injected *s.c.* with 50 μl of each reagent three times a week for the next 17 days. The sizes of growing tumor xenografts were externally measured twice a week, starting on the first day of the drug treatment. The tumor xenograft volume was assessed according to the formula volume = $(a \times b^2)/2$, where a and b are lengths of the major axis and the minor axis of the tumor, respectively [16]. At the end of the drug treatment, mice were sacrificed using cervical dislocation, and tumors were excised at necropsy.

2.7. Western blot analysis

Western blotting was performed to detect cleaved PARP and caspase-9 proteins from HEC-1A cells and tumor xenografts. HEC-1A cells were seeded onto 100∮ cell culture dishes and treated with 20(S)-PPD at final concentrations of 0, 2.5, 5 μM, or paclitaxel at 100 nM. HEC-1A cell was harvested after incubating for 24 h at 37°C. After 20(S)-PPD treatments at different concentrations for 24 h at the end of 20(S)-PPD treatment, HEC-1A cells and tumor xenografts were harvested and lysed in RIPA buffer (pH 7.4) consisting of 50 mM Tris, 150 mM NaCl, 5% sodium deoxycholate, 0.5 M EGTA, and 1% NP-40 for 30 min on ice before HEC-1A cells or tumor tissues were homogenized with a glass tissue homogenizer (Kontes, Vineland, NJ) according to the manufacturer's procedures. Total lysates were centrifuged at 15,000 g for 20 min, and supernatants were collected. The protein concentrations in the supernatants were determined using the BSA protein assay kit. Cell extracts equivalent to 40 μg proteins were loaded into 10% to 15% gradient polyacrylamide-SDS gels. After electrophoresis, the gel was transferred onto an ECL membrane and blocked with 5% (w/v) non-fat

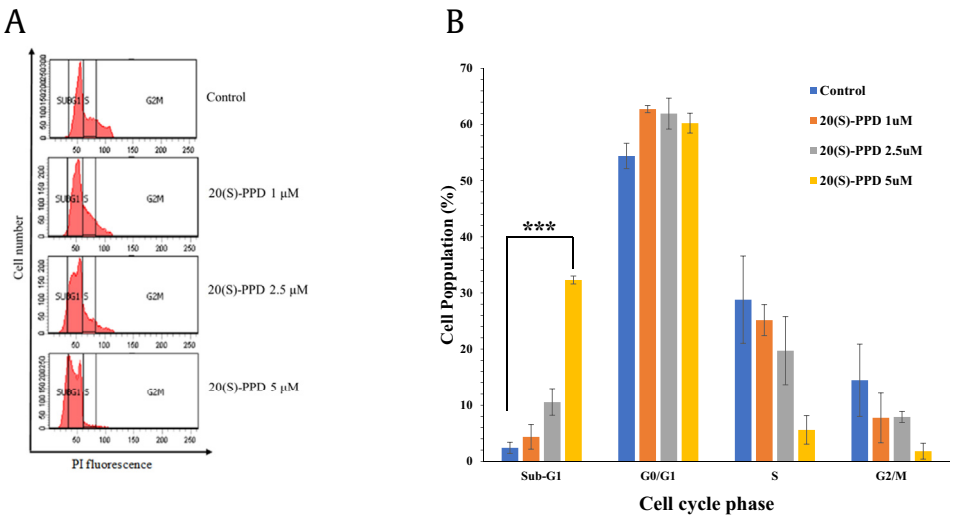

Fig. 3. Sub-G1 arrest induced by 20(S)-PPD. (A) HEC-1A cells were treated with increasing concentrations of 20(S)-PPD (0, 1, 2.5, or 5 μM) for 24 h before PI staining and flow cytometry. (B) Percentages of HEC-1A cells belonging to each cell cycle phase are shown. The results represent triplicate assays (means ± SD). (***$P < 0.001$ vs. control).

milk for 30 min at room temperature. The transferred membrane was incubated with either rabbit anti-PARP antibodies or rabbit anti-caspase-9 antibodies diluted at 1:1000 at 4°C overnight. The membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h, and protein bands were visualized using the HRP reaction with its substrates via ECL chemiluminescence (Model: Fusion SOLO S, VILBER, Collégien, France).

3. Results

3.1. 20(S)-PPD inhibits HEC-1A cell proliferation

Newly synthesized and purified 20(S)-PPD was characterized by high performance liquid chromatography based on the previous procedures (Supplementary Figure 1) [27]. Co-injected standards and synthesized 20(S)-PPDs were separated as a single peak at the same location on the HPLC histogram, indicating that both are identical. Next, HEC-1A cells were treated with three different concentrations of 20(S)-PPD—0, 2.5 μM, 5.0 μM, or 100 nM paclitaxel—for 24 h, and their cytotoxic effects were measured using the MTT assay. In a concentration-dependent manner, HEC-1A cells treated with 20(S)-PPD had rounded morphology and were detached from the bottom of culture dishes. This was also observed with cells under 100 nM paclitaxel treatment (Fig. 2A). Quantitatively, 20(S)-PPD inhibited HEC-1A cell proliferation in a dose-dependent manner with an IC_{50} of 3.5 μM. As expected, 100 nM Taxol treatment manifested approximately 60% inhibition of HEC-1A cell proliferation compared to controls (no 20(S)-PPD) (Fig. 2B). This result demonstrated that 20(S)-PPD has anti-proliferative activity toward HEC-1A cells in an *in vitro* cell culture model.

3.2. Sub-G1 phase arrest of HEC-1A cells is induced by 20(S)-PPD

Here, we investigated whether 20(S)-PPD induced cell cycle arrest in HEC-1A cells. It is now well documented that the sub-G1 phase corresponds to a cell cycle stage at which cells are arrested or stray before entering the apoptotic phase [13]. Our flow cytometric analysis indicated that 1.7% of control cells belonged to the sub-G1 phase, 56% to G1 phase, 23.3% to S phase, and 19% to G2/M phase. However, when treated with 1 μM 20(S)-PPD for 24 h, the percentages of cells in each phase were shifted to 2.8% sub-G1, 63.2% G1, 23.2% S, and 10.9% G2/M. After increasing 20(S)-PPD treatment at 2.5 μM and 5.0 μM, the proportions of cells in sub-G1 were further increased to 12.2% and 31.8%, respectively, (Fig. 3A and B). Thus, 20(S)-PPD clearly induced sub-G1 phase arrest of HEC-1A cells with a corresponding decrease in cell populations either at the S phase or the G2/M phase, implying that apoptotic cell death could be induced by 20(S)-PPD treatment.

3.3. 20(S)-PPD-induced HEC-1A cell apoptosis

Next, apoptotic HEC-1A cell death due to 20(S)-PPD was further confirmed using annexin V/PI staining. One of the early signals for apoptosis is the exposure of phosphatidyl serine, a phospholipid component, after being translocated from the inner leaflet of the cell membrane to the outer leaflet after receiving apoptotic signals [14]. Annexin V binds to the exposed phosphatidyl serine, allowing for quantitative evaluation of early apoptotic events. As shown in Fig. 4A and B, the apoptotic cell population represented by cells either in the Q2 or Q4 quadrants, increased dose-dependently with 20(S)-PPD concentrations. The apoptotic cell portion was 0%, 10.8%, and 58.1% of total cells when treated with 0, 2.5, and 5 μM 20(S)-PPD, respectively. Taxol treatment (100 nM) caused 12.1% of cells to undergo apoptotic cell death. This finding indicates that 20(S)-PPD caused apoptotic cell death in HEC-1A cells.

3.4. Cleaved PARP appearance in HEC-1A cells

To further study the apoptotic activity of 20(S)-PPD, HEC-1A cells were harvested after 20(S)-PPD treatment, lysed in RIPA buffer to obtain total soluble protein extracts, and subjected to western blotting analysis to probe for cleaved PARP. The appearance of cleaved PARP is widely believed to be a cellular indicator of caspase activation underlying the apoptotic cell signaling pathway. As shown in Fig. 5, cleaved PARP fragments of 89 kDa in size emerged after 20(S)-PPD treatment. Their intensities appeared to be proportional to 20(S)-PPD concentrations.

3.5. 20(S)-PPD inhibits the growth of HEC-1A cell xenografts

Having shown that 20(S)-PPD suppresses endometrial cancer cell growth in a cell culture model, we next investigated its

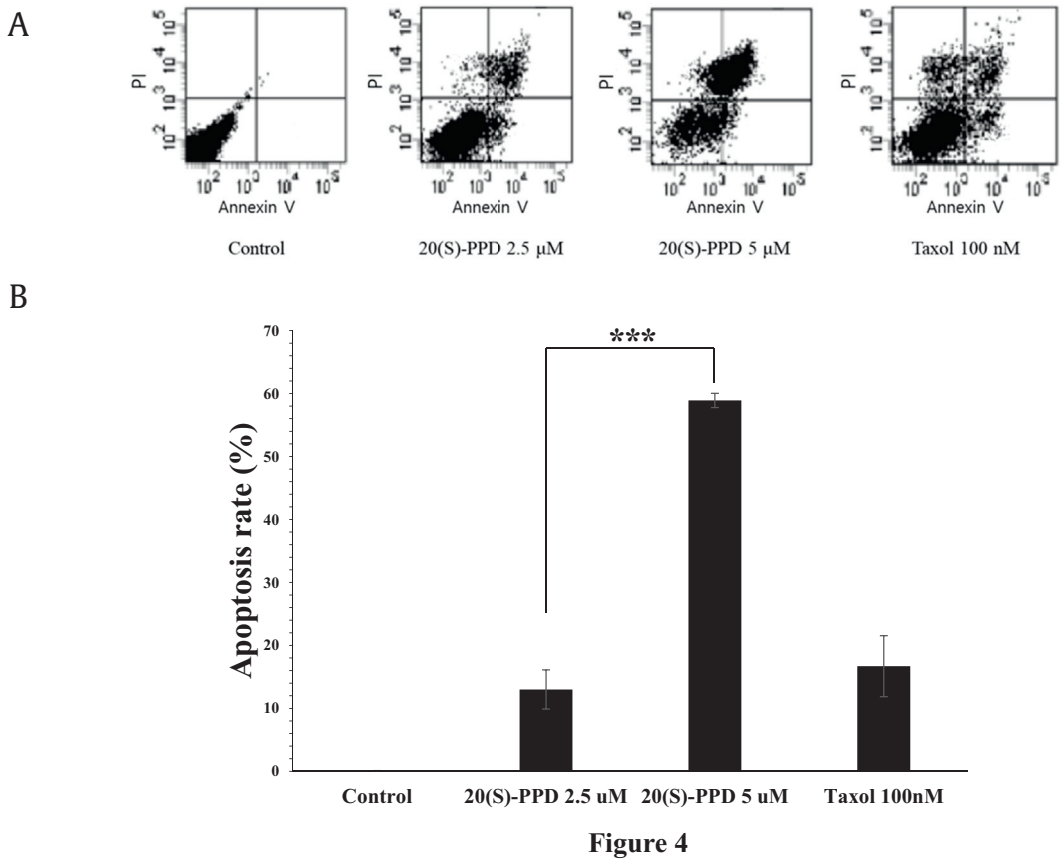

Figure 4

Fig. 4. HEC-1A cell apoptosis induced using 20(S)-PPD. (A) HEC-1A cells were incubated with different concentrations of 20(S)-PPD (0, 2.5, or 5 μM) for 24 h before annexin V-FITC/PI staining and flow cytometry analysis. (B) The percentages of apoptotic cells in Q2 and Q4 quadrants in A are shown. Data are represented as means ± SD of at least triplicate measurements. (***$P < 0.001$ vs. control).

antitumor activity in a xenograft mouse model of endometrial cancer. First, we established HEC-1A cell xenografts in nude mice by injecting HEC-1A cells into the dorsal flank. After three weeks of growth, the tumor size became visible with an average diameter of approximately 7 mm, which is equivalent to approximately 200 mm^3 in volume. The mice were randomly divided and subjected to subcutaneous injections of 20(S)-PPD at different dosages for the next 17 days. The first group of mice received a vehicle solution (0.1% DMSO diluted in PBS), and the second group of mice received paclitaxel at a dose of 10 mg/kg body weight. The last 3 groups of mice received 20(S)-PPD at doses of 10, 20, and 80 mg/kg body weight, respectively. The tumor volume was measured every three days, and the mice were sacrificed at the end of the experiment to excise the tumor xenografts at necropsy. Externally, the tumor volume steadily increased by as much as 235% from approximately 195 mm^3 to 458 mm^3 during the 17 day long dosing experiment, indicating HEC-1A xenografts were established and continued growing in nude mice. However, treatment with 20(S)-PPD suppressed tumor growth in a dosage-dependent manner. At the highest dosage used of 80 mg/kg, tumor growth was suppressed by as much as 18% from approximately 178 mm^3 at day 1 to 146 mm^3 at day 17 (Fig. 6). Importantly, 20(S)-PPD did not cause any death and weight loss in the concentration range used (Table 1), suggesting that 20(S)-PPD effectively inhibits HEC-1A cell growth *in vivo* without apparent normal tissue toxicity.

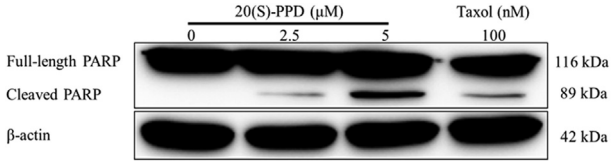

Fig. 5. Apoptotic markers induced by 20(S)-PPD in HEC-1A cells. HEC-1A cells were treated either with increasing concentrations of 20(S)-PPD (0, 1, 2.5, 5 μM) or paclitaxel at 100 nM for 24 h as indicated in Fig. 4. Cells were harvested, homogenized, and separated on SDS gels. Cleaved PARP was visualized using western blotting. β-actin was used as a loading control.

3.6. Cleaved PARP and cleaved caspase-9 expression in HEC-1A tumor xenografts

At the end of the tumor xenograft experiment, the tumor tissues were excised from the mice at necropsy and homogenized to obtain total protein extracts. To confirm whether 20(S)-PPD exerts its antitumor activities via the apoptotic pathway *in vivo*, we used western blotting to probe for the emergence of apoptotic marker molecules. As shown in Fig. 7, the expression of cleaved PARP of 89 kDa in size increased in proportion to 20(S)-PPD concentrations treated. The expression of 35 kDa cleaved caspase-9 also increased in a 20(S)-PPD concentration-dependent manner, confirming the involvement of a caspase-dependent apoptotic pathway in 20(S)-PPD-induced cell death of HEC-1A cell tumor xenografts.

4. Discussion

Ginsenosides, a class of natural product steroid glycosides and triterpene saponins, are found in numerous plant and plant extracts

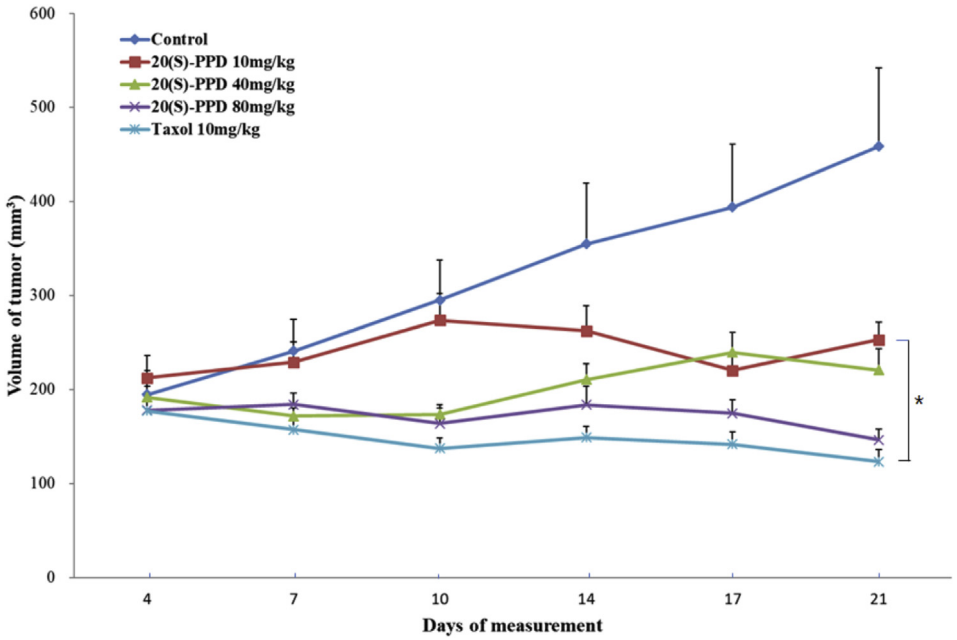

Fig. 6. 20(S)-PPD inhibits HEC-1A cell tumor xenograft growth. Approximately 5×10^6 HEC-1A cells were inoculated into the left flank of BALB/c female nude mice. After sufficient tumor growth was achieved, mice were randomly divided into five groups and injected subcutaneously with various concentrations of 20(S)-PPD (10, 40, or 80 mg/kg), paclitaxel (10 mg/kg), or 0.1% DMSO (controls) for 17 days. The tumor size was externally measured and plotted. $^*P < 0.05$ *vs.* controls.

including ginseng. Owing to their easy preparation and rich biological activity, the ginsenoside framework plays a key role in natural chemical-based therapeutic development by representing an interesting template for combinatorial and medicinal chemistry. Being a novel anti-cancer agent, a certain number of ginsenosides and their aglycone moieties were found to exhibit promising anti-proliferative properties against cancer cells. Studies on the structure-activity relationship of ginsenosides have demonstrated that 20(S)-PPD is one of the most potent anti-cancer compounds among ginsenosides [17,18]. It has been reported that 20(S)-PPD and its glucosides (like ginsenoside Rh2 and compound K) induce apoptosis via the mitochondria-mediated apoptotic pathway in Caco-2, U937, THP-1, and SMMC7721 cancer cells [17,19–21]. Furthermore, 20(S)-PPD could induce programmed cell death in two human glioma cell lines via caspase-dependent and independent pathways [22]. We found evidence for this hypothesis by synthesizing 20(S)-PPD and exploring its anticancer activity to elucidate its underlying anticancer activity mechanisms against endometrial cancer, one of the most frequently encountered gynecological cancers. Our results showed that 20(S)-PPD, but not Rb1, inhibited endometrial cancer cell proliferation in a dose-dependent manner both *in vitro* and *in vivo*. After 20(S)-PPD treatment, we observed (*i*) cellular morphological changes that are frequently encountered in Taxol-treated cells, and (*ii*) we confirmed that this occurred concomitantly with apoptosis induction. Supporting evidence includes annexin V binding and the expression of a plethora of apoptotic proteins including cleaved-PARP and caspase-9 in HEC-1A cells.

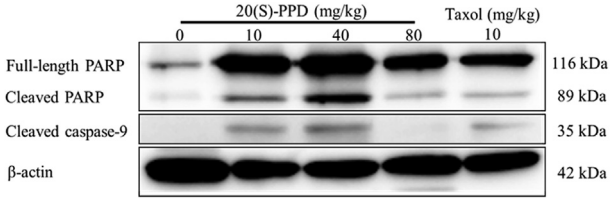

Fig. 7. Apoptotic markers induced by 20(S)-PPD in HEC-1A cell xenografts. HEC-1A tumor tissues were excised from xenograft-bearing mice at the end of the experiment, homogenized, and separated on SDS gels. Cleaved PARP and active caspase-9 were visualized using western blotting. β-actin was used as a loading control.

Ginsenosides are the major bioactive compounds found in ginsengs, and they represent a diverse group of steroidal saponins. More than twenty ginsenosides have been isolated and identified, and novel structures continue to be reported. The two major ginsenoside subtypes have been named protopanaxadiols and

Table 1
Inhibitory effects of 20(S)-PPD on the growth of HEC-1A cell xenografts in nude mice

	Pre-experiment			Post-experiment		
	Weight(g)	Volume(mm^3)	n	Weight(g)	Volume(mm^3)	n
Controls	21.97 ± 0.77	195 ± 26	10	22.48 ± 1.07	458 ± 83	10
20(S)-PPD (10 mg/kg)	22.17 ± 1.14	212 ± 24	10	22.62 ± 1.08	253 ± 19	10
20(S)-PPD (40 mg/kg)	23.42 ± 1.14	192 ± 12	10	23.35 ± 1.03	221 ± 23	10
20(S)-PPD (80 mg/kg)	23.2 ± 1.04	178 ± 16	10	22.77 ± 1.39	146 ± 11	10
Taxol (10 mg/kg)	22.25 ± 0.73	177 ± 13	10	22.64 ± 1.08	123 ± 13	10

HEC-1A cells were inoculated in the flank of nude mice. After the external tumor volumes reached about 200 mm^3, mice were randomly divided and administered 0.1% DMSO (controls), paclitaxel at 10 mg/kg, and different concentrations of 20(S)-PPD (10 mg/kg, 40 mg/kg, or 80 mg/kg). The body weight was measured twice a week, and tumor size was externally measured three times a week for 17 days

protophanaxatriols, and they can give rise to novel metabolites in the body after ingestion [4,6]. 20(S)-protopanaxadiol (20(S)-PPD) is a major metabolite of several protopanaxadiol-type ginsenosides including Rb1, Rb2, and Rc. 20(S)-PPD is mainly produced as a byproduct of processing by human intestinal bacteria, and it is rapidly absorbed into the blood [23]. Although the parent compound ginsenoside Rb1 was also reported to exhibit anticancer activity, a barrier to Rb1 efficacy is its limited cellular uptake ratio which limits its *in vivo* bioavailability [7].

It is important to understand that higher mammals have evolved complex cell death pathways. Many of the pathways feed into apoptotic programs that converge on the mitochondria as a key regulatory component [24]. Our data clearly revealed that 20(S)-PPD has anti-cancer activity by stimulating apoptotic cell death mainly mediated by a caspase-dependent pathway. Our study gathered several lines of supporting data. MTT assay showed that 20(S)-PPD inhibits HEC-1A cell proliferation with an IC_{50} of 3.5 μM. These results show that 20(S)-PPD is a more effective anti-cancer drug than other ginsenoside types [25]. Cell cycle analysis showed that 20(S)-PPD induces sub-G1 arrest in HEC-1A cells. The annexin V binding assay showed that both early-apoptotic and late-apoptotic cell populations increased in a 20(S)-PPD concentration-dependent manner. Western blot analysis also showed that cleaved PARP expression and caspase-9 activation was enhanced after 20(S)-PPD treatment. In addition, the activity of 20(S)-PPD in *in vivo* tumor xenografts was comparable to the cell culture model because 20(S)-PPD inhibited HEC-1A tumor xenograft growth in a dose-dependent manner at the tested concentrations (10, 40 and 80 mg/kg). Thus, all these results support the conclusion that 20(S)-PPD exerts its anti-cancer activity via apoptosis. Notably, it was recently reported that 20(S)-PPD effectively suppressed the NF-κB, JNK, and MAPK/ERK signaling pathways in colon cancer cells [26]. Thus, complex death pathways seemed to be triggered by 20(S)-PPD before triggering apoptotic cell death.

In conclusion, we demonstrated for the first time that 20(S)-PPD induces cytotoxicity in human endometrial cancer via apoptosis. 20(S)-PPD-induced apoptosis is mediated, at least in part, by caspase-dependent intrinsic pathway. These findings suggest that 20(S)-PPD type ginsenosides could be potential anti-cancer agents that target apoptosis. Further studies of the structure-activity relationship and structural modifications can be performed to enhance or improve efficacy without causing side-effects. 20(S)-PPD may be a promising new compound with ample potential benefits for cancer therapy, especially as a combinatorial therapeutic agent.

Conflicts of interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.02.002.

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