RESEARCH ARTICLE

Arch Pharm Res Vol 32, No 1, 49-57, 2009
DOI 10.1007/s12272-009-1117-1

Archives of Pharmacal Research
www.springer.com/12272

Anti-androgen-independent Prostate Cancer Effects of Ginsenoside Metabolites *In Vitro*: Mechanism and Possible Structure-Activity Relationship Investigation

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(Received August 28, 2008/Revised November 25, 2008/Accepted December 2, 2008)

Treatment of androgen-independent prostate cancer (AIPC) remains unsatisfactory. In our present experiment, natural occurring ginsenosides (NOGs) and intestinal bacterial metabolites (IBMs) were employed to investigate their anti-AIPC cell growth activity using PC-3 cells. Our results showed that the IBMs exerted more portent anti-AIPC activity than NOGs, by decreasing survival rate, inhibiting proliferation, inducing apoptosis, and leading to cell cycle arrest in AIPC PC-3 cells. The increase of LogP and decrease of C-6 steric hindrance, which were caused by deglycosylation by intestinal bacteria, may be the reason for the higher anti-AIPC activity of IBMs.

Key words: Ginsenosides, Structure-activity relationship, Anti-androgen-independent prostate cancer, In vitro, Intestinal bacterial metabolites

INTRODUCTION

Although many therapeutic protocols have been reported, treatment of androgen-independent prostate cancer (AIPC) remains unsatisfactory (Aragon-Ching and Dahut, 2007). Thus, novel therapeutic agents to improve the outcome of AIPC therapy was in urgent needed.

Ginseng is increasingly used as a general health tonic in many countries for its wide spectrum of pharmacological effects. It has been reported that ginseng showed the activity of inhibiting tumor growth *in vivo*. Many of the pharmacological effects of ginseng, including antitumor activities, are attributed to triterpene glycosides, known as ginsenosides. Ginsenosides share a similar basic structure, consisting of gonane steroid nucleus having 17 carbon atoms arranged in four rings. According to the existence of the hydroxy group at C-6 or not, ginsenosides are divided into two main categories, the 20(*S*)-protopanaxatriol and 20(*S*)-protopanaxadiol family (Fig. 1). Different sugar moieties attach to the gonane steroid nucleus at C-3, C-6, and/or C-20 through glycosidic bonds. In addition, the deglycosylation of the natural occurring ginsenosides (NOGs) catalyzed by intestinal bacteria, which further contribute to the diversity of the structures, is an important characterization of ginsenosides. Differences in the type, position, and number of sugar moieties of ginsenosides, especially after deglycosylated by intestinal bacteria, can characteristically influence biological responses (Byun et al., 1997; Liu et al., 2000; Odashima et al., 1985; Popovich and Kitts, 2002; Wang et al., 2007). The most significant property, which is influenced by intestinal bacterial metabolism, is that, in contrast to very low absorption of NOGs, their intestinal bacterial metabolites (IBMs), including compound K (C-K, also named as M-1, 20-*O*-(β-d-glucopyranosyl)-20(*S*)-protopanaxadiol), protopanaxadiol (Ppd), 20-*O*-(β-d-glucopyranosyl)-20(*S*)-protopanaxatriol (F_1), and protopanaxatriol (Ppt), are easily absorbed from gut lumen (Hasegawa, 2004; Tawab et al., 2003). Of the antitumor aspect, it has been reported that some high abundant NOGs with more than two sugar moieties including ginsenosides Rb_1, Rb_2, Rc, Rd_2, Re, Rf, Rg_1, Rg_2, and Ro, exert no anti-prostate cancer effects *in vitro* (Kim et al., 2004; Liu et al., 2000), but some rare 20(S)-protopanaxadiol ginsenosides with one or two sugar moieties at C-3

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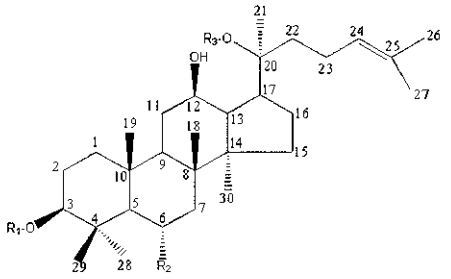

Compound	R_1	R_2	R_3
Protopanaxadiol type:			
Rb_1	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Glc
Rb_2	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Arap
Rg_3	-Glc^{2}-^{1}Glc	-H	-H
Rh_2	-Glc	-H	-H
Rc	-Glc^{2}-^{1}Glc	-H	-Glc^{6}-^{1}Araf
Rd (M10)	-Glc^{2}-^{1}Glc	-H	-Glc
Compound K (C-K, M1)	-H	-H	-Glc
20(S)-protopanaxadiol (Ppd, M12)	-H	-H	-H
Protopanaxatriol type:			
Re	-H	-O-Glc^{2}-^{1}Rha	-Glc
Rf	-H	-O-Glc^{2}-^{1}Glc	-H
Rg_1	-H	-O-Glc	-Glc
Rh_1	-H	-O-Glc	-H
F_1 (M11)	-H	-O-H	-Glc
20(S)-protopanaxatriol (Ppt, M4)	-H	-O-H	-H

Fig. 1. Structure of ginsenosides. Glc: β-D-glucopyranosyl; Arap: α-L-arabinopyranosyl; Araf: α-D-arabinofuranosyl; Rha: α-L-rhamnopyranosyl

position, including Rg_3 and Rh_2, inhibit the growth of both androgen-dependent and -independent prostate cancer cells *in vitro* (Kim et al., 2004; Liu et al., 2000). The aglycone of 20(S)-protopanaxadiol family, protopanaxadiol (Ppd), which is also one minor IBM in human of NOGs, has been reported to have anti-prostate cancer activity *in vitro* in AIPC PC-3 cells (Wang et al., 2008; Wang et al., 2007). Wang et al. have demonstrated that the IC_{50} value of Ppd in PC-3 cells was lower than that of Rg_3 and Rh_2 (Wang et al., 2007). In addition, our previous studies have proved that NOGs might influence the *in vivo* hepatic cytochrome P450 activities via their intestinal metabolites producing after oral administration (Liu et al., 2004; Liu et al., 2006a; Liu et al., 2006b).

These reports prompted us to consider whether other ginsenosides, particularly the IBMs, share similar activities with Rg_3, Rh_2, or Ppd, whether the bioactivity of anti-AIPC of ginsenosides will increase as the sugar moieties located on C-3, C-6, and C-20 of the gonane structure decreases, and whether the biotransformation of ginsenosides in gastrointestinal tract plays an important role in its *in vivo* anti-AIPC activity.

In the present study, with a set of ginsenosides, we found that similar to ginsenoside Ppd (Wang et al., 2008), three IBMs including F_1, CK, and Ppt exerted anti-AIPC activities. We also demonstrated a possible structure-function relationship and possible mechanism for cytotoxic effects in human AIPC PC-3 cells that could be explained on the basis of the hydrophobic character of the ginsenosides. In view of the more potent cytotoxic effects of IBMs than that of NOGs towards human AIPC cells, and the higher absorption from gastrointestinal tract, the deglycosylation by intestinal bacteria may play an important role in their *in vivo* anti-AIPC activities.

MATERIALS AND METHODS

Cell lines

PC-3 cell was obtained from Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI 1640 (Gibco) with 10% fetal bovine serum (FBS, Hangzhou sijiqing biological engineering materials Co., Ltd. China) at 37°C in a 5% CO_2-humidified incubator.

Ginsenosides

Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Ppt, and Ppd was provided by Korea Ginseng Society (Purity >95%). Ginsenosides Rh_1 and CK was provided by Dr. Hideo Hasegawa (Fermenta Herb Institute Inc, Tokyo, Japan) (Purity >98% as assessed by HPLC). Ginsenosides F_1 (Purity >90%) was provided by Dr. Yi-Xuan Zhang (Shenyang Pharmaceutical University, Shenyang, China).

SRB staining assay.

The cytotoxic effects of ginsenosides were assessed by Sulforhodamine B (SRB) assay as described previously (Keepers et al., 1991). PC-3 cells were plated in a 96-well plate at a density of 7500 cells/well in a final volume of 50 μL RPMI 1640 media containing 10% FBS and incubated for 24 h. Then, 50-μL aliquots of each drug dissoluted in RPMI 1640 were added to wells. The final concentration of DMSO for each incubation system was 0.5%. After incubated for 48 h, SRB test was performed to assess cytotoxicity of these compounds. In brief, 50 μL/well 50% ice-cold trichloroacetic acid (TCA, 50 μL) was added. The plate was kept at 4°C for one hour after which was washed five times with cold water, then stained with SRB (Sigma) for 30 min at 37°C. After washing with 1% acetic acid, the bound dye was solubilized with 100 μL Tris base (10 mM, pH 10.5) and then the absorbance at 570 nm was read in a microplate reader (Model 550, Bio-Rad).

Hoechst 33342 staining assay

The Hoechst 33342 staining assay was preformed as previously reported (Cao et al., 2007). In brief, after treatment of PC-3 cells with ginsenosides Ppd (25 μM) and Ppt (50 μM), all the cells was harvest and washed with

PBS twice, and then incubated with Hoechst 33342 (10 μM, Sigma) at 37°C for 30 min. The stained cells were visualized under a fluorescence microscope (Nikon).

Annexin V-PI staining assay

PC-3 cells were seeded in 6-well plates at a density of 1.5×10^5 cells, respectively. After treated with ginsenosides at determined concentration (25 μM and 50 μM for Ppd and Ppt, respectively), both attached and floating cells were harvested and stained with Annexin V-PI according to the manufacturer's procedure (BD Bioscience). The samples were analyzed with a Becton Dickinson FACSVantage SE instrument. Cells that were positive for Annexin V-FITC alone (early apoptosis) and Annexin V-FITC and PI (late apoptosis) were counted.

Mitochondrial membrane potential ($\Delta\Psi_m$) analysis

The uptake of the cationic fluorescent dye Rhodamine 123 has been used for the estimation of mitochondrial membrane potential (Yang et al., 2006). After treatment with ginsenosides at determined concentrations (50 μM for Ppt and 25 μM for Ppd, respectively) for 48 h, control and treated cells were harvested, washed twice with PBS buffer (pH 7.4), and incubated with 0.5 μM Rhodamine 123 (ICN Biomedicals, Inc) at 37°C for 30 min. The fluorescence intensity was measured using an FACSVantage SE flow cytometer (BD) at 490 nm excitation and 520 nm emission wavelengths, and analyzed with the FCS express V3 (De Novo Software). Results were expressed as the fluorescence retained within the cells.

Cell proliferation assay

5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) is equally partitioned among divided daughter cells, permitting the division number of these cells to be determined from fluorescence measurements, providing accurate measurements of the distribution of cells across generations. Proliferative capacity was measured using the CFSE (Beyotime, China) dilution assay as described previously (Lyons and Parish, 1994). In brief, PC-3 cells were labeled with CFSE and then planted on 6-well plate and incubated for 24 h. Then old media was replaced with fresh media containing ginsenosides Ppt (50 μM) and Ppd (25 μM), or 0.5% DMSO control (the final concentration of DMSO for each incubation is 0.5%). After 48-h incubation, cells were harvested and washed with PBS twice. The fluorescence intensity was measured with an FCMScan flow cytometer, and the proliferations index, which indicated the average number of cells that an initial cell became, were calculated with Modfit™ V3.2 software.

Cell cycle distribution

PC-3 cells were seeded in 6-well plate at a density of 1.5×10^5 cells/well. After 24-h incubation followed 48-h 25-μM Ppd or 50-μM Ppt treatment, both attached and floating cells were harvested and stained with cell cycle kit according to the manufacturer's procedure (Genmed Scientifics Inc.). The fluorescence intensity of individual cells was measured by a flow cytometer (FACSVantage SE, BD).

Western blotting

After incubated PC-3 cells with Ppd (25 μM) and Ppt (50 μM) for 48h, all the cells were lysed and the protein concentration was determined using the BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's recommendations.

Twelve percent SDS-PAGE gel electrophoresis and transfer of the separated proteins to PVDF membrane were performed using standard procedures. After being blocked with 5% nonfat dry milk in phosphate-buffered saline over night at 4°C, the blots were incubated with anti-cyclin D1 (Santa Cruz Biotech, Inc., 1:250 dilution), anti-cyclin A (ABZOOM, US., 1:1000 dilution), and β-actin monoclonal antibody (Santa Cruz Biotech, Inc., 1:1000 dilution) overnight at 4°C, washed three times with tris-buffered saline with 0.1% Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibody (Zhongshan Goldenbridge Biotechnology, CO., LTD., 1:10000 dilution). All bands were detected using an ECL Western blot kit (Beyotime, China).

Relationship of logP and anti-cancer activity

LogP values were generated with VCCLAB (Virtual Computational Chemistry Laboratory) (http://www.vcclab.org/lab/alogps/start.html). The correlation analysis and linear regression of Log(IC_{50}) values and LogP was performed to unclose the possible structure-activity relation.

RESULTS

Cytotoxic effect of ginsenoside on PC-3 cells

Results from our study (Fig. 2 and Table I) showed that Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, and Rh_1 exerted no or weak cytotoxic effects on PC-3 cells even at a high concentration of 500 μM. However, the IBMs except Rh_1, including CK, Ppd, F_1 and Ppt displayed a dose-dependent cytotoxic activity on PC-3 cells with an IC_{50} value of 47.0±1.9 μM, 22.5±2.9 μM, 82.2±6.3 μM, and 45.2 ±5.2 μM, respectively. In confirmation of previous studies (Liu et al., 2000), the IC_{50} value of Ppd was similar to the reported 29.3 μM. The cell growth curves in PC-3 cells after treatment of 20(*S*)-protopanaxadiol CK and Ppd was shaper than that of 20(*S*)-protopanaxatiol F_1 and Ppt.

Relationship of LogPs and cytotoxic activities

Compound lipophilicity is a fundamental physicoche-

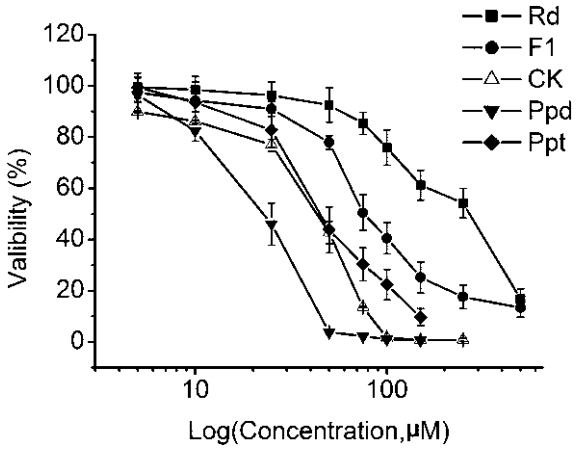

Fig. 2. Growth inhibition effects of ginsenosides Rd, CK, Ppd, F_1, and Ppt on PC-3 cells

Table I. IC_{50} and LogP of ginsenosides

	IC_{50} [a] (μM)	LogP
Protopanaxadiol type		
Rb_1	> 500 (-0.4 ± 2.1)	-0.23
Rb_2	> 500 (9.6 ± 3.2)	-0.02
Rc	> 500 (3.3 ± 1.5)	-0.17
Rd	257.4 ± 25.8	0.73
C-K	47.0 ± 1.9	3.85
Ppd	22.5 ± 2.9	5.37
Protopanaxatriol type		
Re	> 500 (-7.3 ± 2.1)	0.6
Rf	> 500 (-8.7 ± 3.9)	1.13
Rg_1	> 500 (-13.3 ± 4.3)	1.01
Rh_1	> 500 (19.4 ± 1.9)	2.68
F_1	82.2 ± 6.3	2.69
Ppt	45.2 ± 5.2	4.21

[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data were showed as mean ± SD (n=3).

mical property that plays a pivotal role in the pharmacokinetic and pharmacodynamic process of therapeutic drugs. Lipophilicity is expressed in several different ways, including terms such as LogP, cLogP, etc. Here the ALOGPS LogPs for ginsenosides were used to describe the lipophilicity of the molecules. In the present study, we compare the correlation between the cytotoxic effects of the tested ginsenosides and their LogP values. A strong liner relationship was observed between LogP and Log (IC_{50}), which was used to describe the activities of the ginsenosides. The r^2 was 0.9935 ($P < 0.001$) (Fig. 3). This result indicated a high level of correlation between the anti-AIPC activity and the LogP of the ginsenosides (Fig. 3).

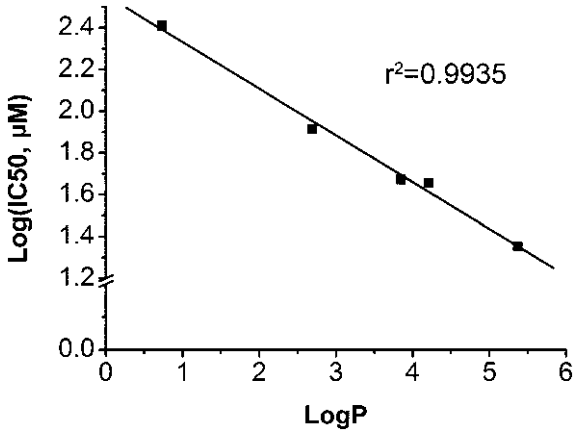

Fig. 3. Correlation of LogP and Log (IC_{50}) for ginsenosides Rd, CK, Ppd, F_1, and Ppt

Apoptosis of PC-3 cells induced by ginsenosides

Since the cell growth inhibitory effects of Ppd and Ppt were observed using the SRB assay, which provided a general view of cell survival, but did not reflect the mechanisms of action, we investigated the effects of Ppd and Ppt on cell apoptosis and proliferation. PC-3 cells was stained with Hoechst 33342 after incubated with Ppd (25 μM) and Ppt (50 μM) for 48 h. The Hoechst 33342 dye stains condensed chromatin of apoptotic cells more brightly than chromatin of normal cells indicating apoptotic cell death occurred after the treatment of the two ginsenosides (Fig. 4A). The Annexin V/PI staining assay also showed that both Ppd at 25 μM and Ppt at 50 μM could increase PC-3 cells apoptosis after 48-h treatment compare to control cells (Fig. 4B). The apoptosis index of PC-3 cells was 317% and 440% after the treatment of Ppt and Ppd.

Ginsenosides induced apoptosis through a mitochondrial-mediated pathway

Due to the disruption of mitochondrial integrity is one of the early events leading to apoptosis (Ly et al., 2003), the effects of Ppd and Ppt on $\Delta\Psi_m$ were investigated. We examined the state of the $\Delta\Psi_m$ after ginsenosides treatment by measuring the relative differences in fluorescence of the cationic dye Rhodamine 123 between control and ginsenosides -treated PC-3 cells. As showed in Fig. 5, after treated with Ppd and Ppt for 48 h, there was a significantly decline in $\Delta\Psi_m$, with positive rate of 82.9% and 61.4% for Ppd and Ppt treatment group, respectively, which was much higher compared to the 7.4% for control group. These results indicated that both the two ginsenosides were capable of decreasing $\Delta\Psi_m$. The results demonstrated the damage to mitochondria membrane potential, which may further activate the intrinsic pathway of apoptosis, may be involved in the apoptosis process induced by

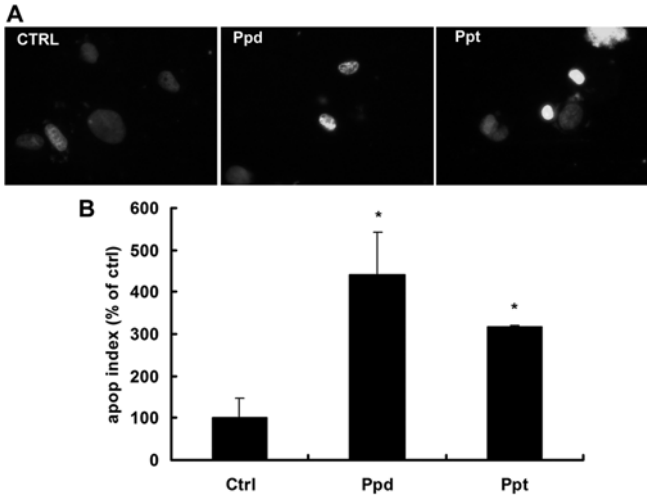

Fig. 4. Induction of apoptosis in PC-3 cells after treatment by ginsenosides Ppd (25 μM) and Ppt (50 μM). (A) Hoechst staining after treated PC-3 cells with Ppd and Ppt. Cells in the treated groups showed condensed of chromatin. (B) The apoptotic index of PC-3 cells after treatment by ginsenosides Ppd (25 μM) and Ppt (50 μM) using annexin V-PI staining assay, calculated in comparison to untreated cells. **P*<0.05 compared to control.

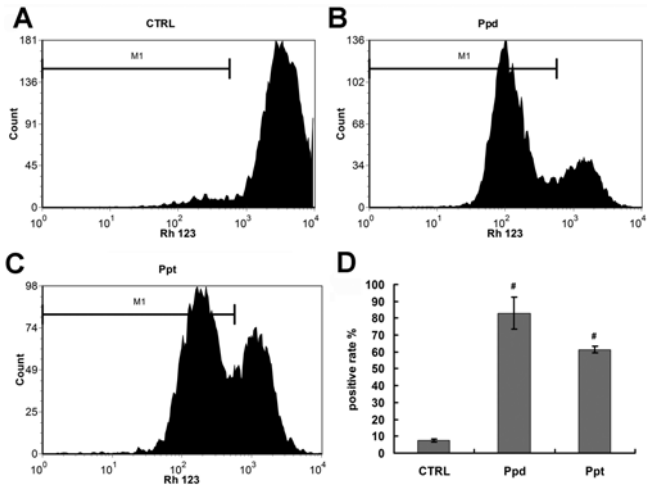

Fig. 5. Mitochondrial transmembrane potential of PC-3 cells after treated with ginsenosides Ppd and Ppt for 48 h. A: Control; B: Ppd; C: Ppt; D: Comparison of the positive rate of PC-3 cells after treated with Ppd and Ppt. # *P*<0.05.

ginsenosides.

Inhibition of cell proliferation after ginsenosides treatment

The stable incorporation of the intracellular dye CSFE into cells is a powerful tool to monitor cell division *in vitro*, which has been extensively utilized to demonstrate the anti-proliferative effect of chemicals. For experiments involving CFSE, curve fits were performed on the CFSE

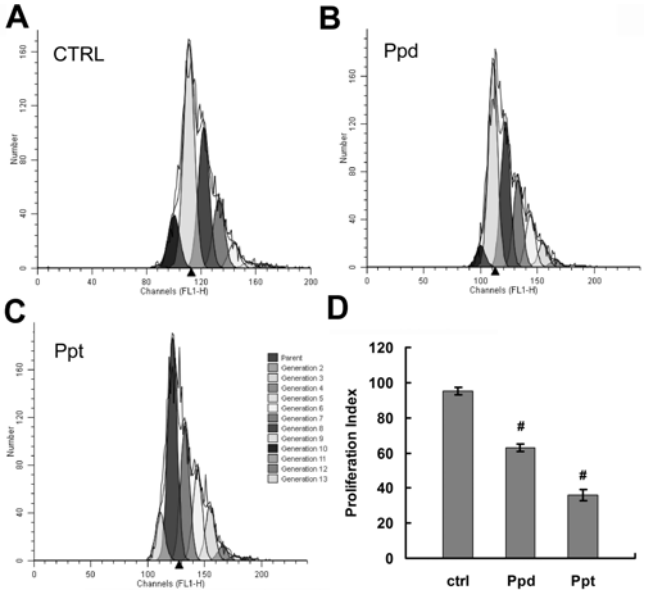

Fig. 6. Proliferation inhibition effects of ginsenosides Ppd and Ppt after 48-h treatment. The FCM data was analyzed and the proliferation index was calculated with Modfit software. The proliferation of PC-3 cells was inhibited by the treatment of ginsenosides. A: Control; B: Ppd; C: Ppt; D: Comparison of the proliferation index of PC-3 cells after Ppd and Ppt treatment. # *P*<0.05.

fluorescence histogram (Fig. 6) to determine the distribution of cells across successive generations of division and calculate the proliferation index using the ModFit^{TM} V3.2. The results showed that treatment of Ppd and Ppt for 48 h, the proliferation index was 63.0 and 36.1, respectively, which was lower than control cells' 95.1, indicating the inhibition against PC-3 proliferation, which might contribute the anti-AIPC effects of ginsenoside.

Cell cycle distribution

To determine whether the inhibition of PC-3 cells' proliferation was a consequence of the cell cycle being arrested at a specific phase, the cell cycle distribution was analyzed after ginsenosides treated. As showed in Fig. 8, a hypodiploid peak of DNA characteristic of apoptosis was observed. Cell cycle of PC-3 cells was arrested in the S phase in response to 25 μM Ppd or 50 μM Ppt treatments. Percentage of cells in the S phase increased in a time-dependent fashion by 32.9% and 27.7% of the total cells 48 h after treatment with Ppd and Ppt, respectively (Fig. 7). In contrast, in control cells, percentages of cells in S phases were 18.5% after 48 h incubation. Compared to the control group, the slight increase of G1 phase cells was also observed in the Ppt treatment group, while the change of the G1 phase in the Ppd group was not obvious. Significant decrease of G2/M phase cells was both detected in the Ppd and Ppt group. These results

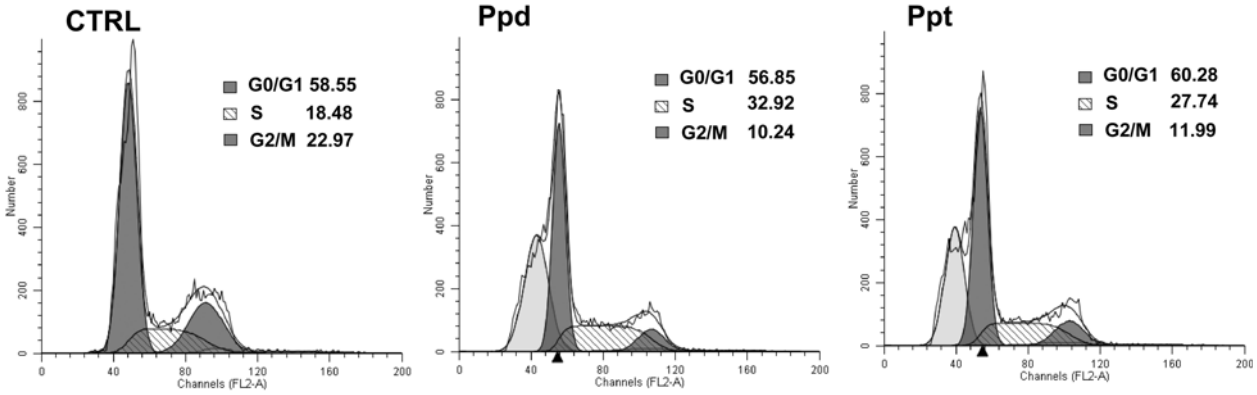

Fig. 7. Effects of ginsenosides Ppt and Ppd on the cell-cycle distribution of PC-3 cell

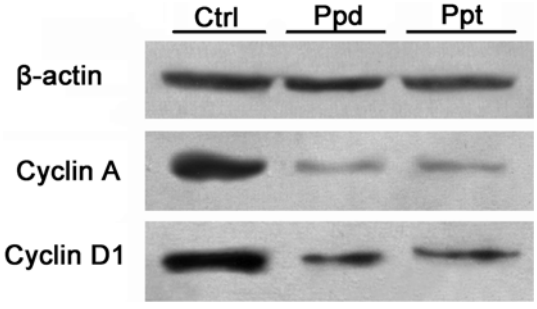

Fig. 8. Western blotting of cyclin D1 and cyclin A in PC-3 cells treated with or without Ppd or Ppt. β-actin blotting was used as control.

clearly indicated that Ppd and Ppt induce the cell cycle arrest in PC-3 cells.

Western blotting of Cyclin D1 and Cyclin A

After 48 h of Ppd or Ppt treatment, the expression of cell cycle regulators was examined to clarify the mechanisms of the cell cycle arrest at G1 and S phase. Cyclin D1 and cyclin A, which involved in the progress of G1 phase and S phase, respectively, were investigated. Both of the expression these two proteins decreased after the treatment of ginsenosides (Fig. 8). These results indicated that the decreased expression of the cyclin by Ppd and Ppt leads to the arrest of the cell cycle.

DISCUSSION

Several NOGs or their IBMs, including Rg_3, Rh_2, and Ppd, have been demonstrated to exert anti-AIPC activities *in vitro* and *in vivo* (Wang et al., 2007, 2008). However, as for some absorbable IBMs of NOGs such as F_1, Rh_1, CK, and Ppt, which might be the really pharmacological components of ginseng (Hasegawa, 2004; Tawab et al., 2003), whether they share similar activities as Rg_3, Rh_2, or Ppd are still not clear. So a systemic study is needed. In the current study, AIPC PC-3 cell was involved to investigate the effects of a set of ginsenosides on human AIPC.

Among all of the NOGs tested, 20(S)-protopanaxadiol ginsenoside Rb_1, Rb_2, Rc, and Rd, as well as 20(*S*)-protopanaxatriol ginsenoside Re, Rf, and Rg_1, together with Rh_1, one of the IBMs which can be detected in human blood after oral administration of ginseng extracts (Tawab et al., 2003), exerted no or weak growth inhibition effects on PC-3 cells even at a high concentration of 500 μM. However, the IBMs except Rh_1, including CK, Ppd, F_1, and Ppt, which can be detected in a higher level in human blood after oral administration of ginseng extracts (Hasegawa et al., 1996; Tawab et al., 2003), displayed a dose-dependent cytotoxic activity on PC-3 cells. Generally, the IBMs exhibited more potent anti-AIPC effects towards human AIPC PC-3 cells than the NOGs, which was similar to the results in other cancer cell lines (Kang et al., 2005; Li et al., 2006; Oh and Lee, 2004; Popovich and Kitts, 2002; Wakabayashi et al., 1998; Wang et al., 2008; Yu et al., 2007).

As for structure and activity relationship investigation, a strong liner relationship between LogPs and Log(IC_{50})s of the ginsenosides investigated was observed, indicating the sugar moieties and C-6 hydroxyl group which may influence the hydrophobicity of the molecules could impact the anti-AIPC efficiency of ginsenosides.

The number of sugar moieties alters the anti-AIPC activity of ginsenosides. The activity of the 20(*S*)-protopanaxadiol family of ginsenosides has been demonstrated to be in the order: Ppd>CK>Rd>Rb_2~Rc~Rb_1. Similar results were also found in the 20(*S*)-protopanaxatriol family, that is in the order: Ppt>F_1>Rh_1>Rg_1~Rf~Re. The previously reported results in THP cells and prostate cancer cells of Ppd, Rh_2, and Rg_3 also consist with the phenomena (Popovich and Kitts, 2002; Wang et al., 2008; Wang et al., 2007). These results demonstrated that the anti-AIPC activities of ginsenosides decline with the increase of

sugar moiety number.

The substitutes at C-6 are of peculiar importance for the anti-AIPC activity of ginsenosides. Ppd and Ppt, as well as CK and F_1, have the same number and position of sugars except the difference of the substitute at C-6 (Fig. 1). However, Ppd and CK exerted more potent cytotoxity than Ppt and F_1 in PC-3 cells, respectively. In addition, Rh_1 and F_1 have the same number of sugar moieties and the same molecular weight, but the cytotoxic activity towards PC-3 cells was dramatically different with an IC_{50} of 82.2 μM for F_1 and above 500 μM for Rh_1. The difference between these two compounds is the position of the sugar moiety, which is at C-20 in F_1 and at C-6 in Rh_1. The number of glycose moiety of Rh_1 was also the same with two 20(*S*)-protopanaxadiol ginsenosides, CK and Rh_2. However, both CK and Rh_2 exerted more potently effects than Rh_1. The differences among these structures also occurred at C-6. Furthermore, although the LogP values of four 20(*S*)-protopanaxatriol ginsenosides, Re, Rf, Rg_1, and Rh_1 were higher than Rd, but none of these three 20(S)-protopanaxatriol ginsenosides exerted anti-AIPC activity. The C-6 substituent is the significant structural difference between these two group ginsenodies. These results suggested that the existence of sugar moiety at C-6 could attenuate the anti-AIPC activities of ginsenosides, which might increase the steric hindrance for these molecules to bind to their targets.

By investigating the structure and function relationship, we propose that the deglycosylation of NOGs catalyzed by intestinal bacteria, which increase LogP and decrease the steric hindrance at C6, may cause the enhancement of anti-AIPC activity of ginsenosides.

Since ginsenosides Ppd and Ppt exhibited the most potent inhibitory effects in 20(*S*)-protopanaxadiol family or 20(S)-protopanaxatriol family, respectively, we chose these two compounds for further investigation to understand the mechanism of the growth inhibition effects on human prostate PC-3 cells by investigating their effects on cell apoptosis and proliferation.

It has been demonstrated that several ginsenosides could inhibit tumor-cell proliferation, induce differentiation and apoptosis, and inhibit tumor-cell invasion and metastasis (Wang et al., 2008; Yue et al., 2007). The Hoechst 33342 staining assay and flow cytometry analysis showed that both Ppd and Ppt could induce apoptosis in PC-3 cells compare to control cells (Fig. 4A & B). The effect of Ppd was similar to the previously reported (Wang et al., 2008). These results indicated that both 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol families of ginsenosides could inhibit the growth of human prostate cancer cell through inducing apoptosis.

The collapse of the mitochondrial transmembrane potential plays an essential role in mediating apoptosis in that it allows the release of apoptotic mediators, such as cytochrome c and the apoptosis-inducing factor, into the cytoplasm (Gross et al., 1999). We examined $\Delta\Psi_m$ as evidence for mitochondrial-membrane permeability. Similar to the effects of ginsenosides Rd in Hela cells (Yang et al., 2006), $\Delta\Psi_m$ in PC-3 cells was decreased significantly by the treatment with Ppd or Ppt (Fig. 6), indicating alterations of the permeability of the outer membrane of mitochondria. The lowered level of $\Delta\Psi_m$ may be participated in the apoptosis led by ginsenosides.

Besides apoptosis, the inhibition of proliferation was another pathway for ginsenosides to inhibit the growth of cancer cells. Rg_3, Rh_2, and Ppd have been reported to have inhibitory effects in PC-3 cells proliferation (Kim et al., 2004; Wang et al., 2008; Wang et al., 2007). Our results showed that Ppd inhibited the proliferation of PC-3 cells, in accordance with previous results. In addition, Ppt also efficiently inhibited the proliferation of PC-3 cells. It is noteworthy that the proliferation inhibition index was lower in Ppt when measured at concentration near IC_{50}.

The blockage of cell cycle at S phase cells was both significant after Ppt or Ppd treatment in our experiments (Fig. 7). Wang et al. reported that 25 μM Ppd treatment of PC-3 cells have no effects on the cell cycle. The different may be due to the longer incubation time in our investigation of 48 h. The blockage indicated that these two ginsenosides may inhibit the DNA synthesis in PC-3 cells. Cyclins are a family of proteins involved in the progression of cells through the cell cycle. The increase of S phase cells may be the result of the decrease of cyclin A (Fig. 8), which binds to S phase Cdk2 and is required for the cell to progress through the S phase. Cyclin D1, which forms a complex CDK4 or CDK6 and is required for cell cycle G1/S transition, was observed to decrease after the treatment of Ppd and Ppt (Fig. 8). However, the increase of G1 phase was not observed in the Ppd treated group. This may be due to the decrease of cyclin A was more than that of cyclin D1.

In summary, to the best of our knowledge, the present studies provide the first experimental evidence that some IBMs of NOGs, including F_1, CK, and Ppt exert anti-AIPC activity. Using a set of ginsenosides, we found there are no significant difference in action mechanisms between 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatiol family in AIPC PC-3 cells, which may both were involved in the induce of cell apoptosis *via* mitochondrial pathway, together with the inhibition of cell proliferation *via* cell cycle arrest. Moreover, there was a possible structure-function relationship existing that the activity will increase as the decrease of the number of sugar moiety attached, particularly at C-6 of the gonane structure. Thus, the action mechanism of these ginsenosides in anti-

AIPC need be further studied in detail, but the obtained information may be useful in developing novel chemotherapeutic or chemopreventive agents against human prostate cancer.

ACKNOWLEDGMENTS

This work was supported by the 973 program (2007CB707802) of the Ministry of Science and Technology of China, National Natural Science Foundation of China (30630075) and Korea Ginseng Society grant. The authors thank Korea Ginseng Society, Dr. Hideo Hasegawa (Fermenta Herb Institute Inc., Tokyo, Japan) and Dr. Yi-Xuan Zhang (ShenYang Pharmaceutical University, Shenyang, China) for the gifts of ginsenosides.

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