

Cytotoxicities of Ginseng Saponins and their Degradation Products against some Cancer Cell Lines

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In order to elucidate the cytotoxicity-structure correlation of ginseng-derived components, several prosapogenins and saponins were prepared from Korean red ginseng (*Panax ginseng*) saponins by acid hydrolysis or alkaline cleavage, and their chemical structures were identified by a combination of spectral and physical methods. Some of these degradation products showed the cytotoxic activities against various cancer cell lines, A549, SK-OV-3, SK-Mel-2, P388, L 1210 and K562. The significant difference in cytotoxicity between stereoisomers was not found and the activity was inversely proportional to the number of sugars linked to saponins. Diol-type prosapogenins and saponins showed higher cytotoxicity than triol-type ones.

Key words : *Panax ginseng*, Cytotoxicity, Ginsenosides, Prosapogenins, Saponins, Structure-cytotoxicity Relationship

INTRODUCTION

Ginseng radix, the root of *Panax ginseng* C. A. Meyer (Araliaceae), has been used as a tonic or preventive and remedy for a variety of pathological conditions in oriental countries for more than several thousands years. Since the beginning of this century, there have been many reports to provide with the scientific evidences for the pharmacological effects of ginseng. In the middle of 1960s, the cytotoxic activity of ginseng alkaloidal fractions against cancer cells was reported for the first time (Woo and Nakamura, 1965). Successively, Hwang (1978), Ahn (1988), Kim (1989) and Katano (1988) reported the cytotoxic effects of ethereal fractions of ginseng, active components of which were revealed to be polyacetylenes. Kitagawa (1984), and Kikuchi (1993) found ginsenoside Rh₁ or Rh₂ to inhibit the growth of some cancer cells. Ginsenosides, the saponins of ginseng, have been regarded as important principles manifesting the pharmacological and biological activity. Even if the acid condition of the stomach and the alkaline medium in the intestines indicate that the absorption, distribution and excretion of ginsenosides orally administrated may occur after complete or par-

tial hydrolysis, little is still known about the pharmacological effects of the metabolites.

In this paper, some cytotoxic ginsenosides and their derivatives, prepared by chemical and enzymatical treatments, were identified by a combination of spectral and physical methods. We evaluated the cytotoxic activities of these substances against several human and murine cancer cell lines and discussed about the relationship between chemical structures and cytotoxicities.

MATERIALS AND METHODS

Plant materials

The red ginseng was prepared by steaming and drying six-years old ginseng root (*Panax ginseng*) provided by Korea Ginseng and Tobacco Research Institute.

Isolation of ginsenosides

A *n*-BuOH fraction (200 g) obtained from red ginseng (*Panax ginseng*) was chromatographed on silica gel column, eluting with CHCl₃-MeOH-H₂O (9 : 3 : 1 → 65 : 35 : 10) and *n*-BuOH-EtOAc-H₂O (15 : 1 : 4), to afford ginsenosides Rb₁ (1, 8.5 g), Re (2, 4.7 g), Rg₁ (3, 7.1 g). A aqueous fraction (110 g) was dissolved in MeOH (800 ml) and treated with ethereal-diazomethane. The reaction mixture was applied over sil-

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ica gel column, using CHCl_3 -MeOH- H_2O (7:3:1) as eluents, to give methyl-esterified ginsenoside Ro (**4**, 2.1 g).

Preparation of ginsenoside Ro, sapogenin and prosapogenins

Compound **4** (2 g) was dissolved in 5% KOH/MeOH (100 ml) and stirred at room temperature for 2 h. The reaction mixture was neutralized with Dowex 50 W \times 8 (H^+) and subjected to silica gel column, eluting with CHCl_3 -MeOH- H_2O (65 : 35 : 10), to yield ginsenoside Ro (**5**, 1.3 g).

Compound **5** (200 mg) was refluxed in 10% HCl/MeOH (50 ml) for 2 h. The reaction solution was treated with Ag_2CO_3 , filtered, concentrated *in vacuo*, and chromatographed with silica gel column, using *n*-hexane-acetone (3:1) as eluents, to produce oleanolic acid (**6**, 55 mg).

KOH/MeOH (10%) was added in aqueous MeOH solution of **5** (1 g) and saponified at 60°C for 3 h. The reaction mixture was neutralized in the same way as above and concentrated *in vacuo*. The residue was dissolved in absolute MeOH (50 ml) and treated with ethereal-diazomethane (20 ml). The methylated solution was purified with silica gel column, eluting with CHCl_3 -MeOH- H_2O (10:3:1), to afford a prosapogenin (**7**), 28,6'-O-dimethyl-oleanolic acid-3-O- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucuronopyranoside (800 mg). In aqueous solution of **7** (400 mg), β -glucosidase (2 g, from almonds, Fluka 49290) was added and stirred at 37°C for 3 days. The reaction mixture was filtered, concentrated *in vacuo*, and applied over silica gel column (CHCl_3 -MeOH- H_2O =12:3:1) to yield another prosapogenin (**8**), 28,6'-O-dimethyl-oleanolic acid-3-O- β -D-glucuronopyranoside (47 mg).

Preparation of panaxadiol (**9**) and panaxatriol (**10**) from *n*-BuOH extract

The *n*-BuOH extract (2 g) was dissolved in 10% H_2SO_4 /MeOH and refluxed for 5 h. The reaction mixture was partitioned between ethyl ether and water. The organic layer was washed with saturated aqueous NaHCO_3 and brine. The solution was dried over magnesium sulfate anhydrous, filtered, and evaporated *in vacuo*. The residue was applied over silica gel column, using EtOAc-benzene (2:5) as eluents, to afford panaxadiol (**9**, 110 mg) and panaxatriol (**10**, 97 mg).

Preparation of diol-type sapogenin and prosapogenins from ginsenoside Rb₁ (**1**)

Compound **1** (4 g) was dissolved in 50% aqueous acetic acid and hydrolyzed at 70°C for 2 h. The reaction solution was concentrated at 60°C and the resi-

due was partitioned with *n*-BuOH and water. The *n*-BuOH layer was concentrated and chromatographed on silica gel column, using CHCl_3 -MeOH- H_2O (10:3:1) as eluents, to produce the racemic mixture of 20 (S)- and 20 (R)-ginsenoside Rg₃ (2.1 g).

In a solution of the racemic mixture (2 g) in pyridine, acetic anhydride was added drop by drop in ice bath and stirred at room temperature for 10 h. The reaction mixture was dispersed in ice water and extracted with EtOAc three times. The organic layer was washed with 5% HCl, saturated aqueous NaHCO_3 and brine, followed by drying over magnesium sulfate anhydrous. The residue was applied over silica gel column, eluting with dichloromethane-EtOAc (8:1) to afford peracetylated-20(S)-ginsenoside Rg₃ (**11**, 930 mg) and peracetylated-20(R)-ginsenoside Rg₃ (**12**, 1.2 g).

Compounds **11** (200 mg) and **12** (200 mg) were dissolved in 5% NaOH/*n*-BuOH (20 ml) in ice bath and stirred at room temperature over night, respectively. After the reaction mixtures were washed with water and evaporated, the residues were applied over silica gel column, using CHCl_3 -MeOH- H_2O (9:3:1) as eluents, to give 20 (S)-ginsenoside Rg₃ (**13**, 58 mg) and 20 (R)-ginsenoside Rg₃ (**14**, 65 mg), respectively.

Compounds **11** (200 mg) and **12** (200 mg) were dissolved in 5% NaOH/*n*-BuOH (40 ml) and stirred at 80°C for 4 h, respectively, being monitored by silica gel TLC. The reaction mixtures were treated by the same procedure as described above and purified by a combination of silica gel column chromatography (CHCl_3 -MeOH- H_2O =10:3:1) and semi-preparative HPLC (Alltech-NH₂, 1 \times 25 cm, CH_3CN - H_2O =9:1, RI detector) to afford 20 (S)-ginsenoside Rh₂ (**15**, 35 mg) and 20 (R)-ginsenoside Rh₂ (**16**, 43 mg). 20 (R)-ginsenoside Rh₂ (**16**): colorless fine crystals (MeOH- H_2O -dioxane); m.p. 208-210°C; $[\alpha]_D^{25}$: -6.2° (c 3.0, pyridine), lit. (Chen *et al.*, 1987) : colorless needles; m.p. 288-290°C; $[\alpha]_D^{25}$: +21.1° (c 0.61, MeOH).

Each 5% NaOH/*n*-BuOH (40 ml) solution of compounds **11** (200 mg) and **12** (200 mg) was stirred at 80°C for 10 h. Each reaction mixture was treated in the same way as described above and was applied over silica gel column, using EtOAc-benzene (1:2) as eluents, to give 20(S)-protopanaxadiol (**17**, 45 mg) and 20(R)-protopanaxadiol (**18**, 50 mg), respectively.

Preparation of triol-type sapogenin and prosapogenins from ginsenoside Re (**2**)

From ginsenoside Re (**2**, 4 g), the similar procedure to that of ginsenoside Rb₁ (**1**) produced 20^s-ginsenoside Rg₂ (**19**, 710 mg, CHCl_3 -MeOH- H_2O =9:3:1), peracetylated-20(S)-ginsenoside Rg₂ (**20**, 950 mg), peracetylated-20(R)-ginsenoside Rg₂ (**21**, 1.3 g), 20(S)-ginsenoside Rg₂ (**22**, 63 mg, CHCl_3 -MeOH- H_2O =8:3:1),

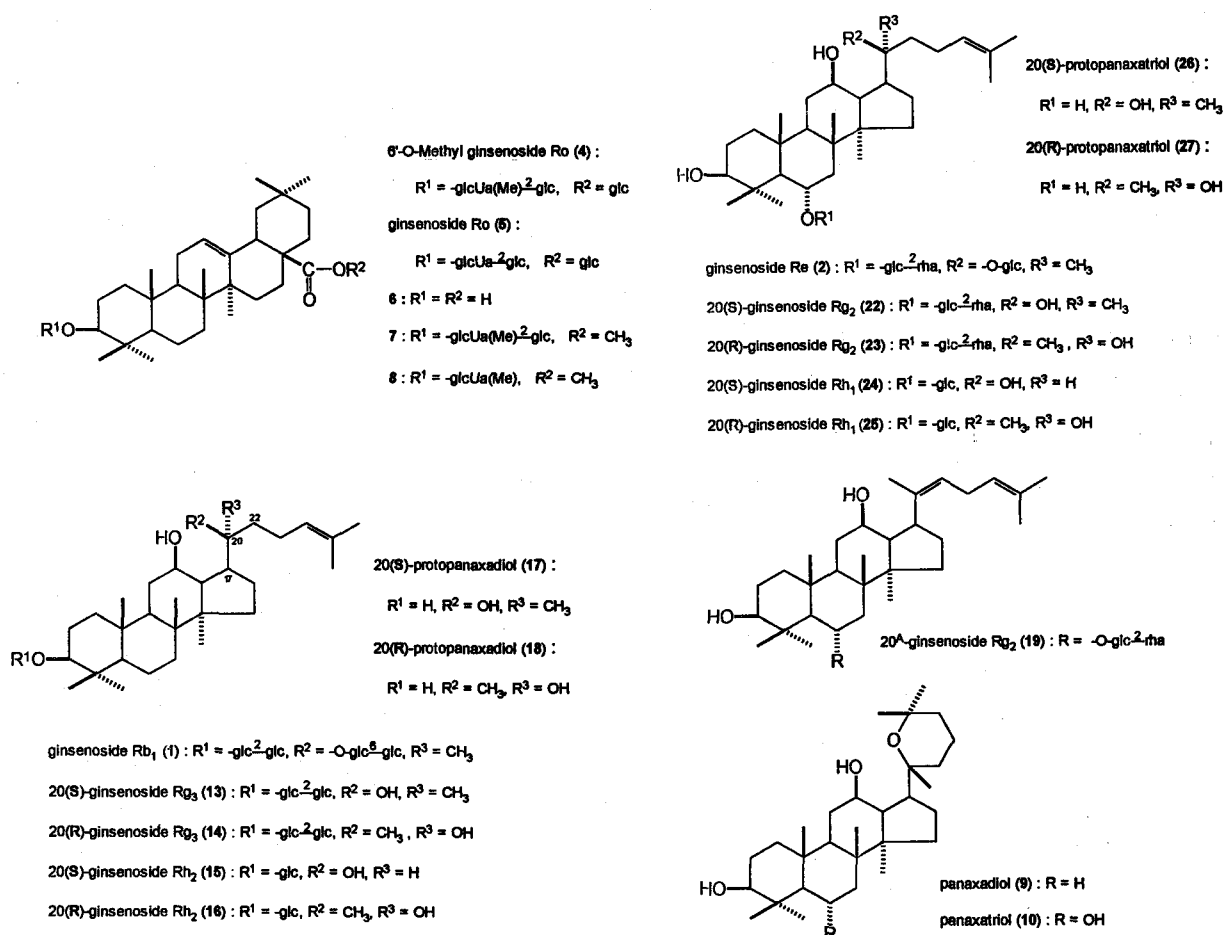


Fig. 1. Structures of saponins, prosapogenins and sapogenins prepared from *Panax ginseng*

20(R)-ginsenoside Rg₂ (23, 72 mg, CHCl₃-MeOH-H₂O=8 : 3 : 1), 20(S)-ginsenoside Rh₁ (24, 41 mg), 20(R)-ginsenoside Rh₁ (25, 47 mg), 20(S)-protopanaxatriol (26, 43 mg) and 20(R)-protopanaxatriol (27, 51 mg).

Cancer cell lines

Cancer cell lines used for cytotoxicity test were as follows : A549 (lung carcinoma, human), SK-OV-3 (ovary adenocarcinoma, human), K562 (chronic leukemia, human), SK-Mel-2 (melanoma, human), P388 (leukemia, murine) and L1210 (leukemia, murine). Each cell was maintained in RPMI 1640 medium supplied with 5% fetal bovine serum and incubated at 37°C in a humidified atmosphere at 5% CO₂.

Evaluation of cytotoxicity

Sulforhodamine B method (Skehan *et al.*, 1990) was applied for evaluation of cytotoxic activity. ED₅₀ value, the concentration of sample caused the 50% inhibition of cell growth, was determined graphically by plotting the net growth rate versus the concentration of test sample.

RESULTS AND DISCUSSION

Ginsenosides Rh₁ and Rh₂ were reported to inhibit the growth of cancer cells (Kitagawa, 1984; Kikuchi, 1993). And Momordin I (*Ampelopsis japonica*), whose structure was similar to that of ginsenoside Ro, was found to be cytotoxic. Even if most ginsenosides are metabolized in stomach or intestine after oral administration, little attention has been paid about the pharmacological effects of the metabolites. This information led us to evaluate the cytotoxicities of degradation products of ginsenoside Rh₁, Rh₂ and Ro.

Aqueous or *n*-BuOH soluble fraction of ginseng extract contain lots of compounds with similar R_f values to that of ginsenoside Ro on silica gel TLC. And ginsenoside Ro has two carboxyl groups at C-28 and C-6' in the molecule. Those factors hindered the isolation of ginsenoside Ro from ginseng root. But, it was easily isolated and purified in the form of methyl ester treated with diazomethane. Alkaline treatment of compound 4 afforded ginsenoside Ro (5) by deacetylation at room temperature, and a prosapogenin (7)

by cleavage of ester-glycosidic linkage at C-28 at high temperature, respectively. The enzymatic hydrolysis of the prosapogenin (7) with β -glucosidase produced another prosapogenin (8) by elimination of terminal glucose at C-2'. Compound 5 gave an oleanolic acid, D-glucuronic acid and D-glucose on complete acidic hydrolysis. The structures of obtained products were identified by comparison of spectral and physical data reported in the literatures.

The major saponins of ginseng root, ginsenoside Rb₁ (1), Re (2), Rg₁ (3) and the mixtures of Rb₂, Rc and Rd, were isolated from *n*-BuOH soluble fraction of ginseng extract by usual procedure.

The hydrolysis of ginsenoside Rb₁ (1) with acetic acid produced a racemic mixture of 20(S)- and 20(R)-ginsenoside Rg₃ by elimination of sugars linked to the C-20. Usually, the racemic mixture is very difficult to be separated into each isomers. The racemic mixture of 20(S)- and 20(R)-ginsenoside Rg₃ wasn't separated by usual silica gel chromatography, either. However, after acetylation of the mixture, each isomer was easily separated with silica gel column chromatography as acetylated compounds, peracetylated-20(S)-ginsenoside Rg₃ (11) and peracetylated-20(R)-ginsenoside Rg₃ (12).

Deacetylation of compounds 11 and 12 with butanolic NaOH at room temperature produced 20(S)-ginsenoside Rg₃ (13) and 20(R)-ginsenoside Rg₃ (14), respectively. While, the same treatment as described above at 80°C, reaction time being varied by TLC, afforded 20(S)-ginsenoside Rh₂ (15) and 20(S)-protopanaxadiol (17) from the former, and 20(R)-ginsenoside Rh₂ (16) and 20(R)-protopanaxadiol (18) from the latter. The configurations of C-20 in each isomer were determined by comparison of specific rotation ($[\alpha]_D$ value) (Kim *et al.*, 1991; Kitagawa *et al.*, 1983; Kaku and Kawashima, 1980) and chemical shifts of neighbouring carbons, C-17, C-21 and C-22, in each ¹³C-NMR spectrum, which were clearly distinguished between 20(S)- and 20(R)-isomers (Asakawa *et al.*, 1977).

20(S)- (15) and 20(R)-ginsenoside Rh₂ (16) were isolated from red ginseng by Kitagawa (1983) and from leaves of *Panax ginseng* by Chen (1987), respectively. Chen reported the $[\alpha]_D$ value of the latter as +21.1° (c 0.61, MeOH), while we determined the value as -6.2° (c 3.0, pyridine). Kitagawa (1983) and Kaku (1980) reported those of 15, 20 (S)-(13), and 20(R)-ginsenoside Rg₃ (14) to be +21.8° (c 0.93, MeOH), +16.5° (c 1.0, MeOH), and -14.0° (c 0.26, pyridine), respectively. Moreover, it is well known 20(R)-type ginsenosides are usually very insoluble in MeOH. Accordingly, it will be desirable to revise the optical characteristics reported by Chen (1987).

Protopanaxadiol and protopanaxatriol, dammarane triterpenoid, are the sapogenins of most ginsenosides

isolated from the root of *Panax ginseng*. Generally, acid hydrolysis of ginsenosides affords panaxadiol or panaxatriol, the artificial sapogenin, by cyclization of the side chain of the sapogenins. So, it is very difficult to obtain the genuine sapogenins. However, we could obtain the genuine sapogenins, protopanaxadiol and protopanaxatriol, by the alkaline treatment with NaOH/*n*-BuOH as well as panaxadiol and panaxatriol by acid hydrolysis with H₂SO₄/MeOH.

From ginsenoside Re (2), one of major triol-type ginsenosides, the similar procedure to that of ginsenoside Rb₁ (1) produced not only peracetylated-20(S)-ginsenoside Rg₂ (20), peracetylated-20(R)-ginsenoside Rg₂ (21), 20(S)-ginsenoside Rg₂ (22), 20(R)-ginsenoside Rg₂ (23), 20(S)-ginsenoside Rh₁ (24), 20(R)-ginsenoside Rh₁ (25), 20(S)-protopanaxatriol (26), and 20(R)-protopanaxatriol (27) but also 20^Δ-ginsenoside Rg₂ (19), whose structure was confirmed by a new triplet proton signal observed at δ 5.64 (*J*=5.64 Hz) in the ¹H-NMR spectrum and two new olefinic carbon signals observed at δ c 140.2 and 123.8 in the ¹³C-NMR spectrum. Their chemical structures were characterized by speculation of several physical and spectral data and direct comparison

Table I. ED₅₀ values (μ M) of several ginseng compounds against some cancer cell lines.

Compounds	A549*	SK-OV-3*	SK-Mel-2*	P388*	L1210*	K562*
Oleanane						
4	> 40	> 40	> 40	> 40	29	32
5	> 40	> 40	> 40	> 40	39	33
6	> 40	> 40	> 40	> 40	33	> 40
7	28	22	22	17	15	16
8	15	13	12	3.2	36	14
Panaxadiol						
1	> 40	> 40	> 40	> 40	> 40	> 40
9	19	22	33	10	11	8.3
13	> 40	> 40	> 40	29	23	> 40
14	> 40	> 40	> 40	> 40	> 40	> 40
15	> 40	> 40	24	> 40	> 40	> 40
16	> 40	> 40	> 40	37	> 40	> 40
17	18	20	13	9.8	10	9.8
18	26	19	22	11	12	18
Panaxatriol						
2	> 40	> 40	> 40	> 40	> 40	> 40
10	33	> 40	39	> 40	31	> 40
19	> 40	37	> 40	> 40	38	> 40
22	> 40	> 40	> 40	> 40	> 40	> 40
23	> 40	> 40	> 40	> 40	> 40	> 40
24	> 40	> 40	> 40	> 40	> 40	> 40
25	> 40	> 40	> 40	> 40	> 40	> 40
26	> 40	> 40	> 40	21	19	34
27	> 40	> 40	> 40	34	32	38

*A549: lung carcinoma (human), SK-OV-3: ovary adenocarcinoma (human), SK-Mel-2: melanoma (human), P388: leukemia (murine), L1210: leukemia (murine), K562: chronic leukemia (human)

of the data reported in the literature.

The cytotoxic activities of obtained compounds against various human and murine cancer cell lines were evaluated by SRB method (Skehan *et al.*, 1990) and the results were shown as ED₅₀ value in Table 1.

Ginsenoside Ro (5) and its methyl ester (4) didn't show significant cytotoxicity. While, a prosapogenin (7), produced by elimination of one glucose at C-28 from ginsenoside Ro (5), exhibited increased cytotoxicity comparing to 4 or 5 and another prosapogenin (8), obtained by elimination of terminal glucose at C-2' from 7, showed higher activity even than 7. But saponin of ginsenoside Ro, oleanolic acid (6), didn't show the activity.

Table I indicated that all of saponins, panaxadiol (9), 20(S)- (17), and 20(R)-protopanaxadiol (18), panaxatriol (10), 20(S)- (26) and 20(R)-protopanaxatriol (27) showed higher cytotoxic activities comparing to their glycosides, regardless of genuineness or stereostructure.

The previous workers (Kitagawa, 1984; Kikuchi, 1993) reported the smaller ED₅₀ values of ginsenoside Rh₁ or Rh₂ in cytotoxicities than those determined by us. The difference, which might be derived from different assay method, shall be revised, afterwards. But it was clear that elimination of sugar from saponins or introduction of double bond into molecule increased the cytotoxicity. Diol-type prosapogenins and saponins showed higher cytotoxicity than triol-type ones, and the significant difference in cytotoxicity between stereoisomers was not found.

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