CYTOTOXICITIES OF GINSENG SAPONINS AND THEIR DEGRADATION PRODUCTS AGAINST SOME CANCEʀ CELL LINES AND STRUCTURE—ACTIVITY RELATIONSHIP

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ABSTRACT

Several Prosapogenins and sapogenins obtained by acid hydrolysis or alkaline cleavage of Korean red ginseng saponins were separated and identified by spectral and physical methods. Some of these degradation products showed the cytotoxic activities against various cancer cell lines, that is, A549, SK – OV – 3, L1210, P388 and K562. The significant difference of activity between stereoisomers was not approved and the activity was inversely proportional to the number of sugars binding to sapogenins. It was clear that diol type prosapogenins and sapogenins were more cytotoxic than triol type ones.

INTRODUCTION

There have been many researches studying the antitumor effects of ginseng root. Woo[1] reported that an alkaloidal fraction inhibited the growth of KB cell and HeLa cell. Hwang[2] extracted the root with petroleum ether and found that the extract was cytotoxic on L1210, L5178Y and HeLa cell. Kim[3] and Katano[4] showed that panaxydol and panaxytriol, among several kinds of polyacetylenes isolated from red ginseng, exhibited a distinguished cytotoxic effect against several kinds of human and murine malignant cells, in vitro.

Meanwhile, the root of *Panax ginseng* contains many kinds of saponins which have been regarded as important principles manifesting the pharmacological and biological activity. Kitagawa [5] reported the cytotoxic effect of ginseng saponin for the first time. He isolated ginsenoside Rh₂ as purified component from red ginseng which showed cytotoxic activities against Lewis lung, Morris hepatoma B16 and HeLa cell. Odashima[6] reported cytotoxic effects of ginsenoside Rh₁ and Rh₂ on the growth and differentiation of B16. Zhang[7] and Kikuchi[8] showed ginsenoside Rh₂ inhibited the growth of HRA and S180 sarcoma, respectively. However, the acid condition of the stomach and the alkaline medium in the testine indicate that the absorption, distribution and excretion of ginsenosides may occur after complete or partial hydrolysis. Little is known about these metabolite, which may be produced during the process of preparation of red ginseng.

Therefore, several cytotoxic ginsenosides, that is ginsenoside Rh₂, Rh₁ and Ro, and their derivatives prepared by chemical and enzymatical treatment were separated and identified by several spectral and physical data. By using these substances we evaluated the cytotoxic activity against several human and murine cancer cell lines, and the relationship between cytotoxicities and structures of isolated substances.

MATERIALS AND METHODS

1. Plant materials

The used red ginseng was prepared by steaming from six years old fresh ginseng provided by Korea Ginseng and Tobacco Research Institute.

2. Cell lines

Cancer cell lines used for cytotoxicity test were as below : 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% FBS and incubated at 37°C in a humidified atmospher at 5% CO_2.

3. Cytotoxicity

SRB method[9] was applied for evaluation of cytotoxic activity. Growth inhibition rate was calculated by following formula :

$$\text{Growth ratio}(\%) = \frac{T - Co}{C - Co} \times 100$$

Co : Initial cell concentration
T : Cell concentration after incubation with sample treatment
C : Cell concentration after incubation without sample treatment

The ED_{50} value was determined graphically by plotting the concentration of the test samples versus the growth inhibition rate with log scale.

4. Isolation of ginsenosides

Ginsenoside Rg₁, Re, Rb₁ and the mixture of Rb₂, Rc and Rd were isolated from red ginseng by usual procedure. Ginsenoside Ro was purified by SiO_2 column chromatography after methylesterification of aquous fraction followed by alkaline hydrolysis. All of the obtained substances were identified by several spectral and physical data.

5. Sapogenin and prosapogenins from ginsenoside Ro

To make a sapogenin, 10% aquous HCl was added in the ginsenoside Ro solution of 50% aquous MeOH and reflux for 2 hrs. The reaction solution was neutralized with Ag₂CO₃ and applied on SiO₂ column eluting with CHCl₃ - MeOH to produce oleanolic acid.(Chart 1)

To make prosapogenins, 10% aquous HCl was added in the ginsenoside Ro solution of 50% aquous MeOH was stirred for 2 hrs at 50°C. The reaction mixture was neutralized with Dowex

50w×8(H⁺ form) and methylated by diazomethane. The final products were purified by SiO₂ column using CHCl₃ - MeOH - H₂O as eluent to afford a prosapogenin methylester, 28, 6' - O - dimethyloleanolic acid 3 - O - β - D - glucopyranosyl(1 →2) - β - D - glucuronopyranoside.

In aquous solution of the prosapogenin mentioned above, β - glucosidase was added and hydrolyzed for 3 days at 37°C. After methylesterification another prosapogenin, 28, 6' - O - dimethyl oleanolic acid 3 - O - β - D - glucuronopyranoside was purified by SiO₂ column chromatography(CHCl₃ - MeOH - H₂O).
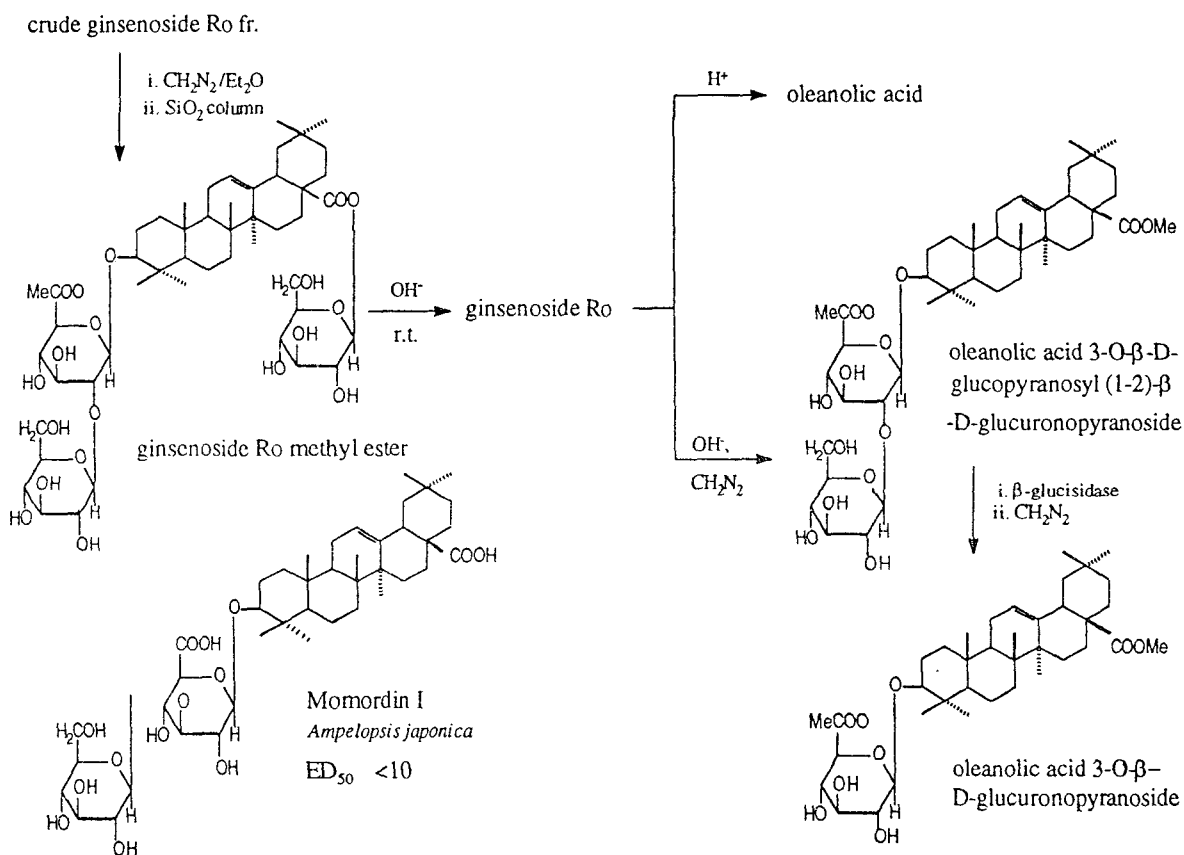

Chart 1. Preparation Procedure of Sapogenin and Sapogenins from Ginsenoside Ro Fraction

6. Panaxadiol and panaxatriol from total saponins

The n - BuOH extract was dissolved in 10% H₂SO₄ in MeOH and refluxed for 5 hrs. The reaction mixture was partitioned between ether and water. The organic phase was washed with saturated aquous NaHCO₃ and brine. The hydrolysis products were applied on SiO₂ column using EtOAc - Benzene as eluents to afford panaxadiol and panaxatriol.

7. Diol - type sapogenin and prosapogenins from ginsenoside Rb₁

The acid hydrolysis of ginsenoside Rb₁ with 50% aquous acetic acid at 70°C produced ⁻20 - ginsenoside Rg₃ and the racemic

mixtures of 20(R&S) - ginsenoside Rg₃, which were separated into 20(R) - ginsenoside Rg₃ and 20(S) - ginsenoside Rg₃ by acetylation (Ac₂O/pyridine) and SiO₂ column chromatography. (Chart 2)

The acetylate of 20(R) - ginsenoside Rg₃ was treated with 5% NaOH/BuOH at r.t. for 1 hr, 40°C for 2 hrs and 90°C for 6 hrs to afford 20(R) - ginsenoside Rg₃, 20(R) - ginsenoside Rh₂, 20(R) - protopanaxadiol, respectively.

20(S) - ginsenoside Rg₃, 20(S) - ginsenoside Rh₂, 20(S) - protopanaxadiol were obtained from the acetylate of 20(S) - ginsenoside Rg₃ by same procedure applied previously. 20(R) - ginsenoside Rh₂ : colorless fine crystals (MeOH - H₂O - dioxane), m.p. 208 - 210°C, [α]ᴅ + 6.2° (c=3.1, pyridine), IRᵥ(K B r ,

ginsenoside Rb1

50% HOAc
70 C, 2hrs

R_1
R_2

20(R&S)-ginsenoside Rg₃

: R_1 = OH or CH₃,
R_2 = CH₃ or OH

Δ20(S)-ginsenoside Rg₃

i. Ac₂O / Py
ii. SiO₂ column

iii. 5% NaOH / BuOH
80 C, 4 - 6 hrs

20(R)⁻-protopanaxadiol
: R_1 = OH, R_2 = CH₃

20(S)--protopanaxadiol
: R_1 = CH₃, R_2 = OH

20(R)-ginsenoside Rh₂
: R_1 = OH, R_2 = CH₃

20(S)-ginsenoside Rh₂
: R_1 = CH₃, R_2 = OH
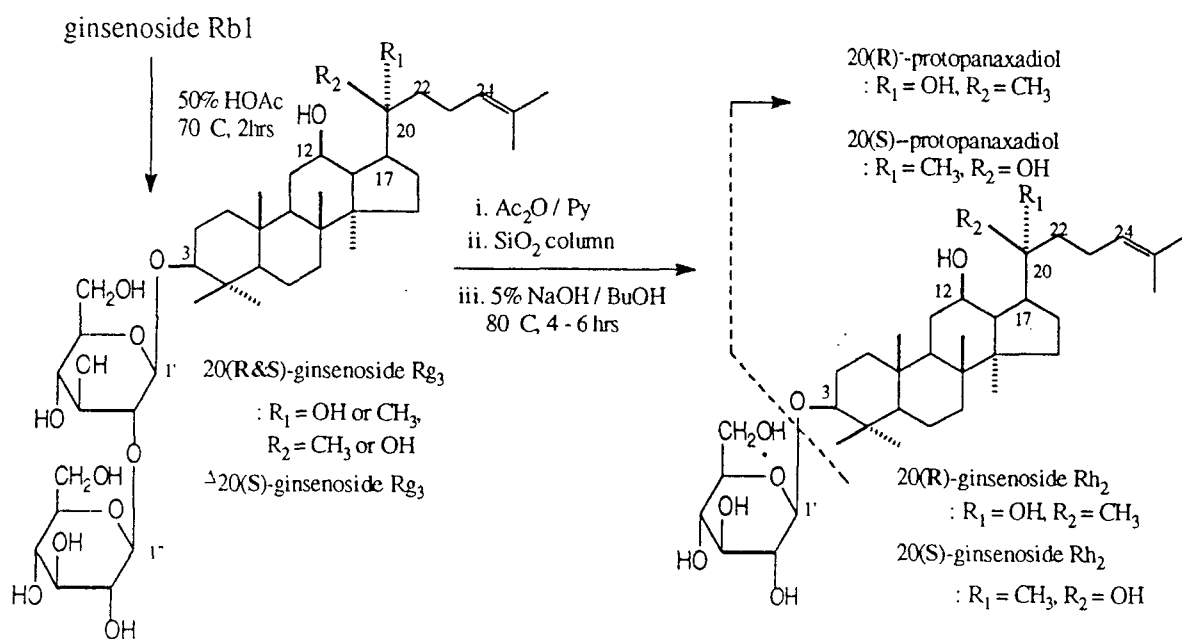
Chart 2. Preparation Procedure of Sapogenin and Sapogenins from Ginsenoside Rb₁

ginsenoside Re, 20-gluco-Rf

50% HOAc
70 C, 2hrs

R_1
R_2

i. Ac₂O / Py
ii. SiO₂ column

iii. 5% NaOH / BuOH
80 C, 4 - 6 hrs

20(R&S)-ginsenoside Rg₂
: R_1 = OH or CH₃, R_2 = CH₃ or OH

Δ20(S)-ginsenoside Rg₂

20(R)⁻-protopanaxatriol
: R_1 = OH, R_2 = CH₃

20(S)--protopanaxatriol
: R_1 = CH₃, R_2 = OH

20(R)-ginsenoside Rh₁
: R_1 = OH, R_2 = CH₃

20(S)-ginsenoside Rh₁
: R_1 = CH₃, R_2 = OH

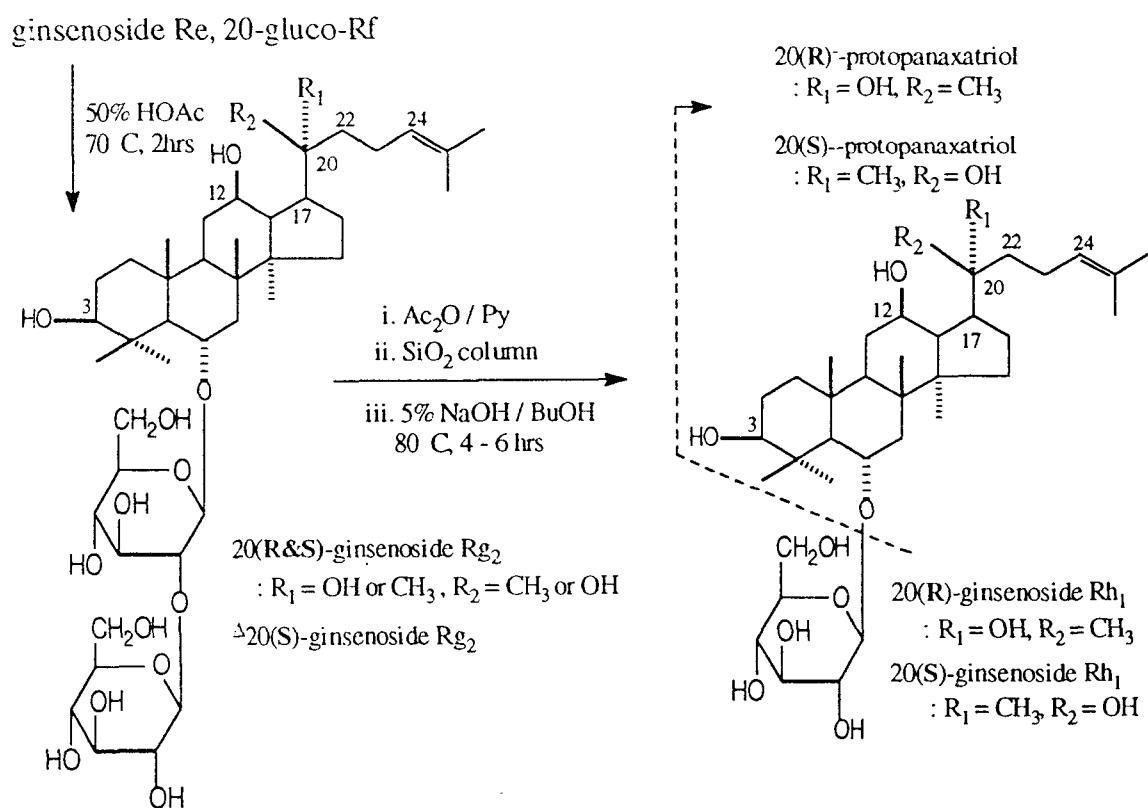Chart 3. Preparation Procedure of Sapogenin and Sapogenins from Ginsenoside Re and 20 - gluco - Rf

max.) 3420, 2940, 1630, pos. FAB MS (m/z) : 623(M + H)⁻, 443, 426, Anal. Calcd. for $C_{36}H_{62}O_8$. H_2O : C, 67.45 ; H, 10.07 Found : C, 67.38 ; H, 9.93, ¹H NMR (300 MHz, d_5-Py., δ) : 0.816, 0.976, 1.005, 1.034, 1.289, 1.384 (CH₃-21), 1.646, 1.689 (all 3H, all s, -CH₃), 3.35 (1H, ddd-like, H-12), 3.92 (1H, dd-like, H-3), 4.93 (1H, d, J = 7.6, H-1'), 5.31 (1H, t, J = 6.8, H-24), ¹³C NMR (75 MHz, d_5-Py, δc) : 15.82(C-19), 16.37 (C-29), 16.77(C-18), 17.31(C-30), 17.69(C-27), 18.46(C-6), 22.75(C-21), 22.95(C-23), 25.62(C-26), 26.62(C-16), 26.70(C-2), 28.13(C-28), 31.42(C-15), 32.15(C-11), 35.16 (C-7), 36.94(C-10), 39.12(C-1), 39.68(C-4), 40.02(C-8), 43.24(C-22), 49.19(C-13), 50.38(C-9), 50.62(C-17), 51.69 (C-14), 56.36(C-5), 63.05(C-6'), 70.85(C-12), 71.65(C-4 '), 72.96(C-20), 75.76(C-2'), 78.34(C-5'), 78.72(C-3'), 88. 77(C-3), 106.94(C-1'), 126.05(C-1'), 130.76(C-25).

8. Triol-type sapogenin and prosapogenins from ginsenoside Re and ginsenoside 20-gluco-Rf

20(R)-ginsenoside Rg₂, 20(R)-ginsenoside Rh₁, 20(R)-protopanaxatriol, 20(S)-ginsenoside Rg₂, 20(S)-ginsenoside Rh₁ and 20(S)-protopanaxatriol were prepared from ginsenoside Re and 20-gluco-Rf by same procedure as diol-type products.

RESULTS AND DISCUSSION

Since 1989 we have been investigating the anticancer agents from natural plants. Several hundreds of Korean plants were collected, extracted, fractionated and evaluated the cytotoxicities against human cancer cell lines. On the course of the research, we found Momordin I isolated from *Ampelopsis japonica* to be cytotoxic, which is the glycoside of oleanolic acid having one glucuronic acid and one arabinose similar to ginsenoside Ro. Therefore, we decided to test the cytotoxicity of ginsenoside Ro.

As mentioned previously, the purification of ginsenoside Ro is not so easy. However, after methylesterification of crude ginsenoside Ro with diazomethane, the ginsenoside Ro methylester could be easily obtained as purified component. Alkaline treatment of the methylester at room temperature and high tempera-

ture afforded purified ginsenoside Ro and prosapogenin, respectively. From the latter another prosapogenin was obtained by the β-glucosidase hydrolysis.

It is usually very difficult to seprarte the racemic mixtures into ecah isomers. However, the racemic mixtures of 20(R&S)-ginsenoside Rg₃ and 20(R&S)-ginsenoside Rg₂ were easily separated by SiO₂ column chromatography after acetylation of the mixtures.

The configuration of C-20 in each isomers were determined by comparision of specific rotation ($[\alpha]_D$ value) and chemical shifts of ¹³C NMR. Especially the chemical shifts of neighbouring carbons to C-20 were clearly distinguished between 20(R) and 20(S) isomers. (Table 1)

Meanwhile, 20(S)-ginsenoside Rh₂ was already isolated from red ginserg[5]. However, 20(R)-ginsenoside Rh₂ was prepared from ginsenoside Rg₃ for the first time[10], even if it was not isolated directly from ginseng. All the physical and spectral data were made out.

The structure of 20-ginsenoside Rg₃ and Rg₂ produced by introduction of a new double bond at C-20 and C-22 were revealed by a new triplet proton signal [δ5.64 (J = 6.0 Hz), δ 5.76 (J = 6.4 Hz), respectively] observed in ¹H NMR spectra.

The cytotoxic activities against various human and murine cancer cell lines of obtained compounds were shown Table 2-4. As shown in table 2, ginsenoside Ro and its methylester didn't show significant cytotoxicity. However, a prosapogenin, Ro1G, produced by elimination of one glucose at C-28 from ginsenoside Ro showed some cytotoxicity and another one, Ro2G, obtained by elimination of terminal glucose at C-2' from Ro1G, was very cytotoxic, especially against P388 (ED₅₀ : 2.1 μg/ml) and L1210 (ED₅₀ : 2.8 μg/ml). Sapogenin of ginsenoside Ro, oleanolic acid, showed mild cytotoxic activity only against L1210.

From the table 3, it was clear that ginsenoside Rb₁, diol-type saponin, was not cytotoxic against any cell lines. 20(R)-and 20(S)-ginsenoside Rg₃, the prosapogenins of ginsenoside Rb₁, was slightly cytotoxic against P388, L1210 and K562. Other prosapogenins, 20(R)- and 20(S)-ginsenoside Rh₂ were more cytotoxic than Rg₃ series.

All of sapogenins, panaxadiol, 20(R)- and 20(S)-protopanaxadiol, showed the most cytotoxic activities among obtained

Table 1. Comparisions of Chemical Shifts(δc) in Various Ginseng Compounds

No of C	20(R)-Rg₃	20(S)-Rg₃	20(R)-Rh₂	20(S)-Rh₂	20(R)-PPD	20(S)-PPD
C-17	50.6	54.7	50.6	54.8	50.7	54.7
C-21	22.6	27.0	22.8	26.8	22.9	26.9
C-22	43.3	35.8	43.2	35.9	43.0	35.8

No of C	20(R)-Rg₂	20(S)-Rg₂	20(R)-Rh₁	20(S)-Rh₁	20(R)-PPT	20(S)-PPT
C-17	50.7	55.2	50.5	54.7	50.6	54.6
C-21	22.8	27.5	22.5	26.8	22.8	26.9
C-22	53.4	36.4	43.2	35.8	43.1	35.7

	A549	SK-OV-3	SK-Mel	P388	L1210	K562
Ro	>40	>40	>40	>40	36.5	31.5
RoMe	>40	>40	>40	>40	28.4	31.5
Ro1G	23.1	18.4	17.5	13.8	11.7	12.9
Ro2G	9.7	8.8	8.2	2.1	2.8	9.5
OLEA	>40	>40	>40	26.0	15.0	23.6

(μg/ml)

Ro : R = H
RoMe : R = -CH₃
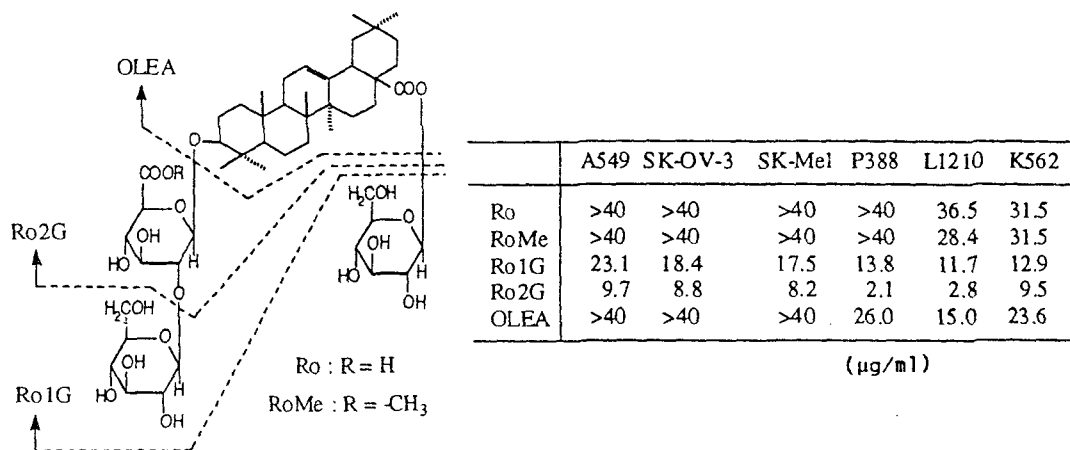
Table 2. ED₅₀ values of Olean - Type Ginsenoside Compounds against Some Cancer Cell Lines

	A549	SK-OV-3	SK-Mel	P388	L1210	K562
Rb₁	>40	>40	>40	>40	>40	>40
20R-Rg₃	>40	>40	>40	22.8	18.2	31.5
20S-Rg₃	>40	>40	>40	29.8	32.7	>40
ΔRg₃	>40	>40	>40	>40	19.5	>40
20R-Rh₂	>40	>40	14.7	27.5	38.6	31.5
20S-Rh₂	39.3	>40	38.5	22.5	31.2	31.5
20R-PPD	8.4	9.1	5.8	4.5	4.8	4.5
20S-PPD	12.2	8.6	10.1	4.9	5.3	8.2
PD	8.7	10.3	14.8	4.7	5.1	3.8

unit : μg/ml
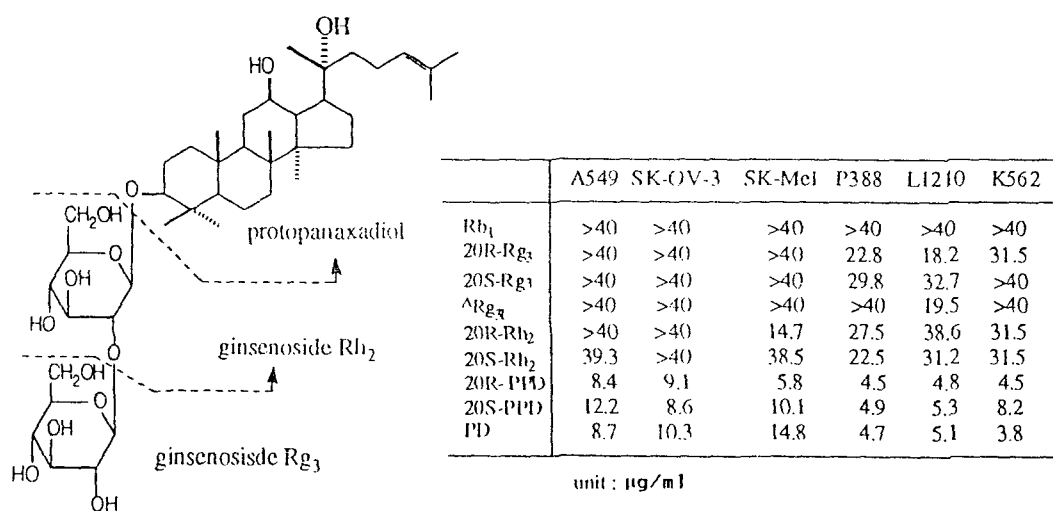
Table 3. ED₅₀ values of Diol - Type Ginsenoside Compounds against Some Cancer Cell Lines

	A549	SK-OV-3	SK-Mel	P388	L1210	K562
Re	>40	>40	>40	>40	>40	>40
20R-Rg₂	>40	>40	>40	>40	>40	>40
20S-Rg₂	>40	>40	>40	>40	>40	>40
ΔRg₂₁	>40	27.8	>40	34.7	29.0	>40
20R-Rh₁	>40	>40	>40	>40	>40	>40
20S-Rh₁	>40	>40	>40	>40	>40	>40
20R-PPT	28.5	34.2	22.3	10.1	9.0	15.5
20S-PPT	27.3	32.6	26.3	16.4	14.5	17.9
PT	14.5	21.5	18.3	28.0	13.9	23.5

unit : μg/ml
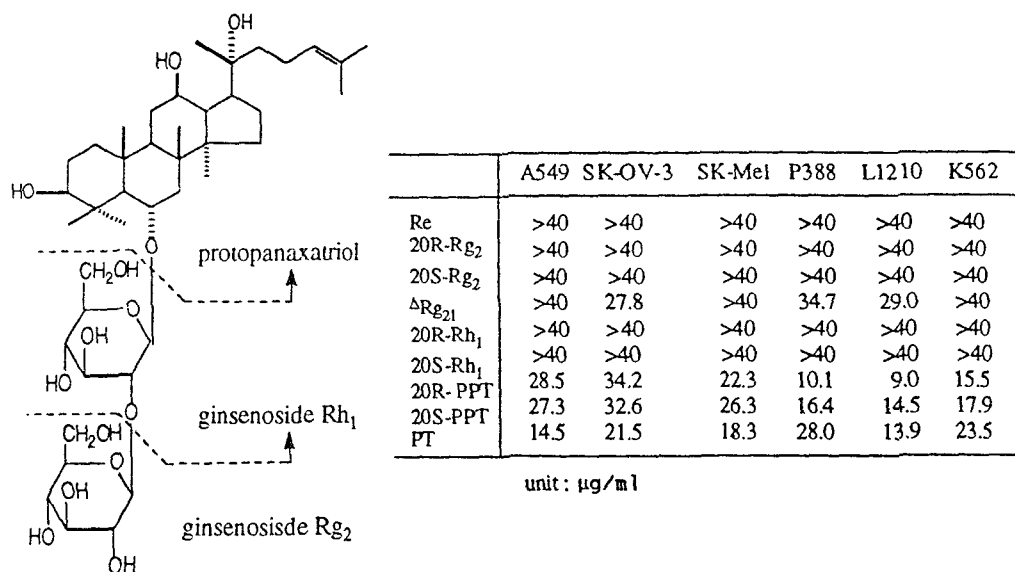
Table 4. ED₅₀ values of Triol - Type Ginsenoside Compounds against Some Cancer Cell Lines

diol - type compounds, regardless of genuineness or stereostructure.

Triol - type saponins, ginsenoside Re, Rg$_2$ and Rh$_1$ were not cytotoxic against all the cell lines. Only ginsenoside Rg21, having one double bond at C - 20 and C - 22, was just a little cytotoxic. Artificial and genuine sapogenins, panaxatriol, 20(**R**) - and 20 (**S**) - protopanaxatriol, showed some cytotoxicity against all the cell lines.

Generally to speak, the significant difference of activity between steroisomers was not approved and the activity was inversely proportional to the number of sugars binding to sapogenins. It was sure that diol type prosapogenins and sapogenins were more cytotoxic than triol type ones.

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수종의 암세포주에 대한 인삼 사포닌 및 그 분해산물의 구조와 세포독성 관계

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한국산 고려 홍삼을 산 또는 알칼리로 가수분해하여, 여러가지 사포게닌과 프로사포게닌을 제조하였으며, 분광학적 데이타와 물리 데이타 등으로부터 이들의 화학 구조를 결정하였다. 이들 중 몇종의 분해산물은 A549, SK - OV - 3, P388, L1210, SK - Mel - 2 및 K562 등의 암세포에 대하여 세포 독성을 나타내었다. Diol계와 triol계 모두 20번 탄소의 절대구조만이 다른 입체 이성체간의 세포독성의 차이는 인정되지 않았으며, diol 계의 물질들이 triol 계 물질보다는 더 높은 활성을 나타내었다. 일반적으로 결합된 탄소의 수가 적을수록 세포독성은 강하여지는 경향을 보였다.