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Cytotoxicity of Compound K (IH-901) and Ginsenoside R_{h2}, Main Biotransformants of Ginseng Saponins by Bifidobacteria, against Some Tumor Cells

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Abstract : When ginsenoside R_{b1} and R_{b2} were anaerobically incubated with human fecal microflora, these ginsenosides were metabolized to compound K (IH-901). When ginsenoside R_{g3} was anaerobically incubated with human fecal microflora, the ginsenoside R_{g3} was metabolized it to ginsenoside R_{h2}. Among ginsenosides, IH-901 and 20(*S*)-ginsenoside R_{h2} exhibited the most potent cyotoxicity against tumor cells: 50% cytotoxic concentrations of IH-901 in the media with and without fetal bovine serum (FBS) were 27.1-31.6 μM and 0.1-0.6 μM, and those of 20(*S*)-ginsenoside R_{h2} were 37.5->50 and 0.7-7.1 μM, respectively. The cytotoxic potency of ginsenosides was IH-901>20(*S*)-ginsenoside R_{h2}≫ 20(*S*)-ginsenoside R_{g3}>ginsenoside R_{b1}≅R_{b2}.

Key words : ginsenosides, 20(*S*)-ginsenoside R_{g3}; 20(*S*)-ginsenoside R_{h2}, compound K, cytotoxicity.

INTRODUCTION

Most herbal medicines are orally administered and their components inevitably come into contact with intestinal microflora in the alimentary tract. These components may be transformed before they are absorbed from the gastrointestinal tract. Studies on the metabolism of herbal medicine components by human intestinal microflora are therefore of great importance in understanding their biological effects.[1,2]

Among herbal medicines, ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae) is frequently used in Asian countries as a traditional medicine. The major components of ginseng are ginsenosides.[3,4] These ginsenosides have been reported to show various biological activities including antiinflammatory activity[5] and antitumor effects.[6,7] The pharmacological actions of these ginsenosides has been explained by the biotransformation of ginsenosides by human intestinal bacteria.[8-12] Transformed 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901, compound K) from ginsenosides R_{b1}, R_{b2} and R_c induces an antimetastatic or anticarcinogenic effect.[13-15] In addition, ginsenosides R_{b1}, R_{b2}, and R_c are transformed to ginsenoside R_{g3} by treatment with mild acid such as stomach acid.[16] Furthermore, ginsenoside R_{g3} is a important component of red ginseng and heat-processed ginseng.[17-18] This ginsenoside R_{g3} was also transformed to ginsenoside R_{h2} by human intestinal bacteria. And this ginsenoside R_{h2} also exhibited the potent cytotoxicity against tumor cells. However, the studies on the cytotoxicity of these transformed ginsenosides were not sufficient, although the cytotoxicity of these transformed ginsenosides against some tumor cells.

Therefore we isolated ginsenosides R_{b1} and R_{b2} from white ginseng and 20(*S*)-ginsenoside R_{g3} from steam-processed ginseng, IH-901 from fermented ginseng and 20(*S*)-ginsenoside R_{h2} fermented steam-processed ginseng, and investigated their cytotoxicity against tumor cells.

MATERIALS AND METHODS

1. Materials and Cell Lines

General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Japan). Tryptic soy broth was purchased from Difco Co. (U.S.A.). The other chemicals were of analytical reagent grade.

Tumor cell lines were purchased from the Korean Cell Bank.

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2. Instruments

Isolated ginsenosides were identified by 500 MHz FT-NMR Spectrometer-U1500 (Varian, U.S.A.), Fisher-Johns melting point apparatus (Fisher Scientific, U.S.A.) and Capillary Electrophoresis-Mass System, V.G. Quttro Q II /HP 1090 LC/Crystal CE System model 300, V.G. Biotech(UK). Quantitative assay of these ginsenosides was performed by HPLC system (Hitachi system, Japan) and TLC scanner Shimadzu model CS-9301PC (Shimadzu, Japan).

3. Isolation of Ginsenosides and Their Metaolites by Human Intestinal Microflora

Ginsenoside R_{b1} (0.2 g) and R_{b2} (0.3 g) from white ginseng (Kyung Dong Market, Seoul, Korea) were isolated according to the previously reported methods.[13]

(1) Ginsenoside R_{b1} (purity, >90%)

white powder, mp 197-198°C (dec.) FAB-MS (*m/z*) 1110 $[M+1]^+$.

(2) Ginsenoside R_{b2} (purity, >90%)

white powder, mp 200-203°C (dec.) FAB-MS (*m/z*) 1080 $[M+1]^+$.

Ginsenoside R_{g3} was isolated from 1 kg of steam-processed ginseng (100°C for 4 h) according to the modified method of Bae *et al.*[19] Steam-processed ginseng was extracted with MeOH, concentrated by rotary evaporator, suspended in distilled water and extracted with BuOH. The BuOH extract (9.4 g) was chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (10:3:1, lower layer) to produce *isomeric* 20(*S*)- and 20(*R*)-ginsenoside R_{g3} (0.6 g). The *isomeric* mixture was suspended in distilled water, and standed at 4°C for overnight. The soluble fraction was concentrated, suspended water in distilled water and standed at 4°C for overnight. The resulting soluble fraction was concentrated and then applied to silica gel column chromatography using $CHCl_3$-MeOH-H_2O (9:3:1, v/v) to give 20(*S*)-ginsenoside R_{g3} (0.21 g).

(3) 20(*S*)-Ginsenoside R_{g3} (purity, >95%)

white powder, mp 248-250°C (dec.) FAB-MS (*m/z*) 786 $[M+1]^+$. ^{1}H-NMR(500 MHz, CD_3OD, δ) 5.38(1H, d, H-1), 5.32(1H, dd, *J*=7.6, 7.3, H-24), 4.94(1H, d, H'-1). ^{13}C-NMR(125 MHz, pyridine-*d*, δ) 130.71(C-25), 126.24(C-24), 105.92(C"-1), 105.03(C'-1), 88.89(C-3), 83.27(C'-2), 78.27(C'-3, C"-3), 78.03(C"-5), 77.89(C"-2), 77.03(C'-5), 72.93(C-20), 71.59(C"-4), 71.54(C'-4), 70.95(C-12), 62.76 (C'-5), 62.65(C"-6), 56.30(C-5), 54.71(C-17), 51.64(C-14), 50.31(C-9), 48.50(C-13), 39.93(C-8), 39.64(C-4), 39.07(C-1), 35.81(C-22), 36.85(C-10), 35.10(C-7), 31.28(C-15), 31.95(C-11), 28.05(C-28), 27.00(C-21), 26.78(C-16), 26.66 (C-2), 25.77(C-26), 22.93(C-23), 18.37(C-6), 17.63(C-27), 16.95(C-30), 16.54(C-18), 16.29(C-29), 15.76(C-19).

Bifidobacterium KK-1[20] was inoculated in TS broth, cultured for 24 h at 37°C, collected, and washed twice with saline. The resulting precipitate (wet weight 10 g) was suspended in 1 liter of water containing 0.5% white ginseng extract was incubated for 24 h at 37°C (fermented ginseng). The fermented ginseng was extracted with BuOH. This BuOH extract was chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (10:3:1, lower layer) to isolate IH-901. IH-901 was chromatographed on a silica gel column using $CHCl_3$-MeOH (15:1) and recrystalized with EtOH (38 mg).

(4) Compound K (IH-901) (purity, >95%)

white powders, mp 219-221°C (dec.) EI-MS (*m/z*) 623 $[M]^+$. ^{1}H-NMR(500 MHz, pyridine-*d*, δ) 5.24(1H, dd, H-24), 5.20(1H, d, *J*=7.7, H'-1). ^{13}C-NMR(100 MHz, pyridine-*d*, δ) 130.7(C-25), 126.3(C-24), 106.0(C'-1), 98.1 (C"-1), 83.3 (C-20), 80.0(C'-3), 79.6(C'-5), 78.3(C"-3), 78.1(C-3, C"-5), 75.4(C'-2), 75.1(C"-2), 71.9(C'-2), 71.7 (C"-4), 71.0(C-12), 63.1(C'-6), 62.6(C"-6), 56.4(C-5), 51.7(C-14,17), 50.6(C-9), 48.6(C-13), 40.1(C-8), 39.5(C-1,4), 35.9(C-22), 37.4(C-10), 35.3(C-7), 31.4(C-15), 32.1(C-11), 28.7 (C-28), 26.1(C-16), 28.2(C-2), 25.8(C-26), 22.4(C-21), 23.0(C-23), 18.8(C-6), 17.7(C-27), 17.1(C-30), 16.2(C-18), 16.4(C-29), 15.8(C-19).

The previously cultured *Bifidobacterium* KK-1 precipitate (wet weight 10 g) was suspended in 1 liter of water containing 0.5% steam-processed ginseng and incubated for 24 h at 37°C (fermented steam-processed ginseng). The fermented steam-processed ginseng was extracted with BuOH. This BuOH extract was chromatographed on a silica gel column using $CHCl_3$-MeOH-H_2O (10:3:1, lower layer) to isolate ginsenoside R_{h2}. The isolated ginsenoside R_{h2} was chromatographed on a silica gel column using $CHCl_3$-MeOH (15:1) and recrystalized with EtOH (22 mg).

(5) 20(S)-Ginsenoside Rh_2 (purity, >95%)

colorless needles, mp 219-221°C (dec.) FAB-MS (*m/z*) 623 $[M]^+$; ^{1}H-NMR(500 MHz, pyridine-*d*, δ) 5.32(1H, dd, *J*=7.0, 7.0, H-24), 4.96(1H, d, *J*=7.7, H'-1), 3.38(1H, dd, *J*=4.5, 11.5, H-3). ^{13}C-NMR(100 MHz, pyridine-*d*, δ) 130.73(C-25), 126.30(C-24), 106.92(C'-1), 88.78(C-3), 78.72(C'-3), 78.34 (C'-5), 75.76(C'-2), 72.94(C-20), 71.65 (C'-4), 70.96(C-12), 63.05(C'-6), 56.35(C-5), 54.77(C-17), 51.69(C-14), 50.38 (C-9), 49.54(C-13), 40.00(C-8), 39.66 (C-4), 39.12(C-1), 36.94(C-10), 35.88(C-22), 35.15(C-7), 32.02(C-11), 31.32 (C-15), 28.14(C-28), 27.05(C-2), 26.83 (C-21), 26.70(C-16), 25.78(C-26), 22.97(C-23), 18.43(C-

6), 17.66(C-27), 17.65(C-30), 16.77(C-18), 16.34(C-29), 15.61(C-19).

4. *In vitro* Cytotoxicity Assay

The *in vitro* cytotoxicity was tested against P388 (mouse lymphoid neoplasma cell line), A549 (human lung carcimoma), HepG2 (human liver hepatoblastoma) and HeLa (human cervix uterine adenocarcinoma) cells by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method of Carmichael *et al.*[21] Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μl volume: 1.5×10^4 cells/well) into 96-well microtiter plate. P338, A549, HepG2 and HeLa cells were cultured for 24 h in media with or without fatal bovine serum (FBS) and treated with the samples. These cells were exposed to the test compounds for 5 d at 37°C. Fifty μl of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 30 min. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plates were read on a microplate reader (540 nm). The 50% cytotoxic concentration (EC_{50}) of tumor cell growth was defined compared with the control cell culture.

5. Assay of Intestinal fecal Activities Transforming Ginseng Saponins to Compound K

About one gram of fresh feces of 92 subjects (56 male and 36 female) was suspended with 20 ml saline and centrifuged at 300×g. The supernatant was centrifuged at 10,000×g for 1 h. The fecal precipitate was suspended with 10-fold 50 mM phosphate buffer and then was used as an enzyme source. The reaction mixture containing 1ml of the human fecal suspension (10 mg) and 1 mg ginseng BuOH extract was incubated at 37°C for 1 h, and the

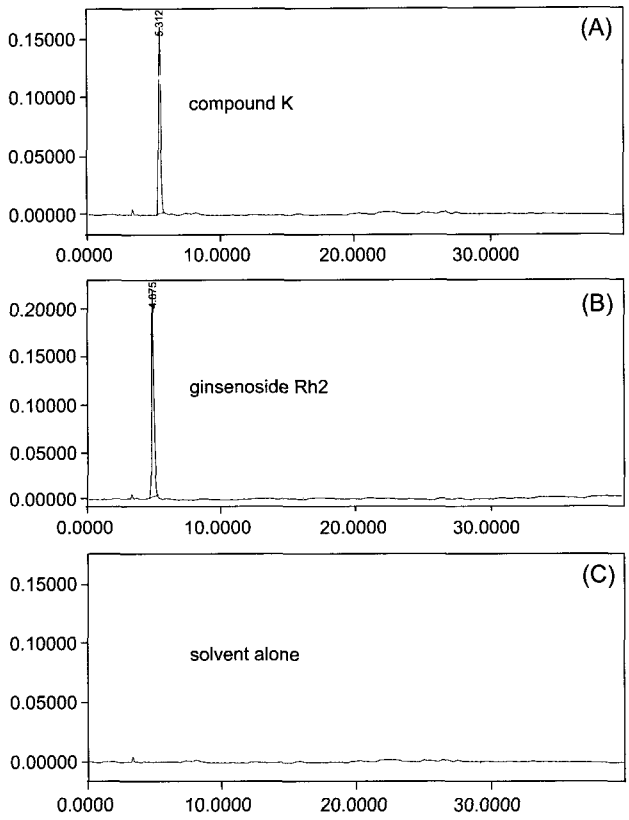


Fig. 1. HPLC chromatogram of ginsenosides isolated from fermented ginsengs. A, compound K; B, ginsenoside Rh2; C, solvent alone. HPLC assay condition-Hitachi HPLC system: column, Lichrosorb NH2 (250×4 mm, 5 μm, Merck); elution solvent, mixtures of solvent A (acetonitrile/water/isopropanol=80:5:15) and solvent B (acetonitrile/water/isopropanol=80:20:15)-gradient profile of solvent A to solvent B from 70:30 to 0:100 for 0-20 min and from 0:100 for 20-40 min; UV detector wavelength, 203 nm. These compounds (20 μl of 0.5 mg/ml (acetonitrile-isopropanol)) were applied to HPLC.

Table 1. The cytotoxicity of ginsenosides against some tumor cells

	EC_{50} (mM)							
	With FBS				Without FBS			
	A549	P388	HeLa	HepG2	A549	P388	HeLa	HepG2
IH-901 (Compound K)	27.9	31.6	27.1	28.8	0.1	–[a]	0.1	0.6
Ginsenoside Rh2	>50	37.6	>50	>50	3.4	–	0.7	7.2
Ginsenoside Rg3	>50	>50	>50	>50	28.9	–	>50	>50
Ginsenoside Rb1	>50	>50	>50	>50	>50	–	>50	>50
Ginsenoside Rb2	>50	>50	>50	>50	>50	–	>50	>50

Each ginsenoside was treated for 48 h in the media with and without fetal bovine serum.
[a]not detectable.
ED_{50}, 50% cytotoxic concentration compared to viability of control.

reaction mixture was extracted twice with 5 ml of ethyl acetate. The ethyl acetate fraction was analyzed with TLC.

6. Thin-Layer Chromatography

TLCs for ginsenosides were performed on silica gel plate with $CHCl_3$: MeOH: H_2O=65:35:10 (lower layer, v/v). The chromatograms of these compounds were quantitatively assayed by TLC.

RESULTS AND DISCUSSION

To investigate the cytotoxocity of some ginsengs against tumor cells, we isolated ginsenoside R_{b1} and R_{b2} from white ginseng, 20(*S*)-ginsenoside R_{g3} from steam processed ginseng (red ginseng), 20(*S*)-ginsenoside R_{h2} from fermented steam-processed ginseng and IH-901 from fermented white ginseng. The isolated ginsenosides comparatively exhibited the high purity (Fig. 1). We also investigated *in vitro* cytotoxic activity of ginsenoside R_{b1}, R_{b2}, R_{g3} and R_{h2} and IH-901 against the tumor cells (Table 1). Ginsenoside R_{b1} and R_{b2} did not exhibit cytotoxicity against the tumor cell lines. 20(*S*)-Ginsenoside R_{g3} only exhibited weak cytotoxicity. IH-901 and 20(*S*)-ginsenoside R_{h2} exhibited the most potent cyotoxicity against tumor cell: 50% cytotoxic concentrations of compound K in the media with and without FBS were 27.1-31.6 μM and 0.1-0.6 μM, and those of 20(*S*)-ginsenoside R_{h2} were 37.5->50 μM and 0.7-7.1 μM, respectively. When these ginsenosides were treated against tumor cells, IH-901 and ginsenoside R_{h2} dose- and time-dependently inhibited the growth of tumor cells (Fig. 2). Particularly, HeLa and HepG2 cells were significantly susceptible for IH-901 and ginsenoside R_{h2}. The cytotoxicity of these ginsenosides was more potent when cultured in the media without FBS, compared to in the media with FBS. These results suggest that FBS may bind ginsenosides and inactivate its

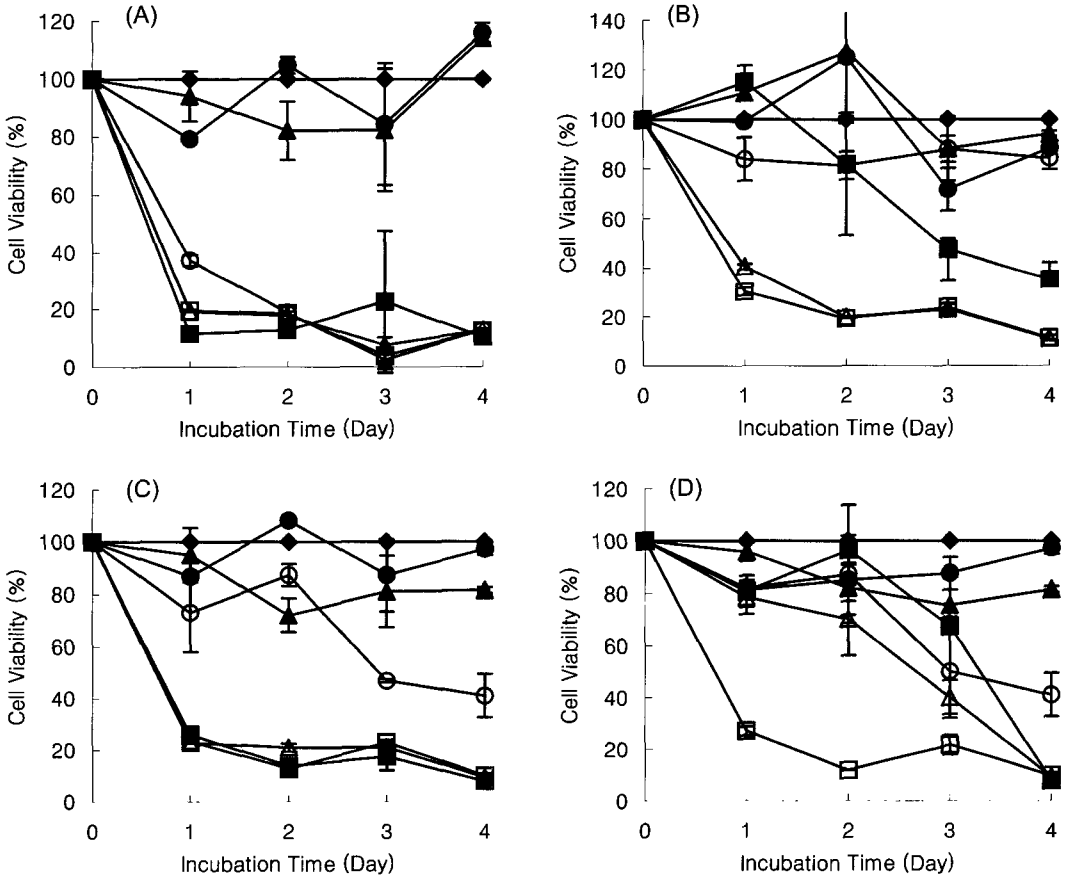
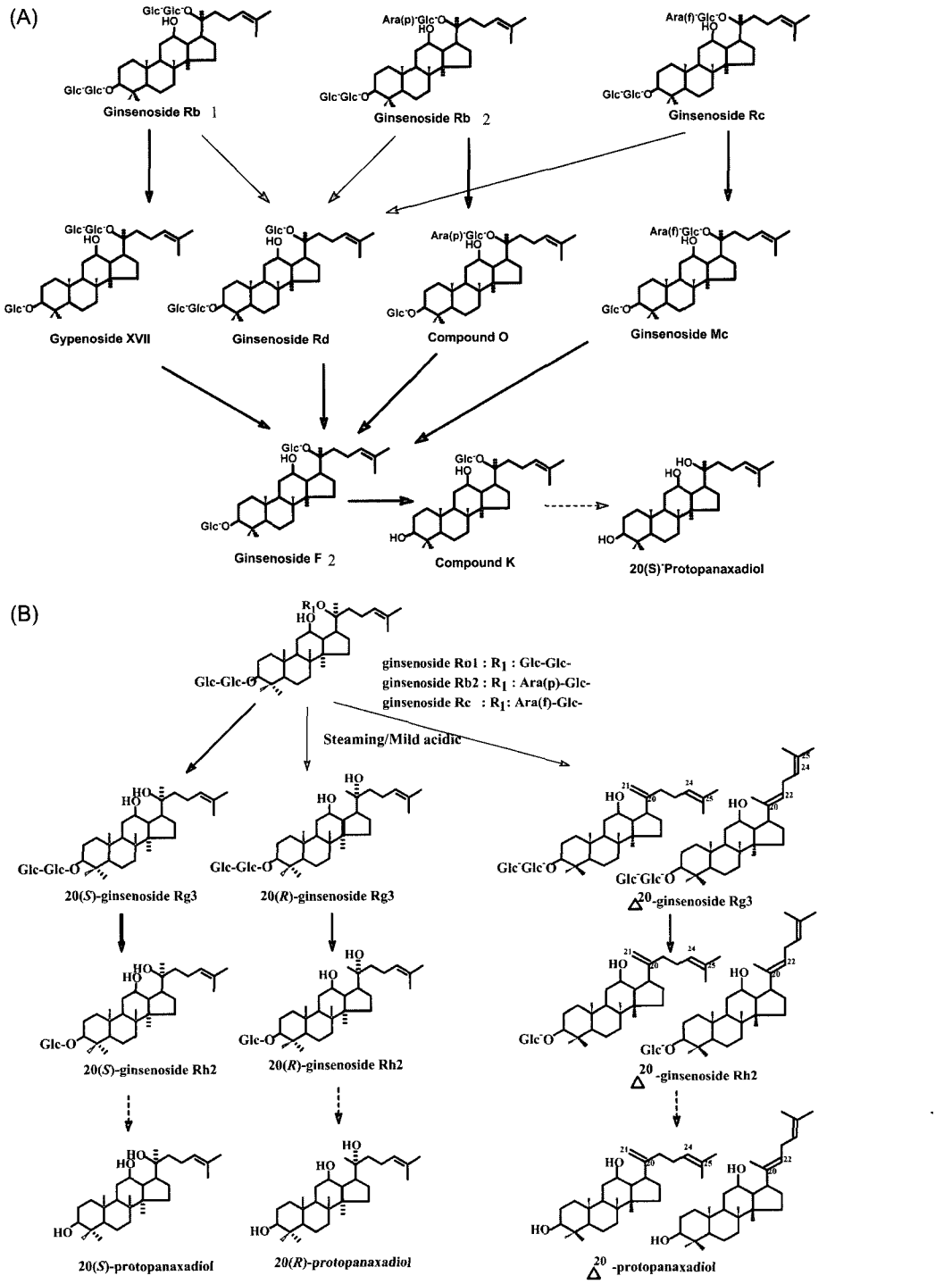


Fig. 2. Dose- and time-dependent viability of HeLa and HepG2 cells treated with IH-901 and ginsenoside Rh2. (A), Viability of HeLa cells treated with IH-901; (B), Viability of HeLa cells treated with ginsenoside Rh2; (C), Viability of HeG2 cells treated with IH-901; (D), Viability of HeG2 cells treated with ginsenoside Rh2. The samples (compound K or ginsenoside Rh2) were treated in the media with or without fetal bovine serum (FBS); ■, 50 mM with FBS; □, 50 mM without FBS; ▲, 5 mM with FBS; △, 5 mM without FBS; ●, 0.5 mM with FBS; ○, 0.5 mM without FBS; ◆, control cells.

cytotoxicity.

The main components of ginseng are ginsenoside R_{b1}, R_{b2} and R_c. These ginsenosides are likely transformed to IH-901 via ginsenoside R_d or gypenoside XVII by human intestinal bacteria[11,13,22] (Scheme 1a). Han *et al.* reported that ginsenoside R_{b1} and R_{b2} were transformed to ginsenoside R_{g3} when these compounds were incubated in mildly acidic conditions (Scheme 1b),[16] and suggested



Scheme 1. The Proposed metabolic pathway of ginsenosides (A) and steamed ginsenosides (B) by human intestinal bacteria. Each metabolic pathway was potently catalyzed by the bacteria listed from bacteria tested in this experiment. ⟶, main pathway; ⟶, minor pathway ; ⇢, trace pathway.

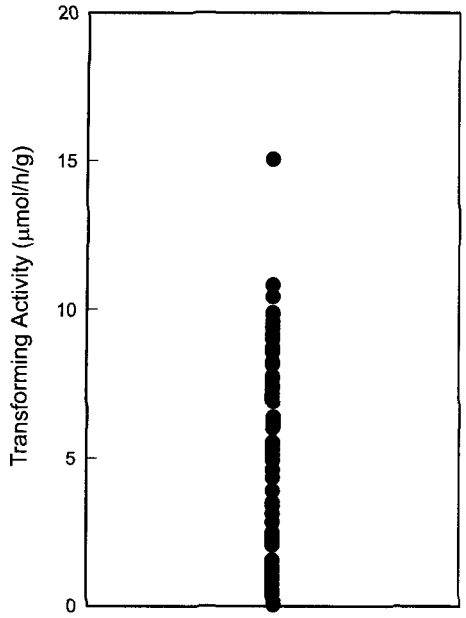


Fig. 3. Fecal metabolic activity transforming components of ginseng to IH-901 by fecal suspension of 92 subjects.

that this transformation of ginsenosides R_{b1} and R_{b2} to ginsenoside R_{g3} could occur during the steam process for red ginseng. Therefore, ginsenoside R_{g3} is a major component of red ginseng rather than of ginseng.[17] When ginsenoside R_{g3} was incubated with human fecal microflora, it was transformed to ginsenoside R_{h2} (3β-*O*-glucopyranosyl-20(*S*)-protopanaxadiol).

In addition, we tested the ginseng saponin transforming activity to IH-901 for 92 human fecal specimens(Fig. 3). Twenty eight percent of fecal specimens almost could not transformed ginseng saponin to IH-901.

We found that, when ginseng saponins were metabolized to IH-901 or 20(*S*)-ginsenoside R_{h2} by human intestinal microflora, the cytotoxicity of ginsenosides against tumor cell lines was increased: The cytotoxic potency of tested ginsenosides againt tested tumor cells was IH-901>20(*S*)-ginsenoside $R_{h2} \gg$ ginsenoside R_{g3}>ginsenoside $R_{b1} \cong R_{b2}$. These results suggest that ginseng saponins should be prodrugs for antitumor actions. If intestinal microflora of somebody did not exhibit ginseng saponin-transforming activity, ginsensides may not be transformed to active compounds, such as IH-901 and ginsenoside R_{h2}. Therefore, we believe that IH-901-or 20(*S*)-ginsenoside R_{h2} enforced ginsengs (fermented ginsengs) can contribute to the treatment and prevention of tumor diseases even if somebody did not exhibit ginseng saponin-transforming activity.

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