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General and Genetic Toxicology of Enzyme-Treated Ginseng Extract

- Toxicology of Ginseng Rh2+ -

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Abstract

Objectives: Ginseng Rh2+ is enzyme-treated ginseng extract containing high amounts of converted ginsenosides, such as compound k, Rh2, Rg3, which have potent anticancer activity. We conducted general and genetic toxicity tests to evaluate the safety of ginseng Rh2+.

Methods: An acute oral toxicity test was performed at a high-level dose of 4,000 mg/kg/day in Sprague-Dawley (SD) rats. A 14-day range-finding study was also conducted to set dose levels for the 90-day study. A subchronic 90-day toxicity study was performed at dose levels of 1,000 and 2,000 mg/kg/day to investigate the no-observed-adverse-effect level (NOAEL) of ginseng Rh2+ and target organs. To identify the mutagenic potential of ginseng Rh2+, we conducted a bacterial reverse mutation test (Ames test) using amino-acid-requiring strains of *Salmonella typhimurium* and *Escherichia coli* (*E. coli*), a chromosome aberration test with Chinese hamster lung (CHL) cells, and an *in vivo* micronucleus test using ICR mice bone marrow as recommended by the Korean Ministry of Food and Drug Safety.

Results: According to the results of the acute oral toxicity study, the approximate lethal dose (ALD) of ginseng Rh2+ was estimated to be higher than 4,000 mg/kg. For the 90-day study, no toxicological effect of ginseng Rh2+ was observed in body-weight changes, food consumption, clinical signs, organ weights, histopathology, ophthalmology, and clinical pathology. The NOAEL of ginseng Rh2+ was established to be 2,000 mg/kg/day, and no target organ was found in this test. In addition, no evidence of mutagenicity was found either on the *in vitro* genotoxicity tests, including the Ames test and the chromosome aberration test, or on the *in vivo* in mice bone marrow micronucleus test.

Conclusion: On the basis of our findings, ginseng Rh2+ is a non-toxic material with no genotoxicity. We expect that ginseng Rh2+ may be used as a novel adjuvant anticancer agent that is safe for long-term administration.

1. Introduction

Ginseng (the root of *Panax ginseng C.A. MEYER*) has been widely used as a traditional medicine in Eastern Asian countries and is today one of the world's most widely used medicinal plants [1]. Ginseng has been reported to have various pharmacological activities, including anti-inflammatory, anti-allergic, anti-fatigue, anti-stress, and anti-cancer activities [2]. The major biologically-active components of ginseng are a series of saponin glycosides collectively known as ginsenosides, a group of steroidal saponins. So far, about 50 kinds of ginsenosides have been identified from the ginseng root, which are defined as protopanaxadiol (PPD) and protopanaxatriol (PPT) according to the dammarane skeleton. Typical PPD-type saponins include the gin-

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Table 1 Quantities of ginsenosides in red ginseng and ginseng Rh2+

Ginsenoside compounds	Rg1	Rb1	Rg3-R	Rg3-S	Rh1	Rh2-R	Rh2-S	Compound K
Red ginseng (mg/g)	3.27	5.85	0.20	0.32	0.96	0.00	0.82	0.83
Ginseng Rh2+ (mg/g)	0.00	1.50	3.71	2.35	3.20	0.45	2.95	5.89

senosdies Rb1, Rb2, Rc, and Rd. The two most abundant PPT-type saponins in *Panax ginseng* are Re and Rg1 [3, 4].

In recent studies, converted ginsenosides, such as compound K, Rh2, Rg3, Rh1, showed anti-cancer activities that were significant compared to those of the major ginsenosides Rb1, Rb2, Rg1, etc. [5-7]. Previously, we demonstrated that enzymatic processing of ginseng could increase the content of converted ginsenosides and reported the anti-carcinogenic effect of enzyme-treated ginseng extracts in HepG2 cell, lung cancer cell and gastric cancer cell models [8-10].

The US National Toxicology Program conducted two-year toxicity and carcinogenicity studies of *Panax ginseng* in rats and mice and concluded *Panax ginseng* is not toxic or tumorigenic [11]. Seely *et al* systematically reviewed the safety of ginseng during pregnancy and lactation [12]. Recently, Park *et al* reported on a subacute oral toxicity of red ginseng extract in rats [13]. Although several studies concerning the toxicity of ginseng have been reported, no toxicological study has been performed on our new ginseng extract, called ginseng Rh2+.

In this study, we conducted general and genetic toxicity tests to evaluate systemically the safety of ginseng Rh2+ and to set criteria for human exposure. The study was conducted in compliance with the good laboratory practices regulations for nonclinical laboratory studies of the Korean Ministry of Food and Drug Safety (MFDS, 2014) and in accordance with the guidelines for toxicity testing of pharmaceuticals (MFDS, 2014) and of the Organization of Economic and Corporation Development (OECD). This research included an acute oral toxicity study, a 14-day range-finding study, a subchronic 90-day toxicity study, a bacterial reverse mutation test, a chromosome aberration test, and an *in vivo* micronucleus test.

2. Material and Methods

For the preparation of ginseng Rh2+, fresh ginseng root (*Panax ginseng C.A. Meyer*, 5 – 6 years old, cultivated at Pocheon-gun, South Korea) was steamed for 2 hours at 100°C, which was followed by drying for 48 hours at 80°C. One hundred kg of this red ginseng (crushed) were boiled for 24 hours at 100°C. After incubation with enzymes (0.9% lactase, 0.1% β-glucosidase) for 48 hours at 40°C, 100 kg of dried red ginseng (ginseng Rh2+) were attained and then filtered using 20 meshes. Standard ginsenosides, including Rg1, Rh1, Rb1, Rg3(S), Rg3(R), Rh2(R), Rh2(S), and com-

pound K, were obtained from ChromaDex Inc. (Irvine, CA) and analyzed using an Acquity ultra-high-performance liquid chromatography (UPLC) system (Waters, Milford, MA) with an Acquity BEH C18 high-performance liquid chromatography (HPLC) column (Fig. 1, Table 1). The above samples were manufactured and provided by MICO Co.

Specific pathogen-free (SPF) Sprague-Dawley (SD) rats and ICR male mice were purchased from a commercial animal breeder (Orient Bio Inc., Seongnam, Gyeongi-do, Korea) and used after 1 week of quarantine and acclimatization. Animals were maintained in a temperature- and humidity-controlled room at 20°C to 25°C and 40% to 60%, respectively, under a 12-hour light-dark cycle and fed a Teklad Certified Irradiated Global 18% Protein Rodent Diet (Harlan Co. Ltd., USA). The food and filtered tap water were supplied ad libitum. All care and handling of the animals were performed according to the "Guide for the Care and Use of Laboratory Animals" NIH publication. Studies were approved by the Institutional Animal Care and Use and Committee of Korea conformity laboratories.

For the acute oral toxicity study, SD rats were randomly assigned to four groups, including a control group, by using a computer-generated (weight-ordered) randomization procedure (5 male and 5 female 8-week-old rats per group). A preliminary study showed that a single oral administration of ginseng Rh2+ had not induce any toxic effect at dose levels of 0, 2,000 and 4,000 mg/kg/day. Based on these results, a dose of 4,000 mg/kg/day was selected as the toxicological limited dose. The middle and the low doses were 2,000 and 1,000 mg/kg/day, respectively. Ginseng Rh2+ was suspended in distilled water, and the volume necessary to administer a dose of 20 mL/kg body weight was calculated. The vehicle control rats received an equivalent volume of distilled water only. For all animals, mortality and clinical signs were recorded at least once daily for 14 days. Clinical signs were observed every hour up to 6 hours after dosing. Body weights were measured on the days of reception, grouping, dosing, and autopsy. On day 14, all animals were anesthetized with CO_2. Gross necropsies were performed on all animals at terminal sacrifice. The tissues and the organs of the thoracic and the abdominal cavities were examined.

The 14-day range-finding study in rats was conducted in accordance with the OECD Guidelines for testing of chemicals in publications TG No. 407. The purpose of this study was primarily to set dose levels for the 90-day study. Previously, a single oral study had been performed at dose levels of 0, 2000, and 4,000 mg/kg/day in one male and one
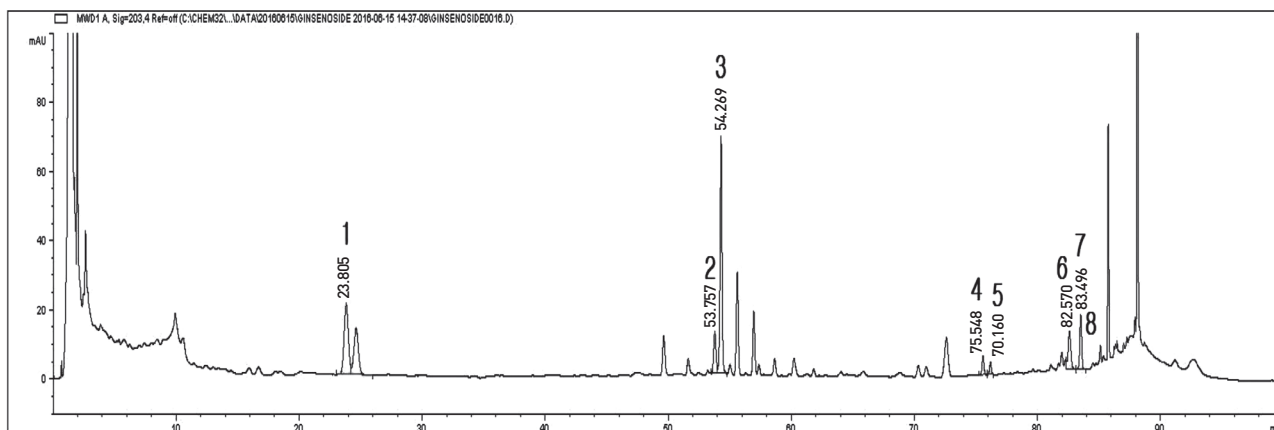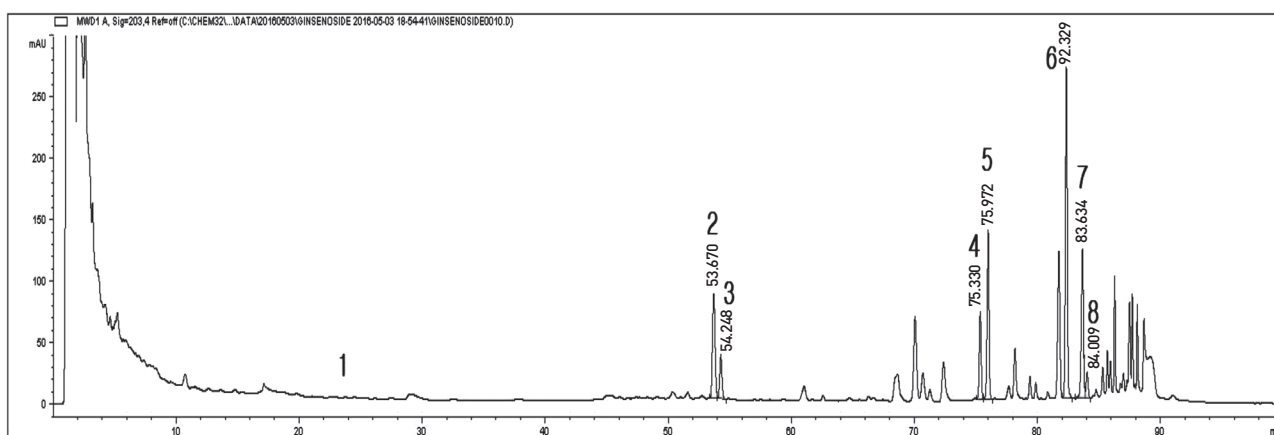
Figure 1 High-performance liquid ultraviolet chromatograms of ginseng root extracts: (A) red ginseng and (B) ginseng Rh2+.

female rat. Salivation was observed in all treated groups. No toxicological signs were found. Based on these results, a dose of 2,000 mg/kg/day was selected as the toxicological limited dose recommended by MFDS guidelines for toxicity testing of pharmaceuticals. The middle and the low doses were set at 1,000 and 500 mg/kg/day, respectively. SD rats were randomly assigned to four groups (5 male and 5 female 5-week-old rats per group).

For this 14-day range-finding study, all doses were administered volumetrically at 10 mL/kg. Evaluations of mortality, clinical signs, body-weight changes, food consumption, and water consumption were recorded for 14 days. Clinical pathology was performed using blood chemistry, hematology (except coagulation), and urinalysis. The animals were sacrificed on day 15 and evaluated for any macroscopic changes. The organs of each animal, including the brain, heart, lungs, kidneys, liver, spleen, thymus, adrenals, prostate, gonads and uterus, were weighed separately.

The subchronic 90-day toxicity study in rats was conducted in accordance with the OECD Guidelines for testing of chemicals in publications TG No. 408 'Repeated Dose

90-day Oral Toxicity Study in Rodents (1998)'. The purpose of this study was to estimate the no-observed-adverse-effect level (NOAEL) of exposure and to identify the target organs. Eighty rats were selected for testing (10 male and 10 female 6-week-old rats in each of 4 groups). All doses were administered volumetrically at 10 mL/kg. Based on the results of the 14-day study, we selected dose levels of 2,000, 1,000, and 500 mg/kg/day as the high, middle, and low doses, respectively.

Prior to the administration of the test substance and the termination of the subchronic 90-day toxicity study, ophthalmological examinations were performed on all rats of the high-dose and the control groups. Evaluations of mortality, clinical signs, body-weight changes, and food consumption were recorded during the experimental period. Clinical pathology was performed using blood chemistry, hematology (including coagulation), and urinalysis. All animals were evaluated for any macroscopic changes. The organs of each animal, including the brain, pituitary, heart, lungs, kidneys, liver, spleen, thymus, adrenals, prostate, gonads and uterus, were weighed separately. Full histopa-

thology was carried out on the preserved organs and tissues of all animals in the control and the high-dose groups. Organs and tissues showing abnormal gross findings from any of the other dosage groups were also examined.

For the preparation of the S9 mixture, rat liver microsomal enzyme (S9) induced by Aroclor 1254 was obtained from Molecular Toxicology Inc. (Boon, NC, USA). Before the experiment, an appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution. The S9 mixture was freshly prepared just prior to use in each test and was kept in a refrigerator until use. In the Ames test, the amount of S9 supernatant was 5% v/v in the S9 mixture. Cofactors were added to the S9 mixture to reach the final concentrations of 8-μmoL $MgCl_2 \cdot 6H_2O$, 33-μmoL KCl, 5-μmoL G-6-P, 4-μmoL nicotinamide adenine dinucleotide phosphate (NADPH), 100-μmoL sodium phosphate buffer (pH 7.4) and 50-μL S9 in the S9 mixture. In the chromosome aberration test, the ingredients and their contents per l mL of the prepared S9 mixture were 800 μL of 8-μmoL $MgCl_2 \cdot 6H_2O$, 33-μmoL KCl, 5-μmoL G-6-P, and 5-μmoL NADP 800 μL and 200 μL of S9 (20% S9, v/v).

The reverse bacterial mutation test (Ames test) was conducted in accordance with the OECD guidelines for the testing of chemicals (July 21, 1997) in publication TG No. 471 'Bacterial Reverse Mutation Test'. The reverse mutation test was performed to investigate the potential of ginseng Rh2+ to induce genetic mutation in histidine-requiring strains of *Salmonella typhimurium* (*S. typhimurium*) TA98, TA100, TA1535, and TA1537 and a tryptophan-requiring strain of *Escherichia coli* (*E. coli*) WP2uvrA (Molecular Toxicology, Inc, Boone, NC, USA) according to the plate incorporation method in the presence and the absence of a metabolic activation system (S9 mixture). The test substance was dissolved in dimethylsulfoxide (DMSO). The positive control factors were dissolved in either distilled water or DMSO and stored at 20°C; these positive control factors were 2-nitrofluorene (2-NF), 2-aminoanthracene (2-AA), sodium azide (SA), Acridine Mutagen ICR191 (ICR-191), 4-nitroquinoline-1-oxide (4NQO), and benzo(a)pyrene (BP).

A dose range-finding test was performed to determine the highest dose for the reverse bacterial mutation test by using five tester strains at 8 dose levels in the range from 5 to 5,000 μg/plate both with and without the S9 mixture. Precipitation was observed on the agar plate at a dose of 1,500 mg/plate or higher for all bacterial strains. No increase in revertant colonies or cytotoxicity was observed. Based on these results, a dose of 5,000 μg/plate was selected as the maximum level.

Furthermore, for the reverse bacterial mutation test, various doses of ginseng Rh2+ were incubated with the tester strains at 37 ± 2°C for 48 hours in the presence or absence of S9 mixture along with vehicle and positive controls containing the following combinations of substances and doses: 2-AA at 1 μg/plate *vs.* TA100 and TA1537 with S9 mixture, at 2 μg /plate *vs.* TA1535, and at 6 μg/plate *vs.* WP2 uvrA with S9 mixture; SA at 0.5 μg/plate *vs.* TA100 and TA1535 without S9 mixture; BP at 1 μg /plate *vs.* TA98 with S9 mixture; 2-NF at 2 μg/plate *vs.* TA98 without S9 mixture; ICR-191 at 0.5 μg/plate *vs.* TA1537 without S9 mixture; 4NQO at 0.5 μg/plate *vs.* WP2 uvrA without S9 mixture.

Each dose of ginseng Rh2+ was tested in triplicate. The results were considered to be positive if a dose-related increase was observed over the range tested and/or a reproducible increase was observed at one or more doses in the mean of revertant colonies per plate in at least one strain with or without the S9 mixture. Cytotoxicity was defined as a clearing or diminution of the background lawn, the appearance of microcolonies, and/or a decrease of > 50% in the number of colonies compared with the relevant vehicle control.

The chromosome aberration test was conducted in accordance with the OECD guidelines for the testing of chemicals (July 21, 1997) in publication TG No. 473 'In vitro Mammalian Chromosome Aberration Test'. The Chinese hamster lung (CHL) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). 4NQO was used as a positive control substance without metabolic activation, as was BP with metabolic activation. We conducted a preliminary study to determine the highest dose by using 8 test dose levels from 5 to 5,000 μg/mL with and without S9 mixture. The highest dose of each treatment series was estimated according to the reduction in the relative increase in cell count (RICC) for cell lines to 45 ± 5% that of the concurrent vehicle control. The main study was assessed *via* two different procedures: a 6-hours treatment followed by an 18-hours recovery (with or without S9 mixture), and a 24-hours continuous treatment. We used replicate, treated cultures at each dose tested. Chromosome aberrations were identified morphologically according to the principles described in the 'Atlas of chromosome aberration by chemicals' (JEMS-MMS, 1988).

In the chromosome aberration test, cells with more than four of the same type of aberration were scored as multiple aberrations. Any metaphase with one or more aberrations, regardless of the type, was classified as an aberration metaphase. Slides were scanned systemically, and each set of metaphases was examined at × 1,000 magnification. Structural chromosome aberrations were evaluated in 150 well-spread metaphases, each containing 23 to 27 chromosomes. The microscopic stage coordinates and each type and number of aberration were recorded for each aberrant metaphase. The results were expressed as the numbers of findings per 100 metaphases. Regardless of the presence of aberrations, an additional 150 metaphases were examined to determine the frequency of diploidy (DP), polyploidy (PP > 37 chromosomes). The results were determined to be positive if a dose-related increase or a reproducible increase was observed at one or more doses in the frequency of aberrant metaphase.

The *in vivo* micronucleus test was conducted in accordance with the OECD guidelines for the testing of chemicals (July 21, 1997) in publication TG No. 474 'Mammalian erythrocyte micronucleus test'. A preliminary range-finding study showed that oral administration of ginseng Rh2+ at doses of 800, 2,000, and 5,000 mg/kg/day did not induce any toxic effect. The highest dose, 5,000 mg/kg/day, was selected as the maximum tolerated dose (MTD), and the middle and the low doses were determined by using a common ratio 2. Ginseng Rh2+ was administered orally twice a day for 2 days to male IRC mice (6, 7-week-old male mice per each of 5 groups). The positive control received

Table 2 Summary of the urinalysis findings for the male rats in the 14-day range-finding study

Parameter		Group (mg/kg/day)*			
		G1 (0)	G2 (500)	G3 (1,000)	G4 (2,000)
Ketones[†]	Negative	3 / 5	5 / 5	0 / 5	5 / 5
	Trace	2 / 5	0 / 5	3 / 5	0 / 5
	1 +	0 / 5	0 / 5	2 / 5	0 / 5
Proteins[‡]	Negative	0 / 5	0 / 5	0 / 5	0 / 5
	Trace	1 / 5	0 / 5	0 / 5	4 / 5
	1 +	4 / 5	5 / 5	5 / 5	1 / 5

*Number of animals with the sign divided by the number of animals examined; [†]Significant difference among groups, $P < 0.05$; [‡]Significant difference among groups, $P < 0.01$.

cyclophosphamide monohydrate (CPA) in normal saline (10 mL/kg/day), which was given at a dose of 70 mg/kg/day by intraperitoneal injection.

Animals used for the *in vivo* micronucleus test were sacrificed by CO_2 gas inhalation at – 24 hours after the final administration, and bone marrow preparations were made using the method of Schmid [14]. Two slides of the cell suspension from each animal were made. Small round or oval bodies, measuring about 1/5 to 1/20 the diameter of a polychromatic erythrocyte (PCE), were counted as micronuclei. The same observer scored a total of 2,000 PCEs per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). The ratio of PCEs to normochromatic erythrocytes (NCE) [PCE/(PCE + NCE)] was calculated by counting 500 cells. The mortality and the clinical signs of animals were checked and recorded during the study period. Body weights were measured on the days of reception, grouping, dosing, and autopsy. The results were determined to be positive if a dose-related increase or a reproducible increase at one or more doses was observed in the frequency of micronucleated polychromatic erythrocytes (MNPCEs).

In the general toxicity test, statistical analyses were performed by using the SPSS 12.0K program (SPSS Inc., Chicago, USA). Means and standard deviations were calculated for all quantitative data. Significance was judged at a probability of $P < 0.05$. Male and female rats were evaluated separately. The body weight changes, food and water consumptions, organ weights and hematological/biochemical values were analyzed by using Levene's test for the homogeneity of variances; then, a one-way analysis of variance (ANOVA) analysis was performed to evaluate the significant differences between the control and the ginseng Rh2+ treated groups. A post-hoc test was performed according to the result of the variance homogeneity (homogeneity: Duncan's test; heterogeneity: Dunnett's T3 test). Categorical variables, such as urinalysis values, were analyzed by using the Chi-square test.

In the chromosome aberration assay, statistical analyses were performed by using SPSS Statistics 22 for Medical Science. Fisher's exact test was used to compare the vehicle and the ginseng Rh2+ treated groups. Differences

were regarded as statistically significant if $P < 0.05$. In the micronucleus test, differences in the numbers of MNPCEs between the treated and the control groups were analyzed using the Kruskal-Wallis' H-test. The Mann-Whitney U-test was used to compare the vehicle and the positive control groups. The difference in the PCE: (PCE + NCE) ratios between the treated and the vehicle control groups was analyzed using the one-way ANOVA and that between the vehicle and the positive control groups was analyzed using the Student's *t*-test (SPSS Statistics 22 for Medical Science).

3. Results

3.1. Acute oral toxicity study in rats

No mortality was observed in any group during the observation period of 14 days. As for clinical symptoms, salivation was observed in two rats that had been administered 4,000 mg/kg/day. However, the symptoms disappeared (the rats recovered) two hours after they had appeared. No other clinical signs of toxicity were observed. In both sexes, the changes in body weight did not differ significantly between the groups ($P > 0.05$). At the time of the scheduled autopsy, no macroscopic abnormalities were observed in any of the organs.

3.2. Fourteen-day range-finding study in rats

No deaths or obvious clinical signs were found in any group throughout the sub-acute experimental period. No significant changes in body weights or in food and water consumptions were found in any group. On the urinalysis, ketones and proteins were statistically different between the male control group and the male treated groups (Table 2). No significant differences in the hematological values and the serum biochemical values were noted, and no abnormal gross findings were observed at necropsy. The relative organ weights of the thyroid glands were statistically greater in female rats that had received a dose of 500 mg/kg/day than they were for the rats in the control group (Table 3).

Table 3 Summary of the relative organ weights for the female rats in the 14-day range-finding study

Parameter	Group (mg/kg/day)*			
	G1 (0)	G2 (500)	G3 (1,000)	G4 (2,000)
Liver	3.1438 ± 0.2412	3.0462 ± 0.1554	3.1304 ± 0.2989	3.1347 ± 0.1294
Kidney (Lt.)	0.4259 ± 0.0499	0.4279 ± 0.0338	0.4232 ± 0.0414	0.4120 ± 0.0161
Kidney (Rt.)	0.4331 ± 0.0608	0.4427 ± 0.0305	0.4379 ± 0.0254	0.4208 ± 0.0104
Heart	0.3898 ± 0.0274	0.4083 ± 0.0281	0.4073 ± 0.0341	0.4003 ± 0.0393
Lung	0.5441 ± 0.0249	0.5370 ± 0.0370	0.5302 ± 0.0249	0.5260 ± 0.0280
Brain	0.9452 ± 0.0670	0.9355 ± 0.0411	0.9277 ± 0.0345	0.9236 ± 0.0404
Thymus	0.2261 ± 0.0358	0.3130† ± 0.0487	0.2666 ± 0.0226	0.2626 ± 0.0531

*Mean ± standard deviation; †Significant difference compared with the control group value, $P < 0.05$.

Table 4 Summary of the clinical chemistry values for the male rats in the 90-day main study

Parameter (UNIT)	Group (mg/kg/day)*			
	G1 (0)	G2 (500)	G3 (1,000)	G4 (2,000)
AST (IU/L)	114 ± 37	114 ± 25	113 ± 25	112 ± 27
ALT (IU/L)	38 ± 6	36 ± 8	38 ± 6	35 ± 6
ALP (IU/L)	211 ± 46	229 ± 61	227 ± 46	229 ± 29
T-BIL (mg/dL)	0.04 ± 0.03	0.05 ± 0.03	0.05 ± 0.02	0.03 ± 0.02
CHO (mg/dL)	67 ± 17	73 ± 11	71 ± 14	79 ± 17
TG (mg/dL)	49.5 ± 28.6	50.1 ± 11.5	47.9 ± 17.1	44.0 ± 18.5
TP (g/dL)	6.3 ± 0.2	6.1 ± 0.2	6.1 ± 0.2	6.3 ± 0.3
ALB (g/dL)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
A/G ratio	0.60 ± 0.05	0.60 ± 0.04	0.61 ± 0.03	0.60 ± 0.06
Mg (mg/dL)	2.2 ± 0.2	2.2 ± 0.1	2.2 ± 0.3	2.3 ± 0.1
Na (mmol/L)	144 ± 2	143 ± 1	142† ± 1	142* ± 2
K (mmol/L)	4.5 ± 0.3	4.7 ± 0.3	4.6 ± 0.1	4.5 ± 0.2
Cl (mmol/L)	106 ± 1	105 ± 2	107 ± 2	107 ± 2

*Mean ± standard deviation; †Significant difference compared with the control group value, $P < 0.05$.
AST, Aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; T-Bil, total bilirubin; CHO, total cholesterol; TG, triglycerides; TP, total protein; ALB, albumin; A/G ratio, albumin/globulin ratio; Mg, magnesium; Na, sodium; K, potassium; Cl, chloride.

### 3.3. Subchronic ninety-day toxicity study in rats

No mortality or abnormal clinical signs were observed in any group throughout the subchronic experimental period. The body weight changes and food consumptions are summarized in Figs. 2, 3, but no significant differences ($P > 0.05$) were found for any group. No ophthalmological changes were noted in any test or control rat. On clinical pathology, the only statistically significant difference noted was that sodium was decreased in male rats treated at doses of 1,000 and 2,000 mg/kg/day compared to the control group (Table 4). The relative organ weights of the liver were significant increased in rats treated at a dose of 2,000 mg/kg/day compared to the control group ($P < 0.05$). No

other significant differences were observed in any of the treated groups (Table 5).

In gross findings, multi-focal pulmonary white spots were found in one control rat, which was identified by histopathology as a perivascular infiltration of inflammatory cells. Atrophic changes of both the testis and epididymis were observed in one male rat treated with a dose of 500 mg/kg/day. That was confirmed by histopathology as atrophy of seminiferous tubule, and aspermia/cell debris of lumen Moreover, a hypothalamic cyst was observed in one female rat treated with a dose of 1,000 mg/kg/day. This finding was identified as a developmental cyst at the pars nervosa. No other differences in macroscopic appearance were ob-
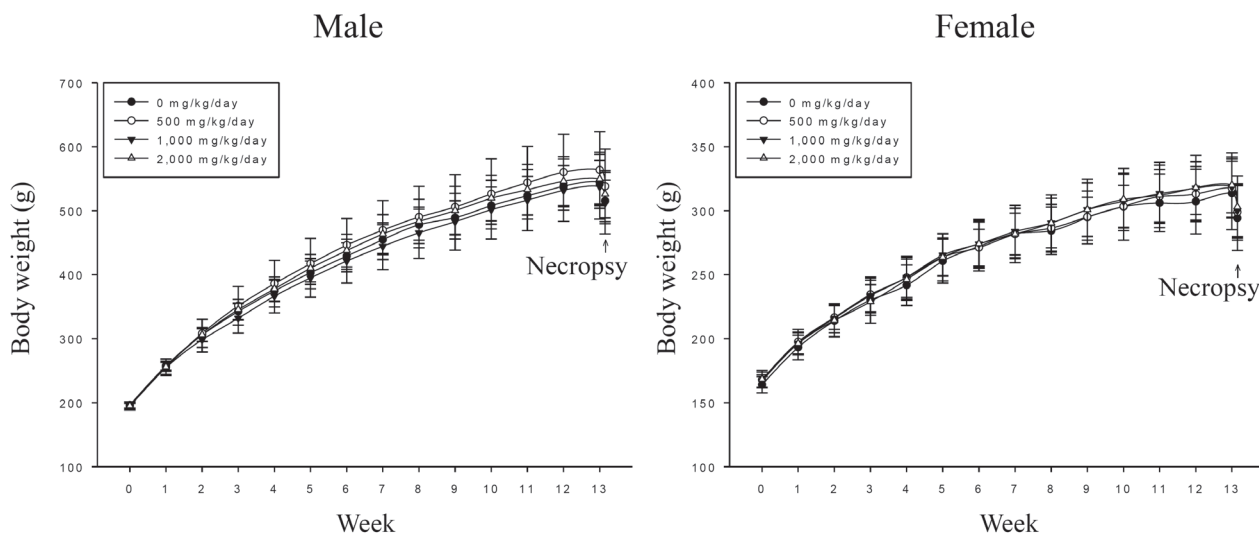
Male

Female

Figure 2 Body weight changes of male and female rats over a period of 13 weeks.
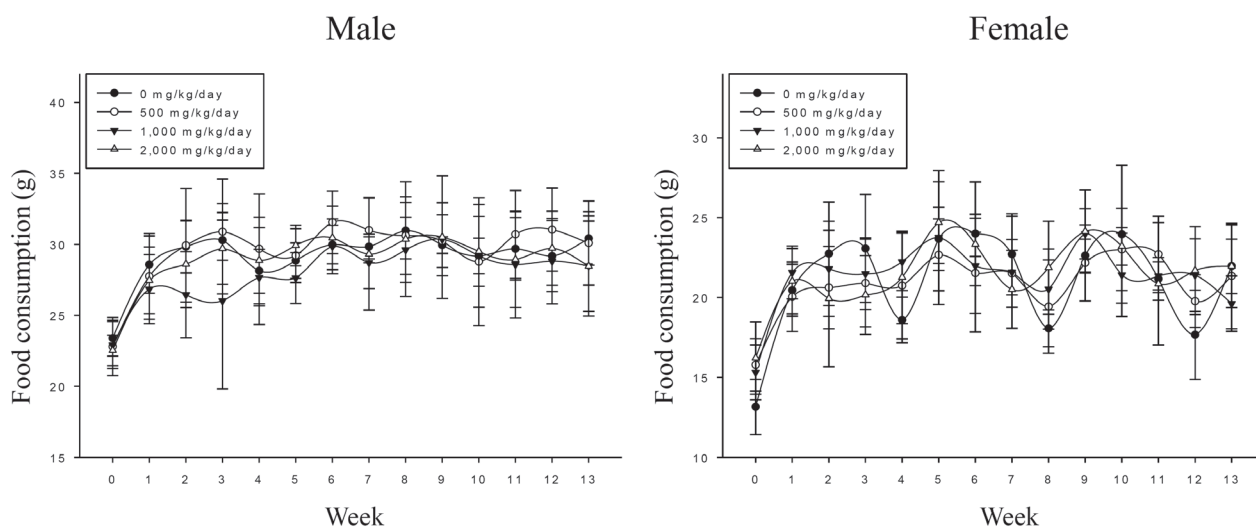
Male

Female

Figure 3 Food consumption of male and female rats over a period of 13 weeks.

served in any of the other groups.

The microscopic finding showed multi-focal perivascular infiltration of inflammatory cells in the lungs in 9 male rats in the control group, 6 male rats treated at a dose of 2,000 mg/kg/day, 7 female rats in the control group, and 9 female rats treated at a dose of 2,000 mg/kg/day. Renal tubular dilatation of the inner stripe was found in one male rat in the control group.

3.4. Bacterial reverse mutation test

Precipitation was observed on the agar plate at a dose of 1,500 mg/plate or more for all bacterial strains. No dose-dependent increase in the mean number of revertant colonies or in the cytotoxicity was observed in any of the *S. typhimurium* or *E. coli* strains with or without the S9 mixture. The positive controls showed significantly increased

numbers of revertant colonies, indicating that the assay was valid (Table 6).

3.5. Chromosome aberration test

In the 6-hours treatment groups (+ S9 mix), the frequency of structural aberrations in all treated and control groups was 0.00%, as was the frequency of numerical aberrations. However, the number of structural aberrations was significantly increased in the positive control group (39.67%). In the 6-hours treatment groups (- S9 mix), the numbers of structural aberrations in all treated groups were not significantly increased compared to that of the vehicle control group. However, a significant increase in the number of structural aberrations was observed in the positive control group (11.33%). In the 24-hours treatment groups (- S9 mix), no structural or numerical aberrations were ob-

Table 5 Summary of the relative organ weights for the female rats in the 90-day main study

Organ	Group (mg/kg/day)*			
	G1 (0)	G2 (500)	G3 (1,000)	G4 (2,000)
Liver	2.3406 ± 0.2172	2.3425 ± 0.0801	2.3548 ± 0.1780	2.5227[†] ± 0.1261
Kidney (Lt.)	0.3088 ± 0.0280	0.2908 ± 0.0168	0.3081 ± 0.0322	0.3029 ± 0.0372
Kidney (Rt.)	0.3221 ± 0.0323	0.2983 ± 0.0112	0.3158 ± 0.0294	0.3112 ± 0.0422
Heart	0.3009 ± 0.0225	0.2848 ± 0.0183	0.2929 ± 0.0168	0.2824 ± 0.0234
Lung	0.3393 ± 0.0378	0.3166 ± 0.0350	0.3496 ± 0.0190	0.3285 ± 0.0324
Brain	0.4219 ± 0.0324	0.3961 ± 0.0334	0.4094 ± 0.0318	0.4012 ± 0.0302

*Mean ± standard deviation (number of animals); †Significant difference compared with the control group value, $P < 0.05$.

Table 6 Summary of the results on the bacterial reverse mutation test

Compound	Dose (µg/plate)	TA100		TA1535		TA98		TA1537		*E.coli* WP2 *uvr*A	
		+ S9 mix	- S9 mix	+ S9 mix	- S9 mix	+ S9 mix	- S9 mix	+ S9 mix	- S9 mix	+ S9 mix	- S9 mix
		Mean revertants/plate		Mean revertants/plate		Mean revertants/plate		Mean revertants/plate		Mean revertants/plate	
Ginseng Rh2+	0	113 ± 14	127 ± 9	11 ± 2	11 ± 2	24 ± 3	17 ± 2	13 ± 2	6 ± 2	23 ± 1	15 ± 2
Ginseng Rh2+	50	114 ± 15	122 ± 16	13 ± 2	13 ± 3	23 ± 4	21 ± 2	12 ± 4	6 ± 1	22 ± 1	17 ± 4
Ginseng Rh2+	150	121 ± 14	124 ± 4	9 ± 3	10 ± 3	28 ± 3	18 ± 3	13 ± 6	5 ± 1	18 ± 3	13 ± 2
Ginseng Rh2+	500	116 ± 7	105 ± 5	7 ± 1	12 ± 2	26 ± 3	16 ± 2	15 ± 4	5 ± 2	19 ± 3	17 ± 2
Ginseng Rh2+	1500*	102 ± 3	105 ± 6	8 ± 1	11 ± 3	23 ± 2	21 ± 3	10 ± 1	6 ± 1	16 ± 2	14 ± 4
Ginseng Rh2+	5000*	117 ± 5	120 ± 11	9 ± 1	11 ± 3	24 ± 2	16 ± 2	11 ± 2	5 ± 2	18 ± 1	16 ± 4
2-AA	1.0	1283 ± 45	–	–	–	–	–	120 ± 25	–	–	–
2-AA	2.0	–	–	146 ± 22	–	–	–	–	–	–	–
B(a)P	1.0	–	–	–	–	344 ± 11	–	–	–	–	–
2-AA	6.0	–	–	–	–	–	–	–	–	124 ± 6	–
SA	0.5	–	469 ± 13	–	387 ± 10	–	–	–	–	–	–
2-NF	2.0	–	–	–	–	–	129 ± 9	–	–	–	–
ICR-191	0.5	–	–	–	–	–	–	–	92 ± 8	–	–
4NQO	0.5	–	–	–	–	–	–	–	–	–	110 ± 6

*Precipitation in the treatment mixture.
B(a)P, benzo(a)pyrene; 4NQO, 4-nitroquinoline-1-oxide.

Table 7 Summary of the results on the chromosome aberration test

Dose (μg/mL)	S9 mix	Time hours[*]	Mean total aberrant metaphases		Mean of PP + ER	RICC (%)
			+ Gaps	- Gaps		
6-h treatment (+ S9 mix)						
0	+	6 - 18	0.00%	0.00%	0.00%	100%
750	+	6 - 18	0.00%	0.00%	0.00%	95%
1,500	+	6 - 18	0.33%	0.00%	0.00%	76%
3,000	+	6 - 18	0.00%	0.00%	0.00%	47%
20 B(a)P	+	6 - 18	40.33%	39.67%[†]	0.00%	61%
6-h treatment (- S9 mix)						
0	–	6 - 18	0.00%	0.00%	0.00%	100%
750	–	6 - 18	0.00%	0.00%	0.00%	78%
1,500	–	6 - 18	0.00%	0.00%	0.00%	61%
3,000	–	6 - 18	1.33%	1.33%	0.00%	42%
0.4 4NQO	–	6 - 18	11.67%	11.33%[†]	0.00%	54%
24-h treatment (- S9 mix)						
0	–	24 - 0	0.00%	0.00%	0.00%	100%
375	–	24 - 0	0.00%	0.00%	0.00%	97%
750	–	24 - 0	0.00%	0.00%	0.00%	68%
1,500	–	24 - 0	0.00%	0.00%	0.00%	44%
0.4 4NQO	–	24 - 0	14.67%	14.67%[†]	0.00%	82%

[*]Treatment time-recovery time (hrs); [†]Significantly different from the negative control at $P < 0.01$ (Fisher's exact test).
PP, polyploidy; RICC, relative increase in cell count; B(a)P, benzo(a)pyrene; 4NQO, 4-nitroquinoline-1-oxide.

Table 8 Summary of the results on the micronucleus test

Dose (mg/kg/day)	Number of animals	MNPCE/2000 PCE (Mean ± SD)	PCE:RBC Ratio (Mean ± SD)	Control (%)
0	6	1.17 ± 0.98	0.53 ± 0.02	100
1,250	6	1.83 ± 1.72	0.53 ± 0.03	101
2,500	6	1.50 ± 1.52	0.54 ± 0.04	102
5,000	6	1.33 ± 0.82	0.54 ± 0.06	103
CPA 70	6	59.50 ± 3.83[*]	0.37 ± 0.02[*]	70

[*]Significantly different from the negative control group at $P < 0.01$.
MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; RBC, red blood cell (polychromatic erythrocytes + normochromatic erythrocytes); SD, standard deviation; CPA, cyclophosphamide monohydrate.

served in any of the treated groups or in the vehicle control group. However, the frequency of structural aberrations was significantly increased in the positive control group (14.6%) (Table 7).

3.6. *In vivo* micronucleus test

No abnormal clinical signs or body weight changes were observed in the treated or the control groups. The frequencies of MNPCE/2000PCE were 1.17 ± 0.98 in the vehicle control, 1.83 ± 1.72 in the 1,250 mg/kg/day group, 1.50 ± 1.52 in the 2,500 mg/kg/day group, and 1.33 ± 0.82 in the 5,000 mg/kg/day group. No statistically significant differences were noted for any of the treated groups, regardless of dose. On the other hand, the frequencies of MN-

PCE/2000 PCE were significantly increased in the positive control group ($P < 0.01$), indicating that the present study had been performed under acceptable experimental conditions. The PCE:red blood cell (RBC) ratios for the treated groups did not differ significantly from that for the vehicle control group, but a significant decreased was observed in the positive control group compared to the vehicle control group ($P < 0.01$) (Table 8).

4. Discussion

Pharmaceutically-active ginsenosides exist as deglycosylated forms. Orally administered, the ginsenosides are extensively metabolized by intestinal microbiota before being absorbed via stepwise cleavage of the sugar moieties. Intestinal bacteria transform major ginsenosides to bioactive metabolites, such as compound K, Rh2, Rh1, etc. However, ginsenosides are poorly absorbed (the absorption rate is as low as 0.1% to 3.7%), and the absorption of the metabolites of ginsenosides depends on the activity of each individual microbial flora [15, 16]. Therefore, many efforts have been made to increase the proportion of metabolic forms of ginsenosides. Enzyme treatment is one of the methods for increasing active metabolites of ginsenosides [17, 18].

Ginseng Rh2+ is an enzyme-treated ginseng extract containing high amounts of converted ginsenosides. Primarily, a HPLC-UV analysis was conducted to generate quantitative profiles of the ginsenoside compositions of both ginseng Rh2+ and red ginseng. As demonstrated in Fig. 1, prominent ginsenoside peaks were present in the chromatograms of ginseng Rh2+ and red ginseng. Minor ginsenosides, including compound K, Rh2, Rg3, and Rh1, were exclusively detected in ginseng Rh2+. By contrast, in red ginseng, the most intensive HPLC peaks were those of Rg1 in the range from 20 to 30 minutes and of Rb1 in the range from 50 to 60 minutes, which were absent or minimal in ginseng Rh2+. Ginseng Rh2+ contained approximately 3.4 mg/g of Rh2, 5.9 mg/g of compound K, and 6.0 mg/g of Rg3 (Table 1). Rg3 is one of the main artifact components of red ginseng and heat-processed ginseng. The fermentation of ginseng (or red ginseng) using enzyme treatment converts PPD major ginsenosides to compound K and Rh2 and PPT major ginsenosides to Rh1 [2, 17]. These results show rich quantities of converted minor ginsenosides in ginseng Rh2+ compared to red ginseng.

As a major metabolite of intestinal bacteria, compound K possesses significant anticancer activities. Many studies have revealed that compound K induces apoptosis by regulating various signaling pathways [19-21]. Compound K also has been reported to inhibit proliferation and to suppress metastasis in several cancer cell lines [22-24]. Recently, Chong et al reported that compounds K and Rg3, as potent angiogenic inhibitors, could be used in combination with other chemotherapeutic cancer agents [25]. Rh2 has also been shown to significantly inhibit growth of cancer, and Rh2 has been reported to induce cell apoptosis and to inhibit tumor growth in numerous types of cancer cells [26-28]. In recent studies, a minor ginsenoside from the PPT group, Rh1, was reported to inhibit the expression of matrix metalloproteinases in human astroglioma cells and hepatocellular carcinoma cells [29, 30] whereas in several studies, major ginsenosides, such as Rb1, Rd, Re, and Rg, did not show any obvious anticancer activities [5-7].

The toxicological potential of ginseng Rh2+ was evaluated in a series of general and genetic toxicity tests. The acute toxicity test was performed in rats at a high-level dose of 4,000 mg/kg/day, which produced no mortality or toxicity-related signs and no significant changes in body weights or gross findings. Therefore, under the conditions of this test, the approximate lethal dose (ALD) of ginseng Rh2+ is thought to be higher than 4,000 mg/kg/day. A 14-day repeated range-finding test was performed for ginseng Rh2+ at doses of 500, 1,000, and 2,000 mg/kg/day. On the urinalysis, the significant differences observed in ketones and proteins were not dose-dependent. The weight of the thymus in female rats treated at a dose of 500 mg/kg/day was slightly increased, but was still within the normal range. Overall, no toxicity-related changes were observed during this test. From the above results, we found the maximum dose to be 2,000 mg/kg/day on the subchronic test.

A subchronic 90-day toxicity study was conducted in rats for doses of 0, 1,000, and 2,000 mg/kg/day of ginseng Rh2+. A number of findings were noted during the course of the 90 days, which were described in detail in the Results section. In the laboratory study, the sodium levels for the male rats treated with doses of 1,000 and 2,000 mg/kg/day were significantly lower ($P < 0.05$) compared to the level for the control group, but all sodium levels were within the normal range. No significant differences were observed in any of the other laboratory values (Table 4). The relative organ weights of the liver were increased in male rats treated at a dose of 2,000 mg/kg/day, but those increases were minimal, and the values were still within the normal range. The results of the liver function test were also in the normal range (Table 4). Macroscopic abnormal findings were found in one rat in the low-dose group and in one rat in the mid-dose groups, suggesting non-treatment-related changes. Abnormal findings on histopathologic examination were observed in both the tested and the control groups, suggesting no correlation with treatment. Therefore, these results indicate that no toxic effect of ginseng Rh2+ was found and that the NOAEL of ginseng Rh2+ is more than 2,000 mg/kg/day; no target organ were found in this study.

The Ames test was performed to investigate the mutagenic potential of ginseng Rh2+ by using amino-acid-requiring strains of *S. typhimurium* and *E. coli*. The main purpose of this test was to detect point mutations, which are the causes of many human diseases including cancer [31]. We found no positive mutagenic responses to ginseng Rh2+ in any of the tester strains compared with the concurrent vehicle control groups both with and without application of the S9 mixture.

Chromosome aberrations are a classical genotoxic response to the tumor initiation and development processes [32, 33]. To determine if ginseng Rh2+ causes structural chromosomal aberrations in CHL cells, we conducted chromosome aberration tests. As described in detail in the Results section, no significant or dose-dependent increases in structural or numerical aberrations were observed in

the groups treated with ginseng Rh2+ with or without the S9 mix. As expected, the positive controls showed a significant increase in the frequency of structural aberrant chromosomes when compared with the vehicle control. These findings confirmed that the methods used in this study were valid.

In the micronucleus test, no statistically-significant treatment-related differences in the incidences of MNPCEs or in the PCE/RBC ratios were noted except for the positive control group, which showed a significantly increased incidence of MNPCEs ($P < 0.01$). Thus, the results of this test demonstrated that ginseng Rh2+ did not induce chromosome aberrations in the bone marrow cells of ICR mice.

In conclusion, ginseng Rh2+ did not show any subchronic toxicity or genotoxicity under the experimental condition in this study. The subchronic toxicity study provided information on the major toxic effects of repeated exposure over a prolonged period of time, identified no possible target organs [34]. Genotoxicity describes the property of chemical or physiological agents that damage deoxyribonucleic acid (DNA) and chromosomes, which may lead to carcinogenic and/or mutagenic potential in humans [35]. Therefore, the findings of our toxicity studies support the long-term use of ginseng Rh2+ to treat safely patients with cancer.

Current chemotherapy for the treatment of patients with cancer is usually long-term. Especially, decreased immunity, as well as the many adverse effects of chemotherapy and surgery, is a well-known limitation of standard cancer treatment [36, 37]. Recent studies have reported that herb medicine can reduce the adverse effects of chemotherapy and improve the quality of life (QOL) for patients with cancer [38, 39]. In particular, ginseng/ginsenoside extracts are known to be well-established potent, bioactive anticancer agents. Recent studies reported the combined effects of compound K and cisplatin in lung cancer cells and breast cancer cells [40, 41]. Wang *et al* reported that ginseng extract enhanced the tumoricidal effects of 5-FU in human colon-cancer cells [39]. In previous studies by our group, ginseng Rh2+ showed anti-proliferative effects in various cancer-cell models. Definite cell inhibition was observed for ginseng Rh2+ compared to a control ginseng extract [8-10]. We expect that ginseng Rh2+ will possibly play an important role as a novel adjuvant anticancer agent.

A limitation of our studies is the fact that the anticancer effects of ginseng Rh2+ were not verified in humans, but in cell models. Further studies are needed if the clinical efficacy of ginseng Rh2+ and its synergetic effects with chemotherapy are to be confirmed. The pharmacokinetics-pharmacodynamics (PK/PD) of ginseng Rh2+ should also be studied.

5. Conculsion

The results of this study suggest that ginseng Rh2+ is a non-toxic material with no genotoxicity. We expect that ginseng Rh2+ may be used as a novel adjuvant anticancer agent that is safe for long-term administration.

Conflict of interest

The authors declare that there are no conflict of interest.

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