



Evaluation of genetic toxicity, acute and sub-chronic oral toxicity and systemic safety of *Agrimonia pilosa* and *Rhus gall* 50% ethanolic extract mixture (APRG64) in vitro and in vivo (rodent and non-rodent animal models)

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Abstract

Agrimonia pilosa (AP) and *Rhus gall* (RG) are traditional medicinal plants. The bioflavonoid composition standardized by HPLC analysis was named APRG64. Despite many studies reported to beneficial bioactivities of AP and RG, very limited range of toxicity tests have documented. So, we did experiment diversely on the toxicity tests of the substance APRG64. Genotoxicity (mammalian chromosomal aberration test, micronucleus test) against APRG64, acute and sub-chronic toxicity test from rodent/non-rodent, and systemic safety pharmacology test were conducted. As a result of the test, genotoxicity against APRG64 was not observed. The NOAEL of rodents was confirmed as 2000 mg/kg/day and non-rodents was confirmed as 500 mg/kg/day. In addition, systemic safety pharmacological toxicity (effects on respiratory system, central nervous system, cardiovascular system) following administration of APRG64 was not observed. Finally, we accomplished ten potential toxicity tests and evaluated extensive safety of APRG64. Consequently, APRG64 may be a promising material for nutraceuticals and natural medicines.

Keywords *Agrimonia pilosa* · *Rhus gall* · Genotoxicity · Systemic safety pharmacological toxicity · Potential toxicity tests · Natural medicines · NOAEL

Jong-Hun Park and Jong-Sung Ra made equal contributions to this work (co-first author).

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Abbreviations

AG	Apigenin-7-O- β -glucuronide
AP	<i>Agrimonia Pilosa</i>
APRG64	AP and RG of each 50% ethanolic extracts mixed 64
CHO-K1	Chinese hamster ovary
EG	Ethyl gallate
FOB	The functional observational battery
GA	Gallic acid
HPLC	High performance liquid chromatography
ICR	CrIjOriCD1
KCL/CRO	Korea Conformity Laboratories
MBP	The mean blood pressure
MFDS	The Ministry of Food and Drug Safety
MMC	Mitomycin C
MNPCE	Micronucleated polychromatic erythrocytes
NCE	Normochromatic erythrocytes
PCE	Polychromatic erythrocytes
PGG	Penta-galloyl glucose
RG	<i>Rhus javanica</i> gall
SOPs	Standard operation procedures
SPF	Specific pathogenic free

Introduction

Traditional medicinal plants have been used for treatment of particular diseases since ancient times and widely used throughout the world. In developing countries approximately 80% of people use traditional medicines, continue to provide humans with new remedies as 50% of all drugs in clinical use in the world are derived from natural products [1]. Despite, the widespread use of plants as medicines, often the use of these resources occurs without proper scientific evidence of their pharmacological properties and toxic potential. Previous study has shown that three quarters of herbal preparations marketed do not contain safety information for adequate consumption. Even if a particular species has low toxicity, its inappropriate use associated with risk factors may lead to serious conditions that are sometimes underreported [2, 3].

Here are two traditional medicinal plants, *Agrimonia pilosa* (AP), also known as hairy agrimony, belongs to the Rosaceae family. It is a perennial herb and widely distributed in Korea and China. The aerial parts of AP were used in folk medicine to treat hematuria, hematemesis, leukorrhea, traumatic injury, diarrhea, stomatitis, tonsillitis, eczema, hemoptysis, hematochezia, anti-viral and gastroenteritis [4–6]. Apigenin (AG) is one of the main flavonoid compounds from AP, has the pharmacological functions of anti-oxidation, anti-inflammation, anti-viral and inhibition of pulmonary fibrosis. Also, it can prevent or reverse liver injury [7, 8]. The gall of *Rhus javanica*,

Rhus gall (RG) is the excrescence produced by parasitic aphids, mainly *Melaphis chinensis* (Bell) Baker, on the leaf of *Rhus javanica* L. [9]. The herb contains Penta-galloyl glucose (PGG), one of the main polyphenolic compounds from RG, has been reported to possess antioxidant activity, anti-diabetic, anti-cancer, and effect on blocking hepatitis C virus entry [10].

Agrimonia pilosa and *Rhus gall* were reported to have anti-viral activity, respectively. Therefore, the antiviral effect is expected to increase more when the two extracts are mixed. The mixture of the two extracts was named APRG64.

This article purposes to evaluate the genetic toxicity, acute and sub-chronic oral toxicity and systemic safety of APRG64. Because the bioflavonoid compounds, AP and PGG of AP and RG have aroused intense interest in the development of pharmacological reagent [7, 10]. Firstly, bacterial reverse mutation test, chromosome aberration test, erythrocyte micronucleus test in mammalian cells were performed for the genetic toxicity evaluation. Secondly, Single dose oral toxicity study, 4-week repeated oral dose range finding study, Repeated dose 13-week oral toxicity and 4-week recovery study were practiced using SD rats to find the NOAEL of APRG64. Lastly, to secure more safety of APRG64, Repeated dose 4-week oral toxicity study in beagle dog was carried out, and central nervous systems, respiratory, and cardiovascular function were assessed through the functional observational battery (FOB), whole body plethysmography system in rats and implanted telemetry in dogs, respectively.

Materials and method

Preparation of extract

Sampling and identification

Dried *Agrimonia pilosa* (AP) and *Rhus gall* (RG) was provided by Kyung Dong Market (Seoul, Korea). The plant was identified and authenticated on the basis of its macroscopic and microscopic characteristics by Dr. Kang, Se Chan, Kyung Hee University (Yongin, Korea). The voucher specimen (NMR-KR-2017-01) was deposited in the Laboratory of Natural Medicine Resources in BioMedical Research Institute, Kyung Hee University.

Extraction

The samples were washed three times with distilled water to remove slats, plants, and sand, and then dry to without direct sunlight, and pulverized. Each crude extract was obtained

by extracting dried AP and RG twice with 50% ethanol for 24 h, at room temperature, respectively. The extracts were concentrated for 16 h at reduced pressure and 40 °C using a rotary evaporator, and then the crude extract was lyophilized to obtain a powder. And then, each 50% ethanolic extracts mixed 6:4 (AP:RG) ratio. A standardized bioflavonoid composition was named APRG64. This mixture stored at $-20\text{ }^{\circ}\text{C}$ until use, and when administered to experimental animals, they were orally mixed with 0.9% normal saline.

HPLC analyses

We conducted the analysis of the dosing formulations of AG and PGG from AP and RG using high performance liquid chromatography (HPLC). HPLC was performed using Shimadzu LC-20AD (Shimadzu, Kyoto, Japan) with and Waters C18 column (5 μm , $4.6\times 250\text{ mm}$). The column oven was maintained at 35 °C, and the mobile phases included solvent A [0.1% formic acid in water (*v/v*)] and solvent B [acetonitrile (*v/v*)]. The elution gradient was as follows: 0–3 min, B 10%; 3–10 min, B 10–20%; 10–20 min, B 20%; 20–30 min, B 2–35%; 30–38 min, B 35–90%; 38–39 min, B 90%; and 39–50 min, B 10%. The flow rate was 1.0 ml/min, and the injection volume was 10 μL for each run.

To analyze the content of AG in AP, the gradient eluted consisted of water with 0.1% formic acid (A) and acetonitrile (B). The mobile phase was used under binary linear gradient conditions as follows: 0–20 min, 5–100% A; 20–40 min, 100–5% A. The flow rate was 1.0 mL/min and injection volume was 10 μL . AP extract (10 mg/mL) and AG (1000 $\mu\text{g}/\text{mL}$) were dissolved methanol with appropriate dilution. Peaks were identified by comparing their retention time and UV–vis spectra with AG and were quantitated using the corresponding AG curve.

In the case of RG content analysis the gradient eluted consisted of 0.1% formic acid (A) and water with acetonitrile (B). The mobile phase was used under binary linear gradient conditions as follows: 0–47 min, 15–23% A. The flow rate was 1.0 mL/min and injection volume was 10 μL RG extract (10 mg/mL) and PGG (1000 $\mu\text{g}/\text{mL}$) were dissolved methanol with appropriate dilution. Peaks were identified by comparing their retention time and UV–vis spectra with PGG and were quantitated using the corresponding standard curve. When performed the individual 10 tests, the content of AG from AP was $14.04\pm 0.69\text{ mg/g}$ and PGG from RG was $230.31\pm 2.30\text{ mg/g}$.

Genotoxicity study

Bacterial reverse mutation study

This study was conducted using the pre-incubation method with and without metabolic activation system (S9) in accordance with the following test guidelines: OECD Guideline for Testing of Chemicals No. 471 ‘Bacterial Reverse Mutation Test’ (Adopted 21st July 1997), Ministry of Food and Drug Safety Notice No. 2015-82 ‘Toxicity Test Standards of Medicine and Medical Supplies’ (Revised 11th November, 2015) and The standard operation procedures (SOPs) of Korea Conformity Laboratories.

Bacterial reverse mutation test was performed by using histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and a tryptophan-requiring strain of *Escherichia coli* (WP2uvrA) in the presence and absence of metabolic activation system (S9 mix). The preliminary range-finding study was performed to determine dose levels at the main study. As a result of preliminary range-finding test, precipitation was observed at 5000 $\mu\text{g}/\text{plate}$ in all bacterial strains. At the main study, the test sample concentration was set at 21, 62, 185, 556, 1667 and 5000 $\mu\text{g}/\text{plate}$, with three plates for each dose. In the test, 0.05 mL of the sample, 0.1 mL of the bacterial suspension and 0.5 mL of either the S9 mix or the phosphate buffer (pH 7.4) were added to 2.0 mL of top agar which contained trace amount of histidine (for *Salmonella*), biotin (for *Salmonella*) and tryptophan (for *Escherichia*) maintained at 45 °C. The mixture was then poured onto a minimal glucose agar plate. The plates were inverted and incubated at 37 °C for 48 h. Dimethyl sulfoxide was used as the solvent control, and sodium azide (NaN_3 , TA1535 in 0.5 $\mu\text{g}/\text{plate}$), 9-aminoacridine hydrochloride hydrate (9-AA, TA1537 in 80 $\mu\text{g}/\text{plate}$), 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, TA98 in 0.1 $\mu\text{g}/\text{plate}$, TA100 in 0.01 $\mu\text{g}/\text{plate}$, WP2uvrA in 0.01 $\mu\text{g}/\text{plate}$) in the absence of S9 mix, and 2-aminoanthracene (2-AA, TA98 in 0.5 $\mu\text{g}/\text{plate}$, TA100 in 1.0 $\mu\text{g}/\text{plate}$, TA1535 in 2.0 $\mu\text{g}/\text{plate}$, TA1537 in 2.0 $\mu\text{g}/\text{plate}$, WP2uvrA in 10 $\mu\text{g}/\text{plate}$) in the presence of S9 mix were used as the positive control. Additionally, 0.1 mL of S9 mix and test substance were mixed for sterility test. The test result was recorded experimental value, average value and standard deviation for the number of revertant colonies per plate. The result was judged as ‘positive’, if there is a dose-dependent increase and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Also, the number of revertant colony should increase more than two times than the negative control group.

Mammalian chromosome aberration study

This study was conducted in accordance with the following test guidelines: Ministry of Food and Drug Safety Notice No. 2015-82 ‘Toxicity Test Standards of Medicine and Medical Supplies’ (Revised 11th November, 2015). OECD Guidelines for the Testing of Chemicals No. 473 ‘In vitro mammalian chromosome aberration test’ (26 September 2014). The standard operation procedures (SOPs) of Korea Conformity Laboratories (KCL/CRO).

In this study was performed the chromosome aberration test to assess the genotoxicity for APRG64 using Chinese Hamster Ovary (CHO-K1) cells in the absence (– S9 mix) and presence (+ S9 mix) of metabolic activation system. F-12 Nutrient Mixture (Gibco, Logan, UT, USA) with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA) and 10% Dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA) were used as culture medium.

The cytotoxicity test (preliminary range-finding test) was conducted to decide the highest treatment concentration for main test, and then the main test was selected 3 concentrations with threefold as follows. Absence metabolic activation system (– S9 mix, 24 h treatment) was 2.29, 6.86, 20.58 µg/mL. Absence metabolic activation system (– S9 mix, 6 h treatment and 18 h recovery) was 6.86, 20.58, 61.73 µg/mL. Presence metabolic activation system (+ S9 mix, 6 h treatment and 18 h recovery) was 6.86, 20.58, 61.73 µg/mL, respectively. Two replicate cultures were used for each concentration level.

In experiment without S9 metabolic activation, the treatment times were 4 and 24 h, respectively. 4×10^4 cells/mL were seeded in 60 mm diameter tissue culture dishes and incubated for 3 days. Before the test substance treatment, the existing culture medium was removed from the culture dish and then 4.80 mL of pre-warmed fresh medium (37 °C) and 0.20 mL of test substance solution were added to each dish which became 5.00 mL. In case of positive control, the existing culture medium was removed from the culture dish and then 4.95 mL medium (37 °C) and 0.05 mL of positive control (Mitomycin C, Sigma Aldrich) were added to each dish which became 5.00 mL. For 6 h treatment and 18 h recovery group, the culture medium was removed at 6 h after treatment and the cells were rinsed once with PBS (Ca^{2+} and Mg^{2+} free Dulbecco’s phosphate buffered saline, DPBS). Then, 5 mL of medium (37 °C) was added and the cells were incubated for additional 18 h until the harvest of metaphase-cells.

In experiment with S9 metabolic activation, the treatment time was 4 h. 4.30 mL of pre-warmed fresh medium (37 °C), 0.20 mL of test substance solution and 0.50 mL of S9 mix were added to each dish which became 5.00 mL. In case of positive control, 4.45 mL of medium (37 °C), 0.05 mL of positive control (Benzo[a]pyrene) and 0.50 mL

of S9 mix were added to each dish which became 5.00 mL. The culture medium was removed at 6 h after treatment and the cells were rinsed once with PBS. Then, 5 mL of pre-warmed fresh culture medium (37 °C) was added and the cells were incubated for additional 18 h until the harvest of metaphase-cells. The chromosome slides were prepared using the harvested cells. Approximately 22 h after treatment, Colcemid (Gibco) was added to each culture plate for a final concentration of 0.2 µg/mL. The cultures were incubated for an additional 2 h. The cells were detached using 1× trypsin solution. The medium containing mitotic cells was centrifuged at 1000 rpm for 5 min. After discarded supernatant, the cell pellets were resuspended in 75 mM potassium chloride (KCl) solution and incubated at 37 °C incubator for 20 min. The cells were fixed three times with Carnoy’s fixative solution (acetic acid:methanol=1:3 v/v) for the slide preparation. The slides were stained with 5% Giemsa (Merck, Darmstadt, Germany) solution for 20 min and observed microscopically. Two slides samples were prepared from each plate.

Mammalian erythrocyte micronucleus study

Mammalian erythrocyte micronucleus test was performed using male and female Specific Pathogen Free (SPF) CrljOri:CD1 (ICR) mice, aged 7–8 weeks, from Orient Bio Co., Ltd. (Seongnam, Korea). Animals were acclimated for 7 days. The only healthy animals were selected for this study by observation of general symptoms during the acclimation period. They were housed in litter downed polycarbonate cage (3–4 animals per cage for preliminary range-finding study, 5 animals per cage for main study) and provided food and water ad libitum. Range of acceptable temperature and humidity for animals were 22.0 ± 0.5 °C and $44.8 \pm 1.9\%$. According to determined dose in preliminary range-finding study, the highest dose in the main study was 2000 mg/kg/day. Test groups consisted of one negative control group (distilled water only), 3 dosing groups (500, 1000, 2000 mg/kg/day) and one positive control group [Mitomycin C (MMC) 2.0 mg/kg]. Each group consisted of five male mice. Because in the preliminary range-finding study, there was no difference in sensitivity to toxicity between male and female, so it was tested as male mice. The test substance was administered by oral (10 mL/kg once every 24 h for 2 days) using sonde and the positive control substance was administered through intraperitoneally to test animals using disposable 1 mL syringe. After dosing, animals examined regularly for clinical signs of toxicity until sacrifice. The thigh bone of the test animal was collected with care to avoid blood contamination in autopsy room within 18–24 h after final administration. The bone marrow was collected in a centrifuge tube by flushing the thigh bone inside with in-activated FBS. The extracted bone marrow was centrifuged at 1000 rpm for

5 min and then resuspended with small aliquots of FBS after discarding the supernatant. The bone marrow was smeared on a slide glass and then dried at room temperature and fixed in methanol for 5 min. Three slides of bone marrow were prepared. The slides were stained in 4% Giemsa staining solution for the scoring of the PCE (polychromatic erythrocytes) to NCE (normochromatic erythrocytes) ratio. The observation of slides was performed with blind method and the PCE/NCE ratio was determined by scoring the number of PCEs and NCEs observed while scoring 500 erythrocytes per animal. In addition, MNPCE (micronucleated polychromatic erythrocytes) from PCEs, stained with acridine orange (40 µg/mL), was scored by analyzing 4000 PCEs per animal.

Oral toxicity study

Single dose toxicity study

Single dose oral toxicity study was performed using male and female SPF CrI:CD (Sprague–Dawley, SD) rats, aged 7 weeks, from Orient Bio Co., Ltd. Female rats were healthy young adults and they were nulliparous and non-pregnant. Animals were acclimated during 6 days after arrival. Only animals with the best appearance were selected for the test after observation during the acclimation period. All animals were housed in wire mesh cages (250 W × 350 L × 180 H mm) and provided food and water ad libitum. Range of acceptable temperature and humidity for animals were 23.5 ± 0.4 °C and $44.7 \pm 1.1\%$. Forty SD rats were equally divided into 4 groups (5/sex/group) and once-daily oral (by gavage in the morning) of the test substance at 500 (low-dose), 1000 (middle-dose), and 2000 (high-dose) mg/kg. Dose levels were determined in accordance with the Ministry of Food and Drug Safety (MFDS) Notification [11]. A high-dose level was set as 2000 mg/kg which is selected as a limited dose generally in single dose toxicity study. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group.

General clinical signs or mortalities of all treated animals were carefully observed continuously during the first half-hour. After that, all animals were observed regularly at 1 h intervals until 6 h on the administration day. From the next day, animals were observed once every day up to 14 days after the administration. Also, individual animals were weighed at the date of acquisition, at grouping, dosing day prior to administration (on the administration day), 1, 4, 7, and 14 days (before necropsy) after the administration. On day 14 after the administration, all surviving animals were anesthetized with CO₂ gas and terminated by exsanguination from the abdominal aorta and caudal vena cava. Complete post-mortem examinations were performed on all

vital organs. This study was approved by the Institutional Animal Care and Use Committee (IACUC) (IA17-00004).

Body weight changes of all animals were analyzed by using the table and figures that were applied to the mean value and the standard deviations. Body weights among the vehicle and dosing groups were compared through the One-way ANOVA and significant difference was conceded in case of the *p* value is under 0.05. Statically analysis was performed in compliance with the SOP of this testing facility and SPSS for windows version 12.0 software (SPSS, Chicago, IL, USA) was used.

Rats: 4-weeks repeated dose toxicity determination study

Four-week repeated oral toxicity determination study was performed using male and female SPF SD rats, aged 5 weeks, from Orient Bio Co., Ltd. Female rats were healthy young adults and they were nulliparous and non-pregnant. Animals were acclimated during 6 days after arrival. Only animals with the best appearance were selected for the test after observation during the acclimation period. All animals were housed in wire mesh cages (250 W × 350 L × 180 H mm) and provided food and water ad libitum. Range of acceptable temperature and humidity for animals were 23.3 ± 0.3 °C and $43.0 \pm 1.6\%$. Forty SD rats were equally divided into 4 groups (5/sex/group) and once-daily oral (by gavage in the morning) of the test substance at 500 (low-dose), 1000 (middle-dose), and 2000 (high-dose) mg/kg for 4 weeks. Dose levels were determined at the results of single dose oral toxicity study, it is considered that toxicological effects related with the test substance were not observed at the dose level of 2000 mg/kg. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group.

During the study period, animals were observed for mortalities, clinical signs, body weights, food and water consumption, eye examination, urinalysis, clinical pathology (hematology and serum biochemistry) and necropsy (organ weights measurement and gross findings). This study was approved by the Institutional Animal Care and Use Committee (IACUC) (IA17-00048).

Statistical analysis was performed in compliance with the SOP of this testing facility and SPSS for windows version 12.0 software (SPSS) was used. The statistical differences among the vehicle control and the all dosing groups were analyzed normally through the parametric multiple comparison procedures or non-parametric multiple comparison procedures. Statistical significance was conceded if the *p* value is under 0.05 and incidence rate was represented by percentage. Analysis of continuous data used the standard one-way ANOVA. If the test showed statistical significance, the data was analyzed through the parametric multiple comparison

to compare the vehicle control group with the experimental groups. If the equal variance was admitted, Duncan's test was used and if not, Dunnett's *T3* test was applied. Analysis of non-continuous data (urinalysis and urine color) was converted by scale conversion as score and then analyzed by Chi-squared test.

Rats: 13-weeks repeated dose toxicity and 4-weeks recovery study

Thirteen-week repeated oral toxicity test and 4-week recovery study was performed using male and female SPF SD rats, aged 5 weeks, from Orient Bio Co., Ltd. Female rats were healthy young adults and they were nulliparous and non-pregnant. Male and female animals were acclimated during 6 and 8 days after arrival, respectively. Only animals with the best appearance were selected for the test after observation during the acclimation period. All animals were housed in wire mesh cages (250 W × 350 L × 180 H mm) and provided food and water ad libitum. Range of acceptable temperature and humidity for animals were 23.0 ± 0.6 °C and $42.8 \pm 2.9\%$. One hundred SD rats were divided into 4 groups (group 1, Vehicle control group, 15 male and female respectively; group 2–3, 500, 1000 mg/kg dosing group, 10 male and female respectively; group 4, 2000 mg/kg dosing group, 15 male and female respectively. Each last consecutive five animals in G1 and G4 groups are assigned to recovery groups.) once-daily oral (by gavage in the morning) of the test substance at 500 (low-dose), 1000 (middle-dose), and 2000 (high-dose) mg/kg were administered for 13 weeks. Dose levels were determined at the results of 4-week repeated oral dose range finding study, it is considered that toxicological effects related with the test substance were not observed at the dose level of 2000 mg/kg. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group.

During the study period, animals were observed for mortalities, clinical signs, body weights, food consumption, ophthalmological examination, clinical pathology (urinalysis, hematology, blood coagulation time and serum biochemistry), necropsy (organ weights measurement and gross findings), and histopathological examination. This study was approved by the Institutional Animal Care and Use Committee (IACUC) (IA17-00569).

Statistical analysis was performed in compliance with the SOP of this testing facility and SPSS for windows version 12.0 software (SPSS) was used. The statistical differences among the vehicle control and the all dosing groups were analyzed normally through the parametric multiple comparison procedures or non-parametric multiple comparison procedures. Statistical significance was conceded if the *p* value is under 0.05 and incidence rate was represented by percentage. Analysis of continuous data used the standard one-way

ANOVA. If the test showed statistical significance, the data was analyzed through the parametric multiple comparison to compare the vehicle control group with the experimental groups. If the equal variance was admitted, Duncan's test was used and if not, Dunnett's *T3*-test was applied. Analysis of non-continuous data (urinalysis and urine color) was converted by scale conversion as score and then analyzed by Chi-squared test.

Beagle dogs: 4-weeks repeated dose determination study

Four-weeks repeated dose determination study was performed using five male and five female beagle dogs, aged 5 months, from Producing Beijing Marshall Biotechnology Co., Ltd. (Beijing, China). Male and female animals were acclimated during for 26 days. The healthy animals selected during the acclimation period. All animals were housed in stainless steel cages (700 W × 750 L × 750 H mm) during the experiment period. Each animal was offered a daily ration of 300 g of solid food (LAB CANINE CHOW, Cargill Agri Purina Korea Inc., Seongnam, Korea). Water was provided ad libitum for municipal tap water purified by reverse osmosis filtering system using water bottles. Range of acceptable temperature and humidity for animals were 20.0 ± 0.4 °C and $46.1 \pm 3.1\%$. Male and female body weight range were 7.80–8.30 kg and 6.82–7.38 kg respectively at the administration time. Eight beagle dogs were equally divided into 4 groups (1/sex/group). once-daily oral, the test substance used in a gelatin capsule at 250 (low-dose), 500 (middle-dose), and 1000 (high-dose) mg/kg were administered for 4 weeks using hand, after opened dog's mouth in a natural condition in cage, gelatin capsules were placed on the rear part of the tongue. Vehicle used sterilized distilled water for control group.

During the study period, animals were observed for mortalities, clinical signs, body weights, food consumption, ophthalmological examination, clinical pathology (urinalysis, hematology and serum biochemistry), necropsy (organ weights measurement and gross findings), and histopathological examination. This study was approved by the Institutional Animal Care and Use Committee (IACUC) (IA17-00740).

The statistical analysis was not performed because only one animal was used per group.

Beagle dogs: 13-weeks repeated dose toxicity and 4-weeks recovery study

Thirteen-week repeated oral dose toxicity test and 4-week recovery study was performed using male and female beagle dogs, aged 5 months, from Producing Beijing Marshall Biotechnology Co., Ltd. (Beijing, China). Male and female animals were acclimated during for 27 days. The healthy

animals selected during the acclimation period. All animals were housed in stainless steel cages (700 W × 750 L × 750 H mm) during the experiment period. Each animal was offered a daily ration of 300 g of solid food (LAB CANINE CHOW, Cargill Agri Purina Korea Inc.). Water was provided ad libitum for municipal tap water purified by reverse osmosis filtering system using water bottles. Range of acceptable temperature and humidity for animals were 21.7 ± 0.5 °C and $52.8 \pm 8.6\%$. Male and female body weight range were 6.84–8.44 kg and 5.60–6.46 kg respectively at the administration time. Sixteen beagle dogs were divided into four groups (three or five/sex/group). Once-daily oral, the test substance used in a gelatin capsule at 250 (low-dose), 500 (middle-dose), and 1000 (high-dose) mg/kg were administered for 4 weeks using hand, after opened dog's mouth in a natural condition in cage, gelatin capsules were placed on the rear part of the tongue. Vehicle used sterilized distilled water for control group. Dose levels were determined at the results of 4-week repeated oral dose range finding study and repeated dose 13-week oral toxicity and 4-week recovery study, it is considered that abnormal clinical finding as salivation was observed in animals at 2000 mg/kg. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group.

During the study period, animals were observed for mortalities, clinical signs, body weights, food consumption, ophthalmological examination, clinical pathology (urinalysis, hematology, blood coagulation time and serum biochemistry), necropsy (organ weights measurement and gross findings), and histopathological examination. This study was approved by the Institutional Animal Care and Use Committee (IACUC) (IA17-00539).

Comparisons between the vehicle control group and the test substance-treated group were generally performed using parametric multiple comparison procedures or non-parametric multiple comparison procedures, and statistically significant when $*p < 0.05$ or $**p < 0.01$. The rate of occurrence was expressed as a percentage. The statistical analysis was performed according to the standard work instructions for the statistical processing of the test institute using SPSS 12.0 K program (SPSS, Chicago, IL, USA), a widely used statistical package.

Systemic safety assays

Rats: central nervous system assay

The central nervous system assay through a FOB was performed using male SPF SD rats, aged 5 weeks, from Orient Bio Co., Ltd. Animals were observed for clinical findings once daily for 6 days of the quarantine-acclimation period. Then they were acclimated to sham dosing and handling once daily for 5 days. All animals were housed in stainless

wire mesh cages, (260 W × 350 D × 210 H mm) and provided food and water ad libitum for municipal tap water purified by reverse osmosis filtering system using water bottles. Range of acceptable temperature and humidity for animals were 21.7 ± 1.3 °C and $54.2 \pm 14.8\%$. Thirty-two SD rats were equally divided into 4 groups (4/sex/group) and once-daily oral (by gavage in the morning) of the test substance at 250 (low-dose), 500 (middle-dose), and 1000 (high-dose) mg/kg. Dose levels were determined as a result of previous study that abnormal clinical finding as salivation was observed in animals at 2000 mg/kg. Thus, 250, 500 and 1000 mg/kg were selected as low, mid and high dose levels, respectively. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group.

During the study period, animals were observed for clinical signs and behavior, body weights, food consumption. Observation points were selected at 0 h (pre-dose) and at 0.5, 1, 3, 6 and 24 h post-dose based on the general change in the concentrations of the test substance in the plasma when dosed intravenously.

The FOB evaluations were conducted by blind method with a person for dosing and two observers. Two observers performed the blind observations, including home cage, open field, hand held and sensory-motor function observations, and the measurement of body temperature according to the "ATTACHMENT. Methods and Criteria for Evaluation of Functional Observational Battery" in the protocol. After the completion of the study, all remaining animals were euthanized in accordance with the SOP specifying the procedure of euthanasia of rodents. The FOB was performed under the condition that the animal response to external noise was reduced by generating white noise of 50 ± 10 dB. To evaluate the effects of the test substance, all parameters of the test substance groups for the FOB at each observation point were compared to those of the control group.

Statistical analysis was performed on FOB parameters using SAS Program (Ver. 9.3, SAS Institute Inc., Cary, NC, USA). Quantitative parameters were analyzed utilizing Bartlett test for homogeneity of variance (significance level: 0.05). One-way ANOVA was employed on homogeneous data (significance level: 0.05). If significant, Dunnett's *t* test was employed for multiple comparisons (significance levels: 0.05 and 0.01, two-tailed). Kruskal–Wallis test was employed on heterogeneous data (significance level: 0.05). All parameters were not statistically significant. Therefore, Steel's test was not applied for multiple comparisons. Rank value parameters were analyzed utilizing Kruskal–Wallis test (significance level: 0.05). If significant, Steel's test was employed for multiple comparisons (significance levels: 0.05 and 0.01, two-tailed).

Rats: respiratory function assay

Respiratory function assay through whole body plethysmography system was performed using male SPF SD rats, aged 5 weeks, from Orient Bio Co., Ltd. Animals were observed for clinical findings once daily for 7 days of the quarantine-acclimation period. Then they were preconditioned to the chambers and sham dosing once daily for 3 days, and animals were placed in the chamber for a certain period of time, so as to be familiar in the administration and unrestrained chambers. All animals were housed in stainless wire mesh cages, (260 W × 350 D × 210 H mm) and provided food and water ad libitum. Range of acceptable temperature and humidity for animals were 21.9 ± 2.5 °C and $49.85 \pm 4.15\%$. Twenty-four SD rats were equally divided into 4 groups (6/group) and once-daily oral (by gavage in the morning) of the test substance at 250 (low-dose), 500 (middle-dose), and 1000 (high-dose) mg/kg. Dose levels were determined as a result of previous study that abnormal clinical finding as salivation was observed in animals at 2000 mg/kg. Thus, 250, 500, and 1000 mg/kg were selected as low, mid and high dose levels, respectively. Dosing volume was adjusted as 10 mL/kg body weight and vehicle used sterilized distilled water for control group. All animals were observed for clinical findings including appearance, behavior and excretion once daily during the experimental period. No abnormal clinical findings were observed in any animal prior to dosing.

Animals were placed individually in the unrestrained chamber prior to dosing to acclimate the animals for approximately 30 min and pre-dose behavior was monitored until data were acquired for a total of 3 min during which their movement was stable. After administration, animals were placed in the unrestrained chamber for about 3 h. The behavior of animals was monitored for 3 min before and after each measuring point, and then transferred back to their cage. They were returned to the unrestrained chambers and acclimated from about 5–6 h post-dose and their behavior was observed for 3 min before and after each measuring point, and then transferred back to their cage. Animals were put in the unrestrained chambers within a reasonable time at 24 h post-dose. After at least 30 min of acclimation, the monitoring of animal behavior was conducted until data were acquired for a total of 3 min during which their movement was stable. After the completion of the study, all remaining animals were euthanized in accordance with the SOP specifying the procedure of euthanasia of rodents.

The monitoring of animal behavior was carried out through video footage transferred from the surveillance cameras and the video footage was recorded at the same time (SRP1650, Samsung Techwin Co., Ltd., Seoul, Korea) to be used as reference material for the

interpretation of the data. When animal behavior was unstable at any measuring point, the corresponding time was recorded and the data obtained at that time were excluded from the analysis.

Measuring points of respiratory parameters were selected at 0 h (pre-dose) and at 0.5, 1, 3, 6 and 24 h post-dose based on the general change in the concentrations of the test substance in the plasma when dosed orally. For the measurement of respiratory parameters (respiratory rate, tidal volume and minute volume), each rat was placed in an unrestrained whole body plethysmography system (Buxco Electronics, Inc., Wilmington, NC, USA). Data were collected continuously for each respiratory parameter and the average values for 10 s were obtained continuously at an interval of 10 s using the FinePointe™ program (Ver. 2.3.1.9, DSI™, IWO Scientific Corporation, Seoul, Korea).

Zero hour (0 h) (pre-dose), 24 h post-dose: The interval in which the behavior of the animal remains stable for a total of 3 min (more than 1 min in minute units). 0.5, 1, 3 and 6 h (post-dose): ± 1 min at each time point (a total of 3 min) Data obtained from unstable animals at each measurement point are excluded from the average value and if less than six data can be selected, then six data from animals stabilized close to the measurement points are selected among the data acquired before and after 3 min of each measurement point. If the number of data that can be selected for 3-min intervals before and after each measurement point is fewer than six, the average value of all the data acquired for 3 min before and after each measurement point is determined.

Statistical analysis was performed on respiratory parameters (respiratory rate, tidal volume and minute volume) using SAS Program (version 9.3, SAS Institute Inc.). Parameters were analyzed using Bartlett test for homogeneity of variance (significance level: 0.05). One-way ANOVA was employed on homogeneous data (significance level: 0.05). All parameters were not statistically significant; therefore, Dunnett's *t* test was not applied for multiple comparisons. Kruskal–Wallis test was employed on heterogeneous data (significance level: 0.05). All parameters were not statistically significant. Therefore, Steel's test was not employed for multiple comparisons.

Dogs: cardiovascular function assay

Cardiovascular function assay was performed using male, 4 beagle dogs, 14 months old, 9.20–12.22 kg. A single dose was administered to the same animal at each of four ascending dose levels (0, 250, 500, and 1000 mg/kg) in the afternoon (13:23–13:51) of each dosing day at 1-week intervals (dose volume was 5 mL/kg). The dosing formulation was administered by gastric intubation using a disposable syringe (50 mL) fitted with a catheter. Evaluation

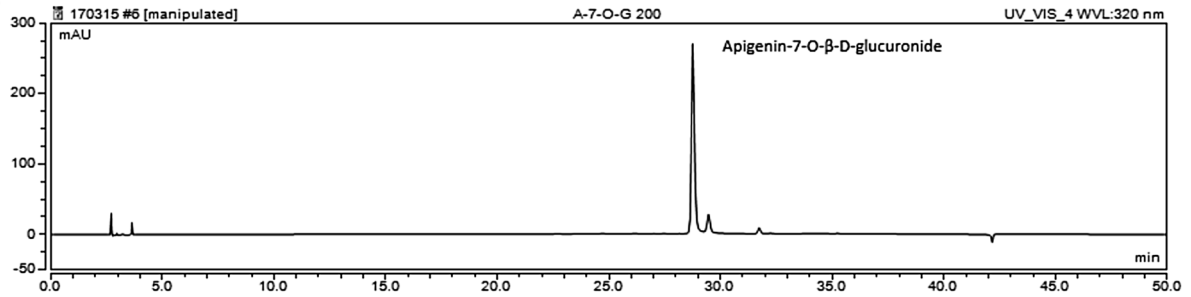
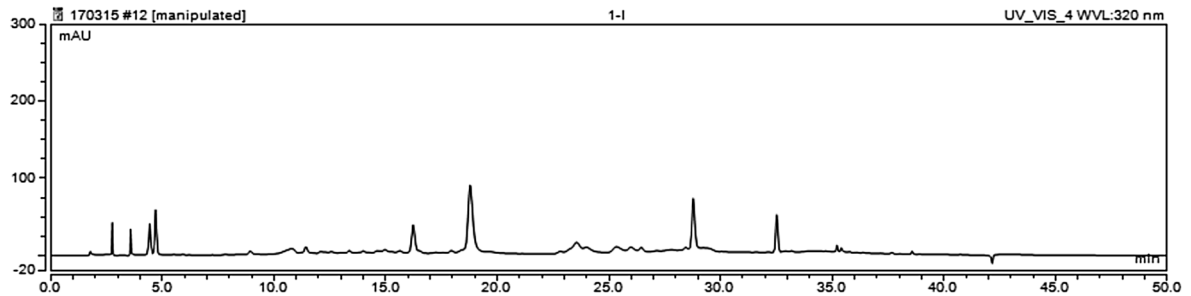
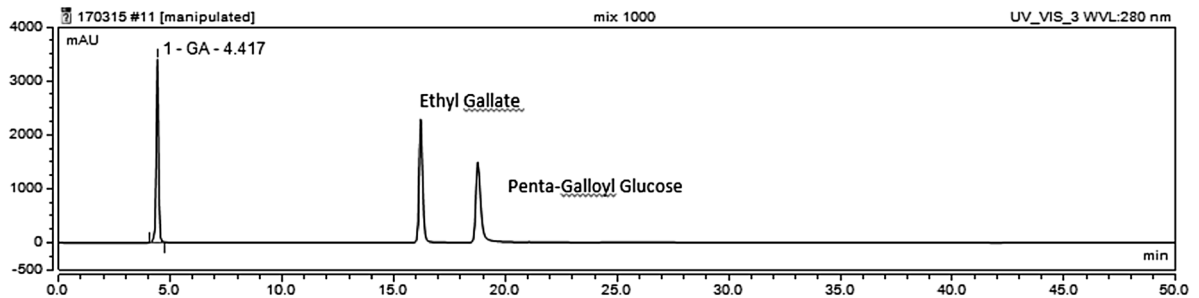
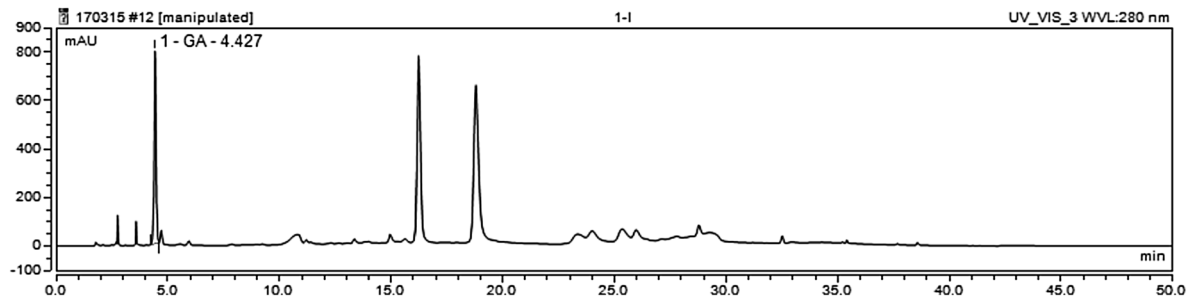
A**[Standard]****[Sample]****B****[Standard]****[Sample]**

Fig. 1 Result of HPLC analysis. **a** apigenin-7-*O*- β -glucuronide (AG) as an indicator and functional ingredient of *Agrimonia Pilosa*. **b** penta-galloyl glucose (PGG) as an indicator and functional ingredient of *Rhus gall*

of cardiovascular parameters were blood pressure, heart rate and electrocardiogram. Measuring time points were selected on the basis of the changes of the concentration

of the general drug in plasma when dosed orally at 0 h (prior to dosing), 0.5, 1, 2, 3, 4, 6, 8 and 24 h post dosing. The data of blood pressure, heart rate, body temperature

Table 1 Bacterial reversion mutation study (main study)

Metabolic activation	Dose ($\mu\text{g}/\text{plate}$)	Revertant colony/plate (Mean \pm SD)				
		Base-pair substitution type			Frameshift type	
		TA100	TA1535	WP2 $uvrA$	TA98	TA1537
S9Mix(–)	0	109 \pm 4.6	12 \pm 2.1	42 \pm 3.1	13 \pm 1.0	9 \pm 1.2
	21	105 \pm 2.1	13 \pm 2.3	41 \pm 3.2	15 \pm 3.1	8 \pm 1.0
	62	101 \pm 1.5	11 \pm 1.0	42 \pm 4.0	16 \pm 1.2	9 \pm 1.0
	185	98 \pm 1.7	12 \pm 1.5	48 \pm 3.5	16 \pm 1.2	7 \pm 1.0
	556	88 \pm 4.4	11 \pm 1.0	42 \pm 8.7	15 \pm 1.0	6 \pm 1.5
	1667	89 \pm 5.7	8 \pm 1.5	24 \pm 1.7	8 \pm 2.1	5 \pm 1.5
	5000 ^a	52 \pm 5.0 ^b	5 \pm 0.6 ^b	5 \pm 2.0 ^b	2 \pm 0.6 ^b	4 \pm 1.5 ^b
S9Mix(+)	0	102 \pm 5.0	14 \pm 1.5	47 \pm 4.0	24 \pm 3.0	12 \pm 1.5
	21	185 \pm 4.0	10 \pm 1.0	44 \pm 4.5	28 \pm 4.4	9 \pm 2.0
	62	102 \pm 7.9	11 \pm 1.5	51 \pm 1.0	25 \pm 3.5	10 \pm 1.5
	185	94 \pm 3.1	14 \pm 1.0	38 \pm 1.5	28 \pm 2.5	11 \pm 2.1
	556	86 \pm 3.5	11 \pm 0.6	37 \pm 6.1	23 \pm 1.2	10 \pm 2.5
	1667	85 \pm 3.5	7 \pm 0.6 ^b	26 \pm 2.0	17 \pm 2.3	8 \pm 2.5
	5000 ^a	84 \pm 2.1	4 \pm 0.6 ^b	10 \pm 1.5 ^b	18 \pm 2.1 ^b	5 \pm 0.6 ^b
Positive controls						
S9Mix(–)	Positive	AF-2	NaN ₃	AF-2	AF-2	9-AA
	Dose	0.01	0.5	0.01	0.1	80
	Mean \pm SD	404 \pm 22.0	447 \pm 21.9	440 \pm 41.8	448 \pm 56.0	2127 \pm 24.9
S9Mix(+)	Positive	2-AA	2-AA	2-AA	2-AA	2-AA
	Dose	1.0	2.0	10	0.5	2.0
	Mean \pm SD	455 \pm 31.4	259 \pm 19.3	393 \pm 19.9	306 \pm 39.9	225 \pm 14.3

Mean \pm SD (number of animals)^aPrecipitation of the test substance^bCytotoxicity

and ECG were obtained from 24 h before administration to 24 h after administration using a data collecting system (Dataquest A.R.T.TM Gold Acquisition Version 4.00, Data Science Int., New Brington, MN, USA) for 20 s at 5 min intervals. The data of blood pressure, heart rate and body temperature at each measuring time point of acquisition were recorded for an average of 20 s. The data obtained before the selection range of the acquisition of data for the measuring time points prior to dosing were used as reference data for the identification and assessment of signal quality.

The data of systolic and diastolic blood pressures, heart rate and body temperature were converted to Microsoft Excel[®] by using a data analysis program (Dataquest A.R.T.TM Gold Analysis Version 4.00, Data Science Int.). The data of systolic and diastolic blood pressures, heart rate and body temperature at each data analysis time point were used to determine the mean value of all data recorded at 5-min intervals in the range of selection of data as described below. The data that were difficult to interpret as signal quality problem due to entry of a person or other external environmental factors were excluded

from the data analysis. The mean blood pressure (MBP) of systolic and diastolic blood pressure data was calculated by the following formula:

$$\text{MBP} = \text{DBP} + (\text{SBP} - \text{DBP})/3$$

Measuring time points	The range of data selected
0 h (prior to dosing)	From approximately 85 min before dosing to approximately 30 min before dosing, data were recorded at 5-min intervals (12 collected data)
0.5 and 1 h post dosing	Data recorded at 5-min intervals for 25 min prior to each time point (6 collected data)
2, 3, 4, 6, 8 and 24 h post dosing	Data recorded at a 5-min intervals for 55 min prior to each time point (12 collected data)

ECG parameters (PR, QRS, QT and QTc intervals) were analyzed (Ponemah physiology platform Version 4.60, DSI/PONEMAH, Data Science Int.) at snapshot times as described below. Five consecutive ECG waveforms with stable baseline

Table 2 The number of cells with chromosome aberrations per 100 metaphase cells

Exposure ^a	S9 mix	Dose (µg/mL)	Number of total chromosome aberrations (mean)		Number of cells with chromosome aberrations (mean)		PP + ER (mean)
			(-)Gap	(+)Gap	(-)Gap	(+)Gap	
			24-0	–	Negative control	0.33	
	–	2.29	0.67	0.67	0.67	0.67	0.00
	–	6.86	0.67	0.67	0.67	0.67	0.00
	–	20.58	1.33	1.33	1.33	1.33	0.00
	–	MMC (0.04)	25.67	26.00	25.67*	26.00	0.00
6-18	–	Negative control	0.00	0.00	0.00	0.00	0.00
		6.86	0.33	0.33	0.33	0.33	0.00
		50.28	0.67	1.00	0.67	1.00	0.00
		61.73	0.67	0.67	0.67	0.67	0.00
		MMC (0.04)	23.00	23.00	23.00*	23.00	0.00
		Negative control	0.67	0.67	0.67	0.67	0.00
		6.86	0.67	0.67	0.67	0.67	0.00
		20.58	0.67	0.67	0.67	1.00	0.00
		61.73	1.00	1.00	1.00	1.00	0.00
		B[a]P (20)	23.33	23.67	23.33*	23.67	0.00

Mean ± SD (number of animals). Significantly different from the negative control at $p < 0.05$

MMC mitomycin C (0.04 µg/mL), B[a]P benzo[a]pyrene (20 µg/mL), PP polyploidy, ER endoreduplication

^aTreatment time-recovery time

recorded over 20 s were selected from each ‘snapshot’ time and then, averaged at each wave interval.

Snapshot: 0 h (prior to dosing) and at 0.5, 1, 2, 3, 4, 6, 8 and 24 h post dosing.

QTc interval was corrected by Fridericia’s formula.

$$QTc = QT / \sqrt[3]{RR}$$

When problems were evident at scheduled snapshot times, the suitable data having no problem of signal quality within the range of data selection at each time point were selected and analyzed.

Statistical analysis was performed using SAS Program (version 9.3, SAS Institute Inc.) on the data including actual measurement values of blood pressure, heart rate, body temperature and measurement values of ECG parameters (PR, QRS, QT and QTc intervals). Time-sequential repeated measures of ANOVA were conducted for the data of blood pressure, heart rate, body temperature and ECG parameters (significance level: 0.05) and since the effect of dose or the effect of interaction between dosage and time was not significant, Dunnett’s t-test was not applied for multiple comparisons between the control and test substance at each measuring time point.

Results

Extract

HPLC analysis

We performed high-pressure liquid chromatography (HPLC) analysis for the composition analysis of APRG64. As shown in Fig. 1, the presence of gallic acid (GA), ethyl gallate (EG), penta-galloyl glucose (PGG), and apigenin-7-O-β-glucuronide (AG) was confirmed. We selected AG as an indicator and functional ingredient of *A. Pilosa* and PGG as an indicator and functional ingredient of *Rhus gall*.

Genotoxicity tests

Bacterial reverse mutation test

As shown in Table 1, cytotoxicity was observed at 5000 µg/plate (TA98, TA100, TA1535, TA1537 and WP2uvrA) in the absence of metabolic activation system [S9 mix(-)]. The number of revertant colonies did not show significant increase to judge positive compared with the number of revertant colonies of negative control group. In the presence of metabolic activation system [S9 mix(+)], cytotoxicity was observed at 5000 µg/plate (TA1537 and WP2uvrA) and

Table 3 Micronuclei frequency and cytotoxicity

Groups	Dose (mg/kg)	Animal No.	MNPCE/4000 PCEs (mean \pm SD, %)	PCE/(PCE + NCE) (mean \pm SD, BW/ day)		
Negative control	Vehicle	0	1	0.25 (0.25 \pm 0.07)	0.53 (0.50 \pm 0.03)	
			2	0.28	0.47	
			3	0.30	0.50	
			4	0.30	0.53	
			5	0.13	0.49	
Test substance	APRG64	500	6	0.28 (0.29 \pm 0.11)	0.43 (0.51 \pm 0.05)	
			7	0.18	0.53	
			8	0.43	0.52	
			9	0.38	0.50	
			10	0.18	0.58	
		1000		11	0.08 (0.32 \pm 0.20)	0.54 (0.52 \pm 0.02)
				12	0.43	0.51
				13	0.15	0.49
				14	0.43	0.53
				15	0.53	0.53
		2000		16	0.35 (0.34 \pm 0.05)	0.48 (0.53 \pm 0.05)
				17	0.38	0.49
				18	0.25	0.50
				19	0.35	0.59
				20	0.38	0.58
Positive control	MMC	2.0	21	8.35 (10.43 \pm 1.56)**	0.49 (0.48 \pm 0.01)	
			22	12.68	0.48	
			23	9.85	0.49	
			24	10.63	0.46	
			25	10.65	0.47	

Vehicle distilled water

Mean \pm SD (number of animals)

MNPCE micronucleated polychromatic erythrocytes, PCE Polychromatic erythrocyte, NCE normochromatic erythrocyte, MMC mitomycin C

Significantly different from the control at ** $p < 0.01$

1667 and 5000 $\mu\text{g}/\text{plate}$ (TA1535). The number of revertant colonies did not show significant increase to judge positive compared with the number of revertant colonies of negative control group.

Mammalian chromosome aberration test

As a result of main test, the number of cells with chromosome aberrations per 100 metaphase cells was 0.33, 0.67, 0.67, 1.33, and 25.67 at negative control, 2.29, 6.86, 20.58 $\mu\text{g}/\text{mL}$ and positive control in the absence of metabolic activation system (24 h treatment group). The test substance did not cause statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Table 2). In the absence of metabolic activation system (6 h treatment and 18 h recovery group), the number of cells with chromosome aberrations per 100 metaphase cells was 0.00,

0.33, 0.67, 0.67, and 23.00 at negative control, 6.86, 20.58, 61.73 $\mu\text{g}/\text{mL}$, and positive control. The test substance did not cause statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Table 2). In the presence of metabolic activation system (6 h treatment and 18 h recovery group), the number of cells with chromosome aberrations per 100 metaphase cells was 0.67, 0.67, 0.67, 1.00, and 23.33 at negative control, 6.86, 20.58, 61.73 $\mu\text{g}/\text{mL}$, and positive control. The test substance did not cause statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Table 2). In the presence and absence of metabolic activation system, the test substance did not cause statistically significant in the number of cells with polyploidy and endoreduplication, when compared with negative control group (Table 2). However, positive controls induced statistically significant increase in the number

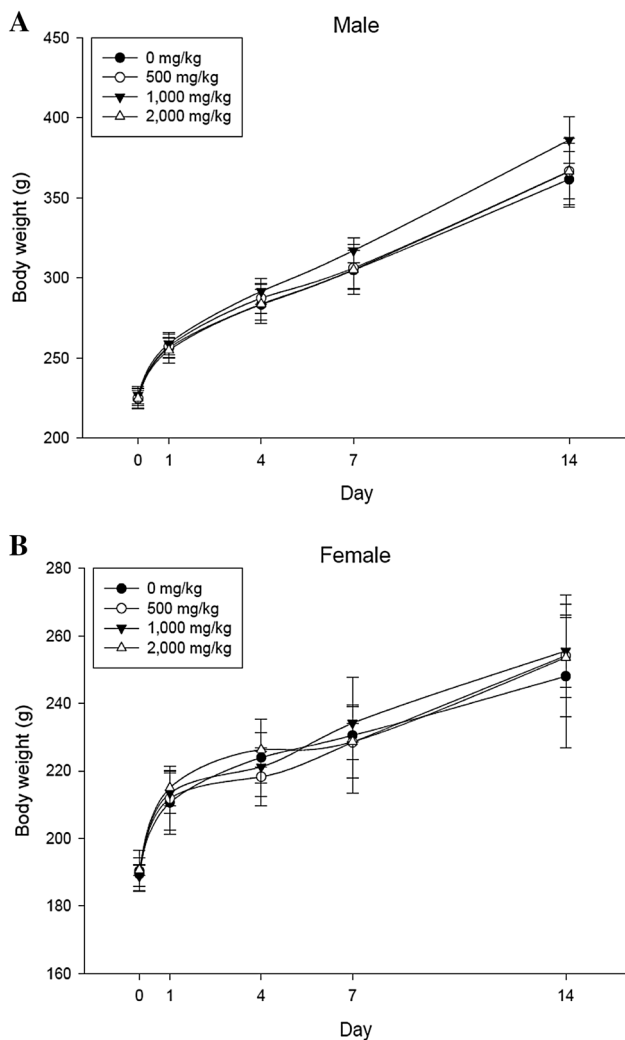


Fig. 2 Single dose toxicity study; body weights of male and female rats. Body weight changes of vehicle control and APRG treated group ($n = 5$ per each groups)

of cells with chromosome aberration when compared with negative control group ($p < 0.05$) (Table 2).

Mammalian erythrocyte micronucleus test

As a result of observation, no special abnormality was observed compared to negative control group, and there was no statistically change in body weight at any group compared to negative control group (data not shown). The results of microscopic observations in the micronucleus assay for mice groups were summarized in the Table 3. There was no statistically increase in frequency of PCEs having micronuclei in administration group compared to negative control group, and clear statistically significant increase in positive control group compared with negative control group in frequency of MNPCE ($p < 0.01$).

Oral toxicity study

Single dose toxicity study

This study was conducted to investigate toxic symptoms and approximate lethal dose (LD) by single oral administration of the test substance, APRG64. There were no mortalities during the experimental period. Some animals in the male and female 2000 mg/kg dosing group showed salivation right after treating the test substance. However, they do not have toxicological significances because it is considered that the secretion of saliva was promoted temporarily. As shown in Fig. 2, in body weight, temporary decreases were observed in female of the vehicle control and the 1000 mg/kg dosing group, respectively. However, it was shown in the vehicle control group and mild change without results that support the decrease in other experimental items. Therefore, they are not considered to be affected by the test substance. As a result of the autopsy after the end of the experiment, no unusual macroscopic findings were observed in all the experimental animals.

Rats: 4-weeks repeated dose toxicity determination study (DRF)

This study was conducted to investigate the no observed adverse effect level (NOAEL) by oral administration of the test substance APRG64 for 4 weeks and to set the dose of 13 weeks repeated oral toxicity study. There were no mortalities in all treated groups during the experimental period. Salivation was observed right after administration in some animals of the male and female 1000 mg/kg dosing groups intermittently and in all animals of the male and female 2000 mg/kg dosing groups consistently. Other unusual clinical signs were not observed (data not shown). And there were no statistically significant differences between the vehicle control and dosing groups during the experimental period, due to weight change, feed intake and water consumption, and ophthalmological examination results.

As shown in Table 4, bilirubin and urine color parameters shown significant differences among male and female groups ($p < 0.01$ or $p < 0.05$). In case of color, observation frequency of yellow or dark yellow color were higher in the male and female 1000 and 2000 mg/kg dosing groups compared with that of the vehicle control group ($p < 0.01$). This can lead to differences in metabolites by sex hormones in males and females [12] Dark yellow urine is only observed during the medication period and is probably due to urine excretion of the test substance or its metabolites. This can lead to an increase in ketone bodies due to urine pigments associated with changes in urine color [13]. In addition, female rats had significant differences among groups in parameters such as glucose, ketone body, specific gravity, occult blood

Table 4 Four-weeks repeated dose toxicity: urinalysis of male rats and female rats

Summary of urinalysis (sex: male)				
Test item	Group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Glucose				
Negative	5/5	5/5	5/5	4/5
Trace	0/5	0/5	0/5	1/5
Bilirubin*				
Negative	5/5	5/5	2/5	0/5
1+	0/5	0/5	2/5	2/5
2+	0/5	0/5	1/5	3/5
Ketone body				
Trace	3/5	3/5	2/5	0/5
1+	2/5	2/5	3/5	5/5
Specific gravity				
1.010	1/5	1/5	0/5	0/5
1.015	2/5	1/5	3/5	0/5
1.020	2/5	3/5	2/5	2/5
1.025	0/5	0/5	0/5	2/5
≥ 1.030	0/5	0/5	0/5	1/5
Occult blood				
Negative	5/5	5/5	3/5	1/5
Trace	0/5	0/5	2/5	3/5
1+	0/5	0/5	0/5	1/5
pH				
7.0	0/5	1/5	0/5	3/5
7.5	1/5	2/5	2/5	2/5
8.0	1/5	1/5	1/5	0/5
Protein				
Negative	2/5	3/5	1/5	1/5
Trace	3/5	2/5	3/5	2/5
1+	0/5	0/5	1/5	2/5
Urobilinogen				
0.2	5/5	5/5	4/5	3/5
1	0/5	0/5	1/5	2/5
Nitrite				
Negative	5/5	5/5	5/5	4/5
Positive	0/5	0/5	0/5	1/5
Leukocyte^a				
–	–	–	–	–
Color^{**}				
Straw	5/5	5/5	2/5	1/5
Yellow	0/5	0/5	2/5	0/5
Dark yellow	0/5	0/5	1/5	4/5
Summary of urinalysis (sex: female)				
Test item	Group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Glucose*				
Negative	5/5	5/5	5/5	5/5
Trace	0/5	0/5	3/5	0/5

Table 4 (continued)

Summary of urinalysis (sex: female)

Test item	Group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Bilirubin**				
Negative	5/5	5/5	0/5	1/5
1+	0/5	0/5	3/5	1/5
2+	0/5	0/5	2/5	3/5
Ketone body**				
Negative	5/5	4/5	0/5	0/5
Trace	0/5	1/5	4/5	3/5
1+	0/5	0/5	1/5	2/5
Specific gravity*				
1.010	4/5	2/5	0/5	0/5
1.015	1/5	1/5	0/5	0/5
1.020	0/5	2/5	4/5	2/5
1.025	0/5	0/5	1/5	0/5
≥ 1.030	0/5	0/5	0/5	3/5
Occult blood*				
Negative	5/5	5/5	2/5	1/5
Trace	0/5	0/5	3/5	3/5
1+	0/5	0/5	0/5	1/5
pH*				
5.0	0/5	0/5	0/5	1/5
6.5	0/5	0/5	0/5	3/5
7.0	0/5	3/5	3/5	1/5
7.5	1/5	0/5	1/5	0/5
8.0	0/5	1/5	1/5	0/5
8.5	4/5	1/5	0/5	0/5
Protein				
Negative	5/5	5/5	2/5	1/5
Trace	0/5	0/5	3/5	2/5
1+	0/5	0/5	0/5	1/5
2+	0/5	0/5	0/5	1/5
Urobilinogen				
0.2	5/5	5/5	4/5	3/5
1	0/5	0/5	1/5	2/5
2	0/5	0/5	0/5	1/5
Nitrite				
Negative	5/5	5/5	3/5	3/5
Positive	0/5	0/5	2/5	2/5
Leukocyte^a				
–	–	–	–	–
Color**				
Straw	5/5	5/5	2/5	0/5
Yellow	0/5	0/5	0/5	2/5
Dark yellow	0/5	0/5	0/5	3/5

Number of animals with the sign/number of animals examined

^aLeukocyte was examined by urine sediment observation because it could not be detected through examination using an urine stickSignificant difference among groups: * $p < 0.05$, ** $p < 0.01$

Table 5 Four-weeks repeated dose toxicity: hematological values of male rats and female rats

Summary of hematological tests				
Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
WBC ¹ (K/ μ L)	7.89 \pm 2.14 (5)	7.68 \pm 1.78 (5)	7.46 \pm 1.22 (5)	9.61 \pm 2.23 (5)
NE ² (K/ μ L)	1.20 \pm 0.20 (5)	1.07 \pm 0.54 (5)	1.14 \pm 0.25 (5)	1.52 \pm 0.78 (5)
EO ³ (K/ μ L)	0.06 \pm 0.01 (5)	0.06 \pm 0.02 (5)	0.05 \pm 0.03 (5)	0.05 \pm 0.03 (5)
BA ⁴ (K/ μ L)	0.00 \pm 0.00 (5)	0.00 \pm 0.00 (5)	0.00 \pm 0.00 (5)	0.00 \pm 0.00 (5)
LY ⁵ (K/ μ L)	6.42 \pm 1.94 (5)	6.34 \pm 1.25 (5)	6.06 \pm 1.03 (5)	7.78 \pm 1.54 (5)
MO ⁶ (K/ μ L)	0.13 \pm 0.04 (5)	0.12 \pm 0.03 (5)	0.13 \pm 0.03 (5)	0.17 \pm 0.06 (5)
LUC ⁷ (K/ μ L)	0.09 \pm 0.04 (5)	0.07 \pm 0.01 (5)	0.07 \pm 0.04 (5)	0.09 \pm 0.01 (5)
NEP ⁸ (%)	15.6 \pm 2.2 (5)	13.4 \pm 3.5 (5)	15.4 \pm 2.8 (5)	15.3 \pm 4.5 (5)
EOP ⁹ (%)	0.9 \pm 0.3 (5)	0.8 \pm 0.3 (5)	0.7 \pm 0.3 (5)	0.5 \pm 0.2 (5)
BAP ¹⁰ (%)	0.0 \pm 0.1 (5)	0.0 \pm 0.0 (5)	0.1 \pm 0.0 (5)	0.0 \pm 0.0 (5)
LYP ¹¹ (%)	80.7 \pm 2.7 (5)	83.1 \pm 2.8 (5)	81.3 \pm 2.7 (5)	81.5 \pm 4.9 (5)
MOP ¹² (%)	1.7 \pm 0.5 (5)	1.6 \pm 0.5 (5)	1.7 \pm 0.2 (5)	1.7 \pm 0.2 (5)
LUP ¹³ (%)	1.1 \pm 0.4 (5)	1.0 \pm 0.2 (5)	0.9 \pm 0.3 (5)	1.0 \pm 0.2 (5)
RBC ¹⁴ (M/ μ L)	7.71 \pm 0.35 (5)	7.52 \pm 0.30 (5)	7.29 \pm 0.35 (5)	7.52 \pm 0.42 (5)
Hb ¹⁵ (g/dL)	15.1 \pm 0.4 (5)	14.4 \pm 0.2** (5)	14.1 \pm 0.3** (5)	14.3 \pm 0.4** (5)
RDW ¹⁶ (%)	11.3 \pm 0.6 (5)	11.4 \pm 0.6 (5)	11.3 \pm 0.2 (5)	11.6 \pm 0.6 (5)
HCT ¹⁷ (%)	45.4 \pm 1.6 (5)	43.8 \pm 0.4 (5)	42.3 \pm 2.1* (5)	43.6 \pm 0.9 (5)
MCV ¹⁸ (fL)	58.9 \pm 2.4 (5)	58.4 \pm 1.9 (5)	58.0 \pm 1.3 (5)	58.0 \pm 2.9 (5)
MCH ¹⁹ (pg)	19.6 \pm 1.0 (5)	19.1 \pm 0.6 (5)	19.4 \pm 0.8 (5)	19.1 \pm 1.1 (5)
MCHC ²⁰ (g/dL)	33.2 \pm 0.7 (5)	32.8 \pm 0.4 (5)	33.5 \pm 1.2 (5)	32.9 \pm 0.6 (5)
Reti ²¹ (%)	2.88 \pm 0.46 (5)	2.96 \pm 0.30 (5)	2.71 \pm 0.33 (5)	2.84 \pm 0.65 (5)
PLT ²² (K/ μ L)	1231 \pm 66 (5)	1269 \pm 101 (5)	1134 \pm 136 (5)	1194 \pm 91 (5)
MPV ²³ (fL)	5.7 \pm 0.2 (5)	5.6 \pm 0.2 (5)	5.7 \pm 0.2 (5)	5.3 \pm 0.2** (5)
Summary of hematological tests				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
WBC ¹ (K/ μ L)	7.45 \pm 0.74 (5)	7.53 \pm 2.89 (5)	6.95 \pm 1.32 (5)	7.97 \pm 2.24 (5)
NE ² (K/ μ L)	0.65 \pm 0.10 (5)	0.89 \pm 0.53 (5)	0.82 \pm 0.36 (5)	1.14 \pm 0.72 (5)
EO ³ (K/ μ L)	0.07 \pm 0.01 (5)	0.07 \pm 0.03 (5)	0.07 \pm 0.02 (5)	0.07 \pm 0.02 (5)
BA ⁴ (K/ μ L)	0.01 \pm 0.00 (5)	0.00 \pm 0.01 (5)	0.00 \pm 0.00 (5)	0.00 \pm 0.00 (5)
LY ⁵ (K/ μ L)	6.48 \pm 0.64 (5)	6.27 \pm 2.24 (5)	5.81 \pm 1.00 (5)	6.48 \pm 1.69 (5)
MO ⁶ (K/ μ L)	0.14 \pm 0.06 (5)	0.15 \pm 0.10 (5)	0.14 \pm 0.07 (5)	0.19 \pm 0.03 (5)
LUC ⁷ (K/ μ L)	0.10 \pm 0.03 (5)	0.13 \pm 0.11 (5)	0.11 \pm 0.08 (5)	0.10 \pm 0.03 (5)
NEP ⁸ (%)	8.7 \pm 0.8 (5)	11.7 \pm 3.9 (5)	11.7 \pm 4.5 (5)	13.6 \pm 5.5 (5)
EOP ⁹ (%)	1.0 \pm 0.2 (5)	1.0 \pm 0.4 (5)	1.0 \pm 0.2 (5)	0.8 \pm 0.2 (5)
BAP ¹⁰ (%)	0.1 \pm 0.0 (5)	0.0 \pm 0.1 (5)	0.1 \pm 0.1 (5)	0.1 \pm 0.1 (5)
LYP ¹¹ (%)	87.0 \pm 1.1 (5)	83.7 \pm 4.2 (5)	83.9 \pm 5.2 (5)	81.8 \pm 5.0 (5)
MOP ¹² (%)	1.8 \pm 0.6 (5)	1.9 \pm 0.5 (5)	1.9 \pm 0.6 (5)	2.4 \pm 0.5 (5)
LUP ¹³ (%)	1.3 \pm 0.4 (5)	1.6 \pm 0.7 (5)	1.5 \pm 0.8 (5)	1.3 \pm 0.4 (5)
RBC ¹⁴ (M/ μ L)	7.74 \pm 0.25 (5)	7.74 \pm 0.33 (5)	7.61 \pm 0.30 (5)	7.51 \pm 0.23 (5)
Hb ¹⁵ (g/dL)	14.8 \pm 0.6 (5)	14.6 \pm 0.5 (5)	14.3 \pm 0.6 (5)	14.1 \pm 0.2 (5)
RDW ¹⁶ (%)	10.7 \pm 0.6 (5)	10.4 \pm 0.2 (5)	10.4 \pm 0.4 (5)	10.7 \pm 0.1 (5)
HCT ¹⁷ (%)	43.9 \pm 1.3 (5)	43.1 \pm 1.4 (5)	42.8 \pm 1.7 (5)	41.6 \pm 0.7 (5)
MCV ¹⁸ (fL)	56.7 \pm 0.3 (5)	55.7 \pm 0.9 (5)	56.2 \pm 0.3 (5)	55.4 \pm 1.0 (5)
MCH ¹⁹ (pg)	19.1 \pm 0.4 (5)	18.9 \pm 0.6 (5)	18.8 \pm 0.1 (5)	18.7 \pm 0.4 (5)
MCHC ²⁰ (g/dL)	33.7 \pm 0.7 (5)	33.9 \pm 0.7 (5)	33.5 \pm 0.3 (5)	33.8 \pm 0.3 (5)

Table 5 (continued)

Summary of hematological tests				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Reti ²¹ (%)	2.18 ± 0.28 (5)	1.88 ± 0.30 (5)	2.04 ± 0.40 (5)	2.28 ± 0.41 (5)
PLT ²² (K/ μ L)	1078 ± 123 (5)	1092 ± 95 (5)	1159 ± 121 (5)	1247 ± 65 (5)
MPV ²³ (fL)	5.7 ± 0.6 (5)	5.5 ± 0.5 (5)	5.4 ± 0.5 (5)	5.7 ± 0.6 (5)

Mean ± SD (number of animals)

1: white blood cell, 2: neutrophil, 3: eosinophil, 4: basophil, 5: lymphocyte, 6: monocyte, 7: large unstained cell, 8: percent of neutrophil, 9: percent of eosinophil, 10: percent of basophil, 11: percent of lymphocyte, 12: percent of monocyte, 13: percent of large unstained cell, 14: red blood cell, 15: hemoglobin, 16: red cell distribution width, 17: hematocrit, 18: mean corpuscular volume, 19: mean corpuscular hemoglobin, 20: mean corpuscular hemoglobin concentration, 21: reticulocyte, 22: platelet, 23: mean platelet volume

Significant difference compared with control group value: * $p < 0.05$, ** $p < 0.01$

and pH ($p < 0.01$ or $p < 0.05$) (Table 4). Especially specific gravity showed a tendency to increase with dose–response correlation, while pH showed a tendency to decrease with dose–response correlation. Bilirubin may produce false positive reactions due to the interference of some compounds or metabolites [14]. Except that, there were no statistically significant differences among groups in other inspection items. Also, urine sediment test on the leukocyte showed that there were no statistically significant differences among groups.

In Table 5, male rats hematological test revealed statistically significantly lower values when compared to the that of vehicle control group in the following parameters: Hb (hemoglobin) value in the male 500, 1000, and 2000 mg/kg dosing group, HCT (hematocrit) value in the male 1000 mg/kg dosing group, and MPV (mean platelet volume) value in the male 2000 mg/kg dosing group (HCT; $p < 0.05$, Hb, MPV; $p < 0.01$). Except that, there were no statistically significant differences among vehicle control and dosing groups in other inspection items.

Also, in male groups (Table 6), statistically significant increases were detected on Cl (chloride) value in the 1000 and 2000 mg/kg dosing groups and ALT (alanine aminotransferase) value in the 2000 mg/kg dosing groups when compared with that of vehicle control group (ALT; $p < 0.05$, Cl; $p < 0.01$). On the other hand, significant decreases were found on BUN (blood urea nitrogen) value in the 500, 1000, and 2000 mg/kg dosing groups, Na (sodium) value in the 1000 and 2000 mg/kg dosing groups, and GLU (glucose) and PRO (protein) values in the 2000 mg/kg dosing group (GLU, PRO; $p < 0.05$, Na, BUN; $p < 0.01$). Other statistically significant differences were not observed between the vehicle control and dosing groups in other inspection items.

At scheduled necropsy, there were no abnormal gross findings in all animals. Also, there were no statistically

significant differences among vehicle control and dosing groups in absolute and relative organ weights.

Rats: 13-weeks repeated dose toxicity and 4-weeks recovery study

There were no mortalities during treatment and recovery period. Salivation was observed right after administration of the test substance in the male and female 1000 and 2000 mg/kg/day dosing groups throughout treatment period. No abnormal clinical signs were detected in other animals and experimental period (including recovery period).

In the body weight change, there were no statistically significant differences between vehicle control and dosing groups during exposure and recovery periods.

Food consumption in the female 1000 and 2000 mg/kg/day dosing group statistically significantly increased on week 5 compared to the that of vehicle control group ($p < 0.05$, data not shown). Except for that, there were no statistically significant differences between vehicle control and dosing groups during exposure and recovery periods. Also, there were no abnormalities detected in all animals of main and recovery groups subjected to the ophthalmological examination.

The urinalysis results (Table 7) as follows:

Main groups In urinalysis, detection frequency of ketone body shown significant differences among female groups, which the change had increase tendency with dose–response relationship ($p < 0.01$, Table 7). Although there was no significant difference, increase tendency of ketone body was also found in male groups. In urine color, significant difference was observed among female groups showing dark yellow, dark brown, and dark green color urine in dosing groups ($p < 0.01$). Dark yellow and dark brown color urine were detected in male dosing groups

Table 6 Four-weeks repeated dose toxicity: serum biochemical values of male rats and female rats

Summary of serum biochemical tests				
Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
AST ¹ (IU/L)	112 ± 17 (5)	112 ± 9 (5)	114 ± 19 (5)	117 ± 19 (5)
ALT ² (IU/L)	40 ± 4 (5)	40 ± 4 (5)	39 ± 5 (5)	48 ± 6* (5)
GGT ³ (IU/L)	0.60 ± 0.55 (5)	0.80 ± 0.45 (5)	0.60 ± 0.55 (5)	1.00 ± 0.00 (5)
ALP ⁴ (IU/L)	456 ± 83 (5)	386 ± 66 (5)	439 ± 66 (5)	521 ± 53 (5)
BIL ⁵ (mg/dL)	0.04 ± 0.02 (5)	0.04 ± 0.01 (5)	0.02 ± 0.01 (5)	0.02 ± 0.02 (5)
BUN ⁶ (mg/dL)	16.5 ± 2.4 (5)	12.9 ± 2.1** (5)	12.1 ± 0.6** (5)	13.6 ± 1.4** (5)
CRE ⁷ (mg/dL)	0.46 ± 0.07 (5)	0.43 ± 0.01 (5)	0.42 ± 0.03 (5)	0.43 ± 0.05 (5)
UA ⁸ (mg/dL)	1.0 ± 0.2 (5)	1.0 ± 0.1 (5)	1.0 ± 0.1 (5)	0.8 ± 0.1 (5)
GLU ⁹ (g/dL)	164 ± 13 (5)	154 ± 23 (5)	149 ± 17 (5)	132 ± 8* (5)
CHO ¹⁰ (mg/dL)	72 ± 12 (5)	67 ± 12 (5)	69 ± 11 (5)	60 ± 8 (5)
TG ¹¹ (mg/dL)	44 ± 22 (5)	50 ± 22 (5)	63 ± 25 (5)	43 ± 8 (5)
PRO ¹² (g/dL)	6.0 ± 0.3 (5)	5.9 ± 0.0 (5)	5.8 ± 0.2 (5)	5.7 ± 0.1* (5)
ALB ¹³ (g/dL)	2.4 ± 0.1 (5)	2.3 ± 0.1 (5)	2.4 ± 0.1 (5)	2.3 ± 0.0 (5)
A/G ratio ¹⁴	0.66 ± 0.06 (5)	0.65 ± 0.02 (5)	0.71 ± 0.03 (5)	0.69 ± 0.03 (5)
LDH ¹⁵ (IU/L)	808 ± 516 (5)	841 ± 326 (5)	743 ± 363 (5)	684 ± 327 (5)
CPK ¹⁶ (U/L)	618 ± 312 (5)	588 ± 197 (5)	502 ± 168 (5)	492 ± 178 (5)
Ca ¹⁷ (mg/dL)	9.7 ± 0.4 (5)	9.8 ± 0.2 (5)	9.4 ± 0.3 (5)	9.6 ± 0.2 (5)
IP ¹⁸ (mg/dL)	8.0 ± 0.5 (5)	7.8 ± 0.2 (5)	7.6 ± 0.2 (5)	8.2 ± 0.2 (5)
Mg ¹⁹ (mg/dL)	2.3 ± 0.1 (5)	2.3 ± 0.1 (5)	2.3 ± 0.2 (5)	2.5 ± 0.2 (5)
Na ²⁰ (mmol/L)	148 ± 1 (5)	149 ± 4 (5)	141 ± 5** (5)	141 ± 3** (5)
K ²¹ (mmol/L)	4.8 ± 0.3 (5)	4.9 ± 0.2 (5)	4.5 ± 0.2 (5)	4.8 ± 0.2 (5)
Cl ²² (mmol/L)	100 ± 1 (5)	99 ± 3 (5)	108 ± 4** (5)	108 ± 2** (5)
Summary of serum biochemical tests				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
AST ¹ (IU/L)	98 ± 12 (5)	115 ± 23 (5)	108 ± 11 (5)	108 ± 19 (5)
ALT ² (IU/L)	26 ± 3 (5)	28 ± 4 (5)	33 ± 10 (5)	32 ± 5 (5)
GGT ³ (IU/L)	1.00 ± 0.00 (5)	1.00 ± 0.00 (5)	0.80 ± 0.45 (5)	1.00 ± 0.00 (5)
ALP ⁴ (IU/L)	237 ± 73 (5)	256 ± 61 (5)	261 ± 94 (5)	257 ± 68 (5)
BIL ⁵ (mg/dL)	0.07 ± 0.01 (5)	0.08 ± 0.02 (5)	0.07 ± 0.01 (5)	0.05 ± 0.03 (5)
BUN ⁶ (mg/dL)	18.4 ± 2.4 (5)	16.7 ± 3.3 (5)	14.0 ± 2.2 (5)	17.9 ± 3.2 (5)
CRE ⁷ (mg/dL)	0.46 ± 0.03 (5)	0.46 ± 0.07 (5)	0.46 ± 0.03 (5)	0.48 ± 0.05 (5)
UA ⁸ (mg/dL)	0.8 ± 0.2 (5)	1.1 ± 0.2 (5)	1.1 ± 0.2 (5)	1.0 ± 0.2 (5)
GLU ⁹ (mg/dL)	138 ± 12 (5)	133 ± 12 (5)	135 ± 14 (5)	128 ± 10 (5)
CHO ¹⁰ (mg/dL)	82 ± 7 (5)	66 ± 15 (5)	69 ± 22 (5)	72 ± 11 (5)
TG ¹¹ (mg/dL)	33 ± 9 (5)	18 ± 6 (5)	25 ± 12 (5)	25 ± 5 (5)
PRO ¹² (g/dL)	6.0 ± 0.2 (5)	6.3 ± 0.4 (5)	6.5 ± 0.2 (5)	6.4 ± 0.3 (5)
ALB ¹³ (g/dL)	2.6 ± 0.1 (5)	2.7 ± 0.2 (5)	2.9 ± 0.2 (5)	2.8 ± 0.2 (5)
A/G ratio ¹⁴	0.76 ± 0.06 (5)	0.75 ± 0.04 (5)	0.78 ± 0.05 (5)	0.79 ± 0.06 (5)
LDH ¹⁵ (IU/L)	615 ± 273 (5)	674 ± 462 (5)	535 ± 357 (5)	551 ± 361 (5)
CPK ¹⁶ (U/L)	436 ± 113 (5)	459 ± 289 (5)	351 ± 130 (5)	405 ± 232 (5)
Ca ¹⁷ (mg/dL)	9.8 ± 0.1 (5)	9.6 ± 0.3 (5)	9.8 ± 0.3 (5)	9.7 ± 0.2 (5)
IP ¹⁸ (mg/dL)	7.6 ± 0.6 (5)	7.4 ± 0.6 (5)	7.4 ± 0.8 (5)	7.2 ± 0.8 (5)
Mg ¹⁹ (mg/dL)	2.4 ± 0.1 (5)	2.5 ± 0.1 (5)	2.5 ± 0.2 (5)	2.4 ± 0.1 (5)
Na ²⁰ (mmol/L)	145 ± 5 (5)	143 ± 6 (5)	140 ± 2 (5)	145 ± 7 (5)
K ²¹ (mmol/L)	4.6 ± 0.2 (5)	4.5 ± 0.5 (5)	4.2 ± 0.2 (5)	4.4 ± 0.4 (5)

Table 6 (continued)

Summary of serum biochemical tests				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Cl ²² (mmol/L)	102 ± 4 (5)	102 ± 8 (5)	108 ± 5 (5)	100 ± 6 (5)

1: aspartate aminotransferase, 2: alanine aminotransferase, 3: gamma(γ)-glutamyl transferase, 4: alkaline phosphatase, 5: total bilirubin, 6: blood urea nitrogen, 7: creatinine, 8: uric acid, 9: glucose, 10: total cholesterol, 11: triglyceride, 12: total protein, 13: albumin, 14: albumin/globulin ratio, 15: lactate dehydrogenase, 16: creatine phosphokinase, 17: calcium, 18: inorganic phosphorus, 19: magnesium, 20: sodium, 21: potassium, 22: chloride

Mean \pm SD (number of animals)

Significant difference compared with control group value: * $p < 0.05$, ** $p < 0.01$

(but no significant difference). Some metabolites are more significant difference in females than males because sex hormones may affect metabolic processes [12]. The occurrence of dark yellow urine is only observed during the sample treatment period, which may be due to urine excretion of the test substance or its metabolites [13]. In addition, female had significant differences among groups in parameters such as occult blood ($p < 0.01$) and nitrite ($p < 0.05$). But, it concluded that trace changes occur or there is no dose-related correlation. Except that, no statistically significant differences were observed among all the male and female groups, regarding urinalysis, urine sediment test, and urine volume.

Recovery groups In urinalysis, urine sediment test, and urine volume, there were no statistically significant differences among groups.

The hematology results (Table 8) as follows:

Main groups Significant increases compared to the control group value were observed in following values; the LY (lymphocyte) value in the female 1000 mg/kg/day dosing group and the WBC (white blood cell), LY and MCV (mean corpuscular volume) value in the female 2000 mg/kg/day dosing group ($p < 0.05$). No other statistically significant differences were observed between vehicle control and dosing groups.

Recovery groups The EO (eosinophil) value in the male 2000 mg/kg/day dosing group was significantly lower than that in the vehicle control group ($p < 0.05$, data not shown). No other statistically significant differences were observed between vehicle control and dosing groups. In blood coagulation test, there were no statistically significant differences between vehicle control and dosing groups in main and recovery groups.

The serum biochemistry results (Table 9) as follows.

Main groups The Na (sodium) value in the female 500, 1000, and 2000 mg/kg/day dosing group was significantly lower when compared to that of vehicle control group ($p < 0.01$). There were no statistically significant differences between vehicle control and dosing groups in other inspection items.

Recovery groups Data not shown, the Cl (chloride) value was statistically significantly decreased in the male 2000 mg/kg/day group compared with that of vehicle control group ($p < 0.05$) and Mg (magnesium) value was significantly increased in the female 2000 mg/kg/day group ($p < 0.01$). No other statistically significant differences were observed in other inspection items.

And at necropsy, there were no abnormal gross findings in all animals in main and recovery groups.

In organ weights results (Table 10) as follows:

Main groups In absolute organ weights, right ovary of the female 1000 mg/kg/day dosing group was significantly heavier than that of vehicle control group ($p < 0.05$). No statistically significant differences in the absolute and relative organ weights were observed between vehicle control and treated groups in both the males and females.

Recovery groups In absolute organ weights, significant decreases were observed in right ovary and lung weight of the female 2000 mg/kg/day group ($p < 0.05$, data not shown). No statistically significant differences were observed in other organ weights.

The histopathological examination results as follows:

Lung Histopathological examination for lung in the vehicle control and 2000 mg/kg/day main and recovery groups of both sexes revealed as follows: focal/multifocal, inflammatory, cell infiltration, bronchiolalveolar was sporadically found in 1–4 animals of each main group, and 0–2 animals of each recovery group. In addition, focal foreign body with alveolar macrophage was confirmed in alveolar of one ani-

Table 7 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: urinalysis of male rats (main study) and urinalysis of female rats (main study)

Summary of urinalysis (sex: male)				
Test item	Main group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Glucose				
Negative	5/5	3/5	5/5	2/5
Trace	0/5	2/5	0/5	2/5
1+	0/5	0/5	0/5	1/5
Bilirubin				
Negative	5/5	4/5	2/5	2/5
1+	0/5	1/5	1/5	0/5
2+	0/5	0/5	2/5	1/5
3+	0/5	0/5	0/5	2/5
Ketone body				
Negative	3/5	1/5	0/5	0/5
Trace	1/5	3/5	1/5	1/5
1+	1/5	1/5	4/5	4/5
Specific gravity				
≤1.005	1/5	2/5	0/5	0/5
1.010	4/5	3/5	3/5	4/5
1.020	0/5	0/5	2/5	0/5
1.025	0/5	0/5	0/5	1/5
Occult blood**				
Negative	2/5	4/5	3/5	2/5
Trace	3/5	1/5	2/5	2/5
2+	0/5	0/5	0/5	1/5
pH				
6.5	0/5	0/5	0/5	1/5
7.0	0/5	0/5	1/5	1/5
7.5	1/5	1/5	0/5	0/5
8.0	0/5	0/5	2/5	0/5
8.5	4/5	4/5	2/5	3/5
Protein				
Negative	3/5	2/5	3/5	2/5
Trace	2/5	2/5	0/5	0/5
1+	0/5	1/5	2/5	2/5
2+	0/5	0/5	0/5	1/5
Urobilinogen				
0.2	5/5	5/5	5/5	3/5
1.0	0/5	0/5	0/5	2/5
Nitrite				
Negative	5/5	5/5	4/5	3/5
Positive	0/5	0/5	1/5	2/5
Leukocyte				
Negative	5/5	5/5	5/5	5/5
Color				
Straw	5/5	3/5	2/5	5/5
Yellow	0/5	2/5	0/5	0/5
Dark yellow	0/5	0/5	2/5	0/5
Dark brown	0/5	0/5	1/5	0/5

Table 7 (continued)

Summary of urinalysis (sex: female)

Test item	Main group (mg/kg/day)			
	G1(0)	G2 (500)	G3 (1000)	G4 (2000)
Glucose				
Negative	5/5	3/5	5/5	4/5
Trace	0/5	2/5	0/5	1/5
Bilirubin				
Negative	5/5	3/5	5/5	1/5
1+	0/5	1/5	0/5	2/5
2+	0/5	1/5	0/5	2/5
Ketone body**				
Negative	5/5	1/5	0/5	0/5
Trace	0/5	4/5	4/5	3/5
1+	0/5	0/5	1/5	2/5
Specific gravity				
1.010	1/5	1/5	0/5	0/5
1.015	2/5	2/5	0/5	1/5
1.020	0/5	0/5	1/5	1/5
1.025	0/5	0/5	1/5	1/5
Occult blood**				
Negative	5/5	4/5	5/5	1/5
Trace	0/5	1/5	0/5	4/5
pH				
6.5	0/5	0/5	3/5	0/5
7.0	0/5	1/5	0/5	2/5
7.5	1/5	1/5	2/5	2/5
8.0	1/5	1/5	0/5	1/5
8.5	3/5	2/5	0/5	0/5
Protein				
Negative	5/5	3/5	4/5	1/5
Trace	0/5	1/5	0/5	1/5
1+	0/5	1/5	1/5	3/5
Urobilinogen				
0.2	5/5	4/5	5/5	4/5
1.0	0/5	1/5	0/5	1/5
Nitrite*				
Negative	5/5	2/5	5/5	2/5
Positive	0/5	3/5	0/5	3/5
Leukocyte				
Negative	5/5	5/5	5/5	3/5
Trace	0/5	0/5	0/5	2/5
Color**				
Colorless	3/5	0/5	0/5	0/5
Straw	2/5	1/5	5/5	1/5
Yellow	0/5	3/5	0/5	0/5
Dark yellow	0/5	1/5	0/5	0/5
Dark brown	0/5	0/5	0/5	3/5

Number of animals with the sign/number of animals examined. Significant difference among groups: * $p < 0.05$, ** $p < 0.01$

Table 8 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: hematological values of male rats (main study) and hematological values of female rats (mains study)

Summary of hematological tests (sex: male)				
Test item	Main group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
WBC ¹ (K/ μ L)	6.66 ± 1.46 (10)	8.57 ± 1.57 (10)	8.53 ± 3.35 (10)	9.33 ± 2.89 (10)
NE ² (K/ μ L)	0.96 ± 0.28 (10)	1.25 ± 0.55 (10)	1.22 ± 0.48 (10)	1.33 ± 0.53 (10)
EO ³ (K/ μ L)	0.06 ± 0.03 (10)	0.10 ± 0.07 (10)	0.09 ± 0.04 (10)	0.08 ± 0.04 (10)
BA ⁴ (K/ μ L)	0.01 ± 0.01 (10)	0.00 ± 0.01 (10)	0.01 ± 0.01 (10)	0.01 ± 0.01 (10)
LY ⁵ (K/ μ L)	5.37 ± 1.29 (10)	6.95 ± 1.47 (10)	6.91 ± 2.93 (10)	7.62 ± 2.30 (10)
MO ⁶ (K/ μ L)	0.14 ± 0.07 (10)	0.18 ± 0.06 (10)	0.17 ± 0.08 (10)	0.17 ± 0.08 (10)
LUC ⁷ (K/ μ L)	0.12 ± 0.08 (10)	0.10 ± 0.03 (10)	0.12 ± 0.08 (10)	0.13 ± 0.07 (10)
NEP ⁸ (%)	14.8 ± 3.9 (10)	14.8 ± 6.1 (10)	15.0 ± 5.2 (10)	14.0 ± 3.0 (10)
EOP ⁹ (%)	1.0 ± 0.5 (10)	1.2 ± 0.8 (10)	1.1 ± 0.4 (10)	0.8 ± 0.3 (10)
BAP ¹⁰ (%)	0.1 ± 0.1 (10)	0.1 ± 0.1 (10)	0.1 ± 0.1 (10)	0.0 ± 0.0 (10)
LYP ¹¹ (%)	80.4 ± 5.0 (10)	80.8 ± 7.2 (10)	80.5 ± 5.2 (10)	82.1 ± 3.7 (10)
MOP ¹² (%)	2.1 ± 0.9 (10)	2.1 ± 0.7 (10)	1.9 ± 0.6 (10)	1.7 ± 0.5 (10)
LUP ¹³ (%)	1.8 ± 0.9 (10)	1.1 ± 0.2 (10)	1.4 ± 0.4 (10)	1.3 ± 0.4 (10)
RBC ¹⁴ (M/ μ L)	7.94 ± 0.28 (10)	7.92 ± 0.35 (10)	7.68 ± 0.55 (10)	7.93 ± 0.34 (10)
Hb ¹⁵ (g/dL)	14.3 ± 0.6 (10)	14.6 ± 0.9 (10)	14.4 ± 0.4 (10)	14.4 ± 0.5 (10)
RDW ¹⁶ (%)	12.8 ± 1.0 (10)	12.7 ± 0.8 (10)	12.7 ± 0.8 (10)	12.5 ± 0.9 (10)
HCT ¹⁷ (%)	44.3 ± 1.9 (10)	44.8 ± 2.7 (10)	43.8 ± 2.4 (10)	44.7 ± 1.4 (10)
MCV ¹⁸ (fL)	55.8 ± 1.5 (10)	56.5 ± 2.1 (10)	57.1 ± 2.7 (10)	56.4 ± 1.4 (10)
MCH ¹⁹ (pg)	18.0 ± 0.5 (10)	18.4 ± 0.8 (10)	18.8 ± 1.2 (10)	18.2 ± 0.4 (10)
MCHC ²⁰ (g/dL)	32.4 ± 0.5 (10)	32.6 ± 1.0 (10)	32.9 ± 1.4 (10)	32.3 ± 0.5 (10)
Retic ²¹ (%)	2.21 ± 0.45 (10)	2.36 ± 0.37 (10)	2.42 ± 0.74 (10)	2.13 ± 0.53 (10)
PLT ²² (K/ μ L)	901 ± 86 (10)	892 ± 150 (10)	927 ± 121 (10)	957 ± 150 (10)
MPV ²³ (fL)	5.0 ± 0.2 (10)	4.9 ± 0.2 (10)	5.0 ± 0.3 (10)	5.1 ± 0.2 (10)

Summary of hematological tests (sex: female)

Test item	Group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
WBC ¹ (K/ μ L)	3.99 ± 0.49 (10)	4.32 ± 1.15 (10)	5.04 ± 1.33 (10)	5.36 ± 1.06* (10)
NE ² (K/ μ L)	0.74 ± 0.15 (10)	0.87 ± 0.60 (10)	0.67 ± 0.21 (10)	0.95 ± 0.34 (10)
EO ³ (K/ μ L)	0.06 ± 0.01 (10)	0.06 ± 0.03 (10)	0.07 ± 0.03 (10)	0.08 ± 0.02 (10)
BA ⁴ (K/ μ L)	0.01 ± 0.01 (10)	0.01 ± 0.01 (10)	0.01 ± 0.01 (10)	0.00 ± 0.01 (10)
LY ⁵ (K/ μ L)	3.08 ± 0.57 (10)	3.24 ± 0.85 (10)	4.13 ± 1.23* (10)	4.17 ± 0.82* (10)
MO ⁶ (K/ μ L)	0.10 ± 0.04 (10)	0.12 ± 0.05 (10)	0.14 ± 0.06 (10)	0.14 ± 0.07 (10)
LUC ⁷ (K/ μ L)	0.02 ± 0.01 (10)	0.02 ± 0.01 (10)	0.02 ± 0.02 (10)	0.02 ± 0.01 (10)
NEP ⁸ (%)	18.9 ± 4.8 (10)	19.6 ± 8.8 (10)	13.8 ± 4.9 (10)	17.6 ± 4.8 (10)
EOP ⁹ (%)	1.4 ± 0.3 (10)	1.5 ± 0.4 (10)	1.5 ± 0.5 (10)	1.4 ± 0.4 (10)
BAP ¹⁰ (%)	0.1 ± 0.1 (10)	0.2 ± 0.1 (10)	0.2 ± 0.2 (10)	0.1 ± 0.1 (10)
LYP ¹¹ (%)	76.7 ± 5.5 (10)	75.7 ± 9.0 (10)	81.4 ± 5.7 (10)	78.0 ± 5.3 (10)
MOP ¹² (%)	2.4 ± 0.9 (10)	2.6 ± 0.7 (10)	2.7 ± 1.2 (10)	2.6 ± 1.2 (10)
LUP ¹³ (%)	0.4 ± 0.1 (10)	0.5 ± 0.3 (10)	0.4 ± 0.2 (10)	0.3 ± 0.2 (10)
RBC ¹⁴ (M/ μ L)	7.70 ± 0.24 (10)	7.58 ± 0.31 (10)	7.61 ± 0.27 (10)	7.41 ± 0.42 (10)
Hb ¹⁵ (g/dL)	14.5 ± 0.4 (10)	14.6 ± 0.5 (10)	14.6 ± 0.3 (10)	14.4 ± 0.7 (10)
RDW ¹⁶ (%)	11.0 ± 0.2 (10)	11.1 ± 0.3 (10)	10.9 ± 0.3 (10)	10.9 ± 0.5 (10)
HCT ¹⁷ (%)	43.6 ± 1.5 (10)	43.2 ± 1.4 (10)	43.3 ± 1.3 (10)	43.0 ± 2.1 (10)
MCV ¹⁸ (fL)	56.6 ± 1.0 (10)	57.0 ± 1.0 (10)	56.8 ± 1.3 (10)	58.1 ± 1.3* (10)
MCH ¹⁹ (pg)	18.9 ± 0.6 (10)	19.3 ± 0.5 (10)	19.2 ± 0.5 (10)	19.4 ± 0.5 (10)

Table 8 (continued)

Summary of hematological tests (sex: female)				
Test item	Group (mg/kg/day)			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
MCHC ²⁰ (g/dL)	33.6±0.6 (10)	33.9±0.6 (10)	33.7±0.7 (10)	33.5±0.6 (10)
Retic ²¹ (%)	1.78±0.35 (10)	1.95±0.30 (10)	1.89±0.42 (10)	1.98±0.50 (10)
PLT ²² (K/μL)	1048±131 (10)	1007±72 (10)	1076±130 (10)	1034±74 (10)
MPV ²³ (fL)	5.1±0.2 (10)	5.0±0.2 (10)	5.1±0.3 (10)	4.9±0.2 (10)

1: white blood cell, 2: neutrophil, 3: eosinophil, 4: basophil, 5: lymphocyte, 6: monocyte, 7: large unstained cell, 8: percent of neutrophil, 9: percent of eosinophil, 10: percent of basophil, 11: percent of lymphocyte, 12: percent of monocyte, 13: percent of large unstained cell, 14: red blood cell, 15: hemoglobin, 16: red cell distribution width, 17: hematocrit, 18: mean corpuscular volume, 19: mean corpuscular hemoglobin, 20: mean corpuscular hemoglobin concentration, 21: reticulocyte, 22: platelet, 23: mean platelet volume

Mean ± SD (Number of animals)

Significant difference compared with the control group value: * $p < 0.05$

mal (animal ID G4-88) in the female 2000 mg/kg/day main group.

Other organs and tissues Histopathological examination for the organs and tissues from the vehicle control and 2000 mg/kg/day groups were conducted. At the results, following lesions were observed solitarily in main groups; focal inflammation in kidney cortex, developmental cyst in pituitary, and unilateral focal atrophy in seminiferous. In recovery groups, multifocal vacuolization in cytoplasmic of liver and cardiomyopathy in heart were solitarily found.

Beagle dogs: 4-weeks repeated dose toxicity determination study (DRF)

This study was conducted to investigate the toxicity of the test substance APRG64 when repeatedly orally administered to non-rodent Beagle dog for 4 weeks, and to establish a capacity setting basis for the subsequent repeat toxicity test. There was no mortality during the study period. No clinical signs related to the administration of test substance were observed. Except for that, anorexia and vomiting were sporadically observed without sex and dose-dependency. As a result of body weight measurement, the decrease of body weight gain was observed only in female 500 and 1000 mg/kg/day administration group. However, this is considered to be a change not within the normal weight range and not related to the administration of the test substance. There were no food consumptions changes related to the administration of test substance were observed. And no abnormal signs related to the administration of test substance were observed in ophthalmoscopy.

The urinalysis results that there were no significant changes related to the administration of test substance

compared with the control group and before administration (data not shown).

In the hematology results (Table 11), increasing tendency of LYP (percent of lymphocyte) values was observed in males at dosing group compared with before administration. Decreasing tendency of retic (reticulocyte) values was observed in females at dosing group compared with before administration. Except for that, there were no significant changes in other items.

And serum biochemical results (Table 12) that increasing tendency of K (potassium) values in males, GLU (glucose) and A/G ratio (albumin/globulin ratio) values in females were observed at dosing group compared with before administration. Decreasing tendency of CHO (total cholesterol) values in males and Ca (calcium) in females was observed at dosing group compared with before administration. Except for that, there were no significant changes in other items.

When necropsy, in male at 1000 mg/kg/day group, small testis (bilateral), epididymis (bilateral) and prostate were observed (data not shown).

In male at 1000 mg/kg/day group, decrease tendency in absolute and relative weights of testis, epididymis and prostate were observed. In females, increasing tendency in absolute and relative weights of thyroid (bilateral) at dosing group were observed compared with control group. Except for that, there were no significant changes in other inspection organs. In histopathological examination, in male at 1000 mg/kg/day group, small testis (bilateral), epididymis (bilateral) and prostate were considered to immature lesion (data not shown).

Beagle dogs: 13-weeks repeated dose toxicity and 4-weeks recovery study

The test substance, APRG64, was orally administered to non-rodent beagle dog for 13 weeks, and the toxicity of the

Table 9 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: serum biochemical values of male rats (main study) and serum biochemical values of female rats (main study)

Summary of serum biochemical tests (sex: male)

Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
AST ¹ (IU/L)	115 ± 32 (10)	123 ± 28 (10)	121 ± 19 (10)	130 ± 30 (10)
ALT ² (IU/L)	053 ± 11 (10)	60 ± 11 (10)	58 ± 5 (10)	60 ± 12 (10)
GGT ³ (IU/L)	0.0 ± 0.0 (10)	0.0 ± 0.0 (10)	0.2 ± 0.4 (10)	0.1 ± 0.3 (10)
ALP ⁴ (IU/L)	315 ± 63 (10)	338 ± 93 (10)	375 ± 85 (10)	347 ± 111 (10)
BIL ⁵ (mg/dL)	0.06 ± 0.04 (10)	0.04 ± 0.03 (10)	0.06 ± 0.04 (10)	0.05 ± 0.03 (10)
BUN ⁶ (mg/dL)	14.5 ± 1.8 (10)	14.4 ± 2.0 (10)	15.3 ± 2.8 (10)	15.2 ± 1.8 (10)
CRE ⁷ (mg/dL)	0.45 ± 0.03 (10)	0.44 ± 0.02 (10)	0.45 ± 0.04 (10)	0.47 ± 0.09 (10)
UA ⁸ (mg/dL)	0.8 ± 0.4 (10)	0.9 ± 0.4 (10)	1.0 ± 0.4 (10)	1.2 ± 0.4 (10)
GLU ⁹ (mg/dL)	215 ± 60 (10)	235 ± 40 (10)	220 ± 58 (10)	218 ± 45 (10)
CHO ¹⁰ (mg/dL)	83 ± 14 (10)	73 ± 9 (10)	72 ± 15 (10)	73 ± 15 (10)
TG ¹¹ (mg/dL)	85 ± 32 (10)	93 ± 47 (10)	92 ± 38 (10)	112 ± 79 (10)
PRO ¹² (g/dL)	6.2 ± 0.4 (10)	6.0 ± 0.2 (10)	6.1 ± 0.2 (10)	6.1 ± 0.2 (10)
ALB ¹³ (g/dL)	2.3 ± 0.1 (10)	2.3 ± 0.1 (10)	2.3 ± 0.1 (10)	2.4 ± 0.1 (10)
A/G ratio ¹⁴	0.60 ± 0.03 (10)	0.64 ± 0.04 (10)	0.63 ± 0.06 (10)	0.64 ± 0.04 (10)
LDH ¹⁵ (IU/L)	719 ± 532 (10)	761 ± 499 (10)	799 ± 639 (10)	877 ± 642 (10)
CPK ¹⁶ (U/L)	376 ± 220 (10)	400 ± 213 (10)	444 ± 296 (10)	460 ± 269 (10)
Ca ¹⁷ (mg/dL)	9.1 ± 0.3 (10)	9.0 ± 0.2 (10)	9.0 ± 0.3 (10)	9.0 ± 0.2 (10)
IP ¹⁸ (mg/dL)	6.8 ± 0.9 (10)	6.7 ± 0.4 (10)	6.5 ± 0.6 (10)	6.5 ± 0.6 (10)
Mg ¹⁹ (mg/dL)	2.1 ± 0.1 (10)	2.2 ± 0.1 (10)	2.2 ± 0.2 (10)	2.2 ± 0.2 (10)
Na ²⁰ (mmol/L)	138 ± 2 (10)	137 ± 3 (10)	139 ± 4 (10)	140 ± 3 (10)
K ²¹ (mmol/L)	4.5 ± 0.4 (10)	4.6 ± 0.4 (10)	4.4 ± 0.5 (10)	4.5 ± 0.5 (10)
Cl ²² (mmol/L)	101 ± 1 (10)	103 ± 2 (10)	103 ± 3 (10)	104 ± 2 (10)

Summary of serum biochemical tests (sex: female)

Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
AST ¹ (IU/L)	104 ± 30 (10)	105 ± 29 (10)	102 ± 19 (10)	138 ± 140 (10)
ALT ² (IU/L)	30 ± 7 (10)	31 ± 14 (10)	30 ± 5 (10)	57 ± 84 (10)
GGT ³ (IU/L)	0.8 ± 0.4 (10)	0.9 ± 0.3 (10)	0.9 ± 0.3 (10)	0.8 ± 0.4 (10)
ALP ⁴ (IU/L)	111 ± 29 (10)	115 ± 47 (10)	118 ± 44 (10)	121 ± 34 (10)
BIL ⁵ (mg/dL)	0.09 ± 0.04 (10)	0.08 ± 0.03 (10)	0.09 ± 0.03 (10)	0.08 ± 0.06 (10)
BUN ⁶ (mg/dL)	16.1 ± 2.0 (10)	16.9 ± 2.8 (10)	17.6 ± 5.0 (10)	17.0 ± 3.6 (10)
CRE ⁷ (mg/dL)	0.56 ± 0.07 (10)	0.56 ± 0.06 (10)	0.61 ± 0.11 (10)	0.54 ± 0.06 (10)
UA ⁸ (mg/dL)	1.0 ± 0.3 (10)	1.1 ± 0.2 (10)	1.2 ± 0.3 (10)	1.0 ± 0.2 (10)
GLU ⁹ (mg/dL)	140 ± 11 (10)	141 ± 14 (10)	136 ± 10 (10)	136 ± 14 (10)
CHO ¹⁰ (mg/dL)	85 ± 15 (10)	82 ± 10 (10)	77 ± 14 (10)	80 ± 15 (10)
TG ¹¹ (mg/dL)	28 ± 9 (10)	36 ± 17 (10)	28 ± 10 (10)	39 ± 29 (10)
PRO ¹² (g/dL)	7.3 ± 0.3 (10)	7.2 ± 0.4 (10)	7.1 ± 0.3 (10)	7.2 ± 0.5 (10)
ALB ¹³ (g/dL)	3.1 ± 0.2 (10)	3.1 ± 0.2 (10)	3.0 ± 0.2 (10)	3.1 ± 0.3 (10)
A/G ratio ¹⁴	0.75 ± 0.03 (10)	0.75 ± 0.04 (10)	0.72 ± 0.06 (10)	0.75 ± 0.06 (10)
LDH ¹⁵ (IU/L)	784 ± 406 (10)	775 ± 397 (10)	776 ± 350 (10)	522 ± 300 (10)
CPK ¹⁶ (U/L)	370 ± 176 (10)	376 ± 178 (10)	370 ± 125 (10)	274 ± 157 (10)
Ca ¹⁷ (mg/dL)	9.6 ± 0.2 (10)	9.6 ± 0.4 (10)	9.5 ± 0.2 (10)	9.7 ± 0.3 (10)
IP ¹⁸ (mg/dL)	6.3 ± 0.9 (10)	6.4 ± 0.5 (10)	5.8 ± 0.3 (10)	6.2 ± 0.7 (10)
Mg ¹⁹ (mg/dL)	2.3 ± 0.1 (10)	2.2 ± 0.1 (10)	2.3 ± 0.2 (10)	2.2 ± 0.1 (10)
Na ²⁰ (mmol/L)	141 ± 4 (10)	138 ± 3** (10)	135 ± 2** (10)	137 ± 2** (10)

Table 9 (continued)

Summary of serum biochemical tests (sex: female)				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
K ²¹ (mmol/L)	4.0 ± 0.2 (10)	4.1 ± 0.3 (10)	4.0 ± 0.3 (10)	4.0 ± 0.2 (10)
Cl ²² (mmol/L)	101 ± 3 (10)	101 ± 2 (10)	103 ± 2 (10)	102 ± 3 (10)

1: aspartate aminotransferase, 2: alanine aminotransferase, 3: gamma(γ)-glutamyl transferase, 4: alkaline phosphatase, 5: total bilirubin, 6: blood urea nitrogen, 7: creatinine, 8: uric acid, 9: glucose, 10: total cholesterol, 11: triglyceride, 12: total protein, 13: albumin, 14: albumin/globulin ratio, 15: lactate dehydrogenase, 16: creatine phosphokinase, 17: calcium, 18: inorganic phosphorus, 19: magnesium, 20: sodium, 21: potassium, 22: chloride

Mean ± SD (number of animals)

Significant difference compared with the control group value: ** $p < 0.01$

substance was investigated and the recovery was evaluated by 4 weeks of the recovery period. There was no mortality during the study period. Salivation was observed in 250 mg/kg/day treated male, 500 and 1000 mg/kg/day treated male and female groups. In addition, compound-colored feces were observed in 500 and 1,000 mg/kg/day treated male and female dogs, and anorexia was observed in male and female dogs treated with 250, 500, and 1000 mg/kg/day. As a result, there was a tendency to decrease feed consumption in the dosing group, and a tendency to lose weight and decrease weight gain was observed. And no abnormal signs related to the administration of test substance were observed in ophthalmoscopy. No changes related to the administration of the test substance were observed as a result of ophthalmological examination, electrocardiogram, and hematological test.

The urinalysis results (Table 13) as follows:

Main groups There was a statistically significant increase ($p < 0.05$) in ketone body and protein in the dosing group males. Urinary proteins are generally found in trace amounts in normal condition, and are known to be more prominent in rats and dogs. Males are known to be more affected than females, and many reports have shown similar trends [15–21].

Recovery groups No changes associated with the administration of the test substance were observed. A significant increase ($p < 0.05$) was observed in the occult blood of the female dosing group compared to the vehicle control group. However, changes not observed in the main groups are not considered to be related to the transfer of test substances.

In the hematology results (Table 14),

Main groups In the blood test at the 4th week of administration, there was a significant decrease in MCH (Mean

corpuscular hemoglobin) levels and female LYP (Percent of lymphocyte) values in males treated with 1000 mg/kg/day as compared with the vehicle control group ($p < 0.05$). NEP (percent of neutrophil) and NE (Neutrophil) levels of females treated with 1000 mg/kg/day were significantly increased compared to the vehicle control group ($p < 0.05$). At the 13th week of administration, there was a significant decrease in male HGB (hemoglobin, $p < 0.05$), HCT (hematocrit, $p < 0.05$), MCV (Mean corpuscular volume, $p < 0.01$), MCH ($p < 0.01$) and females MCHC (Mean corpuscular hemoglobin concentration, $p < 0.01$) levels of compared to the vehicle control group at 1000 mg/kg/day. Also, Retic (% reticulocyte) values of male 500 and 1000 mg/kg/day treated group were significantly increased compared with the vehicle control group ($p < 0.01$).

Recovery groups No abnormal signs related to the administration of test substance were observed in hematology test.

The serum biochemical test results (Table 15) were as follows,

Main groups At the 13th week of administration, a significant increase was observed in male AST (Aspartate aminotransferase) and ALT (Alanine aminotransferase) treated with 1000 mg/kg/day compared to the vehicle control group ($p < 0.01$). And, AST and ALT in female group of 1000 mg/kg/day showed a tendency to increase compared to the vehicle control group. Therefore, histopathological examination was implemented in both females and males to confirm the above results. Except, a significant increase was observed in CRE (creatinine) from the male 250 mg/kg/day group at the 13th week of administration compared to the vehicle control group. However, it was concluded that there was no dose-related correlation.

As a result of blood coagulation time test. (Table 16),

Table 10 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: absolute organ weights of male rats (main study) and absolute organ weights of female rats (main study)

Summary of absolute organ weights				
Organs	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Body weight ^a	535.73 ± 84.83 (10)	535.22 ± 62.92 (10)	490.29 ± 63.21 (10)	489.69 ± 49.94 (10)
Testis (Lt)	1.7813 ± 0.1841 (10)	1.7714 ± 0.2152 (10)	1.7468 ± 0.2339 (10)	1.8673 ± 0.1526 (10)
Testis (Rt)	1.7802 ± 0.2017 (10)	1.7890 ± 0.2088 (10)	1.7431 ± 0.2133 (10)	1.8763 ± 0.1677 (10)
Prostate	0.7915 ± 0.1978 (10)	0.9267 ± 0.1106 (10)	0.8057 ± 0.2603 (10)	0.7445 ± 0.1951 (10)
Spleen	0.8705 ± 0.1257 (10)	0.8918 ± 0.1397 (10)	0.8390 ± 0.1635 (10)	0.8091 ± 0.1094 (10)
Liver	16.0900 ± 2.7647 (10)	17.0546 ± 3.3324 (10)	15.3102 ± 2.3546 (10)	16.0392 ± 1.9907 (10)
Adrenal gland (Lt)	0.0288 ± 0.0042 (10)	0.0300 ± 0.0074 (10)	0.0256 ± 0.0035 (10)	0.0268 ± 0.0049 (10)
Adrenal gland (Rt.)	0.0284 ± 0.0043 (10)	0.0300 ± 0.0051 (10)	0.0260 ± 0.0042 (10)	0.0260 ± 0.0036 (10)
Kidney (Lt.)	1.6634 ± 0.1897 (10)	1.6672 ± 0.1708 (10)	1.5449 ± 0.1997 (10)	1.5439 ± 0.1833 (10)
Kidney (Rt.)	1.7031 ± 0.1927 (10)	1.7161 ± 0.1781 (10)	1.5681 ± 0.1632 (10)	1.5819 ± 0.2258 (10)
Heart	1.6654 ± 0.2586 (10)	1.7142 ± 0.2137 (10)	1.4698 ± 0.1986 (10)	1.5341 ± 0.1801 (10)
Lung	1.7255 ± 0.2003 (10)	1.7596 ± 0.1421 (10)	1.7341 ± 0.2641 (10)	1.7563 ± 0.1444 (10)
Brain	2.1120 ± 0.1244 (10)	2.1616 ± 0.1015 (10)	2.1626 ± 0.1531 (10)	2.1508 ± 0.0684 (10)
Pituitary	0.0115 ± 0.0024 (10)	0.0138 ± 0.0025 (10)	0.0117 ± 0.0026 (10)	0.0124 ± 0.0016 (10)
Thymus	0.3541 ± 0.0959 (10)	0.3052 ± 0.1068 (10)	0.3259 ± 0.1225 (10)	0.2721 ± 0.0641 (10)
Summary of absolute organ weights				
Organs	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (500)	G3 (1000)	G4 (2000)
Body weight ^a	318.86 ± 26.31 (10)	319.01 ± 28.27 (10)	333.19 ± 25.50 (10)	302.30 ± 30.84 (10)
Testis (Lt)	0.0484 ± 0.0125 (10)	0.0488 ± 0.0103 (10)	0.0538 ± 0.0108 (10)	0.0424 ± 0.0104 (10)
Testis (Rt)	0.0421 ± 0.0132 (10)	0.0483 ± 0.0114 (10)	0.0553 ± 0.0076* (10)	0.0437 ± 0.0097 (10)
Prostate	0.6989 ± 0.2967 (10)	0.7401 ± 0.2401 (10)	0.7946 ± 0.3375 (10)	0.6031 ± 0.3655 (10)
Spleen	0.5631 ± 0.0859 (10)	0.5741 ± 0.0538 (10)	0.6382 ± 0.1002 (10)	0.5517 ± 0.1132 (10)
Liver	7.8426 ± 0.8494 (10)	8.0393 ± 0.8365 (10)	8.0891 ± 0.5265 (10)	7.9703 ± 0.8543 (10)
Adrenal gland (Lt)	0.0385 ± 0.0053 (10)	0.0358 ± 0.0068 (10)	0.0387 ± 0.0062 (10)	0.0340 ± 0.0073 (10)
Adrenal gland (Rt.)	0.0391 ± 0.0057 (10)	0.0347 ± 0.0059 (10)	0.0366 ± 0.0081 (10)	0.0351 ± 0.0061 (10)
Kidney (Lt.)	1.0138 ± 0.1077 (10)	1.0473 ± 0.0658 (10)	1.0376 ± 0.0840 (10)	1.0042 ± 0.0722 (10)
Kidney (Rt.)	1.0285 ± 0.1296 (10)	1.0688 ± 0.0952 (10)	1.0497 ± 0.0866 (10)	1.0336 ± 0.0936 (10)
Heart	1.0887 ± 0.0804 (10)	1.0921 ± 0.1448 (10)	1.2278 ± 0.2875 (10)	1.0952 ± 0.0668 (10)
Lung	1.3680 ± 0.1085 (10)	1.3501 ± 0.0796 (10)	1.4233 ± 0.1236 (10)	1.4048 ± 0.1979 (10)
Brain	1.9743 ± 0.0425 (10)	1.9943 ± 0.1132 (10)	2.0501 ± 0.1255 (10)	1.9846 ± 0.0912 (10)
Pituitary	0.0206 ± 0.0034 (10)	0.0184 ± 0.0046 (10)	0.0167 ± 0.0048 (10)	0.0180 ± 0.0042 (10)
Thymus	0.2648 ± 0.0499 (10)	0.2939 ± 0.0636 (10)	0.2872 ± 0.0687 (10)	0.2813 ± 0.0537 (10)

Mean ± SD (Number of animals)

Lt. left, Rt. right

^aAt necropsySignificant difference compared with the control group value: * $p < 0.05$

Table 11 Four-weeks repeated dose toxicity: Hematological values of male dogs and Hematological values of female dogs

Individual data of hematological test								
Animal ID	Group (mg/kg/day) sex: male							
	G1 (0)		G2 (250)		G3 (500)		G4 (1000)	
	256-1		256-2		256-3		256-4	
Test item	Before	After	Before	After	Before	After	Before	After
WBC ¹ (K/ μ L)	7.83	6.33	10.09	7.91	9.57	7.04	13.69	13.12
RBC ² (M/ μ L)	6.81	7.48	6.3	7.5	6.38	7.57	6.24	6.5
HGB ³ (g/dL)	15.6	17.6	13.9	16.4	13.4	16.2	14.5	14.6
HCT ⁴ (%)	48.4	52	42.4	49.4	41.8	49	42.5	44.4
MCV ⁵ (fL)	71.2	69.6	67.4	65.9	65.5	64.8	68	68.2
MCH ⁶ (pg)	22.9	23.5	22.1	21.9	21	21.4	23.2	22.4
MCHC ⁷ (g/dL)	32.1	33.8	32.8	33.2	32.1	33.1	34.1	32.9
RDW ⁸ (%)	12.6	12.9	12.9	13.3	13.6	13.5	12.8	12.8
PLT ⁹ (K/ μ L)	214	189	368	292	298	219	296	346
MPV ¹⁰ (fL)	11.5	11.9	7.9	8.8	9.4	9.6	9.3	9.6
NEP ¹¹ (%)	43.8	49.7	48	43.7	47.1	48.2	54.9	54
LYP ¹² (%)	42.4	40.4	37	45.4	37	39.2	34.2	36.5
MOP ¹³ (%)	7.1	5.2	7.5	6	8.7	7.1	7.6	6.4
EOP ¹⁴ (%)	5.8	3.5	6.1	2.9	6.4	4	2.8	1.8
LUP ¹⁵ (%)	0.5	0.2	0.7	0.8	0.5	0.2	0.3	0.3
BAP ¹⁶ (%)	0.4	1	0.7	1.2	0.3	1.2	0.1	1
LYM ¹⁷ (K/ μ L)	3.32	2.55	3.73	3.59	3.54	2.76	4.68	4.79
MO ¹⁸ (K/ μ L)	0.56	0.33	0.76	0.48	0.83	0.5	1.04	0.84
EO ¹⁹ (K/ μ L)	0.46	0.22	0.62	0.23	0.62	0.28	0.39	0.24
LUC ²⁰ (K/ μ L)	0.04	0.01	0.07	0.07	0.05	0.02	0.04	0.04
Retic ²¹ (K/ μ L)	2.02	1.72	1.45	1.46	1.48	1.24	1.9	1.93

Individual data of hematological test								
Animal ID	Group (mg/kg/day) sex: female							
	G1 (0)		G2 (250)		G3 (500)		G4 (1000)	
	256-5		256-6		256-7		256-8	
Test item	Before	After	Before	After	Before	After	Before	After
WBC ¹ (K/ μ L)	13.93	9.58	11.79	11.21	7.93	9.23	10.24	7.94
RBC ² (M/ μ L)	7.12	7.64	8	7.92	7.31	8.04	7.51	7.79
HGB ³ (g/dL)	16	17.4	17.8	18.3	16.6	18.3	15.2	16.2
HCT ⁴ (%)	49.6	52.6	53.5	53.1	50.9	54.2	48	48.6
MCV ⁵ (fL)	69.7	68.9	66.9	67	69.6	67.4	64	62.4
MCH ⁶ (pg)	22.5	22.8	22.3	23.1	22.7	22.8	20.2	20.8
MCHC ⁷ (g/dL)	32.3	33.2	33.3	34.5	32.5	33.8	31.7	33.4
RDW ⁸ (%)	13.2	13	13.9	12.6	11.8	11.5	15.2	14.2
PLT ⁹ (K/ μ L)	405	310	284	267	258	281	358	293
MPV ¹⁰ (fL)	7.8	8.5	8.6	9.3	8.9	9.4	8.9	8.6
NEP ¹¹ (%)	41.5	46.5	38.3	41.6	54.7	55.8	51.6	51.9
LYP ¹² (%)	44.6	46.5	53.8	51.2	35.8	35.1	38.7	41.3
MOP ¹³ (%)	7.1	4.8	4.5	4.6	6.9	5.7	5.3	4.3
EOP ¹⁴ (%)	6.1	1.4	2.5	1.7	1.8	2	3.4	1.8
LUP ¹⁵ (%)	0.5	0.4	0.4	0.4	0.4	0.2	0.7	0.3
BAP ¹⁶ (%)	0.3	0.4	0.4	0.4	0.3	1.1	0.3	0.4

Table 11 (continued)

Individual data of hematological test								
Animal ID	Group (mg/kg/day) sex: female							
	G1 (0)		G2 (250)		G3 (500)		G4 (1000)	
	256-5		256-6		256-7		256-8	
Test item	Before	After	Before	After	Before	After	Before	After
NE ¹⁷ (K/ μ L)	5.77	4.46	4.52	4.67	4.34	5.15	5.28	4.12
LYM ¹⁸ (K/ μ L)	6.21	4.45	6.34	5.74	2.84	3.24	3.97	3.28
MO ¹⁹ (K/ μ L)	0.99	0.46	0.53	0.52	0.55	0.53	0.54	0.34
EO ²⁰ (K/ μ L)	0.85	0.13	0.29	0.19	0.14	0.18	0.35	0.14
LUC ²¹ (K/ μ L)	0.06	0.04	0.05	0.05	0.03	0.02	0.07	0.02
BA ²² (K/ μ L)	0.04	0.04	0.05	0.05	0.02	0.1	0.03	0.03
Retic ²³ (K/ μ L)	2.85	2.13	2.44	1.94	2.13	0.97	2.51	1.57

1: white blood cell, 2: red blood cell, 3: hemoglobin, 4: hematocrit 5: mean corpuscular volume, 6: mean corpuscular hemoglobin, 7: mean corpuscular hemoglobin concentration, 8: red cell distribution width, 9: platelet, 10: mean platelet volume, 11: percent of neutrophils, 12: percent of lymphocyte 13: percent of monocyte; 14: percent of eosinophil, 15: percent of large unstained cell, 16: percent of basophil, 17 neutrophils, 18: lymphocyte, 19: monocyte, 20: eosinophil, 21: large unstained cell, 22: basophil, 23: reticulocyte

Main groups APTT (active partial thromboplastin time) was significantly increased in 1000 mg/kg/day of male and female compared to vehicle control ($p < 0.01$).

Recovery groups APTT increase tendency was observed in male and female 1000 mg/kg/day.

And at necropsy, black-gray colored mottled was observed in the duodenum of the male 1000 mg/kg/day group and gray colored degeneration was observed in the duodenum of the female 1000 mg/kg/day group, respectively one. These changes are estimated to be due to the test substance. However, there was no toxicological significance because the degree was mild and there was no continuous change in the recovery group.

In absolute organ weights, no statistically significant changes were observed in either the main groups or recovery groups.

Finally, histopathological examination was as follows,

Main groups In both sex animals of 1000 mg/kg/day groups, we observed the central lobular ballooning degeneration in the liver (three male and one female, respectively). Also, in male 500 mg/kg/day group, we observed the liver central lobular rarefaction (one of the three).

Recovery groups In the liver of 1000 mg/kg/day group, the central lobular ballooning degeneration was observed in two male and two female. Thus, NOAEL in beagle dog of the test substance is judged to be 500 mg/kg/day for both male and female, and the target organ is judged to be liver.

Systemic safety assays

Central nervous system assay

This study evaluated the effects on the central nervous system by performing a single oral administration of the test substance to male SD rats at 6 weeks of age and performing functional observational battery. No statistically significant or biologically relevant differences were noted in any parameter in animals at 250 and 500 mg/kg when compared to the control group at any measuring point. Therefore, it is considered that there was no effect of the test substance on the central nervous system. A statistically significant increase in the number of urine pools was noted in animals at 1000 mg/kg when compared to the control group at 1 h post-dose. However, it is considered not to be a test substance-related effect because there was no biologically relevant difference in comparison to the measured values prior to dosing (0 h) and no significant differences in the related parameters such as the number of fecal pellets. No statistically significant or biologically relevant differences were noted in the other parameters when compared to the control group.

In Home Cage Observations (Table 17), no statistically significant or biologically relevant differences in body posture, behavior or ease of removal from cage were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group prior to dosing (0 h) and at 0.5, 1, 3, 6 and 24 h post-dose.

In Open Field Observations (Table 18), no statistically significant or biologically relevant differences in the number

Table 12 Four-weeks repeated dose toxicity: serum biochemical values of male dogs and serum biochemical values of female dogs

Individual data of serum biochemical test

Animal Id	Group (mg/kg/day) Sex: Male							
	G1 (0)		G2 (250)		G3 (500)		G4 (1000)	
	256-1		256-2		256-3		256-4	
	Before	After	Before	After	Before	After	Before	After
ALB ¹ (g/dL)	2.7	3.0	2.4	2.5	2.5	2.6	2.6	2.6
ALP ² (IU/L)	399	347	740	598	219	183	450	320
CA ³ (mg/dL)	10.9	10.9	10.8	10.7	11.1	10.9	10.8	10.2
CHO ⁴ (mg/dL)	176	178	211	192	213	211	191	166
CRE ⁵ (mg/dL)	0.65	0.81	0.64	0.84	0.69	0.83	0.74	0.81
GLU ⁶ (mg/dL)	91	109	105	125	99	112	104	109
AST ⁷ (IU/L)	32	32	38	36	30	31	34	43
ALT ⁸ (IU/L)	22	27	24	32	29	33	20	26
IP ⁹ (mg/dL)	7.2	6.5	7.7	7.0	6.9	6.9	7.2	6.6
TP ¹⁰ (g/dL)	5.6	5.9	5.3	5.4	5.5	5.3	5.8	5.5
BUN ¹¹ (mg/dL)	14.5	12.3	10.1	12.2	15.9	13.1	14.8	12.3
T-BIL ¹² (mg/dL)	0	0.01	0.02	0.01	0	0.02	0.01	0
CPK ¹³ (U/L)	248	243	280	264	274	289	394	305
Na ¹⁴ (mmol/L)	151	152	151	147	150	152	152	152
K ¹⁵ (mmol/L)	5.4	5.2	5.3	5.5	5.2	5.5	5.4	5.7
Cl ¹⁶ (mmol/L)	103	107	107	114	103	104	104	107
A/G ratio ¹⁷	0.93	1.03	0.83	0.86	0.83	0.96	0.81	0.90

Individual data of serum biochemical test

Animal ID	Group (mg/kg/day)							
	G1 (0)		G2 (250)		G3 (500)		G4 (1000)	
	256-5		256-6		256-7		256-8	
	Before	After	Before	After	Before	After	Before	After
ALB ¹ (g/dL)	2.6	2.7	2.7	2.9	2.6	2.8	2.7	2.9
ALP ² (IU/L)	506	434	443	453	259	297	355	243
CA ³ (mg/dL)	10.7	10.7	11.2	10.5	11.1	11.0	10.9	10.7
CHO ⁴ (mg/dL)	151	154	162	190	150	148	194	167
CRE ⁵ (mg/dL)	0.74	0.89	0.63	0.70	0.69	0.78	0.65	0.75
GLU ⁶ (mg/dL)	100	108	86	105	90	104	100	125
AST ⁷ (IU/L)	45	46	38	41	32	29	43	64
ALT ⁸ (IU/L)	27	34	31	37	23	24	26	29
IP ⁹ (mg/dL)	7.2	6.9	7.8	6.0	7.0	6.0	7.6	6.5
TP ¹⁰ (g/dL)	5.6	5.6	5.7	5.9	5.5	5.8	5.6	5.7
BUN ¹¹ (mg/dL)	14.0	14.0	15.5	15.9	12.6	11.4	10.7	11.4
T-BIL ¹² (mg/dL)	0	0.04	0.03	0.02	0.02	0.01	0.02	0.04
TG ¹³ (mg/dL)	53	37	37	48	31	30	46	34
CPK ¹⁴ (U/L)	534	474	270	285	275	212	345	447
Na ¹⁵ (mmol/L)	150	153	151	156	152	153	150	149
K ¹⁶ (mmol/L)	5.8	5.4	5.7	5.3	5.5	5.3	5.6	5.1
Cl ¹⁷ (mmol/L)	103	105	103	102	105	107	105	109
A/G ratio ¹⁸	0.87	0.93	0.90	0.97	0.90	0.93	0.93	1.04

1: albumin, 2 alkaline phosphatase, 3: calcium, 4: total cholesterol; 5: creatinine, 6: glucose, 7: aspartate aminotransferase, 8: alanine aminotransferase, 9: inorganic phosphorus, 10: total protein, 11: blood urea nitrogen, 12: total bilirubin, 13: triglyceride, 14: creatine phosphokinase, 15: sodium, 16: potassium, 17: chloride, 18: albumin/globulin ratio

Table 13 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: Urinalysis of male dogs (main study) and urinalysis of female dogs (Main study)

Summary of urinalysis (sex: male)				
Test item	Main group (mg/kg/day)			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
Glucose				
Negative	5/5	3/3	3/3	5/5
Bilirubin				
Negative	1/5	1/3	1/3	0/5
1+	3/5	1/3	2/3	3/5
2+	1/5	1/3	0/3	2/5
Ketone body*				
Negative	4/5	2/3	0/3	1/5
Trace	1/5	2/3	1/3	4/5
1+	0/5	1/3	2/3	0/5
Specific gravity				
1.010	2/5	2/3	0/3	1/5
1.015	0/5	0/3	0/3	2/5
1.020	1/5	0/3	0/3	0/5
1.025	0/5	0/3	1/3	2/5
1.030	2/5	1/3	2/3	0/5
Occult blood				
Negative	3/5	2/3	3/3	2/5
Trace	0/5	0/3	0/3	3/5
1+	1/5	0/3	0/3	0/5
2+	1/5	1/3	0/3	0/5
pH				
5.5	2/5	1/3	2/3	1/5
6.0	2/5	1/3	2/3	0/5
6.5	0/5	0/3	1/3	1/5
7.0	1/5	0/3	0/3	2/5
8.0	0/5	1/3	0/3	1/5
8.5	2/5	1/3	0/3	0/5
Protein*				
Negative	4/5	1/3	3/3	1/5
Trace	0/5	0/3	0/3	4/5
1+	1/5	2/3	0/3	0/5
Urobilinogen				
0.2	0/5	0/3	0/3	0/5
Nitrite				
Negative	3/5	2/3	2/3	3/5
Positive	2/5	1/3	1/3	2/5
Leukocyte				
Negative	2/5	1/3	2/3	0/5
Trace	0/5	0/3	1/3	2/5
1+	2/5	1/3	0/3	2/5
2+	0/5	0/3	0/3	1/5
3+	1/5	1/3	0/3	0/5
Color				
Straw	5/5	3/3	3/3	5/5

Table 13 (continued)

Summary of urinalysis (sex: female)

Test item	Main group (mg/kg/day)			
	G1(0)	G2 (250)	G3 (500)	G4 (1000)
Glucose				
Negative	5/5	3/3	3/3	5/5
Bilirubin				
Negative	5/5	2/3	3/3	3/5
1+	0/5	1/3	0/3	2/5
Ketone body				
Negative	3/5	0/3	1/3	2/5
Trace	0/5	2/3	1/3	1/5
1+	2/5	1/3	1/3	2/5
Specific gravity				
≤ 1.005	1/5	0/3	0/3	2/5
1.010	3/5	1/3	1/3	0/5
1.015	0/5	0/3	1/3	3/5
1.020	1/5	1/3	0/3	0/5
1.030	0/5	1/3	1/3	0/5
Occult blood				
Negative	2/5	1/3	3/3	0/5
Trace	1/5	1/3	0/3	4/5
1+	0/5	0/3	0/3	1/5
2+	1/5	0/3	0/3	0/5
3+	1/5	1/3	0/3	0/5
pH				
6.0	0/5	1/3	0/3	0/5
6.5	0/5	0/3	1/3	0/5
7.0	0/5	1/3	1/3	2/5
7.5	2/5	1/3	0/3	2/5
8.0	1/5	0/3	0/3	0/5
8.5	2/5	0/3	1/3	1/5
Protein				
Negative	4/5	3/3	2/3	5/5
Trace	1/5	0/3	1/3	0/5
Urobilinogen				
0.2	5/5	3/3	3/3	5/5
Nitrite				
Negative	2/5	2/3	1/3	3/5
Positive	3/5	1/3	2/3	2/5
Leukocyte				
Negative	5/5	2/3	3/3	5/5
2+	0/5	1/3	0/3	0/5
Color				
Straw	5/5	3/3	3/3	5/5

Number of animals with the sign/number of animals examined

Significant difference compared with the control group value, * $p < 0.05$

Table 14 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: hematological values of male dogs and hematological values of female dogs

Summary of hematological test				
Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
WBC ¹ (K/ μ L)	8.10 \pm 1.51 (5)	8.92 \pm 1.32 (3)	7.61 \pm 1.27 (3)	8.35 \pm 1.31 (5)
RBC ² (M/ μ L)	7.46 \pm 0.33 (5)	7.60 \pm 0.62 (3)	7.05 \pm 0.45 (3)	7.32 \pm 0.55 (5)
HGB ³ (g/dL)	17.22 \pm 0.70 (5)	17.43 \pm 0.67 (3)	15.80 \pm 1.11 (3)	15.30 \pm 1.07* (5)
HCT ⁴ (%)	53.90 \pm 1.50 (5)	54.43 \pm 2.06 (3)	49.97 \pm 2.94 (3)	48.86 \pm 4.00* (5)
MCV ⁵ (fL)	72.34 \pm 2.00 (5)	71.77 \pm 3.08 (3)	70.83 \pm 1.07 (3)	66.72 \pm 2.08** (5)
MCH ⁶ (pg)	23.10 \pm 0.75 (5)	23.00 \pm 0.96 (3)	22.40 \pm 0.36 (3)	20.92 \pm 0.92** (5)
MCHC ⁷ (g/dL)	31.92 \pm 0.48 (5)	32.07 \pm 0.15 (3)	31.63 \pm 0.38 (3)	31.38 \pm 0.83 (5)
RDW ⁸ (%)	12.94 \pm 0.25 (5)	12.43 \pm 0.38 (3)	12.57 \pm 0.15 (3)	12.58 \pm 0.27 (5)
PLT ⁹ (K/ μ L)	215.40 \pm 77.01 (5)	236.00 \pm 63.50 (3)	233.00 \pm 156.89 (3)	322.60 \pm 42.88 (5)
MPV ¹⁰ (fL)	10.10 \pm 1.69 (5)	9.90 \pm 1.59 (3)	8.80 \pm 0.78 (3)	8.68 \pm 0.72 (5)
NEP ¹¹ (%)	52.46 \pm 4.49 (5)	50.07 \pm 6.12 (3)	49.30 \pm 3.00 (3)	53.90 \pm 6.37 (5)
LYP ¹² (%)	34.58 \pm 6.03 (5)	39.87 \pm 5.71 (3)	40.90 \pm 2.78 (3)	35.78 \pm 5.69 (5)
MOP ¹³ (%)	8.14 \pm 2.33 (5)	5.70 \pm 2.48 (3)	5.80 \pm 0.82 (3)	6.32 \pm 1.74 (5)
EOP ¹⁴ (%)	3.76 \pm 2.07 (5)	3.50 \pm 1.14 (3)	3.20 \pm 1.61 (3)	3.12 \pm 0.75 (5)
LUP ¹⁵ (%)	0.24 \pm 0.17 (5)	0.23 \pm 0.06 (3)	0.20 \pm 0.10 (3)	0.16 \pm 0.05 (5)
BAP ¹⁶ (%)	0.76 \pm 0.39 (5)	0.60 \pm 0.35 (3)	0.60 \pm 0.17 (3)	0.72 \pm 0.56 (5)
NE ¹⁷ (K/ μ L)	4.29 \pm 1.14 (5)	4.43 \pm 0.53 (3)	3.73 \pm 0.41 (3)	4.52 \pm 0.97 (5)
LY ¹⁸ (K/ μ L)	2.75 \pm 0.39 (5)	3.56 \pm 0.71 (3)	3.12 \pm 0.62 (3)	2.97 \pm 0.51 (5)
MO ¹⁹ (K/ μ L)	0.66 \pm 0.19 (5)	0.53 \pm 0.31 (3)	0.45 \pm 0.13 (3)	0.54 \pm 0.21 (5)
EO ²⁰ (K/ μ L)	0.32 \pm 0.23 (5)	0.32 \pm 0.16 (3)	0.26 \pm 0.17 (3)	0.26 \pm 0.08 (5)
LUC ²¹ (K/ μ L)	0.02 \pm 0.02 (5)	0.02 \pm 0.00 (3)	0.01 \pm 0.01 (3)	0.01 \pm 0.01 (5)
BA ²² (K/ μ L)	0.06 \pm 0.03 (5)	0.05 \pm 0.03 (3)	0.04 \pm 0.01 (3)	0.06 \pm 0.04 (5)
Retic ²³ (K/ μ L)	0.89 \pm 0.12 (5)	1.01 \pm 0.05 (3)	1.31 \pm 0.39** (3)	1.55 \pm 0.09** (5)

Summary of hematological test

Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
WBC ¹ (K/ μ L)	9.40 \pm 3.32 (5)	9.63 \pm 2.69 (3)	8.02 \pm 1.66 (3)	7.56 \pm 2.80 (5)
RBC ² (M/ μ L)	7.56 \pm 0.51 (5)	6.80 \pm 0.55 (3)	7.09 \pm 0.82 (3)	7.71 \pm 0.36 (5)
HGB ³ (g/dL)	17.42 \pm 1.21 (5)	15.57 \pm 1.65 (3)	16.40 \pm 1.18 (3)	17.28 \pm 0.97 (5)
HCT ⁴ (%)	53.38 \pm 3.18 (5)	48.10 \pm 4.64 (3)	51.03 \pm 3.78 (3)	54.54 \pm 2.53 (5)
MCV ⁵ (fL)	70.64 \pm 1.43 (5)	70.63 \pm 2.02 (3)	72.23 \pm 3.20 (3)	70.74 \pm 1.01 (5)
MCH ⁶ (pg)	23.04 \pm 0.52 (5)	22.83 \pm 0.90 (3)	23.23 \pm 1.05 (3)	22.40 \pm 0.44 (5)
MCHC ⁷ (g/dL)	32.62 \pm 0.37 (5)	32.30 \pm 0.36 (3)	32.17 \pm 0.21 (3)	31.68 \pm 0.36** (5)
RDW ⁸ (%)	12.86 \pm 0.46 (5)	12.43 \pm 0.40 (3)	12.50 \pm 0.60 (3)	12.08 \pm 0.29 (5)
PLT ⁹ (K/ μ L)	208.00 \pm 112.75 (5)	254.33 \pm 46.44 (3)	132.67 \pm 130.37 (3)	226.00 \pm 181.81 (5)
MPV ¹⁰ (fL)	9.14 \pm 1.13 (5)	9.10 \pm 0.26 (3)	10.07 \pm 2.27 (3)	10.00 \pm 2.87 (5)
NEP ¹¹ (%)	55.90 \pm 7.29 (5)	61.40 \pm 4.19 (3)	50.43 \pm 6.43 (3)	54.84 \pm 6.05 (5)
LYP ¹² (%)	34.14 \pm 6.54 (5)	29.97 \pm 3.09 (3)	40.73 \pm 4.43 (3)	36.26 \pm 6.42 (5)
MOP ¹³ (%)	5.52 \pm 1.44 (5)	5.70 \pm 0.95 (3)	5.83 \pm 4.39 (3)	4.32 \pm 0.91 (5)
EOP ¹⁴ (%)	3.20 \pm 1.65 (5)	2.33 \pm 0.35 (3)	2.10 \pm 0.62 (3)	3.46 \pm 1.51 (5)
LUP ¹⁵ (%)	0.38 \pm 0.18 (5)	0.13 \pm 0.06 (3)	0.27 \pm 0.06 (3)	0.22 \pm 0.08 (5)
BAP ¹⁶ (%)	0.90 \pm 0.51 (5)	0.43 \pm 0.06 (3)	0.67 \pm 0.23 (3)	0.92 \pm 0.58 (5)
NE ¹⁷ (K/ μ L)	5.42 \pm 2.58 (5)	5.98 \pm 2.00 (3)	4.09 \pm 1.17 (3)	4.19 \pm 1.64 (5)
LY ¹⁸ (K/ μ L)	3.07 \pm 0.71 (5)	2.83 \pm 0.52 (3)	3.27 \pm 0.83 (3)	2.72 \pm 1.15 (5)
MO ¹⁹ (K/ μ L)	0.53 \pm 0.29 (5)	0.54 \pm 0.13 (3)	0.42 \pm 0.22 (3)	0.32 \pm 0.12 (5)

Table 14 (continued)

Summary of hematological test				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
EO ²⁰ (K/ μ L)	0.28 \pm 0.16 (5)	0.22 \pm 0.05 (3)	0.17 \pm 0.06 (3)	0.25 \pm 0.11 (5)
LUC ²¹ (K/ μ L)	0.03 \pm 0.02 (5)	0.01 \pm 0.01 (3)	0.02 \pm 0.01 (3)	0.02 \pm 0.01 (5)
BA ²² (K/ μ L)	0.07 \pm 0.02 (5)	0.04 \pm 0.02 (3)	0.05 \pm 0.02 (3)	0.06 \pm 0.03 (5)
Retic ²³ (K/ μ L)	0.92 \pm 0.32 (5)	0.84 \pm 0.13 (3)	1.02 \pm 0.32 (3)	1.18 \pm 0.36 (5)

1: white blood cell, 2: red blood cell, 3: hemoglobin, 4: hematocrit, 5: mean corpuscular volume, 6: mean corpuscular hemoglobin, 7: mean corpuscular hemoglobin concentration, 8: red cell distribution width, 9: platelet, 10: mean platelet volume, 11: percent of neutrophil, 12: percent of lymphocyte, 13: percent of monocyte, 14: percent of eosinophil, 15: percent of large unstained cell, 16: percent of basophil, 17: neutrophil, 18: lymphocyte, 19: monocyte, 20: eosinophil, 21: large unstained cell, 22: basophil, 23: reticulocyte

Mean \pm SD (number of animals)

Significant difference compared with the control group value, * $p < 0.05$, ** $p < 0.01$

of unit areas crossed, rearing counts, respiration rate, stereotypy, unusual behavior, tremor, convulsion, gait, arousal level or number of fecal pellets and urine pools were noted in any animal at 250 and 500 mg/kg when compared to the control group prior to dosing (0 h) and at 0.5, 1, 3, 6, and 24 h post-dose. A statistically significant increase ($p < 0.05$) in the number of urine pools was noted in animals at 1000 mg/kg at 1 h post-dose when compared to the control group, and no significant difference was noted at other observation time points. No statistically significant or biologically relevant differences were noted in any animal at 1000 mg/kg when compared to the control group prior to dosing (0 h) and at 0.5, 1, 3, 6, and 24 h post-dose.

In Hand Held Observations, no statistically significant or biologically relevant differences in fur and skin appearance, piloerection, contaminated abdominal region, lacrimation, salivation, palpebral closure, pupil size, pupillary reflex, muscle tone or extensor-thrust reflex were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group prior to dosing (0 h) and at 0.5, 1, 3, 6 and 24 h post-dose (data not shown).

In sensori-motor function observations (data not shown), no statistically significant or biologically relevant differences in the visual, touch, click and tail pinch responses, aerial righting reflex, hindlimb landing foot splay, forelimb grip strength or hindlimb grip strength were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control

group prior to dosing (0 h) and at 0.5, 1, 3, 6, and 24 h post-dose.

Also, in determined of body temperature (data not shown), no statistically significant or biologically relevant difference in rectal temperature was noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group prior to dosing (0 h) and at 0.5, 1, 3, 6 and 24 h post-dose.

No change in the test substance 250, 500, and 1000 mg/kg administration groups was observed in all parameters of FOB. From the above results, it can be concluded that the test substance had no effect on the central nervous system at doses of 250, 500, and 1000 mg/kg in a single oral administration using the rats under the test conditions.

Respiratory function assay

This study evaluated respiratory effects by measuring the respiratory rate, tidal volume, and minute volume under no anesthesia and no restraint conditions after single oral administration of the test substance, APRG64, to male SD rats at 6 weeks of age. No statistically significant or biologically relevant differences in the respiratory rate, tidal volume or minute volume were noted in the animals at 250, 500, and 1000 mg/kg when compared to the control group at any measuring point. Therefore, it is considered that there were no effects of the test substance after the administration.

Table 15 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: serum biochemical values of male dogs and serum biochemical values of female dogs

Summary of serum biochemical test				
Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
ALB ¹ (g/dL)	2.86 ± 0.11 (5)	3.03 ± 0.12 (3)	2.70 ± 0.17 (3)	2.98 ± 0.33 (5)
ALP ² (IU/L)	264.20 ± 91.85 (5)	216.33 ± 59.87 (3)	187.00 ± 51.45 (3)	316.40 ± 69.24 (5)
CA ³ (mg/dL)	10.48 ± 0.24 (5)	10.33 ± 0.32 (3)	10.07 ± 0.35 (3)	10.22 ± 0.26 (5)
CHO ⁴ (mg/dL)	205.80 ± 40.12 (5)	190.67 ± 31.82 (3)	198.33 ± 57.83 (3)	192.00 ± 38.46 (5)
CRE ⁵ (mg/dL)	0.85 ± 0.03 (5)	0.94 ± 0.02** (3)	0.85 ± 0.01 (3)	0.82 ± 0.05 (5)
GLU ⁶ (mg/dL)	94.80 ± 8.70 (5)	99.67 ± 0.58 (3)	89.67 ± 0.58 (3)	92.60 ± 6.47 (5)
AST ⁷ (IU/L)	35.40 ± 3.65 (5)	38.67 ± 3.21 (3)	38.00 ± 3.46 (3)	52.20 ± 6.53** (5)
ALT ⁸ (IU/L)	36.20 ± 5.89 (5)	37.33 ± 3.21 (3)	34.00 ± 4.36 (3)	61.20 ± 15.90** (5)
IP ⁹ (mg/dL)	5.90 ± 0.23 (5)	5.33 ± 0.15 (3)	5.40 ± 0.53 (3)	5.06 ± 0.60 (5)
TP ¹⁰ (g/dL)	6.18 ± 0.31 (5)	6.17 ± 0.23 (3)	6.00 ± 0.26 (3)	6.00 ± 0.19 (5)
BUN ¹¹ (mg/dL)	17.62 ± 1.41 (5)	16.00 ± 2.36 (3)	20.03 ± 4.58 (3)	16.38 ± 2.30 (5)
T-BIL ¹² (mg/dL)	0.02 ± 0.03 (5)	0.05 ± 0.03 (3)	0.02 ± 0.01 (3)	0.04 ± 0.02 (5)
TG ¹³ (mg/dL)	45.80 ± 17.99 (5)	27.00 ± 5.57 (3)	42.33 ± 24.34 (3)	24.00 ± 7.81 (5)
CPK ¹⁴ (U/L)	226.40 ± 80.20 (5)	201.00 ± 16.70 (3)	212.00 ± 58.97 (3)	195.40 ± 45.33 (5)
Na ¹⁵ (mmol/L)	143.80 ± 4.32 (5)	144.00 ± 0.00 (3)	142.33 ± 3.51 (3)	143.40 ± 0.55 (5)
K ¹⁶ (mmol/L)	4.80 ± 0.21 (5)	4.77 ± 0.32 (3)	4.90 ± 0.20 (3)	4.94 ± 0.26 (5)
Cl ¹⁷ (mmol/L)	108.40 ± 2.70 (5)	106.00 ± 1.00 (3)	108.33 ± 4.04 (3)	105.60 ± 1.34 (5)
A/G ratio ¹⁸	0.87 ± 0.10 (5)	0.97 ± 0.00 (3)	0.82 ± 0.03 (3)	1.02 ± 0.28 (5)
Summary of serum biochemical test				
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
ALB ¹ (g/dL)	2.88 ± 0.08 (5)	2.80 ± 0.10 (3)	2.83 ± 0.06 (3)	2.86 ± 0.17 (5)
ALP ² (IU/L)	231.00 ± 104.26 (5)	181.33 ± 26.73 (3)	152.67 ± 26.03 (3)	237.60 ± 42.05 (5)
CA ³ (mg/dL)	10.34 ± 0.38 (5)	10.07 ± 0.21 (3)	10.40 ± 0.30 (3)	10.38 ± 0.30 (5)
CHO ⁴ (mg/dL)	167.00 ± 20.22 (5)	209.33 ± 35.81 (3)	168.67 ± 32.01 (3)	189.60 ± 30.25 (5)
CRE ⁵ (mg/dL)	0.75 ± 0.10 (5)	0.82 ± 0.06 (3)	0.78 ± 0.05 (3)	0.83 ± 0.23 (5)
GLU ⁶ (mg/dL)	88.80 ± 7.66 (5)	92.00 ± 1.00 (3)	94.33 ± 3.06 (3)	91.60 ± 9.02 (5)
AST ⁷ (IU/L)	38.40 ± 2.97 (5)	40.00 ± 0.00 (3)	46.67 ± 10.60 (3)	71.00 ± 37.70 (5)
ALT ⁸ (IU/L)	34.00 ± 2.92 (5)	38.67 ± 1.15 (3)	34.00 ± 1.73 (3)	53.20 ± 16.57 (5)
IP ⁹ (mg/dL)	5.18 ± 0.40 (5)	5.27 ± 0.31 (3)	5.67 ± 0.51 (3)	5.42 ± 0.30 (5)
TP ¹⁰ (g/dL)	6.10 ± 0.16 (5)	5.87 ± 0.21 (3)	6.07 ± 0.15 (3)	6.20 ± 0.20 (5)
BUN ¹¹ (mg/dL)	16.26 ± 3.79 (5)	18.27 ± 3.09 (3)	16.60 ± 1.47 (3)	15.44 ± 4.02 (5)
T-BIL ¹² (mg/dL)	0.05 ± 0.02 (5)	0.05 ± 0.02 (3)	0.04 ± 0.02 (3)	0.06 ± 0.03 (5)
TG ¹³ (mg/dL)	27.60 ± 7.57 (5)	30.67 ± 10.50 (3)	29.00 ± 7.94 (3)	22.20 ± 8.44 (5)
CPK ¹⁴ (U/L)	191.20 ± 39.07 (5)	185.00 ± 54.78 (3)	214.33 ± 109.99 (3)	306.80 ± 142.52 (5)
Na ¹⁵ (mmol/L)	145.80 ± 1.10 (5)	145.33 ± 2.08 (3)	143.33 ± 0.58 (3)	144.60 ± 4.77 (5)
K ¹⁶ (mmol/L)	4.54 ± 0.23 (5)	4.53 ± 0.15 (3)	4.70 ± 0.10 (3)	4.74 ± 0.15 (5)
Cl ¹⁷ (mmol/L)	106.20 ± 1.92 (5)	108.00 ± 2.00 (3)	106.00 ± 2.65 (3)	106.60 ± 4.04 (5)
A/G ratio ¹⁸	0.90 ± 0.07 (5)	0.91 ± 0.02 (3)	0.88 ± 0.08 (3)	0.86 ± 0.08 (5)

1: albumin, 2 alkaline phosphatase, 3: calcium, 4: total cholesterol; 5: creatinine, 6: glucose, 7: aspartate aminotransferase, 8: alanine aminotransferase, 9: inorganic phosphorus, 10: total protein, 11: blood urea nitrogen, 12: total bilirubin, 13: triglyceride, 14: creatine phosphokinase, 15: sodium, 16: potassium, 17: chloride, 18: albumin/globulin ratio

Mean ± SD (number of animals)

Significant difference compared with the control group value: ** $p < 0.01$

Table 16 Thirteen-weeks repeated dose toxicity and four-weeks recovery study: blood coagulation values of dogs

Summary of blood coagulation time test (s)				
Test item	Group (mg/kg/day) sex: male			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
PT ¹	6.84 ± 0.64 (5)	6.90 ± 0.91 (3)	6.75 ± 0.60 (3)	6.87 ± 0.49 (5)
APTT ²	13.52 ± 1.37 (5)	14.13 ± 0.64 (3)	15.73 ± 2.25 (3)	31.48** ± 7.45 (5)
Test item	Group (mg/kg/day) sex: female			
	G1 (0)	G2 (250)	G3 (500)	G4 (1000)
PT ¹	7.23 ± 0.87 (5)	6.50 ± 0.46 (3)	6.50 ± 0.09 (3)	7.41 ± 1.05 (5)
APTT ²	14.72 ± 4.59 (5)	18.20 ± 0.26 (3)	18.10 ± 1.73 (3)	25.24** ± 4.84 (5)

1: Prothrombin time, 2: Active partial thromboplastin time

Mean ± SD (number of animals)

Significant difference compared with the control group value: ***p* < 0.01

Table 17 Home cage observations

Summary data of home cage observations						
Group/dose (mg/kg)	Time after administration (h)					
	0	0.5	1	3	6	24
Body posture (ranked, normal score; 3)						
G1 (0)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)
G2 (250)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)
G3 (500)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)
G4 (1000)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)	3.0 ± 0 (8)
Behavior (ranked, normal score; 0)						
G1 (0)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G2 (250)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G3 (500)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G4 (1000)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
Ease of removal from cage (ranked, normal score; 0)						
G1 (0)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G2 (250)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G3 (500)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)
G4 (1000)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)	0.0 ± 0 (8)

No statistically significant differences were noted in the control group (G1) and the test substance groups (G2~G4). Mean ± SD (number of animals)

Table 18 Open field observations

Summary data of open field observations						
Group/dose (mg/kg)	Time after administration (h)					
	0	0.5	1	3	6	24
Number of unit areas crossed (quantitative value./3 min)						
G1 (0)	86.9 ± 18.6 (8)	43.9 ± 18.6 (8)	22.6 ± 21.5 (8)	10.1 ± 18.6 (8)	6.6 ± 12.0 (8)	11.3 ± 18.5 (8)
G2 (250)	91.9 ± 19.5 (8)	22.4 ± 12.3 (8)	10.6 ± 14.0 (8)	2.9 ± 2.0 (8)	1.3 ± 1.0 (8)	3.6 ± 2.1 (8)
G3 (500)	71.0 ± 25.5 (8)	20.6 ± 17.7 (8)	5.9 ± 4.3 (8)	2.3 ± 0.7 (8)	2.1 ± 1.1 (8)	1.8 ± 1.2 (8)
G4 (1000)	77.9 ± 17.3 (8)	27.6 ± 21.1 (8)	13.4 ± 14.7 (8)	2.8 ± 2.5 (8)	2.0 ± 0.9 (8)	6.9 ± 10.4 (8)
Rearing counts (quantitative value./3 min)						
G1 (0)	13.3 ± 6.9 (8)	6.6 ± 7.7 (8)	5.4 ± 6.5 (8)	1.8 ± 4.2 (8)	1.6 ± 4.2 (8)	1.4 ± 2.4 (8)
G2 (250)	13.5 ± 4.8 (8)	2.9 ± 3.3 (8)	1.8 ± 3.2 (8)	0.4 ± 0.5 (8)	0.0 ± 0.0 (8)	0.5 ± 0.9 (8)
G3 (500)	7.6 ± 6.2 (8)	2.3 ± 3.1 (8)	1.5 ± 1.8 (8)	0.0 ± 0.0 (8)	0.0 ± 0.0 (8)	0.0 ± 0.0 (8)
G4 (1000)	8.9 ± 3.7 (8)	1.6 ± 2.6 (8)	0.4 ± 0.5 (8)	0.1 ± 0.4 (8)	0.0 ± 0.0 (8)	0.8 ± 1.8 (8)
Number of fecal pellets (quantitative value./3 min)						
G1 (0)	1.0 ± 1.8 (8)	2.3 ± 2.4 (8)	1.4 ± 1.1 (8)	1.8 ± 2.2 (8)	1.6 ± 2.1 (8)	2.1 ± 1.4 (8)
G2 (250)	1.6 ± 1.8 (8)	3.0 ± 1.7 (8)	1.1 ± 1.6 (8)	2.0 ± 2.4 (8)	3.1 ± 1.1 (8)	2.5 ± 1.5 (8)
G3 (500)	2.1 ± 1.6 (8)	2.9 ± 2.3 (8)	1.6 ± 1.8 (8)	2.4 ± 1.8 (8)	3.5 ± 2.0 (8)	4.1 ± 1.6 (8)
G4 (1000)	2.3 ± 1.6 (8)	3.3 ± 3.5 (8)	1.5 ± 1.5 (8)	1.6 ± 2.3 (8)	2.9 ± 2.4 (8)	3.1 ± 2.1 (8)
Number of urine pools (quantitative value./3 min)						
G1 (0)	0.6 ± 0.5 (8)	0.5 ± 0.5 (8)	0.4 ± 0.5 (8)	0.4 ± 0.5 (8)	0.3 ± 0.5 (8)	0.6 ± 0.5 (8)
G2 (250)	0.8 ± 0.5 (8)	0.8 ± 0.5 (8)	0.8 ± 0.5 (8)	0.8 ± 0.5 (8)	0.6 ± 0.5 (8)	0.8 ± 0.5 (8)
G3 (500)	0.6 ± 0.5 (8)	0.8 ± 0.5 (8)	0.8 ± 0.5 (8)	0.6 ± 0.5 (8)	0.9 ± 0.4 (8)	0.9 ± 0.4 (8)
G4 (1000)	0.9 ± 0.4 (8)	0.9 ± 0.4 (8)	1.0 ± 0.0* (8)	0.8 ± 0.5 (8)	0.8 ± 0.5 (8)	0.5 ± 0.5 (8)

Mean ± SD (number of animals)

Significant difference compared with the control group value, * $p < 0.05$

In the respiratory rate (Fig. 3a), no statistically significant or biologically relevant differences in the respiratory rate were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group at 0 h (pre-dose) and at 0.5, 1, 3, 6, and 24 h post-dose.

Tidal volume results (Fig. 3b) that no statistically significant or biologically relevant differences in the tidal volume were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group at 0 h (pre-dose) and at 0.5, 1, 3, 6, and 24 h post-dose.

In minute volume (Fig. 3c), no statistically significant or biologically relevant differences in the minute volume were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group at 0 h (pre-dose) and at 0.5, 1, 3, 6, and 24 h post-dose.

From the above results, it was concluded that APRG64, which is a test substance, has no effect on the respiratory system at doses of 250, 500, and 1000 mg/kg in a single oral administration using the rats under the test conditions.

Cardiovascular function assay

This test was conducted to evaluate the effects on the cardiovascular system by blood pressure, heart rate and electrocardiogram after oral administration of the test substance to four male beagle dogs with no anesthesia and no restraint and inserted with a remote transmitter. In cardiovascular parameters, no statistically significant differences or biologically relevant differences in the blood pressure (systolic, diastolic and mean blood pressures), heart rate, and electrocardiogram parameters (PR, QRS, QT and QTc intervals) were noted in animals dosed at 250, 500, and 1000 mg/kg at any measuring time point when compared to the control values. Therefore, it is considered that there were no effects of the test substance on the blood pressure (systolic, diastolic and mean blood pressures), heart rate and electrocardiogram parameters (PR, QRS, QT and QTc intervals) after administration.

In blood pressure and heart rate results (Fig. 4) were follow as: No statistically significant differences or biologically relevant differences in the blood pressure (systolic, diastolic and mean blood pressures) and heart rate were noted in

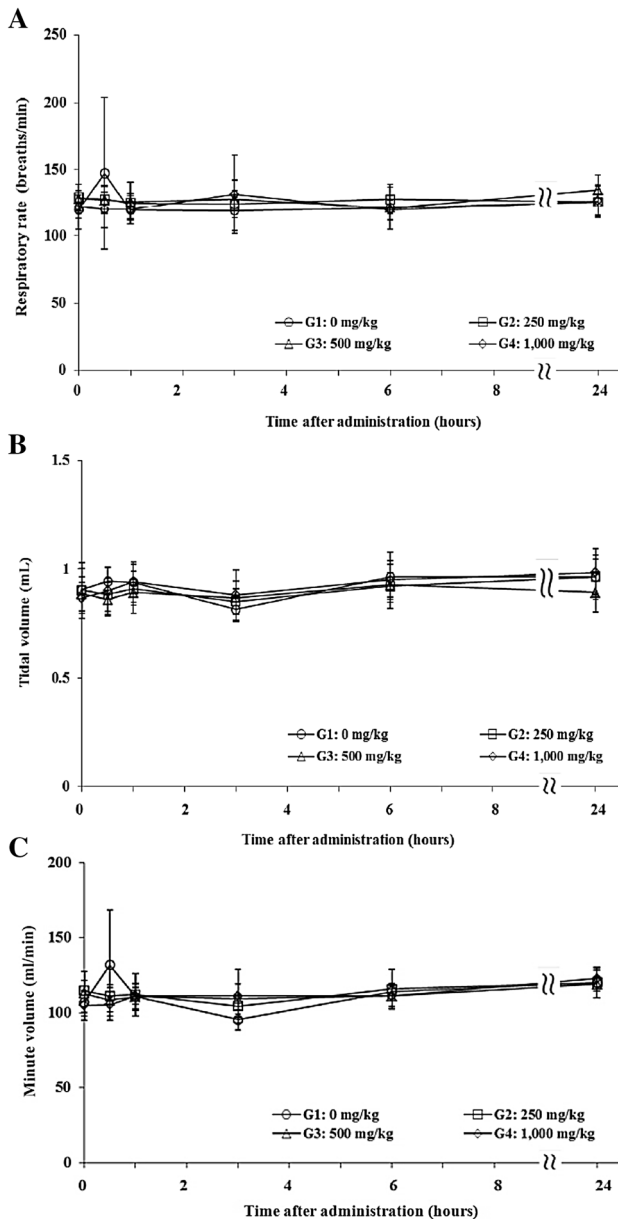


Fig. 3 Respiratory function. **a** respiratory rate, **b** tidal volume, **c** minute volume. No statistically significant or biologically relevant differences were noted in any animal at 250, 500, and 1000 mg/kg when compared to the control group at 0 h (pre-dose) and at 0.5, 1, 3, 6, and 24 h post-dose

animals dosed at 250, 500, and 1000 mg/kg at any measuring time point when compared to the control values.

The result of electrocardiogram (Fig. 5) were follow as: No statistically significant differences or biologically relevant differences in the electrocardiogram parameters (PR, QRS, QT and QTc intervals) were noted in animals dosed at

250, 500, and 1000 mg/kg at any measuring time point when compared to the control values.

Discussion

We conducted a genotoxic and general toxicity test to determine the safety of APRG64, which we intend to develop as a new natural product medicine. First, the results of the three genotoxicity tests are as follows. As a result of the bacterial reverse mutation test (Ames test) using the direct method and the metabolic activation method, it was judged that APRG64 did not induce a reverse mutation under the test conditions set in this study. As a result of chromosome aberration test using CHO-K1 cell derived from Chinese hamster, APRG64 did not induce chromosome aberration under the test conditions set in this study. In addition, an erythrocyte micronucleus induction test using an ICR mouse was carried out, and it was confirmed that the micronucleus was not induced in the bone marrow cells of the mice under the conditions of this test.

General toxicity studies consist of a single dose with rodent, 4-week repeated oral dose toxicity determination study (DRF), and 13-week repeated oral dose toxicity and 4-week recovery study with rodent and non-rodent. In addition, the central nervous system, respiratory system, and cardiovascular function safety study was conducted. The rats were dosed once orally to determine the approximate lethal dose (LD). No toxic symptoms were observed and the approximate LD was > 2000 mg/kg. Based on this result, the high-dose was set at 2000 mg/kg/day and the 4-week repeated dose toxicity determination study (DRF) was performed. No toxicological changes were observed by APRG64. Thereafter, the NOAEL was assumed to be 2000 mg/kg/day, and conducted to 13-week repeated dose toxicity and 4-week recovery study. In conclusion, the NOAEL of APRG64 for rodents was confirmed to be 2000 mg/kg/day, and no toxic target organ was observed. At the same time, DRF was performed on non-rodents beagle dogs. Since no toxicity 1000 mg/kg/day of APRG64 was observed when repeated oral administration for 4 weeks, the groups of 13-week repeated dose toxicity and 4-week recovery studies was set at concentrations of 250, 500, 1000 mg/kg/day. Under this study condition, it was confirmed that there is no effect in the central nervous system, respiratory system and cardiovascular test conditions. However, we confirmed the central lobular balloon degeneration of the liver at 1000 mg/kg. Thus, the NOAEL of APRG64 for beagle dogs was 500 mg/kg/day for both males and females, and the toxic target organ was judged to be liver.

The results of this safety study will be of great help in the subsequent pharmacological use and development of APRG64.

Fig. 4 Blood pressure and heart rate. **a** Systolic blood pressure, **b** diastolic blood pressure, **c** mean blood pressure, **d** heart rate. No statistically significant differences or biologically relevant differences in the blood pressure (systolic, diastolic and mean blood pressures), heart rate, and electrocardiogram parameters were noted in animals dosed at 250, 500, and 1000 mg/kg at any measuring time point when compared to the control values

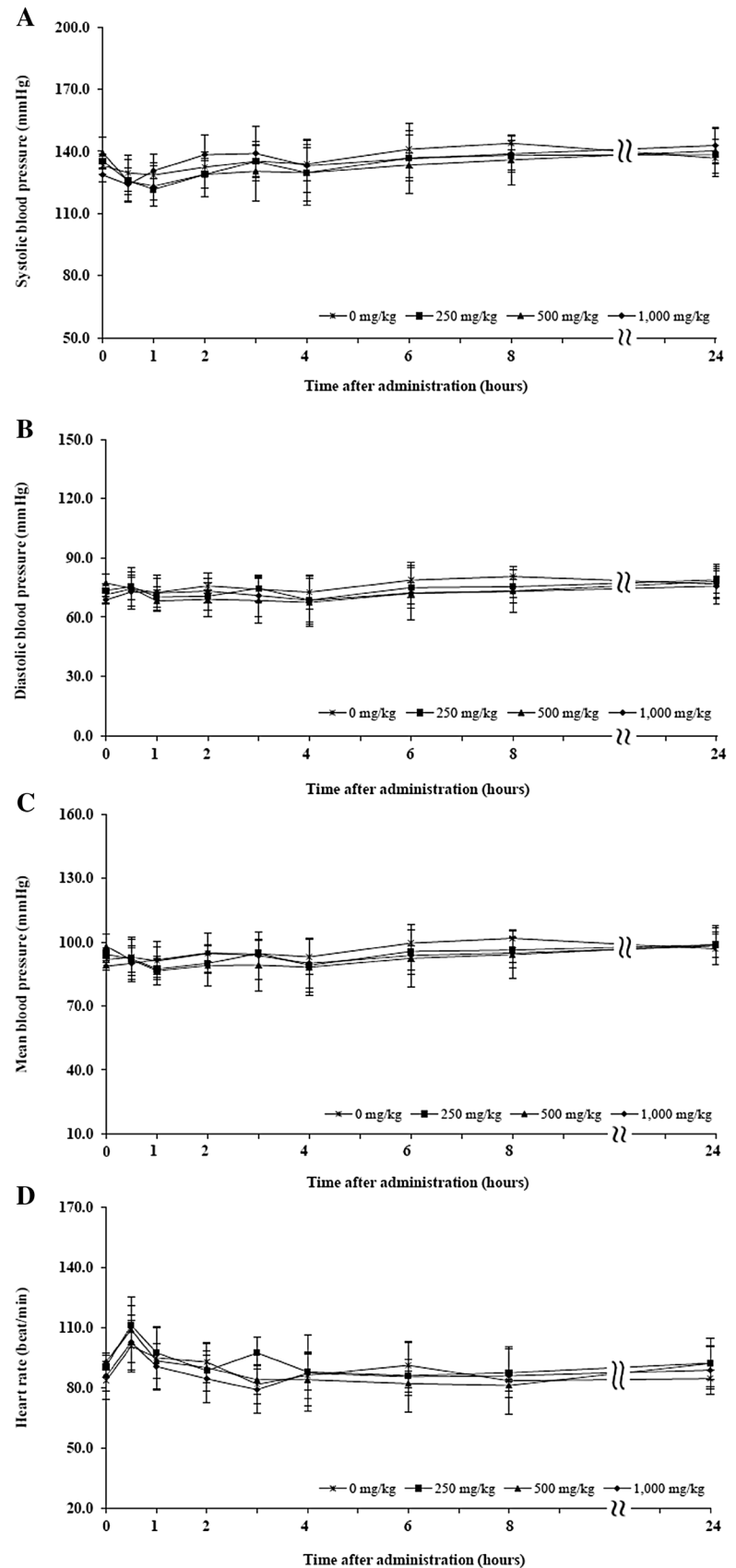
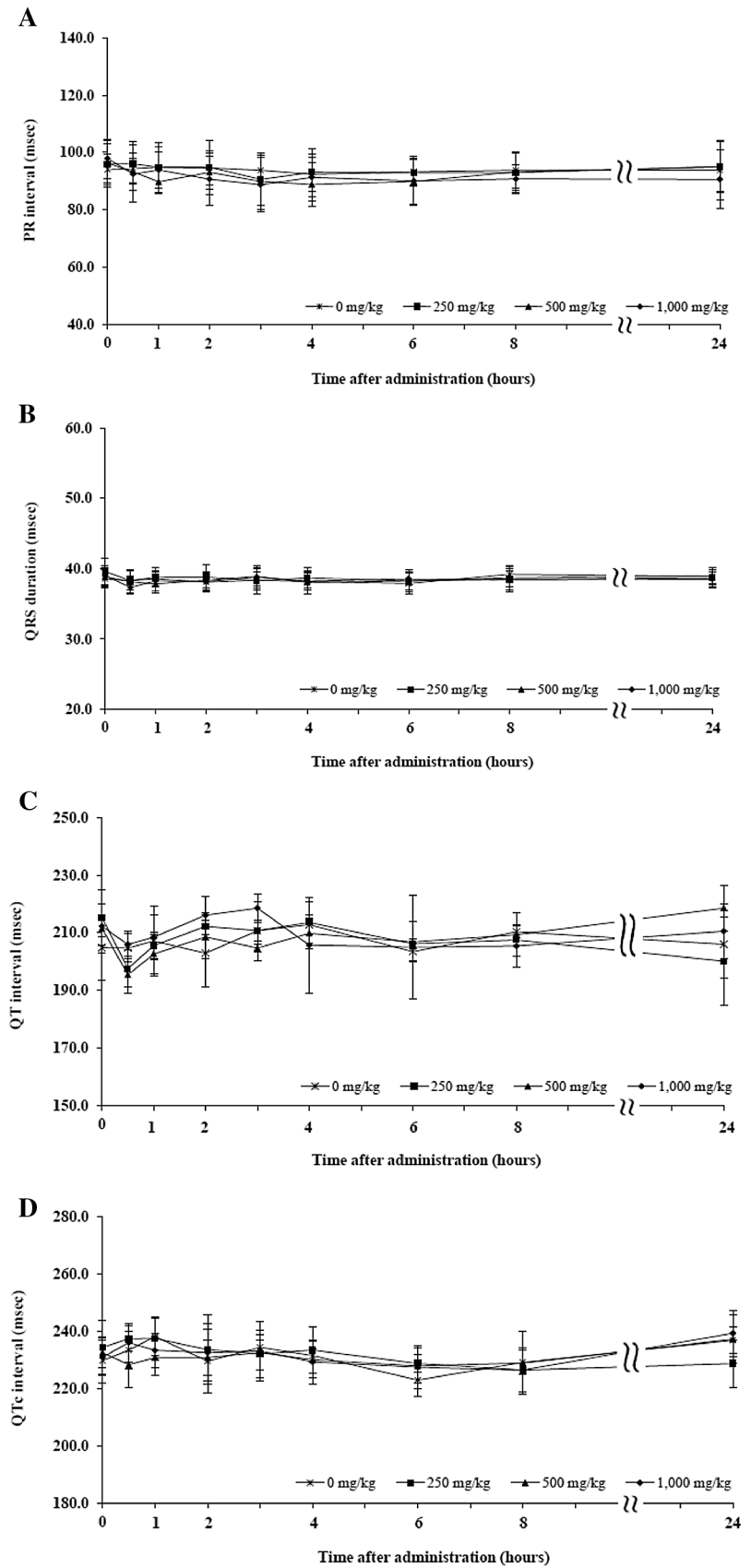


Fig. 5 Electrocardiogram. **a** PR interval, **b** QRS duration, **c** QT interval, **d** QTc interval. No statistically significant differences or biologically relevant differences in the electrocardiogram parameters (PR, QRS, QT and QTc intervals) were noted in animals dosed at 250, 500, and 1000 mg/kg at any measuring time point when compared to the control values



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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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