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RESEARCH ARTICLE

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Assessment of the 4-week repeated-dose oral toxicity and genotoxicity of GHX02

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Abstract

Herbal medicines are widely utilized for disease prevention and health promotion. GHX02 consists of mixtures including Gwaruin (*Trichosanthes kirilowii*), Haengin (*Prunus armeniaca*), Hwangryeon (*Coptis japonica*) and Hwangkeum (*Scutellaria baicalensis*). It has been purported to have therapeutic effectiveness in cases of severe bronchitis. Non-clinical safety testing comprised a single-dose oral toxicity study and a 28-day repeated-dose oral toxicity study with a 14-day recovery period, and genotoxicity was assessed by a bacterial reverse mutation test, in vitro chromosomal aberration test, in vivo mouse bone marrow micronucleus test and single cell gel electrophoresis assay (comet assay). In the single-dose oral toxicity study, the approximate lethal dosage is estimated to be higher than 5000 mg/kg in rats. Thus, the dosage levels were set at 0, 1250, 2500 and 5000 mg/kg/day in the 28-day repeated-dose oral toxicity study, and 10 male rats and 10 female rats/dose were administered GHX02. No clinical signs of toxicological significance were recorded in any animal during the dosing and the observation period in the single-dose study. The no-observed-adverse-effect level of GHX02 was 5000 mg/kg/day when administered orally for 28 days to male and female Sprague-Dawley rats. Despite increases in the frequencies of cells with numerical chromosomal aberration in the in vitro test, the increases were not considered relevant to the in vivo genetic risk. Except for the increase of in vitro numerical chromosomal aberration, clear negative results were obtained from other genetic toxicity studies.

KEYWORDS

28-day repeated-dose toxicity study, genotoxicity, GHX02, herbal medicine, single oral dose toxicity study

1 | INTRODUCTION

Herbal medicine has been widely used to prevent disease and promote health in various countries including South Korea, with early

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use for symptom relief documented in the Joseon dynasty text Dongui Bogam in about 1613. According to the World Health Organization (WHO, 2005), approximately 80% of individuals internationally currently have a reliance on herbal medicine for their primary healthcare. Herbal products generally comprise multiplex mixtures of various organic substances that can originate from any raw material or processed contain parts of herbs including root, stems, leaves, seeds and flowers (Bent, 2008).

Acute bronchitis constitutes a short-term inflammation of the bronchi of the lungs, with severe coughing representing the primary symptom. In the clinic, it is important to distinguish acute bronchitis from pneumonia, common cold and asthma because no specific method exists for diagnosing acute bronchitis (Albert, 2010). Moreover, although the majority of cases are generated by viruses (>90%) with only <10% resulting from bacterial infections (Steinman, Sauaia, Maselli, Houck, & Gonzales, 2004), antibiotics are frequently administered to patients thereby providing minimal disease relief albeit resulting in various adverse effects related to the development of bacterial resistance (Smith, Fahey, Smucny, & Becker, 2014).

A variety of herbal products is available for treating acute bronchitis, some of which are based on combinations of various active ingredients. In particular, GHX02 is the extract composed of a mixture of

TABLE 1 Single-dose oral toxicity of GHX02

Sex	Group/dose (mg/kg)	Body weight (mean ± SD)			Clinical signs	Mortality (dead/total)
		Day 0	Day 7	Day 14		
Male	G1 (0)	173.0 ± 5.0	257.4 ± 11.8	315.7 ± 21.1	N	0% (0/5)
	G2 (5000)	173.7 ± 3.6	263.9 ± 6.4	329.5 ± 14.3	N	0% (0/5)
Female	G1 (0)	134.4 ± 3.3	191.0 ± 10.4	218.0 ± 11.7	N	0% (0/5)
	G2 (5000)	134.6 ± 4.2	183.6 ± 8.8	210.4 ± 13.8	N	0% (0/5)

N, normal.

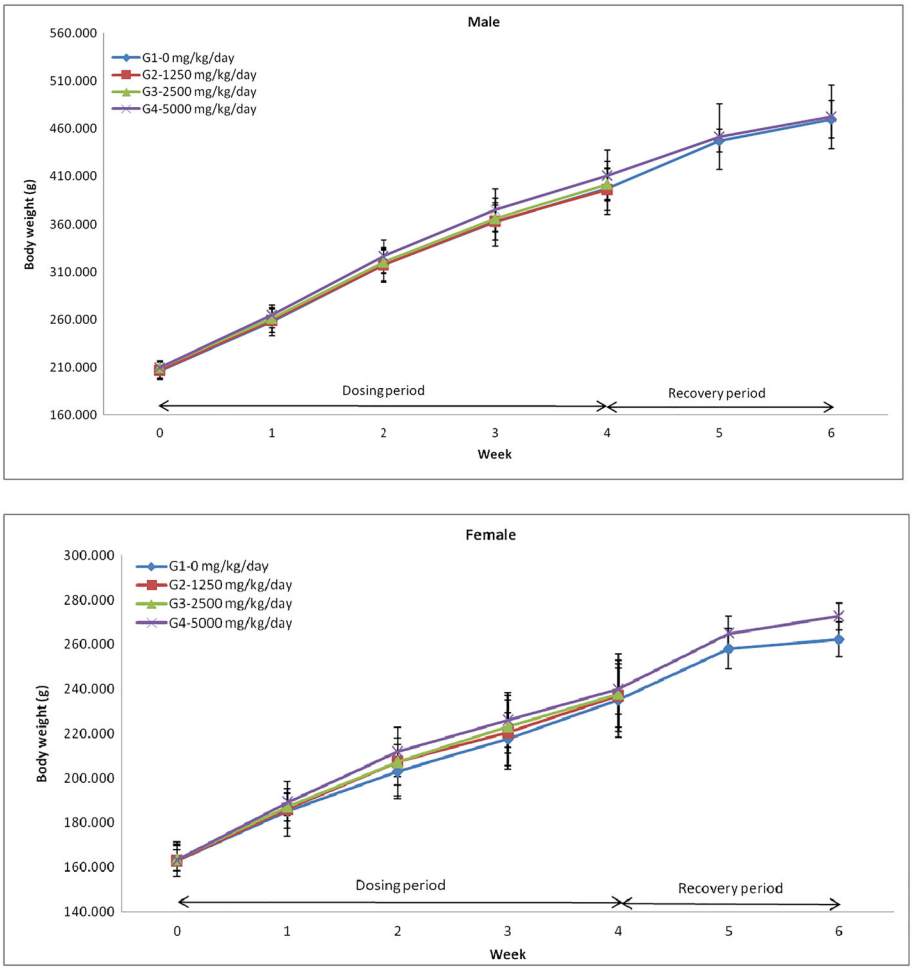

FIGURE 1 Body weight (mean ± SD) of male and female rats treated with 0, 1250, 2500 and 5000 mg/kg/day in the 28-day repeated-dose oral toxicity study of GHX02 with a 14-day recovery period. *Significantly different from the vehicle control at $P < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

roots and seeds of four herbs: Gwaruin (*Trichosanthes kirilowii*); Haengin (*Prunus armeniaca*); Hwangryeon (*Coptis japonica*); and Hwangkeum (*Scutellaria baicalensis*). The components of GHX02 have been traditionally used to improve acute bronchitis with few side effects, as suggested by empirical evidence (Lyu et al., 2018).

A series of studies were performed on GHX02 to produce toxicity data to support future clinical studies. Although GHX02 has been used as an herbal medicine in Korea, its toxicological data, which is essential for Investigational New Drug (IND) application, have not yet been submitted for IND review. Therefore, we evaluated the safety of GHX02 and got approval of phase II clinical trials for the treatment of acute bronchitis, multicenter-based, including dose-finding, double-blind, randomized placebo-controlled trial of two different doses of GHX02. ClinicalTrials.gov Identifier: NCT03310385.

In our studies, the safety of GHX02 was estimated by a single-dose oral toxicity study and a 28-day repeated-dose oral toxicity study using Sprague-Dawley rats with a 14-day recovery period. In addition, genotoxicity was assessed using a bacterial reverse mutation test (Ames test), in vitro chromosomal aberration test, in vivo mouse micronucleus assay and single cell gel electrophoresis assay (comet assay).

2 | MATERIALS AND METHODS

2.1 | Preparation of GHX02

GHX02, soft brown extract, was provided by Hankookshinyak Pharmaceutical Co., Ltd. produced using the standards of Good Manufacturing Practice. GHX02 for oral administration or treatment was formulated in sterile distilled water. The components, parts and ratio of GHX02 are described in a previous paper (Lyu et al., 2018).

2.2 | Experimental animals and husbandry

All toxicological studies were carried out by Chemon Inc. under Good Laboratory Practice (GLP) conditions. Specific pathogen-free Sprague-Dawley rats (Crl:CD(SD)) were used for general toxicity studies and the comet assay. Specific pathogen-free ICR mice (CrljOri:CD1(ICR)) were used for the bone marrow micronucleus assay. The rats and mice used for the general toxicity and bone marrow micronucleus tests were gained from Orientbio Inc., and the rats for the comet assay were obtained from Samtako Inc. The studies were approved by the Institutional Animal Care and Use Committee (IACUC). Animals were housed in the laboratory animal facility at a temperature of 22 ± 3°C and a relative humidity of 50% ± 20%. The animal rooms were maintained under a 12-hour light-dark cycle, and 10-20 air changes per hour. Animals were supplied irradiation-sterilized pellet feed (Teklad Certified Irradiated Global 18% Protein Rodent Diet, 2918C; Envigo RMS, Inc.) along with tap water disinfected using an ultraviolet sterilizer and ultrafiltration ad libitum. All animals were acclimated for 7 days before the start of the experiment. For the single-dose oral toxicity study, the animals were housed in stainless steel cages with mesh bottom. For the repeated-dose studies and the micronucleus test, the animals were housed in a solid bottom polycarbonate cage with bedding.

2.3 | Single-dose oral toxicity study

Six-week-old Sprague-Dawley male and female rats (n = 5 per sex and group) were orally treated with GHX02 at 0 and 5000 mg/kg. Animals were housed in stainless steel cages with mesh bottoms. No more than three animals were housed per cage during the quarantine and

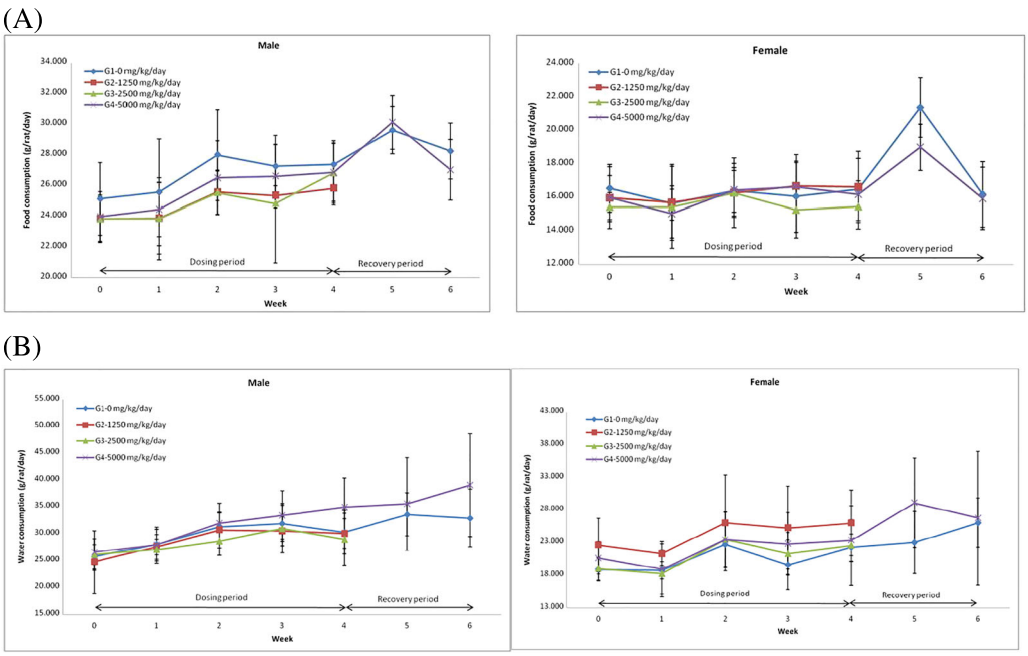

FIGURE 2 Effect of GHX02 on the weekly food and water consumption after oral administration in male and female rats for 28 days. A, Weekly food consumption. B, Weekly water consumption. Data are expressed as the mean ± SD [Colour figure can be viewed at wileyonlinelibrary.com]

acclimation period, and the animals were individually housed during the dosing and the observation period. The clinical signs and mortality were monitored constantly for the first 30 minutes, and then every hour until 6 hours after oral treatment and daily for 14 days subsequently. During the 15-day experimental period, the body weight of all group rats was recorded, and gross findings were observed at necropsy.

2.4 | 28-Day repeated-dose oral toxicity study

The 28-day repeated-dose oral toxicity study was performed according to OECD TG407. The high dose was set at 5000 mg/kg/day as GHX02 is a mixture of many compounds of unknown concentration, and there were no toxic signs in the single-dose study. Six-week old Sprague-Dawley rats (n = 10 per sex and group) were orally administered with GHX02 at 0, 625, 1250, 2500 and 5000 mg/kg/day for 28 days to male and female rats in this study. Five animals/sex were added to the vehicle control group and the high-dose group (5000 mg/kg/day) to evaluate the recovery potential. The body weight ranges at the initiation of dosing were 192.42-226.39 g for males and 150.23-175.29 g for females, respectively.

Animals were checked once a day to observe any clinical signs and mortalities, and the type, date of occurrence and severity of signs were recorded individually. The body weights of all group rats were recorded before the initiation of dosing (day 1) and once a week during the experimental period. Before necropsy, all group rats were fasted overnight and the body weight of all group rats were

TABLE 2 Urinalysis and sediments in male and female rats

		Dose (mg/kg/day)											
		Male						Female					
		Main groups				Recovery groups		Main groups				Recovery groups	
Parameter		0	1250	2500	5000	0	5000	0	1250	2500	5000	0	5000
Glucose	Negative	5	5	5	5	5	5	5	5	5	5	5	5
Bilirubin	Negative	5	5	5	5	5	5	5	5	5	5	5	5
Ketone body	Negative	4	5	1	0	0	0	5	5	5	1	5	5
	Trace	1	0	2	4	1	2	0	0	0	4*	0	0
	15 mg/dL	0	0	2	1*	4	3					0	0
Specific gravity	≤1.005	0	0	0	0	0	0	1	2	0	0	1	0
	1.010	4	5	1	1	2	1	4	3	5	4	4	5
	1.015	1	0	3	2	0	3	0	0	0	1	0	0
	1.020	0	0	1	1	3	1					0	0
	1.025	0	0	0	1								
pH	7.0	0	0	0	1	0	0	0	0	0	0	0	0
	7.5	0	0	1	0	0	0	0	0	0	0	0	0
	8.0	3	1	0	0	0	0	1	2	0	2	0	1
	8.5	2	4	4	4	5	5	4	3	5	3	5	4
Protein	Negative	4	4	1	2	1	1	5	5	5	5	5	5
	15 mg/dL	1	1	4	3	4	4					0	0
Urobilinogen	0.2 EU/dL	5	5	5	5	5	5	5	5	5	5	5	5
Nitrite	Negative	5	5	5	5	5	5	5	5	5	5	5	5
Occult blood	Negative	5	4	5	5	4	4	5	5	5	5	5	5
	Trace	0	1	0	0	1	1					0	0
Clarity	Clear	5	5	5	5	5	5	5	5	5	5	5	5
Color yellow		5	5	5	5	5	5	5	5	5	5	5	5
Volume (mL) ± SD		18.8 ±2.7	13.4 ±6.7	16.0 ±2.6	15.0 ±5.7	11.4 ±2.1	14.6 ±7.1	11.0 ±4.0	14.6 ±4.3	9.2 ±1.8	11.2 ±4.3	11.6 ±2.6	14.0 ±7.4
Sediment													
RBC	0	5	5	5	5	5	5	5	5	5	5	5	5
WBC	0	5	5	5	5	5	5	5	5	5	5	5	5
Epithelial cells	0/20 fields	5	5	5	5	5	5	5	5	5	5	5	5
Casts	0	5	5	5	5	5	5	5	5	5	5	5	5
No. of animals		5	5	5	5	5	5	5	5	5	5	5	5

RBC, red blood cell count; WBC, white blood cell count.

*Significant difference at the $P < .05$ level compared with the vehicle control.

recorded at necropsy. Food and water intake was checked on the same days as the body weight measurements were recorded. The ophthalmological observation examination was processed during the last week of the experimental period using a fundus camera (Keeler Instruments Inc.), and the eyes of all animals were macroscopically examined.

Urinalysis was performed during the last week of observation; all animals were individually housed in a stainless-steel cage cleaned and disinfected with 70% alcohol. Urine samples were collected and 0.3 mL of fresh urine was taken for analysis. Urine samples were analyzed for glucose, bilirubin, ketone body, specific gravity, pH, protein, urobilinogen, nitrite, occult blood, urine color and clarity using an automatic analyzer (Clinitek Advantus; Siemens).

At necropsy, animals were anesthetized by inhalation of 3%-5% isoflurane (Terel liquid; Kyongbo Pharma. Co., Ltd.). Blood samples were collected from the posterior vena cava for hematological and serum biochemical testing. Approximately 1 mL blood was placed in a CBC bottle (Vacutainer 3 mL; BD) with anticoagulant EDTA-2K. Hematology parameters were measured using a Coulter counter (ADVIA 2120; Siemens), including red blood cell count (RBC), hemoglobin distribution width, hemoglobin concentration, hematocrit, platelet count (PLT), mean corpuscular volume, mean cell hemoglobin,

TABLE 3 Hematological values of GHX02 in male rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Male						
RBC (10^6/μL)	7.95 ± 0.22	8.10 ± 0.23	7.86 ± 0.28	7.99 ± 0.32	8.09 ± 0.57	8.33 ± 0.28
HGB (g/dL)	15.7 ± 0.6	15.7 ± 0.5	15.3 ± 0.4	15.3 ± 0.7	15.1 ± 0.9	15.3 ± 0.2
HCT (%)	48.6 ± 1.9	49.1 ± 1.7	47.6 ± 1.2	47.8 ± 2.3	46.6 ± 2.4	48.2 ± 0.6
MCV (fL)	61.1 ± 2.2	60.6 ± 2.1	60.6 ± 1.7	59.9 ± 1.5	57.7 ± 2.8	57.8 ± 1.4
MCH (pg)	19.7 ± 0.6	19.4 ± 0.5	19.5 ± 0.6	19.2 ± 0.5	18.8 ± 1.1	18.4 ± 0.5
MCHC (g/dL)	32.3 ± 0.4	32.0 ± 0.3	32.1 ± 0.3	32.1 ± 0.3	32.5 ± 0.4	31.9 ± 0.5
RDW (%)	12.0 ± 0.5	11.7 ± 0.4	11.5 ± 0.3	11.7 ± 0.7	11.9 ± 0.5	12.1 ± 0.6
HDW (g/dL)	2.48 ± 0.11	2.44 ± 0.15	2.39 ± 0.24	2.32 ± 0.22	2.59 ± 0.27	2.62 ± 0.19
RET (%)	3.46 ± 1.05	3.09 ± 0.54	2.84 ± 0.48	2.85 ± 0.42	2.45 ± 0.57	2.87 ± 0.44
PLT (10^3/μL)	1162.5 ± 119.2	1143.8 ± 64.9	1095.5 ± 76.2	1049.8 ± 104.0*	1032.8 ± 59.8	1122.4 ± 209.9
MPV (fL)	6.01 ± 0.85	5.77 ± 0.55	6.07 ± 0.59	5.71 ± 0.32	6.52 ± 0.28	6.16 ± 0.17*
WBC (10^3/μL)	10.49 ± 3.23	12.28 ± 3.08	9.70 ± 2.42	9.01 ± 1.42	10.67 ± 1.86	12.96 ± 2.70
NEU (%)	18.3 ± 11.1	13.9 ± 3.2	15.7 ± 3.7	16.5 ± 4.1	17.4 ± 6.3	14.5 ± 3.8
NEU (10^3/μL)	1.7 ± 0.7	1.7 ± 0.5	1.5 ± 0.3	1.5 ± 0.4	1.8 ± 0.6	1.9 ± 0.6
LYM (%)	76.4 ± 12.4	81.9 ± 3.6	80.1 ± 3.6	78.0 ± 3.4	78.4 ± 6.0	81.3 ± 4.2
LYM (10^3/μL)	8.3 ± 2.7	10.1 ± 2.8	7.8 ± 2.2	7.0 ± 1.1	8.4 ± 1.7	10.5 ± 2.3
MONO (%)	3.55 ± 1.87	2.61 ± 0.83	2.67 ± 0.98	3.80 ± 1.29	2.88 ± 1.45	2.38 ± 0.77
MONO (10^3/μL)	0.34 ± 0.15	0.30 ± 0.07	0.27 ± 0.13	0.35 ± 0.16	0.30 ± 0.13	0.30 ± 0.11
EOS (%)	0.72 ± 0.30	0.54 ± 0.24	0.62 ± 0.25	0.74 ± 0.26	0.72 ± 0.16	0.94 ± 0.48
EOS (10^3/μL)	0.08 ± 0.04	0.07 ± 0.03	0.06 ± 0.02	0.07 ± 0.02	0.08 ± 0.03	0.12 ± 0.07
BASO (%)	0.23 ± 0.09	0.29 ± 0.07	0.22 ± 0.08	0.21 ± 0.07	0.22 ± 0.11	0.24 ± 0.13
BASO (10^3/μL)	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02
LUC (%)	0.73 ± 0.26	0.71 ± 0.22	0.72 ± 0.29	0.78 ± 0.34	0.48 ± 0.18	0.64 ± 0.13
LUC (10^3/μL)	0.08 ± 0.03	0.09 ± 0.03	0.08 ± 0.05	0.07 ± 0.04	0.05 ± 0.02	0.09 ± 0.04
PT (s)	8.0 ± 0.3	8.1 ± 0.2	8.3 ± 0.3	8.1 ± 0.5	8.7 ± 0.4	8.3 ± 0.5
APTT (s)	14.6 ± 1.0	14.4 ± 0.8	14.8 ± 1.6	14.7 ± 1.3	15.6 ± 0.5	15.8 ± 1.7
No. of animals	10	10	10	10	5	5

APTT, active partial thromboplastin time; BASO, basophils; EOS, eosinophils; HCT, hematocrit; HDW, hemoglobin distribution width; HGB, hemoglobin; LUC, large unstained cells; LYM, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEU, neutrophils; PLT, platelet; PT, prothrombin time; RBC, red blood cell count; RDW, red cell distribution width; RET, reticulocyte; WBC, white blood cell count.

*Significant difference at the $P < .05$ level compared with the vehicle control.

mean cell hemoglobin concentration, red cell distribution width, mean platelet volume (MPV), reticulocytes, white blood cell count and white blood cell count differential count (neutrophils, basophils, monocytes, lymphocytes, eosinophils and large unstained cells).

Serum biochemical parameters were measured using a serum biochemistry analyzer (AU680; Beckman Coulter). About 2 mL of the blood sample was added into a 5 mL Vacutainer tube (SST™ II Advance; BD) that contained clot activator. The blood was coagulated by remaining at room temperature for 15-20 minutes and then centrifuged for 10 minutes (3000 rpm, 1902 Relative Centrifugal Force (RCF), Combi-514R; Hanil) to collect serum sample. The parameters examined were aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine phosphokinase, reticulocytes, total bilirubin, glucose, total cholesterol, triglyceride, total protein, albumin, albumin/globulin ratio, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium ion and chloride ion.

After the blood sampling, animals were killed by exsanguination from the abdominal aorta and posterior vena cava. Gross findings were recorded including the body surface, subcutis, head and all organs in the abdominal and thoracic cavities. Following this, organs were weighed using an electronic balance (Sartorius AG): kidney (both), spleen, liver, thymus, heart, lung, brain and adrenal gland. Organ weights (%) relative to the terminal body weights were also calculated. Tissue slides were prepared from fixed tissues with gross findings of all animals, and histopathological examination was

TABLE 4 Hematological values of GHX02 in female rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Female						
RBC (10^6/μL)	7.65 ± 0.21	7.66 ± 0.32	7.63 ± 0.29	7.54 ± 0.24	7.65 ± 0.26	7.42 ± 0.14
HGB (g/dL)	15.0 ± 0.4	14.8 ± 0.4	14.7 ± 0.5	14.7 ± 0.5	14.3 ± 0.4	14.2 ± 0.4
HCT (%)	45.1 ± 1.2	45.0 ± 1.6	44.7 ± 1.1	44.5 ± 1.3	44.0 ± 1.2	43.2 ± 1.1
MCV (fL)	59.1 ± 1.2	58.8 ± 1.1	58.6 ± 1.2	58.9 ± 0.8	57.5 ± 1.3	58.3 ± 1.0
MCH (pg)	19.6 ± 0.4	19.4 ± 0.4	19.3 ± 0.5	19.5 ± 0.3	18.7 ± 0.6	19.2 ± 0.3
MCHC (g/dL)	33.2 ± 0.4	33.0 ± 0.3	33.0 ± 0.5	33.0 ± 0.3	32.5 ± 0.4	32.9 ± 0.3
RDW (%)	10.9 ± 0.2	10.9 ± 0.2	10.7 ± 0.2	10.8 ± 0.3	11.2 ± 0.3	11.3 ± 0.3
HDW (g/dL)	2.27 ± 0.11	2.24 ± 0.14	2.25 ± 0.08	2.24 ± 0.11	2.33 ± 0.08	2.27 ± 0.12
RET (%)	2.44 ± 0.30	2.35 ± 0.64	2.22 ± 0.59	2.29 ± 0.44	2.32 ± 0.34	2.75 ± 0.41
PLT (10^3/μL)	1008.2 ± 127.6	1077.6 ± 158.3	1048.1 ± 172.7	1060.9 ± 139.5	1009.0 ± 95.0	934.8 ± 69.7
MPV (fL)	6.28 ± 0.47	6.12 ± 0.51	6.03 ± 0.47	5.89 ± 0.49	5.84 ± 0.26	6.20 ± 0.42
WBC (10^3/μL)	9.61 ± 2.25	10.01 ± 2.80	9.45 ± 1.59	10.28 ± 2.31	6.55 ± 0.62	5.30 ± 2.11
NEU (%)	10.6 ± 4.5	7.3 ± 1.7	7.7 ± 2.3	8.1 ± 2.6	14.6 ± 2.7	16.6 ± 7.1
NEU (10^3/μL)	1.0 ± 0.3	0.7 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	0.9 ± 0.1	0.8 ± 0.2
LYM (%)	84.8 ± 4.8	88.0 ± 2.4	86.8 ± 1.8	86.9 ± 2.4	80.4 ± 3.3	78.1 ± 7.9
LYM (10^3/μL)	8.2 ± 2.1	8.8 ± 2.4	8.2 ± 1.5	8.9 ± 2.1	5.3 ± 0.7	4.3 ± 2.1
MONO (%)	2.84 ± 0.76	2.72 ± 0.90	3.42 ± 1.05	3.03 ± 0.69	2.76 ± 0.64	3.20 ± 1.89
MONO (10^3/μL)	0.27 ± 0.10	0.27 ± 0.11	0.32 ± 0.10	0.31 ± 0.10	0.18 ± 0.05	0.15 ± 0.05
EOS (%)	0.83 ± 0.22	0.83 ± 0.17	0.94 ± 0.36	0.68 ± 0.16	1.36 ± 0.60	1.34 ± 0.51
EOS (10^3/μL)	0.08 ± 0.02	0.08 ± 0.03	0.09 ± 0.04	0.07 ± 0.02	0.09 ± 0.04	0.06 ± 0.02
BASO (%)	0.22 ± 0.06	0.25 ± 0.10	0.25 ± 0.05	0.25 ± 0.07	0.12 ± 0.04	0.18 ± 0.08
BASO (10^3/μL)	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
LUC (%)	0.80 ± 0.39	0.88 ± 0.21	0.95 ± 0.39	1.14 ± 0.32	0.68 ± 0.32	0.58 ± 0.22
LUC (10^3/μL)	0.08 ± 0.04	0.09 ± 0.03	0.09 ± 0.04	0.12 ± 0.05	0.05 ± 0.02	0.03 ± 0.02
PT (s)	7.7 ± 0.2	7.4 ± 0.2	7.5 ± 0.2	7.5 ± 0.2	7.7 ± 0.1	7.8 ± 0.2
APTT (s)	12.5 ± 0.8	12.0 ± 0.9	12.3 ± 1.0	12.5 ± 1.0	14.4 ± 1.0	14.6 ± 0.6
No. of animals	10	10	10	10	5	5

APTT, active partial thromboplastin time; BASO, basophils; EOS, eosinophils; HCT, hematocrit; HDW, hemoglobin distribution width; HGB, hemoglobin; LUC, large unstained cells; LYM, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEU, neutrophils; PLT, platelet; PT, prothrombin time; RBC, red blood cell count; RDW, red cell distribution width; RET, reticulocyte; WBC, white blood cell count.

performed. The histopathological findings were processed by the Pristima® (xybion) program. The diagnostic terms in the lexicon of Pristima® were used primarily.

2.5 | Bacterial reverse mutation test

The mutagenic potential of GHX02 was evaluated by bacterial reverse mutation assay according to OECD TG471. The four histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98 and TA1537 along with a tryptophan auxotroph strain of *Escherichia coli*, WP2 *uvr*A (pKM101), were used for bacterial reverse mutation testing (Green & Muriel, 1976; Maron & Ames, 1983). The metabolic activation system consisted of the cofactor-supplemented post-mitochondrial fraction (S9) of liver homogenate from rats pretreated with phenobarbital and 5,6-benzoflavone (Claxton et al., 1987). The test strains were exposed to the test article using the preincubation method. Based on the results of a range-finding test conducted on the test article, dose ranges were determined using the five test strains in both the presence and absence of the metabolic activation system with three plates per dose. In this study, the highest dose of this study was set at 5000 μg/plate for all test strains, and six-serial diluted concentrations (5000, 2500, 1250, 625, 313 and 0 μg/plate) were tested in the main study. The colonies were counted using an automated ProtoCOL 3 colony counter (Synbiosis).

2.6 | In vitro chromosomal aberration test

The in vitro chromosomal aberration test was performed according to OECD TG473. Chinese hamster lung cells (CHL/IU) were obtained from the American Type Culture Collection. The cells were maintained with Eagle's minimum essential medium supplied with 10% fetal bovine serum and used for chemical treatment (Koyama, Utakoji, & Ono, 1970). The cells were cultured at 37 ± 1°C and 5% CO_2-95% air using a humidified incubator (MCO-20AIC; Sanyo) and cell culture flasks (culture surface 75 cm^2). Dose ranges of this study were determined by the cytotoxicity (ascertained via relative population doubling) observed in a range-finding test. The highest dose was chosen to produce <50% relative population doubling. The cells were plated in 60 mm culture plates at a density of 2.5 × 10^5 cells/plate and incubated for approximately 24 hours before chemical treatment. The cells were treated with each dose and incubated for 24 hours. After incubation, the cells were harvested and slides were prepared (Ishidate, 1981; Sofuni, 1998). In total, 100 metaphases per culture (200

TABLE 5 Serum biochemical values of GHX02 in male rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Male						
AST (U/L)	109.9 ± 16.3	107.1 ± 17.5	107.6 ± 25.9	112.1 ± 33.8	111.9 ± 19.7	118.1 ± 8.1
ALT (U/L)	28.6 ± 3.2	27.6 ± 3.3	30.5 ± 6.6	32.6 ± 6.2	31.6 ± 6.8	26.8 ± 4.8
ALP (U/L)	185.0 ± 29.6	196.3 ± 20.5	205.5 ± 35.0	178.8 ± 27.3	153.6 ± 25.1	151.0 ± 53.2
CPK (U/L)	462.2 ± 164.5	395.5 ± 133.0	397.5 ± 180.4	336.6 ± 116.6	540.0 ± 115.9	645.0 ± 256.2
TBIL (mg/dL)	0.12 ± 0.02	0.11 ± 0.03	0.12 ± 0.03	0.12 ± 0.02	0.10 ± 0.02	0.10 ± 0.01
Glucose (mg/dL)	111.1 ± 11.0	108.9 ± 7.3	117.4 ± 19.9	105.4 ± 8.2	107.2 ± 7.0	107.9 ± 5.9
TCHO (mg/dL)	69.0 ± 15.5	62.3 ± 12.7	62.8 ± 8.5	63.9 ± 10.6	68.6 ± 27.9	65.6 ± 16.0
TG (mg/dL)	55.3 ± 17.8	53.7 ± 21.3	48.2 ± 18.3	59.9 ± 32.9	43.6 ± 15.8	64.6 ± 22.1
TP (g/dL)	5.89 ± 0.33	5.93 ± 0.19	5.92 ± 0.16	6.03 ± 0.20	5.85 ± 0.23	5.90 ± 0.22
Albumin (g/dL)	2.93 ± 0.10	2.94 ± 0.12	2.94 ± 0.06	2.99 ± 0.07	2.98 ± 0.09	3.04 ± 0.12
A/G ratio	1.00 ± 0.07	0.98 ± 0.05	0.99 ± 0.04	0.98 ± 0.04	1.04 ± 0.07	1.07 ± 0.08
BUN (mg/dL)	12.6 ± 1.0	11.1 ± 1.5	11.6 ± 2.7	11.0 ± 1.3	13.7 ± 3.2	12.8 ± 1.0
Creatinine (mg/dL)	0.40 ± 0.02	0.38 ± 0.02	0.38 ± 0.02	0.37 ± 0.02	0.38 ± 0.03	0.40 ± 0.05
IP (mg/dL)	7.99 ± 0.44	7.90 ± 0.55	7.90 ± 0.69	7.88 ± 0.53	7.36 ± 0.60	7.71 ± 0.44
Ca^{2+} (mg/dL)	9.75 ± 0.18	9.81 ± 0.25	9.74 ± 0.21	9.64 ± 0.33	9.17 ± 0.19	9.24 ± 0.17
Na^+ (mmol/L)	136.9 ± 1.1	137.1 ± 1.0	136.3 ± 1.1	137.3 ± 1.0	140.3 ± 2.3	141.6 ± 1.2
K^+ (mmol/L)	4.40 ± 0.17	4.62 ± 0.18*	4.30 ± 0.24	4.42 ± 0.25	4.74 ± 0.17	4.76 ± 0.18
Cl^- (mmol/L)	98.7 ± 1.0	100.0 ± 1.6	98.9 ± 1.2	99.4 ± 1.1	100.7 ± 2.2	102.4 ± 1.5
No. of animals	10	10	10	10	5	5

A/G, albumin/globulin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatine phosphokinase; IP, inorganic phosphorus; TBIL, total bilirubin; TCHO, total cholesterol; TG, triglyceride; TP, total protein.

*Significant difference at $P < .05$ level compared with the vehicle control.

metaphases per dose) were evaluated for chromosome aberrations. The slides of continuous treatment series were not observed because a positive result was obtained in the short-duration treatment series. The results are represented as the mean percentage of metaphases with structural or numerical aberrations per 200 metaphases.

2.7 | In vivo mouse bone marrow micronucleus test

The in vivo micronucleus test was performed according to OECD TG474. Five or seven male ICR mice (8 weeks old, 33.3-37.9 g) per dose were orally administered with GHX02 at doses of 1250, 2500 and 5000 mg/kg/day. The animals of the positive control group were treated with mitomycin C (Sigma-Aldrich Co.) once intraperitoneally. Animals were killed 24 hours after the final administration. The bone marrow smears of mice were prepared and counting of micronuclei was performed according to the previous study (Schmid, 1975). The frequency of micronucleated polychromatic erythrocytes (MNPCE) was determined by counting the number of MNPCEs per 2000 PCEs per animal.

2.8 | In vivo alkaline comet assay

This study was performed as a follow-up study to confirm the positive result of the in vitro chromosomal aberration test. This in vivo comet assay was performed according to OECD T G489. Six male Sprague-Dawley rats (8 weeks old, 258.13-290.16 g) were orally administered the test article once a day for three consecutive days at doses of 0, 1250, 2500 and 5000 mg/kg/day. The animals of the positive control group were treated with ethyl methane sulfonate (200 mg/kg/day) once orally on the day of the third administration. All animals were killed approximately 3 hours after the final administration and a cell suspension generated from a portion of the left lobe of the liver was used to make specimens. Electrophoresis was conducted on the following day (Silva, de Freitas, Marinho, Speit, & Erdtmann, 2000). According to the "Atlas of Comet Assay Images," cells were classified into scorable, non-scorable and hedgehog; only scorable cells were analyzed (JEMS-MMS, 2014). A total of 150 cells were counted per animal and the median value of the percentage tail DNA was calculated (Olive, Banáth, & Durand, 1990).

TABLE 6 Serum biochemical values of GHX02 in female rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Female						
AST (U/L)	112.2 ± 23.0	106.0 ± 32.7	107.3 ± 17.6	107.1 ± 22.1	84.0 ± 13.5	78.3 ± 11.2
ALT (U/L)	28.3 ± 4.4	27.0 ± 3.9	26.3 ± 5.8	23.0 ± 4.0	27.7 ± 5.3	20.1 ± 4.5*
ALP (U/L)	143.1 ± 28.4	125.6 ± 40.0	122.9 ± 38.1	126.9 ± 30.9	93.1 ± 24.0	85.8 ± 40.7
CPK (U/L)	409.1 ± 188.4	373.8 ± 215.8	334.8 ± 114.3	373.1 ± 260.5	285.2 ± 126.7	261.8 ± 99.4
TBIL (mg/dL)	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.04	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.03
Glucose (mg/dL)	105.2 ± 13.4	110.2 ± 17.9	106.8 ± 13.6	103.8 ± 9.4	111.8 ± 8.4	114.1 ± 10.1
TCHO (mg/dL)	73.2 ± 9.8	78.2 ± 11.8	70.6 ± 16.7	69.4 ± 12.7	73.8 ± 8.9	78.2 ± 18.3
TG (mg/dL)	38.9 ± 10.6	52.6 ± 28.1	37.5 ± 12.8	28.7 ± 4.6	31.4 ± 9.3	33.2 ± 7.8
TP (g/dL)	5.92 ± 0.23	6.15 ± 0.28	6.06 ± 0.33	6.18 ± 0.27	6.39 ± 0.09	6.32 ± 0.18
Albumin (g/dL)	3.12 ± 0.16	3.17 ± 0.15	3.21 ± 0.19	3.27 ± 0.20	3.49 ± 0.11	3.44 ± 0.19
A/G ratio	1.11 ± 0.05	1.07 ± 0.04	1.13 ± 0.07	1.13 ± 0.07	1.20 ± 0.05	1.20 ± 0.10
BUN (mg/dL)	17.9 ± 3.0	17.3 ± 2.2	16.7 ± 2.9	15.6 ± 3.3	16.7 ± 1.5	13.6 ± 0.9**
Creatinine (mg/dL)	0.46 ± 0.04	0.45 ± 0.03	0.47 ± 0.04	0.47 ± 0.04	0.44 ± 0.03	0.44 ± 0.02
IP (mg/dL)	7.98 ± 0.69	7.60 ± 0.57	8.27 ± 0.75	7.92 ± 0.37	6.73 ± 0.75	5.99 ± 0.37
Ca^{2+} (mg/dL)	9.83 ± 0.28	9.83 ± 0.32	9.88 ± 0.32	9.83 ± 0.29	9.66 ± 0.14	9.47 ± 0.31
Na^{+} (mmol/L)	135.3 ± 1.9	135.2 ± 1.3	135.3 ± 0.7	135.5 ± 1.1	140.9 ± 0.5	140.0 ± 0.8
K^{+} (mmol/L)	4.27 ± 0.36	4.28 ± 0.31	4.27 ± 0.40	4.19 ± 0.23	4.23 ± 0.17	4.08 ± 0.18
Cl^{-} (mmol/L)	98.8 ± 1.7	100.1 ± 2.0	99.6 ± 2.1	100.5 ± 1.4	104.7 ± 0.7	104.5 ± 1.0
No. of animals	10	10	10	10	5	5

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; TBIL, total bilirubin; TCHO, total cholesterol; TG, triglyceride; TP, total protein; A/G, albumin/globulin; BUN, blood urea nitrogen; IP, inorganic phosphorus.

Significant difference at

*$P < .05$ or**$P < .01$ levels compared with the vehicle control.

2.9 | Statistical analysis

Data were statistically analyzed using SAS (version 9.3; SAS Institute Inc.) for the single-dose oral toxicity study, chromosomal aberration test, and micronucleus test; SPSS 10.1K (IBM Corp.) was used for the repeated-dose oral toxicity study and in vivo alkaline comet assay. The significance level was set at $P < .05$. Body weights (for the single-dose oral toxicity study) were assumed to be normally distributed and analyzed using the Student's *t*-test. Body weights (for the micronucleus test) and PCE/RBC ratio were assumed to be normally distributed and analyzed using one-way analysis of variance (ANOVA). The homogeneity of variance was tested using the Bartlett test. When ANOVA was significant and the assumption of homogeneity of variance was met, the negative and positive groups were compared using the Student's *t*-test.

TABLE 7 Absolute and relative organ weights in male rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Male						
Body weights (g)[a]	363.29 ± 31.85	369.18 ± 21.62	377.59 ± 16.89	383.35 ± 24.66	440.35 ± 12.71	441.63 ± 30.22
Adrenal gland (left)	0.0340 ± 0.0072	0.0348 ± 0.0069	0.0345 ± 0.0038	0.0329 ± 0.0049	0.0351 ± 0.0055	0.0306 ± 0.0045
% of body weight	0.0093 ± 0.0013	0.0094 ± 0.0016	0.0091 ± 0.0010	0.0085 ± 0.0009	0.0080 ± 0.0012	0.0069 ± 0.0007
Adrenal gland (right)	0.0320 ± 0.0066	0.0334 ± 0.0059	0.0321 ± 0.0033	0.0312 ± 0.0055	0.0327 ± 0.0038	0.0287 ± 0.0045
% of body weight	0.0088 ± 0.0012	0.0090 ± 0.0013	0.0085 ± 0.0009	0.0081 ± 0.0010	0.0074 ± 0.0007	0.0065 ± 0.0007
Pituitary gland	0.0116 ± 0.0015	0.0124 ± 0.0009	0.0122 ± 0.0012	0.0128 ± 0.0023	0.0141 ± 0.0017	0.0125 ± 0.0018
% of body weight	0.0032 ± 0.0003	0.0034 ± 0.0003	0.0032 ± 0.0003	0.0033 ± 0.0005	0.0032 ± 0.0005	0.0028 ± 0.0003
Thymus	0.5237 ± 0.1510	0.5891 ± 0.1479	0.5800 ± 0.1130	0.5413 ± 0.0746	0.4491 ± 0.1304	0.4941 ± 0.1212
% of body weight	0.1430 ± 0.0372	0.1603 ± 0.0419	0.1533 ± 0.0278	0.1413 ± 0.0188	0.1024 ± 0.0316	0.1111 ± 0.0231
Prostate gland	0.5029 ± 0.0967	0.5072 ± 0.1124	0.4672 ± 0.0807	0.5257 ± 0.0669	0.6725 ± 0.0899	0.5604 ± 0.0818
% of body weight	0.1388 ± 0.0273	0.1381 ± 0.0329	0.1239 ± 0.0215	0.1382 ± 0.0239	0.1525 ± 0.0172	0.1277 ± 0.0239
Testis (left)	1.6548 ± 0.1095	1.6764 ± 0.1060	1.6583 ± 0.1187	1.7285 ± 0.1359	1.6937 ± 0.1375	1.8541 ± 0.1578
% of body weight	0.4570 ± 0.0287	0.4556 ± 0.0403	0.4392 ± 0.0256	0.4528 ± 0.0494	0.3852 ± 0.0376	0.4218 ± 0.0497
Testis (right)	1.6438 ± 0.1175	1.6902 ± 0.1163	1.6732 ± 0.1136	1.7444 ± 0.1543	1.6990 ± 0.1022	1.8360 ± 0.1592
% of body weight	0.4540 ± 0.0315	0.4595 ± 0.0432	0.4433 ± 0.0256	0.4569 ± 0.0523	0.3861 ± 0.0264	0.4176 ± 0.0487
Epididymis (left)	0.4755 ± 0.0264	0.4827 ± 0.0363	0.4651 ± 0.0245	0.5105 ± 0.0335*	0.6092 ± 0.0523	0.6398 ± 0.0794
% of body weight	0.1317 ± 0.0131	0.1314 ± 0.0148	0.1234 ± 0.0091	0.1338 ± 0.0135	0.1386 ± 0.0146	0.1455 ± 0.0211
Epididymis (right)	0.4825 ± 0.0327	0.4775 ± 0.0270	0.4777 ± 0.0465	0.5153 ± 0.0426	0.6415 ± 0.0262	0.6401 ± 0.0772
% of body weight	0.1334 ± 0.0110	0.1299 ± 0.0127	0.1265 ± 0.0109	0.1351 ± 0.0155	0.1458 ± 0.0075	0.1457 ± 0.0217
Spleen	0.8101 ± 0.2144	0.8111 ± 0.0898	0.7642 ± 0.0988	0.8049 ± 0.1222	0.8116 ± 0.1140	0.8208 ± 0.0590
% of body weight	0.2240 ± 0.0638	0.2203 ± 0.0264	0.2023 ± 0.0236	0.2101 ± 0.0314	0.1848 ± 0.0290	0.1859 ± 0.0073
Kidney (left)	1.3493 ± 0.1448	1.3875 ± 0.1101	1.4221 ± 0.1339	1.4342 ± 0.0988	1.5635 ± 0.0765	1.5241 ± 0.1476
% of body weight	0.3719 ± 0.0301	0.3756 ± 0.0130	0.3763 ± 0.0268	0.3750 ± 0.0281	0.3551 ± 0.0162	0.3446 ± 0.0136
Kidney (right)	1.3337 ± 0.1687	1.3672 ± 0.1011	1.4771 ± 0.1876	1.4157 ± 0.1095	1.5948 ± 0.1077	1.5363 ± 0.1704
% of body weight	0.3673 ± 0.0349	0.3704 ± 0.0192	0.3906 ± 0.0400	0.3696 ± 0.0227	0.3623 ± 0.0238	0.3472 ± 0.0214
Heart	1.2717 ± 0.1521	1.3088 ± 0.1262	1.3840 ± 0.0780	1.3582 ± 0.0956	1.4089 ± 0.1156	1.5723 ± 0.1948
% to body weight	0.3493 ± 0.0138	0.3541 ± 0.0201	0.3668 ± 0.0182	0.3547 ± 0.0195	0.3207 ± 0.0350	0.3552 ± 0.0263
Lung	1.5112 ± 0.1077	1.4543 ± 0.1189	1.5317 ± 0.0908	1.5156 ± 0.1335	1.6142 ± 0.1750	1.5509 ± 0.0818
% of body weight	0.4172 ± 0.0263	0.3945 ± 0.0310	0.4064 ± 0.0303	0.3962 ± 0.0350	0.3674 ± 0.0480	0.3517 ± 0.0155
Brain	2.0354 ± 0.0979	2.1289 ± 0.0720	2.0319 ± 0.1191	2.1080 ± 0.1071	2.0853 ± 0.0597	2.1688 ± 0.0978
% of body weight	0.5624 ± 0.0328	0.5788 ± 0.0447	0.5384 ± 0.0275	0.5517 ± 0.0425	0.4740 ± 0.0241	0.4927 ± 0.0367
Liver	10.8878 ± 1.2300	11.3656 ± 1.2219	11.2731 ± 0.9244	11.5953 ± 1.1745	11.8723 ± 0.8323	12.7927 ± 1.3199
% of body weight	2.9957 ± 0.1711	3.0732 ± 0.2037	2.9862 ± 0.2158	3.0199 ± 0.1521	2.6981 ± 0.2064	2.8931 ± 0.1731
No. of animals	10	10	10	10	5	5

[a]Body weights were measured immediately prior to necropsy after overnight fast.

*Significant difference at $P < .05$ level compared with the vehicle control.

Body weights, food and water intake, hematological and serum biochemistry data, organ weights (for repeated-dose oral toxicity test), and median percentage tail DNA (for the comet assay) were assumed to be normally distributed and analyzed using one-way ANOVA. The homogeneity of variance was tested using the Levene test. When ANOVA was significant and the assumption of homogeneity of variance was met, Duncan's multiple range test was used as a post hoc test to determine which group was significantly different from the negative control group.

For the urinalysis data, the rank transformation was performed and analyzed using the Mann-Whitney *U*-test, to determine which group significantly differed from the control group. Fisher's exact test was used for the chromosome aberration test to compare the frequency of aberrant cells between negative control and treated groups. Dose-responsiveness was tested using the Cochran-Armitage trend test. For the frequencies of micronuclei, the Kastenbaum and Bowman (1970) test was analyzed. Negative and positive groups were compared using the Student's *t*-test.

TABLE 8 Absolute and relative organ weights in female rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Female						
Body weights (g)[a]	221.37 ± 13.95	224.62 ± 14.97	227.34 ± 14.13	223.51 ± 12.80	245.12 ± 8.30	257.00 ± 5.26[a]
Ovary (left)	0.0447 ± 0.0084	0.0439 ± 0.0072	0.0418 ± 0.0089	0.0460 ± 0.0102	0.0439 ± 0.0067	0.0490 ± 0.0098
% of body weight	0.0201 ± 0.0032	0.0196 ± 0.0034	0.0185 ± 0.0043	0.0207 ± 0.0052	0.0179 ± 0.0031	0.0191 ± 0.0042
Ovary (right)	0.0436 ± 0.0069	0.0453 ± 0.0086	0.0500 ± 0.0083	0.0464 ± 0.0083	0.0408 ± 0.0079	0.0530 ± 0.0098
% of body weight	0.0197 ± 0.0032	0.0202 ± 0.0035	0.0221 ± 0.0041	0.0208 ± 0.0036	0.0166 ± 0.0029	0.0207 ± 0.0041
Adrenal gland (left)	0.0362 ± 0.0044	0.0333 ± 0.0034	0.0343 ± 0.0040	0.0370 ± 0.0059	0.0338 ± 0.0038	0.0356 ± 0.0038
% of body weight	0.0164 ± 0.0024	0.0149 ± 0.0016	0.0151 ± 0.0019	0.0165 ± 0.0023	0.0138 ± 0.0017	0.0139 ± 0.0015
Adrenal gland (right)	0.0360 ± 0.0051	0.0323 ± 0.0018	0.0336 ± 0.0033	0.0345 ± 0.0053	0.0314 ± 0.0024	0.0342 ± 0.0029
% of body weight	0.0163 ± 0.0024	0.0145 ± 0.0013	0.0148 ± 0.0014	0.0154 ± 0.0021	0.0128 ± 0.0011	0.0133 ± 0.0010
Pituitary gland	0.0145 ± 0.0024	0.0144 ± 0.0013	0.0148 ± 0.0013	0.0151 ± 0.0025	0.0175 ± 0.0042	0.0159 ± 0.0012
% of body weight	0.0065 ± 0.0010	0.0064 ± 0.0004	0.0065 ± 0.0008	0.0067 ± 0.0009	0.0071 ± 0.0018	0.0062 ± 0.0005
Thymus	0.3902 ± 0.0502	0.4466 ± 0.0825	0.4010 ± 0.1193	0.4086 ± 0.0764	0.3317 ± 0.0726	0.4590 ± 0.1050
% of body weight	0.1767 ± 0.0235	0.1984 ± 0.0321	0.1744 ± 0.0457	0.1831 ± 0.0342	0.1354 ± 0.0296	0.1790 ± 0.0426
Uterus (with cervix)	0.6626 ± 0.1965	0.6330 ± 0.2132	0.5680 ± 0.1132	0.7010 ± 0.3434	0.5073 ± 0.0780	0.5142 ± 0.0939
% of body weight	0.3006 ± 0.0927	0.2811 ± 0.0901	0.2492 ± 0.0438	0.3144 ± 0.1545	0.2068 ± 0.0305	0.2004 ± 0.0387
Spleen	0.4835 ± 0.0408	0.4701 ± 0.0622	0.4781 ± 0.0536	0.5037 ± 0.0697	0.5460 ± 0.0253	0.5503 ± 0.0464
% of body weight	0.2188 ± 0.0185	0.2098 ± 0.0279	0.2100 ± 0.0162	0.2249 ± 0.0254	0.2230 ± 0.0128	0.2140 ± 0.0160
Kidney (left)	0.8399C0.0638	0.7872 ± 0.0551	0.8349 ± 0.0664	0.8203 ± 0.0720	0.8510 ± 0.0938	0.8818 ± 0.0691
% of body weight	0.3798 ± 0.0242	0.3514 ± 0.0277	0.3674 ± 0.0214	0.3668 ± 0.0220	0.3469 ± 0.0331	0.3430 ± 0.0253
Kidney (right)	0.8740 ± 0.0767	0.8119 ± 0.0803	0.8610 ± 0.0711	0.8326 ± 0.0627	0.8826 ± 0.0854	0.8842 ± 0.0722
% of body weight	0.3950 ± 0.0268	0.3618 ± 0.0299[a]	0.3790 ± 0.0244	0.3725 ± 0.0197	0.3599 ± 0.0305	0.3442 ± 0.0291
Heart	0.8230 ± 0.0704	0.8638 ± 0.0906	0.8456 ± 0.0910	0.8000 ± 0.0661	0.8654 ± 0.0273	0.9092 ± 0.0220[a]
% of body weight	0.3721 ± 0.0275	0.3840 ± 0.0218	0.3729 ± 0.0421	0.3582 ± 0.0252	0.3531 ± 0.0069	0.3538 ± 0.0084
Lung	1.1194 ± 0.0810	1.0940 ± 0.0358	1.0994 ± 0.1213	1.1433 ± 0.0497	1.2018 ± 0.0643	1.2057 ± 0.0664
% of body weight	0.5063 ± 0.0308	0.4888 ± 0.0328	0.4846 ± 0.0533	0.5122 ± 0.0187	0.4911 ± 0.0378	0.4693 ± 0.0267
Brain	1.8941 ± 0.0606	1.9042 ± 0.0968	1.9220 ± 0.0420	1.9447 ± 0.0659	1.9544 ± 0.0778	1.9613 ± 0.0727
% of body weight	0.8582 ± 0.0522	0.8512 ± 0.0718	0.8484 ± 0.0553	0.8728 ± 0.0592	0.7980 ± 0.0403	0.7636 ± 0.0375
Liver	6.3116 ± 0.5547	6.3648 ± 0.7227	6.3941 ± 0.4378	6.6211 ± 0.4146	6.6580 ± 0.3665	6.6968 ± 0.3032
% of body weight	2.8488 ± 0.1150	2.8328 ± 0.2455	2.8131 ± 0.0967	2.9655 ± 0.1613	2.7152 ± 0.0897	2.6047 ± 0.0651
No. of animals	10	10	10	10	5	5

[a]Body weights were measured immediately prior to necropsy after overnight fast.

*Significant difference at $P < .05$ level compared with the vehicle control.

3 | RESULTS

3.1 | Single-dose oral toxicity study

There were no unscheduled deaths. There were no test article-related clinical signs or body weight changes. The histopathological examination was not performed, as there were no organs with a gross lesion. Thus, the approximate lethal dosage of GHX02 was higher than 5000 mg/kg in male and female rats (Table 1).

3.2 | 28-Day repeated-dose oral toxicity study

No GHX02-related deaths were found during the 28-day exposure period. Compound-colored stool was observed in the 5000 mg/kg/day group in both sexes during the administration period. No significant changes were found in body weight gain, food and water intake. In the recovery groups, the body weight was temporarily increased in the 5000 mg/kg/day group in females ($P < .05$) (Figure 1). Nevertheless, the extent of the increase was minor. After the recovery period, no significant changes were found in food and water intake. No abnormalities were found in the ophthalmological examination in any animal both in the main and the recovery group (Figure 2).

In urinalysis, the levels of ketone bodies were significantly increased in males and females at the dose of 5000 mg/kg/day ($P < .05$) (Table 2). The significant decrease in PLT (male, in the 5000 mg/kg/day group) was not considered of toxicological significance because the changes were not accompanied by correlated findings related to MPV. The MPV of male rats was significantly lower in the 5000 mg/kg/day group during the recovery period, but there was no significant change in PLT level (Tables 3 and 4).

The results of serum biochemistry in comparison with the negative control are shown in Tables 5 and 6. The level of K^+ significantly increased in the males of the main study groups at 1250 mg/kg/day ($P < .05$), but the change was not associated with GHX02 as there was no dose-related response. In the recovery group, the levels of ALT and BUN significantly decreased in females in the 5000 mg/kg/day group, respectively ($P < .05$ and $P < .01$). However, these decreases were not observed in the main study groups and no differences were detected in the liver and kidney upon autopsy and histopathology assessment.

The absolute and relative weights of various organs are shown in Tables 7 and 8. The absolute weight of the left epididymis in the 5000 mg/kg/day male group significantly increased ($P < .05$) whereas the relative weight of the right kidney in the 1250 mg/kg/day female group significantly decreased ($P < .05$). In the recovery group, the absolute weight of the heart significantly increased in females in the 5000 mg/kg/day group due to an increase of fasting body weights. The decreased relative weight of the heart was observed only in the recovery group but it was considered to fall within the normal ranges (Han et al., 2010).

The histopathological results in male and female rats are shown in Table 9. Based on the histopathological results, there were no abnormalities attributable to the administration of GHX02. Hydronephrosis in the kidney was observed in all male groups albeit without a dose-related response. The retention of clear fluid was observed in the female uterus of all female groups but was not attributed to GHX02; rather, this probably resulted from natural change during the estrous cycle.

3.3 | Bacterial reverse mutation test

Precipitation was seen on all test plates at 2500 and 5000 μg/plate at the time of plate scoring. There was no microbial colonization found

TABLE 9 Necropsy findings in male and female rats

	Main groups (mg/kg/day)				Recovery groups (mg/kg/day)	
Parameter	G1 (0)	G2 (1250)	G3 (2500)	G4 (5000)	G1 (0)	G4 (5000)
Male						
Mandibular lymph node						
Agenesis	1	0	0	0		
Thymus						
Extraction of estimated site	1	0	0	0		
Decreased size					1	0
Kidney						
Right hydronephrosis	1	0	2	1		
Stomach						
Partial black spots of glandular stomach	0	0	0	1		
Jejunum						
Partial bulging					1	0
Spleen						
Increased size of tail					1	0
White discoloration of increased size region					1	0
Thyroid gland						
Decreased size on the right					1	0
Seminal vesicle						
Decreased size on the right					0	1
No. gross findings	8	10	8	8	2	4
No. of animals	10	10	10	10	5	5
Female						
Uterus						
Retention of clear fluid	5	4	1	2		
Jejunum						
Partial bulging	0	0	1	0		
No. gross findings	5	6	8	8	5	5
No. of animals	10	10	10	10	5	5

TABLE 10 Bacterial reverse mutation test

	Base-pair substitution type						Frameshift type			
	TA100		TA1535		WP2 uvrA (pKM101)		TA98		TA1537	
Concentration (μg/plate)	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix
0	99 ± 3	86 ± 2	11 ± 1	12 ± 2	151 ± 5	103 ± 3	35 ± 1	18 ± 1	19 ± 1	8 ± 1
313	108 ± 1	84 ± 3	10 ± 1	14 ± 2	139 ± 5	110 ± 2	35 ± 1	19 ± 1	19 ± 1	10 ± 1
625	115 ± 2	86 ± 1	10 ± 1	13 ± 1	126 ± 4	109 ± 7	33 ± 3	19 ± 1	18 ± 1	10 ± 1
1250	110 ± 6	86 ± 3	11 ± 2	15 ± 2	120 ± 1	120 ± 5	29 ± 1	17 ± 1	18 ± 1	12 ± 2
2500	110 ± 3	92 ± 3	12 ± 1	13 ± 1	128 ± 3	121 ± 2	29 ± 1	20 ± 2	21 ± 2	11 ± 2
5000	102 ± 3	97 ± 4	12 ± 1	11 ± 1	128 ± 6	122 ± 4	30 ± 1	18 ± 1	19 ± 2	12 ± 1
Mutagenicity	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive control (μg/plate)	2-AA 2.0 782 ± 12	SA 1.5 678 ± 29	2-AA 3.0 145 ± 3	SA 1.5 457 ± 7	2-AA 2.0 515 ± 7	4NQO 0.1 574 ± 2	2-AA 1.0 307 ± 10	2-NF 5.0 683 ± 19	2-AA 3.0 181 ± 3	9-AA 80 563 ± 3

2-AA, 2-aminoanthracene; SA, sodium azide; 4NQO, 4-nitroquinoline N-oxide; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoanthracene.

due to contamination in any of the plates for the sterility check of the test article and S9 mix. There was neither an increase of revertants nor cytotoxicity in TA100, TA1535, TA98, TA1537 and WP2 *uvr*A (pKM101) at any dose level of the test article both in the presence and the absence of the metabolic activation system. The mean revertant of the positive control for each test strain exhibited a clear increase over the mean revertant of the negative control for that strain (Table 10).

3.4 | In vitro chromosomal aberration test

There was no statistically significant increase in the frequencies of aberrant metaphases with structural aberrations at any dose of the test article compared with the concurrent negative control. The frequency of metaphases with numerical aberrations, polyploidy (PP) or endoreduplication (ER), significantly increased at all dose levels compared with the negative control ($P < .01$). The positive control articles induced clear positive responses ($P < .01$) (Table 11).

3.5 | In vivo micronucleus test

There were no unscheduled deaths or animals with any clinical signs. The frequency of MNPCEs was not increased at all dose levels of GHX02 while mitomycin C induced a clear increase in the frequency of MNPCE ($P < .01$). The ratios of PCE/RBC were not significantly different at all dose levels of GHX02 (Table 12).

3.6 | In vivo alkaline comet assay

No statistically significant or dose-related increase was observed in the median of percentage tail DNA at all in the GHX02 administration group compared with that of the negative control group. The values were within the distribution of histological control data. The statistically significant increase was observed in the positive controls compared with the negative controls ($P < .01$) (Table 13).

4 | DISCUSSION

In our studies, the safety of GHX02 was estimated by single-dose oral toxicity study and a 28-day repeated-dose oral toxicity study using Sprague-Dawley rats with a 14-day recovery period. In addition, genotoxicity was assessed using a bacterial reverse mutation test (Ames test), in vitro chromosomal aberration test, in vivo mouse micronucleus assay and single cell gel electrophoresis assay (comet assay).

In the single-dose oral toxicity study, there was no GHX02-related toxicity. In the 28-day repeated-dose oral toxicity study, though there were statistically significant changes in some parameters, they were not considered adverse as they were within the normal range or showed a lack of a dose relationship, or they were not accompanied by any significant changes in the relevant parameters.

In the chromosomal aberration study, the frequency of numerical aberrations increased in all dose groups compared with the concurrent negative control. Numerical chromosome aberrations were PP and ER. From these results, an interesting observation was the induction of ER in the presence of the S9 mix and PP in the absence of the S9 mix in a

TABLE 11 In vitro chromosome aberration test

			Percentage of cells showing aberrations							
				Chromosome type		Chromatid type				
Dose (μg/mL)	Time (h)[a]	S9 mix	Observed cells	csb	cse	ctb	cte	others	Structural aberrations (%)	Numerical aberrations (%)
0	6 + 18	+	200	0	0	0.5	0	0	0.5	0.5
350		+	200	0	0	0	0	0	0	4.0**
700		+	200	0	0	0	0	0	0	11.5**
1400		+	200	0	0	0.5	1.0	0	1.5	22.0**
Positive control										
B[a]P 20		+	200	0	0	6.0	26.0	0	28.5**	0
0	6 − 18	−	200	0	0	0	0	0	0	0.5
350		−	200	0	0	0	0	0	0	6.0**
700		−	200	0	0	0	0	0	0	10.5**
1400		−	200	0	0	0	0	0	0	17.5**
Positive control										
MMC 0.1		−	200	0	0	5.5	22.0	0	25.0**	0

csb, chromosome break; cse, chromosome exchange; ctb, chromatide break; cte, chromatide exchange; other, metaphases with >10 aberrations or with chromosome fragmentation; B[a]P, benzo[*a*]pyrene; MMC, mitomycin C.

[a]Treatment + recovery time.

**Significantly different from the vehicle control group at $P < .01$.

TABLE 12 In vivo micronucleus test

Dose (mg/kg)	Body weight (h after dosing)		MNPCE/2000PCE (%)	PCE/NCE ratio (%)
	0	24		
0	35.5 ± 1.47	35.0 ± 1.83	0.060 ± 0.055	30.3 ± 2.63
1250	35.4 ± 1.35	35.2 ± 1.71	0.050 ± 0.050	32.6 ± 2.89
2500	35.4 ± 1.26	34.8 ± 1.15	0.080 ± 0.027	29.3 ± 2.79
5000	34.9 ± 1.41	34.3 ± 1.53	0.040 ± 0.042	31.4 ± 2.81
Positive control				
MMC 2	35.3 ± 1.12	33.8 ± 0.80	5.670 ± 0.930**	29.4 ± 2.05

MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, monochromatic erythrocyte; MMC, mitomycin C.

**Significantly different from the vehicle control group at $P < .01$.

TABLE 13 In vivo comet assay

Dose (mg/kg/day)	% Tail DNA (median, mean ± SD)	% Tail DNA (mean ± SD)	Tail length (μm, mean ± SD)	Tail moment (mean ± SD)
0	0.30 ± 0.25	1.80 ± 0.48	33.52 ± 0.58	0.26 ± 0.06
1250	0.25 ± 0.15	2.00 ± 0.30	32.59 ± 1.72	0.28 ± 0.05
2500	0.33 ± 0.43	1.99 ± 0.56	31.93 ± 1.69	0.28 ± 0.08
5000	0.25 ± 0.07	2.30 ± 0.45	33.67 ± 0.79	0.35 ± 0.08
Positive control				
EMS 200	18.94 ± 1.62**	19.06 ± 1.68**	52.41 ± 2.31**	3.37 ± 0.32**

EMS, ethyl methanesulfonate.

**Significantly different from the vehicle control group at $P < .01$.

dose-dependent manner. ER is caused by successive cycles of replication without segregation of daughter chromatids and is rarely observed in animal cells. ER is usually induced by chemicals that inhibit topoisomerase II (Pastor, Cantero, Campanella, & Cortés, 2005; Sumner, 1998; Sutou & Tokuyama, 1974). Some flavonoids (genistein, fisetin, luteolin and quercetin) are known as DNA topoisomerase inhibitors (Yamashita & Kawanishi, 2000; Salti et al., 2000; Olaharski, Mondrala, & Eastmond, 2005). PP is defined as an exact multiple of the haploid chromosome number and is induced by inhibition of DNA topoisomerase. In this in vitro chromosome aberration test, PP is only defined as ≥37 centromeres.

The mechanisms of numerical aberration may involve topoisomerase inhibition or cytoskeleton disturbance, DNA damage, disruption of cellular checkpoint controls (Cortés, Mateos, Pastor, & Dominguez, 2004). The increase of PP indicates that a chemical has the potential to induce numerical aberration. Although aneugens may induce PP, such an increase of PP does not indicate aneugenic potential, but rather may reflect either cell cycle perturbation or cytotoxicity. In addition, OECD TG473 is not designed to measure numerical aberrations and the guideline recommends an in vitro micronucleus test (OECD TG487) for the detection of aneuploidy. Therefore, the increase of numerical aberrations in the in vitro study may not be directly relevant to the in vivo genetic risk. This argument may be supported by the fact that GHX02 did not result in any positive response in any of the in vivo genetic toxicity studies conducted in this project.

5 | CONCLUSION

The toxicity of GHX02 was evaluated by a single oral toxicity study, a 28-day repeated-oral toxicity study, in vitro and in vivo. GHX02 was not found to exhibit mutagenic or genotoxic potential. Moreover, no significant changes were observed in GHX02-treated groups compared with the vehicle control group on histopathological examination by a 28-day repeated-dose oral toxicity study. Therefore, it was concluded that GHX02 administration up to 5000 mg/kg/day yielded no adverse effects and that the no-observed-adverse-effect level was 5000 mg/kg/day in both sexes. Furthermore, our toxicological data of GHX02 contributed to allow us to get IND and approval of phase II (ClinicalTrials.gov Identifier: NCT03310385).

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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