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Toxicological evaluations of colostrum ultrafiltrate

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ABSTRACT

Colostrum has been consumed safely for many years as a food collected directly from cows. More recently, an ultrafiltrated bovine colostrum product has been developed; however, its safety in toxicology studies has not been extensively evaluated. To assess the safety of bovine colostrum ultrafiltrate, in accordance with internationally accepted standards, the genotoxic potential was investigated in a bacterial reverse mutation test, an in vitro chromosomal aberration test, and an in vivo mammalian micronucleus test. No mutagenicity or genotoxic activity was observed in these three tests. A 90-day repeated-dose oral toxicity study in Hsd.Han Wistar rats was conducted at doses of 0, 1050, 2100, and 4200 mg/kg bw/day by gavage. After 90 days of continuous exposure, no mortality or treatment-related adverse effects were observed, and no target organs were identified. The no-observed-adverse-effect level (NOAEL) was determined to be 4200 mg/kg bw/day, the highest dose tested.

1. Introduction

Colostrum is the milk produced by mammals prior to and several days after parturition; it provides nutrients in different concentrations than the milk that comes in several days later. The nutrients in colostrum stimulate the development of the gastrointestinal system and maturation of the immune system in the neonate (Boix-Amoros et al., 2016). Cows produce colostrum with similar nutrients as humans; however, due to calves' more rapid growth relative to humans, bovine colostrum typically has higher levels of growth factors, protein, and calcium (Solomons, 2002). Evidence exists that humans have a long tradition of consuming bovine colostrum, particularly in Europe, Africa, and the Indian subcontinent (Solomons, 2002). A novel preparation of colostrum, colostrum ultrafiltrate (CUF), has been developed utilizing ultrafiltration to concentrate small molecular weight components.

More than 400 different proteins have been identified in native (i.e., nonfractionated) colostrum and are divided among various fractions (Nissen et al., 2012). The dominant proteins in native colostrum are caseins, immunoglobulins, and β-lactoglobulin, but many other proteins are present with lower abundance, such as lactoferrin, cytokines, complement factors, acute-phase proteins, milk fat globule membrane proteins, and various peptides. Fractionation techniques result in isolation of unique fraction profiles comprised of subsets of total colostrum proteins, including low abundance proteins that may be concentrated in the fraction relative to native colostrum; however, quantitative analysis for direct comparison has not been performed. CUF is a mixture of native colostrum and a unique subset of concentrated low molecular weight (≤10 kDa) proteins.

While one toxicological evaluation of native bovine colostrum has been conducted (Davis et al., 2007), to the best of our knowledge, no toxicological or safety studies have been conducted with CUF. In the study by Davis et al., a 90-day toxicological evaluation of 3% and 10% bovine colostrum in the diet of rats did not produce any abnormalities in biochemical, physical, or histopathological parameters. This assessment did not include genotoxicity studies, and neither is the test item analogous to CUF nor the route of administration ideal for expected consumption patterns of CUF in dietary supplements and functional foods.

Abbreviations: CUF, colostrum ultrafiltrate; DME, Dulbecco's modified Eagle's; DMSO, dimethyl sulfoxide; FOB, functional observation battery; GLP, Good Laboratory Practice; IACUC, Institutional Animal Care and Use Committee; MPCE, micronucleated polychromatic erythrocytes; NOAEL, no observed adverse effect level; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; SOP, standard operating procedure; SPF, specific pathogen-free

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Native colostrum use has also been studied in many clinical trials with minimal adverse events or safety concerns in diverse populations such as athletes, pre-term infants, and children with short bowel syndrome (Aunsholt et al., 2014; Jones et al., 2016; Juhl et al., 2018). However, most of these studies are of short duration and few include safety or adverse event end points. Therefore, due to the unique concentration of low molecular weight proteins in CUF compared to native colostrum and the need for primary safety data, a battery of toxicological studies was conducted in order to assess the safety and potential toxicity of repeated exposure to CUF and to determine a no-observed-adverse-effect level (NOAEL).

2. Material and methods

2.1. Test item

The test item, CUF (lot number PB170207GRAS), is a yellow to white powder without disagreeable odor, supplied by the sponsor (4Life Research USA, Sandy, Utah), and positive identification was made by the laboratory based on the provided analytical certificate.

Product specifications for parameters such as identity (FTIR spectrum), density, color, and aroma have been established and consistently met in accordance with current Good Manufacturing Practice. Raw colostrum is collected from cows in the southwestern United States that are certified free from exposure to antibiotics, pesticide and herbicide residues, genetically modified organisms, gene therapy, and exogenous estradiol or prolactin. The raw colostrum and high protein colostrum powder raw materials used in the manufacture of CUF are food grade materials supplied by qualified producers under adherence to applicable US Department of Agriculture (USDA) regulations. Production of CUF also involves adherence to applicable USDA regulations for milk pasteurization. Production begins with separating the fat from the raw colostrum, which is then concentrated as a low molecular weight fraction through ultrafiltration. A high protein colostrum powder is added to the ultrafiltrated colostrum, followed by drying and sifting of the mixture. No chemicals or solvents are utilized in the production of CUF.

Nutritional analysis of CUF per the Official Methods of AOAC International showed that 100 g typically contains 44 g carbohydrate (of which 34 g are sugar), 42 g protein (of which approximately 15 g are immunoglobulin G), 8.2 g ash, 4.7 g moisture, 1.1 g fatty acids, and 50 mg cholesterol. Of the 20 amino acids, all are present except glutamine and asparagine. The individual amino acid with highest concentration in CUF is glutamic acid, with an average of 7.45 mg/100 g, and the lowest is cysteine, 0.48 mg/100 g.

2.2. Bacterial reverse mutation test

2.2.1. Test procedures

In accordance with Organisation for Economic Cooperation and Development (OECD) test guideline 471 (OECD, 1997) and Principles of Good Laboratory Practice (GLP) (OECD, 1998a), the mutagenic potential of CUF was assessed utilizing procedures established by Ames et al. (1975), Maron and Ames (Maron and Ames (1983), Kier et al. (1986), and Venitt and Parry (1984) and the laboratory's standard operating procedure (SOP). Bacterial tester strains *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2 *uvrA* (Moltox, Inc., Boone, NC, USA) were utilized in the study.

Preliminary solubility and concentration range finding tests (plate incorporation method) were conducted using ultrapure water (Millipore, Direct Q5; ASTM Type I prepared in the lab) and dimethyl sulfoxide (DMSO; Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA) as vehicles. Based on the preliminary test results, the GLP initial (standard plate incorporation procedure) and confirmatory (preincubation procedure) bacterial reverse mutation tests were conducted in triplicate, using ultrapure water as the vehicle at test item concentrations of 5000, 1600, 500, 160, 50, and 16 μg/plate with and without metabolic activation. Positive controls for strains without metabolic activation were: 4-nitro-1,2-phenylenediamine (Merck Life Science GmbH, Eppelheim, Germany) for *S. typhimurium* TA98, sodium azide (Merck Life Science GmbH, Eppelheim, Germany) for *S. typhimurium* TA100 and TA1535, 9-aminoacridine (Merck Life Science GmbH, Eppelheim, Germany) for *S. typhimurium* TA1537, and methyl methanesulfonate (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA) for *E. coli* WP2 *uvrA*. The positive control for metabolic activation with S9-mix for all strains was 2-aminoanthracene (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA). Negative controls were DMSO and ultrapure water. All controls were chosen according to the cited guideline and literature. The sensitivity, reliability, and promutagen activation potential of the S9-mix (prepared in the laboratory with rat liver S9 fraction, Moltox, Inc., Boone, NC, USA) were certified by the supplier using known controls and further investigated with the positive control solutions. The test solution was freshly prepared at the beginning of each experiment.

2.2.2. Analysis of data

Manual counting determined the colony numbers, and from this, the mean values, standard deviations, and mutation rates were calculated. According to the established criteria of the laboratory, the test item was considered mutagenic if:

- A concentration-related increase in revertant colonies occurred; AND/OR
- A reproducible biologically relevant positive response for at least one dose group occurred in at least one strain with or without metabolic activation.

An increase was considered biologically relevant if:

- The number of reversions in strains *S. typhimurium* TA98 and/or TA100 and/or *E. coli* WP2 *uvrA* was at least 2-fold greater than the reversion rate of the negative vehicle-control AND/OR
- The number of reversions in strains *S. typhimurium* TA1535 and/or TA1537 was at least 3-fold greater than the reversion rate of the negative vehicle-control.

The test item was considered non-mutagenic if the criteria for a mutagenic response were not observed. Because biological relevance was the criterion applied for the interpretation of results, no statistical evaluation was conducted.

2.3. In vitro mammalian chromosomal aberration test

2.3.1. Test procedures

The clastogenic potential of CUF was assessed in accordance with OECD GLP (OECD, 1998a) and test guideline 473 (OECD, 2016a) utilizing the laboratory's SOPs and based on procedures described by Preston et al. (1981) and Brusick (Brusick (1989). Male Chinese hamster lung V79 cells (European Collection of Authenticated Cell Cultures; Salisbury, England) grown in supplemented Dulbecco's modified Eagle's (DME) medium (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA) were utilized as the test system.

The positive control without S9 metabolic activation was chosen as ethyl methanesulfonate (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA) because it is a known, widely used mutagen and clastogen according to the referred literature and the test facility's broad historical database; cyclophosphamide (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA) was chosen for use with S9 according to the cited guideline. DME was chosen as the negative control and vehicle for the test item and positive controls based on the results of a preliminary

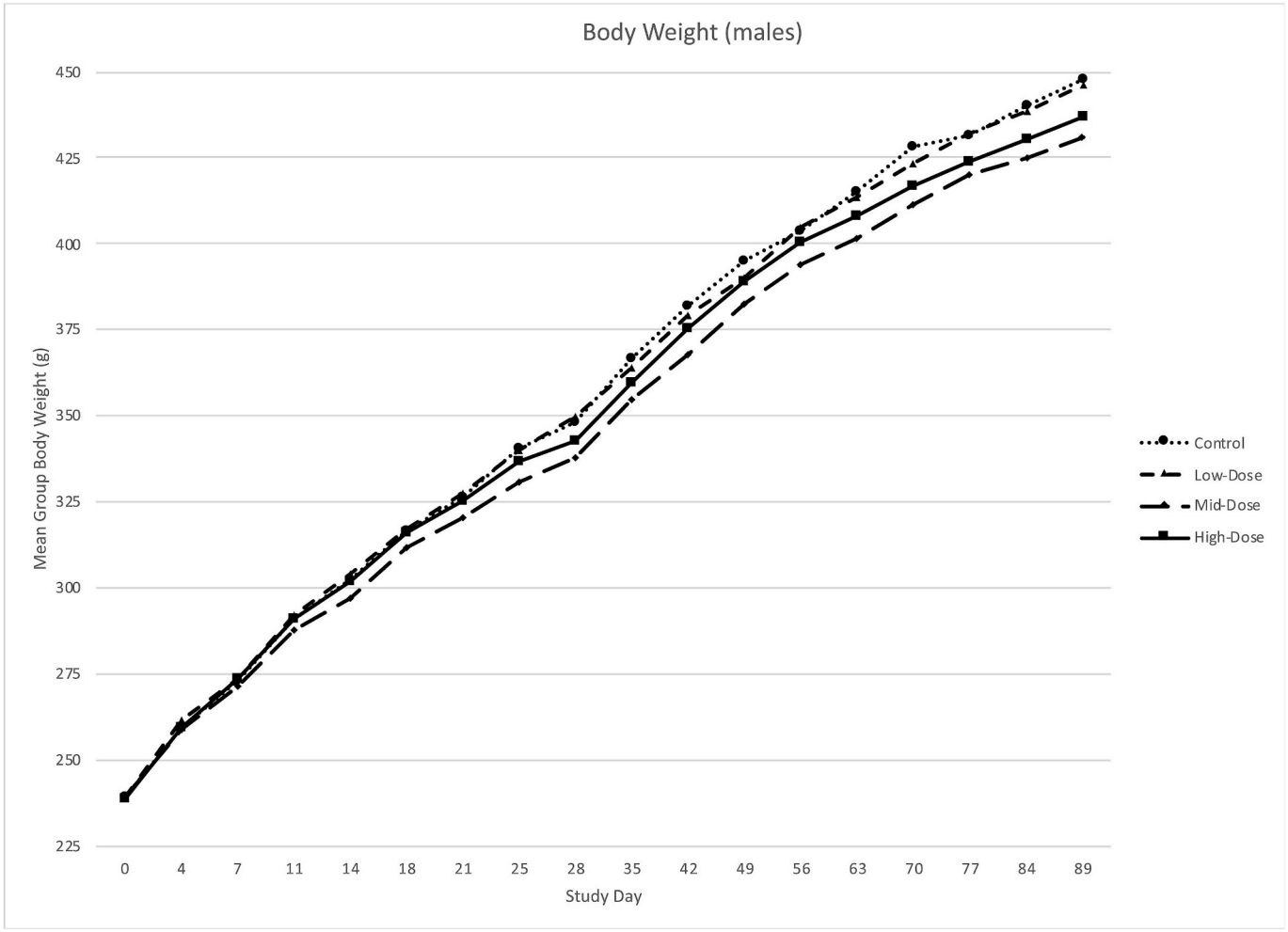

Fig. 1. Body weight (males).

solubility test and due to its compatibility with the survival of the V79 cells and the S9 activity, which was confirmed by the historical control database of the laboratory.

Test solution concentrations for the main chromosomal aberration assay were chosen based on a GLP preliminary cytotoxicity test, and the test solutions, positive controls, and S9 mix were freshly prepared directly prior to treatment of cells. In two independent experiments conducted in duplicate with concurrent positive and negative controls, at least 300 (150 per duplicate) well-spread metaphases were analyzed following exposure to test item concentrations of 1250, 2500, and 5000 µg/mL at treatment/sampling intervals of 3/20 h (Experiment A without and with S9-mix), 20/20 and 20/28 h (Experiment B without S9-mix), and 3/28 h (Experiment B with S9-mix).

2.3.2. Analysis of data

Statistical analyses were performed with SPSS PC + software (SPSS, Inc., Chicago, IL, USA). Fisher exact and CHI^2 tests were utilized to evaluate the number of aberrations and the number of cells with aberrations in the treatment and positive control groups compared to the concurrent negative control, as well as historical controls. A *P*-value of < 0.05 was considered statistically significant in both tests.

2.4. In vivo mammalian micronucleus test

2.4.1. Test procedures

The in vivo genotoxic potential of CUF was assessed in mouse bone marrow according to OECD GLP (OECD, 1998a) and test guideline 474 (OECD, 2016b) and procedures described by Salamone (Salamone and Heddle, 1983) and Heddle (Heddle et al., 1983). Specific pathogen free (SPF) male CRL:NMRI BR mice (Toxi-coop, Budapest, Hungary), 8 weeks old and weighing 30.7–39.3 g at the start of treatment were utilized in the study under the permission of the Institutional Animal Care and Use Committee (IACUC) of the laboratory. The animals were acclimatized and housed under environmental conditions in accordance with OECD guideline 474, and food (ssniff® SM R/M-Z + H complete diet for rats and mice, ssniff Spezialdiäten GmbH, Soest, Germany) and potable tap water were provided *ad libitum*.

A preliminary toxicity test was conducted in male and female mice. Based on these results, the main test was conducted in 5 groups of 5 male mice as follows: the test item was administered twice by gavage, at 24-h intervals, at doses of 0 (negative control), 500, 1000, and 2000 mg/kg bw, and the positive control, cyclophosphamide (Sigma-Aldrich (subsidiary of MERCK KGaA), Schnelldorf, Germany and St. Louis, MO USA), was administered once by intraperitoneal injection at 60 mg/kg bw. The negative control and vehicle for the test item was distilled water (Parma Product Kft., Budapest, Hungary), and the vehicle for the positive control was sterile water (NATURLAND Kft., Budapest, Hungary). The homogenous dosing solutions were freshly prepared on the day of the experiments and administered within 2 h at a constant dose volume of 10 mL/kg bw. Immediately after dosing and at regular intervals until euthanasia, all mice were examined for visible reactions to treatment. Bone marrow sampling (from femurs) was made 24 h after the second treatment, immediately following euthanasia (by cervical dislocation), for scoring of 4000 polychromatic erythrocytes (PCE) for micronucleated PCE (MPCE).

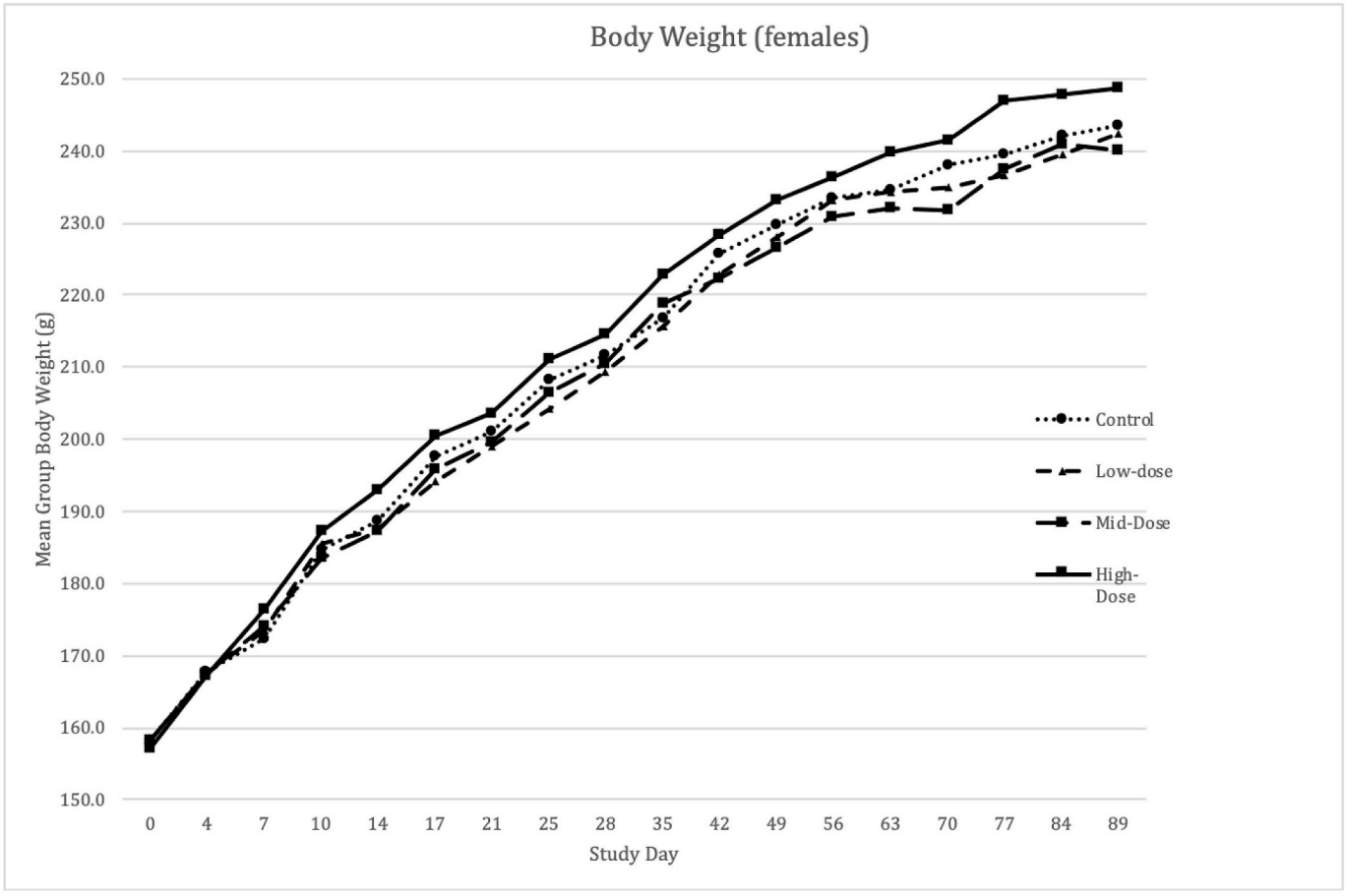

Fig. 2. Body weight (females).

Table 1
Body weight gain.

Group, mg/kg bw/day		Body weight gain (g) between days																	
	Days	0–4	4–7	7–11	11–14	14–18	18–21	21–25	25–28	28–35	35–42	42–49	49–56	56–63	63–70	70–77	77–84	84–89	0–89
Females																			
Control (n = 10)	Mean	9.6	4.5	12.3	4.2	8.7	3.5	7.3	3.5	5.0	8.9	4.1	3.7	1.2	3.5	1.3	2.6	1.3	85.2
	SD	3.1	2.5	2.7	3.6	3.1	3.2	2.1	2.3	3.7	4.1	2.8	3.2	4.8	3.1	3.7	3.8	5.5	17.1
1050 (n = 10)	Mean	9.0	6.2	11.9	2.2	6.6	4.8	5.0	5.4	6.3	7.1	5.1	5.3	1.0	0.5	1.9	2.8	3.0	84.1
	SD	3.2	3.6	2.4	3.3	3.1	5.6	3.0	2.9	2.1	4.2	4.3	4.7	3.9	3.5	2.6	2.7	3.4	14.9
2100 (n = 10)	Mean	9.1	6.7	9.4	3.8	8.4	3.7	7.0	4.1	8.2	3.4	4.5	4.2	1.1	−0.3	5.7	3.5	−0.8	81.7
	SD	3.3	2.8	2.5	3.1	2.6	3.8	4.0	3.5	2.4	3.1	4.0	2.7	2.4	3.5	3.0	1.8	2.7	13.2
	SS									*	**					**			
4200 (n = 10)	Mean	10.2	9.1	10.9	5.9	7.5	3.1	7.4	3.3	8.4	5.5	4.8	3.3	3.5	1.6	5.4	0.8	1.0	91.7
	SD	5.1	3.7	3.6	2.5	2.0	2.8	2.7	2.2	3.3	4.2	2.7	3.1	3.8	4.8	3.7	2.7	4.7	6.3
Test for Significance	SS		**							*						*			
Males																			
Control (n = 10)	Mean	20.2	13.5	18	11.7	13.9	9.8	14.6	7.4	18.4	15.5	12.9	8.7	11.4	13	3.6	8.6	7.3	208.5
	SD	3.9	3.4	4.8	2.3	2.8	1.6	2.4	3.1	2.4	4.0	3.1	4.9	2.8	3.2	3.6	2.5	3.4	13.7
1050 (n = 10)	Mean	22.3	12.2	18.6	12	13	10.3	12.5	9.6	14.2	15.3	10.9	14.5	8.7	9.8	8.9	6.7	7.5	207.0
	SD	4.8	3.3	3.1	3.2	2.4	3.5	3.6	3.2	4.4	3.4	4.8	5.1	3.7	5.8	5.8	4.4	2.8	24.2
	SS									*			**			**			
2100 (n = 10)	Mean	19.5	12.6	16.2	9.6	14.5	8.6	10.6	6.8	16.8	13.3	14.6	11.3	7.9	9.6	8.8	5.2	5.6	191.5
	SD	2.0	3.0	4.5	3.1	3.3	3.4	3.4	3.0	4.1	3.7	4.4	3.6	4.5	4.7	3.0	4.6	5.3	20.3
	SS							*								**			
4200 (n = 10)	Mean	20.5	14.7	17.1	11	13.9	9.4	11.4	6	16.8	16	13.8	11	7.8	8.7	7.3	6.7	6.5	198.6
	SD	4.9	3.3	4.8	2.8	3.2	2.9	4.2	2.5	3.5	4.1	3.3	3.2	4.1	2.9	3.1	3.3	2.2	25.8
Test for Significance	SS															*			

Abbreviations: DN, Duncan's multiple range test; NS, not significant; SD, standard deviation; SS, statistical significance.
*$p < 0.05$ and **$p < 0.01$.

Table 2
Mean food consumption.

Group, mg/kg bw/day		Daily mean food consumption (g/animal/day)												
	Days	0–7	7–14	14–21	21–28	28–35	35–42	42–49	49–56	56–63	63–70	70–77	77–84	84–89
	Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13
Females														
Control (n = 10)	Mean	17.5	17.9	18.3	17.9	18.1	17.8	17.4	17.1	16.2	16.2	16.1	15.8	14.6
	SD	1.69	1.94	1.91	1.92	1.74	1.74	1.56	1.25	1.41	1.38	1.53	1.40	1.81
1050 (n = 10)	Mean	17.5	17.6	17.7	17.2	17.8	17.5	17.0	17.3	16.0	15.7	15.7	15.4	14.0
	SD	1.65	1.91	1.78	1.68	1.72	1.92	1.60	2.05	1.83	2.06	1.80	1.88	2.05
2100 (n = 10)	Mean	16.3	16.8	17.4	16.8	17.5	16.4	16.0	15.9	15.0	14.9	15.3	14.8	13.1
	SD	1.15	1.47	1.59	1.62	2.16	1.92	1.94	1.63	1.74	1.72	1.51	1.48	1.38
4200 (n = 10)	Mean	17.1	18.1	18.1	17.2	17.9	16.9	16.8	16.5	15.3	15.5	15.8	15.1	15.6
	SD	1.65	2.02	2.13	1.99	2.36	1.67	2.22	2.42	2.80	2.97	2.98	2.70	6.03
Test for Significance		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Males														
Control (n = 10)	Mean	25.1	26.1	26.6	26.4	26.1	25.2	26.0	26.0	25.3	25.3	24.4	24.0	22.8
	SD	2.01	2.22	1.73	1.64	1.73	1.76	1.56	2.28	1.71	1.99	2.20	1.83	1.86
1050 (n = 10)	Mean	24.1	25.7	26.3	25.2	25.1	24.5	24.7	25.1	24.2	24.0	24.1	23.4	22.4
	SD	1.34	1.25	1.45	1.55	1.44	1.42	1.47	1.12	1.30	1.60	1.36	1.67	1.88
2100 (n = 10)	Mean	24.1	24.6	24.8	24.0	23.6	23.3	24.1	24.4	23.0	22.7	22.8	21.7	19.7
	SD	1.43	2.58	2.20	2.76	2.51	2.43	2.15	2.21	2.26	1.51	1.71	1.80	2.64
	SS				*	*				*	**		*	**
4200 (n = 10)	Mean	23.7	24.3	24.9	23.9	23.5	23.3	23.4	23.2	21.9	21.5	21.8	20.8	19.4
	SD	1.54	2.00	2.18	1.98	2.01	2.40	2.67	2.51	2.25	2.04	2.86	2.11	2.12
	SS				*	**		*	**	**	**	*	**	**
Test for Significance		NS	NS	NS	DN	DN	NS	DN	DN	DN	DN	DN	DN	DN

Abbreviations: DN, Duncan's multiple range test; NS, not significant;; SD, standard deviation; SS, statistical significane.
*p < 0.05.
**p < 0.01.

2.4.2. Analysis of data

Statistical analysis was performed with SPSS PC + software (SPSS, Inc., Chicago, IL, USA) using Kruskall Wallis non-parametric analysis of variance test for frequencies of MPCE in the test and positive control groups compared to the corresponding negative control group, as well as historical control values. A *P*-value of < 0.05 was considered statistically significant.

2.5. 90-Day repeated-dose oral toxicity study in rats

2.5.1. Test procedures

The 90-day repeated-dose oral toxicity study was conducted in general compliance with OECD GLP (OECD, 1998a) and in accordance with OECD test guideline 408 (OECD, 1998b). SPF Hsd.Han Wistar rats (Toxi-coop, Budapest, Hungary), seven to eight weeks old and weighing 231–255 g (males) and 144–172 g (females) were acclimatized and housed under environmental conditions in accordance with OECD guideline 408. Food (ssniff® SM R/M-Z + H complete diet for rats and mice, ssniff Spezialdiäten GmbH, Soest, Germany) and potable tap water were provided *ad libitum*. The study was conducted under the permission of the IACUC of the laboratory and in compliance with the National Research Council Guide for Care and Use of Laboratory Animals (NRC, 2011) and the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act, 1998 XXVIII) regulating animal protection.

Four groups of 10 rats/sex/group were administered the test item by gavage at doses of 0 (vehicle-control), 1050, 2100, and 4200 mg/kg bw/day in order to examine the potential health hazards and target organs, and to determine a NOAEL. The vehicle-control was distilled water (Parma Product Kft, Budapest, Hungary). Because no biomarkers have been validated for identification of this material, all dose formulations were prepared fresh, by careful weight measurement, each day just prior to administration and administered within four hours at a constant dosing volume of 14 mL/kg bw. Dose selection was based on data from an OECD 407 compliant 14-day repeated-dose oral toxicity study in Hsd.Han Wistar rats in which no mortalities or toxic effects were observed in any of the examined study parameters up to the high-dose of 4200 mg/kg bw/day (the maximum feasible dose due to solubility of the test item).

Following the cited OECD 408 guideline (as a minimum standard), all listed observations and examinations were conducted. Body weight gains and feed efficiency were calculated for each measurement interval and for the study overall. The functional observation battery (FOB) conducted during the final week of treatment utilized a modification of the method of Irwin (1968). Following an overnight fast after the last treatment, animals were placed under deep narcosis induced with Isofluran CP® anesthesia (Medicus Partner Kft, Biatorbágy, Hungary). Blood samples for clinical pathology examinations were collected from the retro-orbital venous plexus and the animals were sacrificed by exsanguination from the abdominal aorta. All animals were weighed prior to sacrifice for calculation of organ to body weight ratios at necropsy, and, following gross pathological examinations and organ weight measurements, tissues were preserved in 4% formaldehyde solution, except testes and epididymides, which were fixed in modified Davidson solution (prepared in the laboratory) and then stored in 4% formaldehyde solution (Reanal Gyógyszer és Finomvegyszergyár Zrt., Budapest, Hungary). The preserved and fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, and stained with hematoxylin and eosin (InnoLab Bt., Dunakeszi, Hungary) for full histopathological examination of the control and high-dose groups and any organs showing macroscopic changes at the necropsy in the mid- and

Table 3
Feed efficiency.

Group, mg/kg bw/day		Feed efficiency (g food/g bwg)													
	Days	0–7	7–14	14–21	21–28	28–35	35–42	42–49	49–56	56–63	63–70	70–77	77–84	84–89	0–89
	Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	1–13
Females	Mean	9.55	8.29	11.48	12.34	41.74	17.21	38.96	31.18	26.23	31.87	53.03	33.69	21.19	18.33
Control	SD	3.61	2.79	4.07	3.25	35.86	8.37	35.33	14.88	9.55	14.32	38.41	12.70	10.81	3.29
	n[a]	10	10	10	10	10	10	9	9	5	8	6	8	6	10
1050	Mean	9.45	9.81	14.52	14.07	21.47	35.40	31.33	27.69	27.80	54.09	38.36	39.20	28.62	18.09
	SD	4.50	3.83	11.64	8.37	6.05	39.52	38.58	12.96	8.14	42.19	16.49	34.69	28.17	3.11
	n[a]	10	10	10	10	10	10	8	9	5	4	7	7	8	10
2100	Mean	8.08	11.18	10.71	12.86	16.02	28.08	31.01	32.72	59.92	41.41	27.54	39.22	51.00	17.67
	SD	3.21	8.17	2.89	5.49	4.53	9.18	29.71	27.07	40.59	15.91	24.97	24.14	14.63	2.62
	n[a]	10	10	10	10	10	8	8	9	6	4	10	10	5	10
	SS					*								*	
4200	Mean	6.55	7.91	13.51	12.80	17.12	32.51	39.61	29.97	39.45	21.44	22.17	57.96	19.57	16.14
	SD	1.38	2.42	5.19	5.00	6.79	35.75	34.83	14.21	32.58	7.71	12.46	35.20	7.66	1.90
	n[a]	10	10	10	10	10	9	10	8	8	5	9	5	6	10
	SS	**				*						*			
Test for Significance		U	NS	NS	NS	U	NS	NS	NS	NS	NS	U	NS	U	NS
Males	Mean	5.29	6.45	7.94	8.64	10.04	12.08	14.96	21.97	16.56	14.64	65.19	20.82	29.11	10.85
Control	SD	0.59	1.86	1.05	1.66	1.20	3.17	4.18	9.90	5.14	4.80	46.46	5.10	20.07	0.63
	n[a]	10	10	10	10	10	10	10	9	10	10	9	10	10	10
1050	Mean	4.97	6.00	8.07	8.28	14.26	12.06	20.15	13.50	17.90	23.01	20.35	25.46	23.73	10.66
	SD	0.57	0.81	1.39	1.71	7.28	4.46	12.44	4.45	3.61	12.97	11.53	14.05	9.39	1.05
	n[a]	10	10	10	10	10	10	10	10	9	10	9	9	10	10
	SS											**			
2100	Mean	5.33	6.94	8.18	10.54	10.16	13.14	12.39	16.80	21.48	20.79	20.47	30.58	25.99	10.94
	SD	0.72	1.21	3.11	3.40	1.77	3.74	3.45	6.47	10.92	10.70	8.53	14.89	18.25	1.08
	n[a]	10	10	10	10	10	10	10	10	9	10	10	9	9	10
	SS											**			
4200	Mean	4.82	6.37	7.64	10.79	10.29	10.65	12.36	15.76	21.28	19.38	26.38	26.72	23.85	10.35
	SD	0.71	1.64	1.28	4.85	2.83	2.10	2.69	4.27	12.50	8.58	18.42	12.00	10.55	1.38
	n[a]	10	10	10	10	10	10	10	10	9	10	10	10	10	10
	SS											*			
Test for Significance		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	U	NS	NS	NS

Abbreviations: NS, not significant; SD, standard deviation; SS, statistical significance; U, Mann-Whitney *U* test versus control.
$^{*}P < 0.05$ and $^{**}P < 0.01$.
[a] Group 'n's were reduced for some group weeks due to individual animals either having no (0) or negative body weight gain.

low-dose groups.

2.5.2. Analysis of data

Male and female rats were evaluated separately, and statistical analyses were performed for body weight, food consumption, feed efficiency, clinical pathology, and organ weight using SPSS PC + software (SPSS, Inc., Chicago, IL, USA). Bartlett's homogeneity of variance test was used to assess heterogeneity of variance between groups and was followed by a one-way analysis of variance if no significant heterogeneity was predicted. Duncan's Multiple Range test was used to assess the significance of inter-group differences if a positive result was obtained. Where significant heterogeneity was found, the normal distribution of data was examined by Kolmogorov-Smirnov test. In case of a non-normal distribution, the non-parametric method of Kruskal-Wallis One-Way analysis of variance was used. If there was a positive result, the inter-group comparisons were performed using the Mann-Whitney *U* test. A *P*-value of < 0.05 was considered statistically significant in all tests. Frequencies of occurrence by sex and dose were calculated for clinical signs and ophthalmoscopic and gross and histopathological findings, but statistical analysis was not conducted for these non-quantitative observations.

3. Results

3.1. Bacterial reverse mutation test

No background inhibition and no concentration-related or biologically relevant increases in revertant colony numbers of any of the five tester strains were observed, at any concentration level, either in the presence or absence of the S9-mix (see Supplemental Tables 1 and 2).

3.2. In vitro mammalian chromosomal aberration test

No statistically significant differences or dose-response relationships were observed between treatment and concurrent or historical solvent controls, and no biologically significant increases in structural chromosome aberration frequencies were observed up to the maximum concentration of 5000 µg/mL under the conditions of Experiments A or B (see Supplemental Table 3). No biologically relevant increases in the rate of polyploid or endoreduplicated metaphases occurred in either experiment under the applied conditions.

3.3. In vivo mammalian micronucleus test

No adverse reactions to treatment were observed nor were there any mortalities during 48 h of observation over the course of two administrations of the test item at doses of 0 (control), 500, 1000, and 2000 mg/kg bw.

No biologically or statistically significant increases in the frequency of MPCEs were seen in any treatment group compared to the concurrent negative control. The mean frequencies of MPCEs of the concurrent negative control and groups of mice treated with the test item were statistically significant with respect to historical control means, but remained within the 95% confidence interval of the historical controls (see Supplemental Table 4). The proportion of immature among total erythrocytes in the 500 and 1000 mg/kg bw groups compared to the

Table 4
Clinical chemistry.

Group, mg/kg bw/day		ALT	AST	ALP	TBIL	CREA	UREA	GLUC	CHOL	Pi	Ca++	Na+	K+	Cl−	ALB	TPROT	A/G
		[U/L]	[U/L]	[U/L]	[μmol/L]	[μmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[mmol/L]	[g/L]	[g/L]	
Female																	
Control	Mean	56.80	90.36	42.10	1.79	24.37	6.66	6.41	2.45	1.63	2.66	142.30	4.00	105.53	38.16	64.23	1.47
(n = 10)	SD	7.01	7.94	14.34	0.28	2.50	1.07	0.70	0.71	0.16	0.12	0.82	0.22	1.26	2.04	4.59	0.09
1050 (n = 10)	Mean	57.53	86.06	41.60	1.69	23.48	6.09	6.54	2.24	1.51	2.64	141.80	3.99	104.96	38.86	65.86	1.45
	SD	11.35	12.68	13.36	0.38	2.08	0.80	0.64	0.46	0.23	0.03	0.92	0.14	0.70	1.98	4.62	0.12
2100 (n = 10)	Mean	52.87	91.32	37.70	1.30	27.30	6.73	5.84	2.12	1.40	2.58	142.10	4.07	105.99	36.71	63.39	1.37
	SD	8.33	15.33	6.77	0.20	2.20	0.35	0.53	0.51	0.27	0.08	0.88	0.32	0.78	1.37	2.08	0.09
	SS				**	**		*		*							*
4200 (n = 10)	Mean	49.94	87.09	40.50	1.59	24.53	6.29	5.73	2.65	1.65	2.65	141.80	4.02	105.68	38.26	66.75	1.34
	SD	8.07	12.63	11.93	0.23	2.28	0.50	0.57	0.38	0.13	0.05	1.14	0.37	1.45	1.26	2.94	0.10
	SS							*									*
Test for significance		NS	NS	NS	DN	DN	NS	DN	NS	DN	NS	NS	NS	NS	NS	NS	DN
Historical Control Range		23.4–87.7	71.0–141.8	25.0–126	1.23–3.30	25.6–40.8	4.25–8.50	4.44–7.55	1.35–3.39	0.93–2.01	2.51–2.77	135–148	3.42–4.35	103.3–109.6	33.2–41.0	57.1–74.5	1.1–1.5
Males																	
Control (n = 10)	Mean	55.15	94.93	89.90	1.35	22.52	6.29	6.61	2.21	1.91	2.63	142.50	4.51	104.67	34.76	62.54	1.26
	SD	7.18	9.84	18.99	0.32	2.22	1.03	0.49	0.42	0.17	0.06	0.71	0.19	0.89	0.70	2.16	0.08
1050 (n = 10)	Mean	56.96	91.65	89.10	1.54	26.02	6.92	6.49	2.01	1.83	2.63	142.50	4.27	105.24	35.03	62.93	1.27
	SD	9.86	12.85	10.45	0.31	2.46	0.81	0.47	0.35	0.29	0.06	0.97	0.14	0.58	0.85	2.81	0.12
	SS					**							**				
2100 (n = 10)	Mean	58.62	104.98	89.70	1.42	22.84	6.13	6.44	2.01	1.79	2.57	141.80	4.34	104.73	35.44	63.73	1.26
	SD	10.67	16.35	25.89	0.22	1.70	0.80	0.54	0.42	0.18	0.07	0.79	0.21	0.54	1.06	2.09	0.05
	SS										*		*				
4200 (n = 10)	Mean	58.30	97.55	85.60	1.52	23.14	6.63	6.46	2.16	1.76	2.61	142.10	4.36	105.16	35.53	63.68	1.27
	SD	16.53	13.65	19.18	0.42	2.52	0.84	0.39	0.47	0.12	0.05	0.57	0.12	0.84	1.25	2.55	0.12
	SS												*				
Test for significance		NS	NS	NS	NS	DN	NS	NS	NS	NS	DN	NS	DN	NS	NS	NS	NS
Historical Control Range		28.0–86.2	67.9–135.7	56–184	0.71–2.79	18.5–37.2	3.60–9.26	4.58–8.24	1.27–2.44	1.40–2.41	2.40–2.80	140–146	3.84–5.04	102.7–106.9	33.0–37.4	56.8–68.0	1.0–1.4

Abbreviations: DN, Duncan's multiple range test; NS, not significant; SD, standard deviation; SS, statistical significance.
*$p < 0.05$.
**$p < 0.01$.

Table 5
Absolute organ weights (females).

Group, mg/kg bw/day		Organ weight (g)									
		Body weight	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Uterus	Ovaries	Adrenal glands
Control	Mean	233.0	1.96	6.22	1.58	0.73	0.35	0.52	1.06	0.119	0.084
	SD	20.04	0.09	0.63	0.15	0.06	0.08	0.06	0.46	0.019	0.013
1050 (n = 10)	Mean	230.8	1.94	6.44	1.59	0.73	0.31	0.45	0.76	0.115	0.081
	SD	13.81	0.09	0.68	0.14	0.07	0.06	0.06	0.07	0.033	0.012
	SS							*	*		
2100 (n = 10)	Mean	230.1	1.93	5.96	1.49	0.70	0.36	0.44	0.65	0.125	0.079
	SD	14.51	0.07	0.49	0.11	0.06	0.04	0.06	0.14	0.030	0.009
	SS							**	*		
4200 (n = 10)	Mean	237.7	1.91	7.17	1.70	0.75	0.35	0.49	0.98	0.144	0.090
	SD	7.48	0.06	0.73	0.11	0.04	0.07	0.06	0.23	0.028	0.016
	SS			**							
Test for Significance		NS	NS	DN	NS	NS	NS	DN	U	NS	NS
Historical Control Range		206–285	1.75–2.18	5.30–7.97	1.23–2.00	0.66–0.96	0.24–0.54	0.33–0.75	0.40–2.07	0.098–0.208	0.063–0.113

Abbreviations: DN, Duncan's multiple range test; NS, not significant; SD, standard deviation; SS, statistical significance; U, Mann-Whitney U - test versus control.
*p < 0.05.
**p < 0.01.

Table 6
Relative organ weights (females).

Organ weight relative to body weight (%)											
Group, mg/kg bw/day		Body weight	Brain	Liver	Kidneys	Heart	Thymus	Spleen	Uterus	Ovaries	Adrenal glands
Control	Mean	233.0	0.845	2.675	0.677	0.313	0.150	0.223	0.454	0.0518	0.0359
	SD	20.04	0.049	0.246	0.031	0.024	0.038	0.022	0.188	0.0104	0.0048
1050 (n = 10)	Mean	230.8	0.841	2.794	0.690	0.317	0.134	0.196	0.332	0.0502	0.0350
	SD	13.81	0.022	0.263	0.039	0.020	0.023	0.022	0.034	0.0144	0.0042
	SS							*			
2100 (n = 10)	Mean	230.1	0.842	2.593	0.648	0.305	0.157	0.191	0.285	0.0544	0.0346
	SD	14.51	0.053	0.175	0.043	0.015	0.017	0.021	0.066	0.0128	0.0045
	SS							**	*		
4200 (n = 10)	Mean	237.7	0.805	3.018	0.714	0.317	0.149	0.205	0.413	0.0607	0.0377
	SD	7.48	0.037	0.307	0.047	0.020	0.026	0.024	0.102	0.0117	0.0062
	SS			**							
Test for Significance		NS	NS	DN	NS	NS	NS	DN	U	NS	NS
Historical Control Range		206–285	0.681–0.943	2.172–3.214	0.530–0.752	0.273–0.396	0.093–0.217	0.140–0.298	0.167–0.852	0.043–0.086	0.026–0.044

Abbreviations: DN, Duncan's multiple range test; NS, not significant; SD, standard deviation; SS, statistical significance; U, Mann-Whitney U - test versus control.
*p < 0.05.
**p < 0.01.

control group and was similar but was slightly decreased, without biological or statistical significance, in the 2000 mg/kg bw group.

3.4. 90-Day repeated dose oral toxicity study in rats

3.4.1. Clinical observations and ophthalmology

There were no mortalities in any group, and no differences were observed between the test item groups and controls during daily cage-side observations, weekly detailed clinical observations, or the FOB with the exception of one mid-dose male animal with brownish-red hairs around the right eye on days 5–21. There were no changes observed on ophthalmologic examination for any treatment or control groups.

3.4.2. Body weights and food consumption

Mean body weights were similar in the control and treatment groups (male and female) throughout the study (see Figs. 1 and 2) with only a few minor and transient statistically significant increases and decreases in mean body weight gains observed with respect to control in the treated male groups and the mid- and high-dose female groups (see Table 1). The mean daily food consumption for males in the 1050 mg/kg bw/d group and all female groups were similar to controls during the entire observation period. Beginning on week 4 and continuing the majority of weeks thereafter, slight, but statistically significant lower mean food consumption was observed for males in the 2100 and 4200 mg/kg bw/day groups (see Table 2). Some statistically significant transient changes in feed efficiency were observed in both sexes during the study while overall feed efficiency was similar to controls among the dose groups and sexes (see Table 3).

3.4.3. Clinical pathology

Statistically significant differences in hematology parameters relative to controls were noted in all male test groups for slightly lower mean percentage of white blood cells. In females, the only statistically significant difference was in the high-dose group for which the mean percentage of monocytes was slightly higher than the control group (see Supplemental Table 5). All observed hematological alterations remained within the historical control ranges of the laboratory. There

Table 7
Relative organ weights (females).

Organ weight and body weight relative to brain weight (%)										
Group, mg/kg bw/day		Body weight	Liver	Kidneys	Heart	Thymus	Spleen	Uterus	Ovaries	Adrenal glands
Control	Mean	11876.3	317.44	80.44	37.05	17.71	26.44	53.97	6.10	4.27
	SD	696.13	32.50	6.64	2.62	4.07	2.50	22.72	1.06	0.65
1050 (n = 10)	Mean	11893.2	332.30	82.12	37.71	15.93	23.30	39.44	5.96	4.16
	SD	302.18	32.84	5.52	2.71	2.84	2.75	3.66	1.68	0.52
	SS						*	*		
2100 (n = 10)	Mean	11918.1	308.66	77.05	36.34	18.65	22.76	33.86	6.49	4.11
	SD	783.81	24.45	5.37	3.06	1.68	2.99	7.93	1.60	0.42
	SS						*	*		
4200 (n = 10)	Mean	12450.0	375.70	88.89	39.39	18.56	25.46	51.36	7.57	4.70
	SD	581.32	41.55	6.48	2.63	3.59	3.08	12.57	1.52	0.91
	SS		**	**					*	
Test for Significance		NS	DN	DN	NS	NS	DN	U	DN	NS
Historical Control Range		10600.0–14685.7	276.04–412.95	65.08–104.71	33.65–49.74	11.82–27.55	17.84–38.34	18.96–99.52	5.19–10.79	3.12–5.49

Abbreviations: DN, Duncan's multiple range test; NS, not significant; SD, standard deviation; SS, statistical significance; U, Mann-Whitney U - test versus control.
*$p < 0.05$.
**$p < 0.01$.

Table 8
Histopathology findings.

Organs	Group, mg/kg bw/day	Control (n = 10)	1050 (N/A)	2100 (N/A)	4200 (n = 10)
	Observations	(Incidence of observations per group)			
Females					
	Animals without microscopic findings	4/10	N/A	N/A	1/10
Liver	Focal fibrosis on the Glisson capsule	0/10	/	1†/1	0/10
Lungs	Alveolar emphysema	0/10	/	/	1•/10
	Hyperplasia of BALT	1/10	/	/	2†/10
Ovaries	Cyst	0/10	/	1/1	0/10
Uterus	Dilatation	5/10	/	/	7/10
Males					
	Animals without microscopic findings	8/10	N/A	N/A	7/10
Epididymides	Lack of mature spermatocytes	0/10	/	/	1/10
Kidneys	Pyelectasia	0/10	1/1	0/1	1/10
	Cyst	0/10	0/1	1/1	0/10
Lungs	Alveolar emphysema	1•/10	/	/	1•/10
	Hyperplasia of BALT	2†/10	/	/	0/10
Testes	Decreased intensity of spermatogenesis	0/10	/	/	1/10

Incidence = Number of animals with observations/number of animals examined.
/Not examined.
• minimal severity; † mild severity.
Abbreviation: BALT, Bronchus associated lymphoid tissue; N/A, not applicable.

were also several statistically significant results, all of which remained within the historical control ranges, in the clinical chemistry parameters of both sexes as compared to controls as shown in Table 4.

3.4.4. Gross pathology and organ weights

A slight right-sided dilation, due to fluid accumulation, of the renal pelvis was observed in one low-dose and one high-dose male at necropsy. A renal cyst was observed in one mid-dose male and smaller than normal testes and epididymides were observed in a single high-dose male. In the female groups, hydrometra of slight, moderate, or marked degree was observed in 5, 4, 1, and 7 of 10 animals of the control, low-, mid-, and high-dose groups, respectively. A diaphragmatic hernia and an ovarian cyst were observed in a single mid-dose female.

The only statistically significant differences observed in organ weights of males with respect to the control were for the thymus with slightly lower, dose-related absolute mean thymus weights observed in the mid- and high-dose groups (0.36 ± 0.10 and 0.34 ± 0.60 g, respectively, versus 0.45 ± 0.07 g; $p < 0.05$) and slightly lower thymus relative to both body (0.082 ± 0.01% versus 0.104 ± 0.02%; $p < 0.05$) and brain (16.01 ± 2.69% versus 21.37 ± 4.04%; $p < 0.05$) weights observed in the high-dose group (see Supplemental Table 6). In females, statistically significant changes were observed in absolute and relative (to body and brain weights) liver, spleen, and uterine weights and in kidney and ovary weights relative to brain weights (see Tables 5–7).

3.4.5. Histopathology

A few microscopic changes were observed in the kidneys, lungs, testes, and epididymides of male animals and liver, lungs, and uteri of female animals. These changes are presented in Table 8.

4. Discussion and conclusions

The battery of genetic toxicity studies conducted as part of the current work did not raise concerns of genotoxic potential of CUF. All

validity and acceptability criteria of the conducted tests were fulfilled, and all results were unequivocally negative in accordance with cited guidelines and/or laboratory criteria. The test item did not exhibit mutagenic activity by base pair changes or frameshifts in the genomes of the strains used in the bacterial reverse mutation test and was judged to lack clastogenic activity in Chinese hamster lung cells in the in vitro mammalian chromosomal aberration test. In the in vivo micronucleus test, no genotoxic activity of CUF was observed in PCEs obtained from bone marrow of mice treated up to the limit dose.

In the 90-day toxicological evaluation of colostrum in rats by Davis et al., the high-dose was set at 10-fold the rat equivalent of the highest reported human dose in studies (60 g per day) in order to attempt to induce some toxicity while the lower dose was set to represent a typical human consumption level. The study included biomarkers (bone mineral density and content, IGF-1) to assess potential effects of colostrum on growth. The lack of toxicity from this study provides evidence that colostrum is unlikely to cause adverse effects (Davis et al., 2007). Therefore, to further investigate the oral toxicity of CUF specifically, we conducted the current 90-day study based on the results of an unpublished 14-day repeated-dose range-finding study in rats, in which the highest dose tested (4200 mg/kg bw/day) did not produce toxic effects.

In the current work, the brownish red hairs observed around the right eye of one male rat in the mid-dose group were not considered toxicologically relevant as this finding was present in only one mid-dose animal, is a common observation in experimental rats of this strain and age, and is indicative of over production of porphyrin secretions in the Harderian gland (dos Reis et al., 2005). The statistically significant increases and decreases in mean body weight gains and mean feed efficiency observed in the various dose groups/sex during the study were not considered to be test item-related due to their minimal change from control and transient and sporadic occurrence without overall effects on mean body weight or body weight development. The statistically significant decreases in mean food consumption in mid- and high-dose males were also of minimal change from control without overall effects on mean body weight or body weight development and, therefore, were not considered toxicologically relevant.

While the statistically significant decreases in the albumin/globulin ratio of female animals appeared dose-related, there were no dose-related alterations in albumin or globulin. In addition, the decreases were within historical ranges and without clinical correlates or biological relevance, as were the statistically significant decreases in glucose. The remaining statistically significant alterations in clinical pathology parameters were considered unrelated to administration of the test item and without toxicological or biological relevance due to absence of any discernible dose relationships, their occurrence in only one sex (with the exception of increased creatinine in low-dose males and mid-dose females), the low magnitudes of the changes (remaining well within the historical control ranges of the laboratory), and the absence of correlating histopathology.

The statistically significant decreases in absolute and relative thymus weights observed in male animals, while dose-related, remained well within historical control ranges and lacked correlating histopathology; therefore, they were not considered to have any toxicological relevance. The statistically significant alterations in absolute and relative organ weights observed in the female groups lacked dose relationships or correlating histopathology and were within historical control ranges and, thus, were considered sporadic occurrences unrelated to administration of the test item and without toxicological relevance. The microscopic findings observed in this study were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and, therefore, were considered unrelated to administration of CUF.

In conclusion, the genetic toxicological studies provide evidence that CUF does not possess mutagenic, clastogenic, or in vivo genotoxic potentials under the applied test systems up to the maximum recommended test concentrations or limit dose, respectively, nor does it cause mortality in oral toxicology studies. No adverse effects were seen, and no target organs were identified in male or female Hsd.Han: Wistar rats after 90 days of oral administration of CUF at doses of 1050, 2100, or 4200 mg/kg bw/day. Based on the observations in this 90-day study, the NOAEL was determined to be 4200 mg/kg bw/day.

Declaration of conflicting interest

AIBMR Life Sciences, Inc. (Seattle, WA, USA) was contracted by the study sponsor, as an independent third party, to determine appropriate study protocols and dose selections, place the studies, approve the study plans, and monitor the toxicological studies herein described and to analyze and interpret the resulting data and prepare the manuscript. Toxi-Coop Zrt., with test facilities in Budapest, Hungary, was contracted by AIBMR to develop the study plans and conduct, analyze, interpret, and report the results of the toxicological studies herein described. The authors declare no additional conflicts of interest in regard to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

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Transparency document

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