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Safety evaluation of whey derived beta-lactoglobulin, Lacprodan® BLG

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ABSTRACT

The safety of Lacprodan® BLG, a whey-based protein, was evaluated with respect to genotoxicity and sub-chronic toxicity according to regulatory requirements. Lacprodan® BLG did not show any mutagenic potential in a bacterial reverse mutation assay or any clastogenic or aneugenic potential in an *in vitro* micronucleus assay performed in human lymphocytes. In a sub-chronic toxicity study, groups of 10 male and 10 female Wistar rats received the test item orally by gavage for 90 days at dose levels of 100, 300 and 1000 mg/kg bw/day. A control group, also including 10 male and 10 female rats, received sterile water, as vehicle. No treatment-related clinical observations or toxicological effects on body or organ weights, food consumption, ophthalmic effects, hematology, clinical chemistry, fertility, urinalysis, or pathology were identified. Therefore, the no-observed-adverse-effect level (NOAEL) for Lacprodan® BLG in the 90-day toxicity study was established as 1000 mg/kg bw/day, corresponding to the highest dose level administered.

1. Introduction

Whey proteins have long been recognized as a protein source of superior nutritional quality in terms of digestibility and bioavailability [1]. Whey proteins are known to contain various biologically active components, *e.g.* essential amino acids and bioactive peptides, which can potentially benefit human health. These components may affect diverse biological processes such as muscle metabolism, bone growth, antimicrobial activity, and immune regulation [2,3].

Beta-lactoglobulin (BLG) is the most abundant whey protein in bovine milk, accounting for more than 50 % of the total whey protein [2,3]. Bovine BLG is a relatively small protein of about 162 amino acid residues in a single peptide chain with a molecular weight of approximately 18 kDa.

Because of its effects on various biological processes, BLG has been widely studied in the food industry. BLG has a high content of branched-chain amino acids, particularly leucine, known to be important in stimulation of muscle protein synthesis, and is therefore of relevance to athletes and for maintenance of muscle mass during aging and disease [4–7]. Among many other physiological effects suggested in the literature, BLG may also play a role in the transport and accumulation of lipid-soluble biological components such as fatty acids and retinoids [8].

Lacprodan® BLG is a protein fraction isolated from bovine whey that contains more than 90 % pure BLG. The protein is produced from sweet whey, the by-product from raw milk used to produce cheese, utilizing commonly known processes of the dairy industry. Lacprodan® BLG is intended for use in both medical nutrition and food, especially sports nutrition. These consumer groups currently consume large amounts of whey protein isolates because of the high content of essential amino acids and, in particular, branched-chain amino acids.

In medical nutrition, dietary supplementation is commonly used for patients who are unable to meet normal dietary requirements and/or may have specific nutritional needs. Supplementation of BLG to patients may in particular help prevent muscle loss and improve recovery after illness. In sports nutrition, protein supplementation is commonly used post-exercise to improve endurance, increase muscle mass gains, and speed up recovery after training.

The safety of a novel food such as a whey protein must be evaluated according to regulatory requirements. Therefore, the aim of the present study was to evaluate the safety of Lacprodan® BLG in terms of genotoxic effects as well as sub-chronic toxicity. The potential mutagenic and clastogenic effects of Lacprodan® BLG were addressed using the bacterial reverse mutation assay and the *in vitro* micronucleus assay. In addition, a 13-week sub-chronic rodent toxicity study was conducted to

Abbreviations: BLG, beta-lactoglobulin; bw, body weight; GLP, good laboratory practice; NOAEL, no-observed-adverse-effect level.

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explore the general toxicity of Lacprodan® BLG.

2. Materials and methods

2.1. Test item

The test item Lacprodan® BLG (Batch J44DIMNI005), is a white to yellow pale powder produced from bovine milk by Arla Foods Ingredients P/S Group, Viby J, Denmark. Lacprodan® BLG is produced from a whey protein isolate solution by crystallisation of BLG and separation of BLG crystals from this solution. BLG is subsequently spray-dried into a powder.

The total protein content of Lacprodan® BLG is minimum 86 % using the nitrogen factor of 6.38. The pure BLG content is more than 90 % of the total protein. Furthermore, the rest of Lacprodan® BLG consists mainly of ash (maximum 5 %), moisture (maximum 5.5 %), and fat and lactose (maximum 1 %). The batch used for the toxicity studies (J44DIMNI005) was in compliance with these specifications.

For the *in vitro* studies, Lacprodan® BLG was dissolved and diluted in distilled water for the bacterial reverse mutation assay and in cell culture medium for the mammalian micronucleus assay, respectively. For the animal studies, Lacprodan® BLG was dissolved in sterile water and given to the animals by oral gavage. Prior to the animal treatment period, the samples were analyzed for stability. Based on the results of stability testing, Lacprodan® BLG in water is considered stable at storage for up to 11 days at 2–8 °C (recovery after storage did not differ from start value by more than 15 %).

2.2. Chemicals

Sodium azide (NaN_3) (CAS No. 26628-22-8), methylmethanesulfonate (MMS) (CAS No. 66-27-3), 2-aminoanthracene (2-AA) (CAS No. 613-13-8), cyclophosphamide (CPA) (CAS No. 50-18-0), and colchicine (CAS No. 64-86-8) were obtained from Sigma-Aldrich. 4-nitro-o-phenylene-diamine (4-NOPD) (CAS No. 99-56-9) was from Fluka. These chemicals were used as positive controls in the genotoxicity studies.

2.3. Bacterial strains

S. typhimurium test strains TA98, TA1535 and E. coli WP2 uvrA from MOLTOX Inc. (NC, USA) and *S. typhimurium* TA100 and TA1535 from Xenometrix AG (Switzerland) were used for the bacterial reverse mutation assay. They were stored as stock cultures in ampoules with nutrient broth (OXOID) supplemented with approximately 8% v/v dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.4. Human lymphocytes

Human peripheral blood lymphocytes from healthy non-smoking donors were used for the *in vitro* micronucleus assay. In order to reduce inter-individual variability, blood was collected only from a single donor. Blood samples were drawn by venous puncture, collected in heparinized tubes and stored under sterile conditions at 4 °C for a maximum of 4 h prior to use. Whole blood samples were cultured in RPMI 1640 medium supplemented with 15 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2.4 µg/mL phytohemagglutinin (PHA).

2.5. Animals

Male and female Wistar rats, strain Crl:WI(Han), were from Charles River (Sulzfeld, Germany). Prior to treatment, they were acclimated for five days. The rats were kept in groups of five animals in polysulfone cages on Altromin saw fibre bedding at controlled temperature 22 ± 3 °C and humidity 55 ± 10 % with a 12-h light:12-h dark cycle. They were given a standard diet (Altromin 1324) and tap water *ad libitum*. The animals were derived from a controlled full-barrier maintained breeding system and were bred for experimental purposes according to the German Act on Animal Welfare. The experiment was performed in an AAALAC-accredited laboratory (Eurofins BioPharma) and was reviewed and accepted according to German animal protection law. Furthermore, the study was authorized by the Bavarian animal welfare administration. Procedures and facilities in the test laboratory comply with the requirements of Directive 2010/63/EU.

2.6. Bacterial reverse mutation assay

The bacterial reverse mutation assay was performed at Eurofins BioPharma (Munich, Germany) in accordance with Good Laboratory Practice (GLP) and OECD Test Guideline 471, "Bacterial Reverse Mutation Test", adopted 21 July 1997 [9]. The assay was conducted in two independent experiments carried out in *S. typhimurium* strain TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 uvrA using the standard plate incorporation (Experiment I) and the pre-incubation (Experiment II) methods, respectively. Several different concentrations of the test item, appropriate positive controls and distilled water as negative control were tested in triplicate with and without a metabolic activation system (S9 mix). Based on a pre-experiment to evaluate cytotoxicity and according to the OECD guideline, 5000 µg/plate of Lacprodan® BLG was selected as the maximum concentration. The test item was then tested at 31.6, 100, 316, 2500 and 5000 µg/plate. The positive controls, when testing without S9 mix, were 4-NOPD for *S. typhimurium* TA98 and TA1537, NaN_3 for *S. typhimurium* TA100 and TA1535, and MMS for *E. coli* WP2 uvrA. In the presence of S9 mix, 2-AA was used as positive control for all strains of bacteria.

The bacterial strains were cultured for 12 h at 37 °C in either nutrient broth medium or Luria Bertani medium, to the late exponential or early stationary phase of growth (approximately 1×10^9 cells/mL). For the plate incorporation method, 100 µL test solution, 500 µL S9 mix or phosphate-buffer, 100 µL bacterial suspension, and 2000 µL top agar, containing L-histidine and biotin for *S. typhimurium* and tryptophan for *E. coli*, were mixed and poured onto a minimal agar plate. For the pre-incubation method, 100 µL of the test solution was pre-incubated with the tester strains (100 µL) and S9 mix or phosphate-buffer (500 µL) for 60 min at 37 °C. After adding the top agar (2000 µL), the mix was poured onto a minimal agar plate. After solidification, the plates were incubated at 37 °C for at least 48 h prior to counting.

For the counting of colonies, a ProtoCOL colony counter (Meintrup DWS Laborgeräte GmbH, Germany) was used. Bacterial strains with a low spontaneous mutation frequency like TA1535 and TA1537 were counted manually. The test item was considered to be mutagenic if there was a clear and dose-related increase in the number of revertants, and/or a biologically relevant increase in at least one of the dose groups in at least one strain with or without S9 mix. A biologically relevant increase required that the number of revertants were at least twice as high for TA98, TA100 and WP2 uvrA, or at least three times higher for TA1535 and TA1537 when compared to the control.

2.7. In vitro micronucleus assay using human lymphocytes

The micronucleus assay was performed at Eurofins BioPharma (Munich, Germany) according to GLP and OECD Test Guideline 487, "*In Vitro* Mammalian Cell Micronucleus Test'', adopted 29 July, 2016 [10]. The assay was designed to assess the potential of Lacprodan® BLG to induce micronuclei in cultured human peripheral blood lymphocytes. A pre-experiment (4 h incubation) was carried out in concentrations ranging from 3.9 mg/mL up to 2000 mg/mL with and without S9 mix to establish the toxicity and appropriate test concentrations for the main study. The cytokinesis block proliferation index (CBPI) was used for the evaluation of cytotoxicity:

$$CBPI = ((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3)) / n$$

C_1: mononucleate cells; C_2: binucleate cells; C_x: multinucleate cells; n: total number of cells

In the main micronucleus test, two experiments were performed, one with short-term exposure of 4 h (experiment I) and one with long-term exposure of 44 h (experiment II). Based on the pre-experiment and according to the OECD guideline, 2000 mg/mL was selected as the highest concentration. Each concentration was tested in duplicate. The concentrations used in experiment I and II were 125, 250, 500, 1000, 1500 and 2000 μg/mL. MMS and CPA were used as positive controls in the absence of S9 mix, and culture medium as negative control.

In experiment I, lymphocytes stimulated with PHA, were treated with the test item for 4 h with and without S9 mix, then washed twice with PBS + 10 % FBS and incubated in complete culture medium with 6 μg/mL cytochalasin B for 40 h–42 h at 37 °C and 5% CO_2. At the end of incubation, the medium was removed, and the cells treated with a hypotonic solution (0.075 M KCl) and centrifuged. The cells were resuspended in a 1:1 fixation:0.9 % NaCl solution, centrifuged and then fixed with a 3:1 methanol:glacial acetic acid solution. Subsequently, the cell suspension was placed on microscope slides and stained with acridine orange solution for microscopic analysis.

In experiment II, PHA-stimulated lymphocytes were exposed to the test item in complete culture medium without S9 mix for one hour prior to the addition of 6 μg/mL cytochalasin B and further incubation for 43 h at 37 °C. At the end of treatment, the medium was removed and the cells prepared for microscopic analysis in the same way as in experiment I.

For microscopic analysis, the following concentrations were selected for experiment I: 1000, 1500 and 2000 μg/mL (without S9 mix) and 500, 1000, 1500 and 2000 μg/mL (with S9 mix). For experiment II, microscopic analysis was conducted on the 1000, 1500 and 2000 μg/mL concentrations (without S9 mix). At least 2000 binucleated cells per concentration were analyzed for micronuclei according to the criteria of Fenech [11].

The test item was considered to be positive if there was a statistically significant, dose-dependent increase in micronucleated cells that exceeded historical control limits.

2.8. Repeated dose toxicity: Preliminary fourteen-day study in rats

The preliminary study was performed at BSL Bioservice (Munich, Germany) and followed the procedures as indicated in OECD Test Guideline 407, "Repeated Dose 28-Day Oral Toxicity Study in Rodents", adopted 16 October 2008 [12]. The study was conducted as a dose range-finding study to provide information for the 90-day toxicity study in the rat. Test item formulations were prepared (using water as a vehicle) freshly on each day of administration. Groups of 3 male and 3 female Wistar rats, strain Crl:WI(Han) received the test item by oral gavage for 14 days at dose levels of 100, 300 and 1000 mg/kg bw per day at an application volume of 5 mL/kg bw (referred to as low-, medium- and high-dose groups, respectively). A control group received sterile water, the vehicle used in the study, at the same application volume. The animals were 7–8 weeks old at start of the treatment period corresponding to a body weight of 185−238 g for the males and 153−176 g for the females.

Clinical signs, body weight and food consumption were monitored during the study. At the end of the treatment period, all animals were subjected to necropsy and investigations of hematology and clinical biochemistry parameters.

2.9. Repeated dose toxicity: Thirteen-week study in rats

The thirteen-week study in rats was performed at BSL Bioservice (Munich, Germany) according to GLP and OECD Test Guideline 408, "Repeated Dose 90-Day Oral Toxicity Study in Rodents'', adopted 27 June 2018 [13]. The study has been modified in order to identify potential neurotoxic, immunological, reproductive organ, and endocrine-mediated effects. This included the effect on several parameters that may be indicative of neurotoxicity, such as changes in clinical signs and functional observational battery tests. It also included the effect on several parameters that may indicate an immunological effect, such as changes in spleen and thymus and other organs of the immune system (*e.g.* bone marrow, lymph nodes), as well as changes in the total and differential leukocyte cell numbers. Furthermore, a range of reproductive and endocrine-mediated effects, such as determination of thyroid hormones was investigated in the study.

80 healthy animals (40 males and 40 females) were weighed and randomly assigned to treatment groups such that the most homogenous variation in body weight was achieved. The animals were 7–8 weeks old at start of the treatment period corresponding to a body weight of 159−191 g for the males and 130−167 g for the females. Test item formulations were prepared (using water as a vehicle) at least every 11 days and stored protected from light at 2−8 °C. Control animals received water.

Groups of 10 male and 10 female animals received the test item orally by gavage for 90 days at 100, 300, and 1000 mg/kg bw per day at an application volume of 5 mL/kg bw (referred to as low-, medium- and high-dose groups, respectively). A control group, also including 10 male and 10 female Wistar rats, received sterile water, at the same application volume. The selection of dose levels was based on the outcome of the 14-day range-finding non-GLP study in rats (described in 2.8 and 3.3), where treatment with doses up to 1000 mg/kg bw per day was well tolerated and not associated with any adverse effects. Dose volumes were adjusted based on weekly body weight measurements.

As further described in the following, investigations of clinical condition, functional observations, body weight, food consumption, fertility, hematology, clinical biochemistry, urinalysis, pathology, organ weight and histopathology were undertaken as part of the study. The animals were weighed on the first day of treatment and weekly thereafter. Food consumption was measured weekly.

The animals were observed at least twice daily for morbidity and mortality (once per day in weekends) and once per day for signs of gross toxicity. Detailed clinical observations were performed prior to the first administration and at least once a week thereafter. Ophthalmic examination was made on all animals prior to the start of treatment and during week 13. Once before the first treatment and once during week 13, all animals were subjected to multiple detailed behavioral observations using a functional observational battery of tests [14].

In the last two weeks of treatment, the estrous cycle of all female animals was monitored by evaluation of vaginal smears. At necropsy, (one day after the last exposure), left epididymis, left testis, and left vas deferens were separated and used for evaluation of sperm parameters. Epididymal sperm motility and testicular sperm count was evaluated and sperm morphology slides were prepared from all male animals.

At the end of treatment and after overnight fasting, blood from the abdominal aorta was collected in EDTA-coated tubes for hematology, citrate tubes for coagulation, and serum separator tubes for clinical chemistry. Serum samples were collected for the measurement of T3, T4, TSH, FSH, LH, estradiol and testosterone. The measured blood parameters can be seen in Table 3.

Urine samples were also collected at the end of treatment and analyzed qualitatively for appearance, specific gravity, nitrite, pH, protein, glucose, ketones, urobilinogen, bilirubin, erythrocytes, and leukocytes.

One day after the last administration (study day 91), all animals were sacrificed using anesthesia (ketamine/xylazine) and subjected to a detailed gross necropsy. Vaginal smears were examined on the day of necropsy to determine the stage of estrous cycle.

The organs listed in Table 4 were collected and weighed before fixation, except for thyroid/parathyroid, which were weighed after fixation. Prostate was weighed together with seminal vesicles and coagulating glands and uterus was weighed with cervix. In addition, tissues with gross lesions were collected and preserved, as well as aorta,

cecum, colon, duodenum, esophagus, eyes, femur, ileum, jejunum, lungs, lymph nodes (axillary, mesenteric, mandibular), mammary gland (male and female), oviducts, pancreas, rectum, salivary glands (parotis, sublingual, submandibular), sciatic nerve, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), sternum (with bone marrow), stomach, tongue, trachea, ureters, urinary bladder, and vagina. The tissues were preserved in 4 % buffered formaldehyde, except epididymides, eyes, and testes which were fixed in Modified Davidson's fixative for 24 h and then transferred to 70 % ethanol.

The fixed organs and tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and examined by light microscopy. Histopathological examinations were performed on all control and high dose animals at the GLP-certified contract laboratory AnaPath GmbH (Switzerland). In addition, any gross lesion identified macroscopically was examined in all animals.

2.10. Statistical analyses

2.10.1. Genotoxicity studies

According to the OECD guideline for bacterial reverse mutation assay, the biological relevance of the results should be considered first, and a statistical evaluation of the results is not required. Statistical analyses were therefore not performed for this assay. The *in vitro* micronucleus assay was evaluated by the non-parametric chi-square test and the chi-square test for trend, and results were considered significant when $p < 0.05$.

2.10.2. Repeated dose toxicity studies

Male and female data were analyzed separately. For the majority of parameters, measurements were only made at necropsy after day 90, including parameters of hematology, blood coagulation and clinical chemistry, and absolute and relative organ weights. Parameters like body weight gain and food consumption were calculated for each animal as the difference in weight measured from one week to the next and as the difference in weight from the first to the last week of the study. The relative organ weights were calculated in relation to the brain and body weight (measured at necropsy) and are presented as percentage.

All quantitative data (body weight, food consumption, parameters of hematology, blood coagulation and clinical chemistry, and absolute and relative organ weights) were analyzed for normality and homogeneity of variance. Parametric data were then analyzed using a one-way analysis of variance (ANOVA). If significant differences were found ($P < 0.05$), the Lacprodan® BLG-treated groups were compared with the control group using a post-hoc Dunnett's test. Non-parametric data were analyzed using Kruskal-Wallis test, and in case significant differences were found ($P < 0.05$), the Lacprodan® BLG-treated groups were compared with the control group using a post-hoc Dunn's test. These statistics were performed with Ascentos 1.3.4 or GraphPad Prism V.6.01

Table 1
Bacterial reverse mutation results after Lacprodan® BLG exposure.

Test substance	Dose (μg/plate)	Average number of revertant colonies/plate (n = 3)									
		TA98		TA100		TA1535		TA1537		WP2uvrA	
		Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
BLG with S9											
	0	29 ± 2.5	36 ± 7.1	116 ± 11.2	81 ± 11.8	11 ± 2.6	13 ± 1.5	18 ± 6.7	31 ± 2.0	70 ± 4.9	54 ± 3.8
	31.6	30 ± 3.1	33 ± 5.0	108 ± 16.6	85 ± 12.7	11 ± 2.1	13 ± 0.6	20 ± 1.5	28 ± 8.5	61 ± 7.6	59 ± 2.9
	100	25 ± 2.5	39 ± 2.6	117 ± 6.1	78 ± 13.1	12 ± 0.6	11 ± 1.2	18 ± 0.6	25 ± 3.8	68 ± 1.5	61 ± 7.0
	316	32 ± 2.6	42 ± 1.0	116 ± 5.6	94 ± 2.1	15 ± 3.1	11 ± 0.6	16 ± 2.6	25 ± 5.3	68 ± 5.3	54 ± 3.5
	1000	34 ± 10.6	41 ± 1.2	110 ± 5.2	111 ± 5.1	11 ± 0.6	13 ± 3.0	18 ± 3.2	21 ± 4.5	74 ± 2.1	47 ± 8.3
	2500	28 ± 4.0	40 ± 1.0	105 ± 4.6	101 ± 19.1	10 ± 2.0	11 ± 2.1	16 ± 3.1	21 ± 3.2	74 ± 16.2	48 ± 8.2
	5000	29 ± 9.6	32 ± 4.6	119 ± 13.3	92 ± 11.3	10 ± 3.1	12 ± 3.0	20 ± 2.1	22 ± 5.3	58 ± 3.5	52 ± 2.1
Positive control											
2-AA with S9	2.5	508 ± 41.1	2024 ± 593	1823 ± 356	1177 ± 75	206 ± 36.7	91 ± 57.9	299 ± 50.0	131 ± 27.1	228 ± 106.4	139 ± 27.7
BLG without S9											
	0	28 ± 4.4	30 ± 2.1	104 ± 5.5	121 ± 10.0	12 ± 2.1	12 ± 0.6	15 ± 4.6	23 ± 2.9	48 ± 5.1	48 ± 2.1
	31.6	26 ± 2.5	34 ± 2.3	111 ± 31.9	95 ± 18.6	18 ± 2.6	11 ± 1.0	17 ± 4.2	21 ± 1.0	47 ± 3.8	56 ± 9.0
	100	23 ± 4.5	32 ± 5.6	103 ± 17.0	89 ± 8.9	16 ± 2.5	12 ± 3.5	17 ± 5.1	22 ± 5.0	48 ± 7.2	53 ± 4.4
	316	31 ± 10.8	34 ± 5.1	121 ± 7.0	90 ± 10.1	18 ± 0.6	12 ± 2.6	16 ± 5.0	22 ± 2.0	54 ± 8.7	57 ± 1.2
	1000	30 ± 2.9	28 ± 3.5	115 ± 5.3	84 ± 16.4	13 ± 3.1	10 ± 2.1	14 ± 1.0	24 ± 5.3	56 ± 2.5	50 ± 3.5
	2500	35 ± 6.0	33 ± 3.5	115 ± 14.2	90 ± 3.5	17 ± 4.4	10 ± 3.6	14 ± 1.5	20 ± 4.4	58 ± 11.5	46 ± 1.2
	5000	26 ± 4.5	37 ± 4.2	103 ± 24.8	92 ± 26.5	15 ± 4.5	15 ± 4.0	14 ± 2.1	20 ± 1.5	54 ± 2.5	47 ± 9.5
Positive controls											
4-NOPD without S9	10	676 ± 56.2	603 ± 176.3								
NaN_3 without S9	10			666 ± 49.4	661 ± 77.1	1138 ± 38	854 ± 97.0				
4-NOPD without S9	40							225 ± 62.9	140 ± 18.4		
MMS without S9	1 (μL/plate)									466 ± 13.6	510 ± 76.5

Results are expressed as mean ± SD. 2-AA = 2-aminoanthracene; 4-NOPD = 4-nitro-o-phenylene-diamine; NaN_3 = sodium azide; MMS = methylmethanesulfonate.

software. Results were considered significant when $p < 0.05$.

3. Results

3.1. Bacterial reverse mutation assay

The results of the two experiments (plate incorporation and pre-incubation test) are presented in Table 1. The negative controls were within the historical control range and all positive controls showed a distinct increase in revertants compared to negative controls.

No precipitation or cytotoxic effects were observed in any of the experiments with and without S9 mix. No biologically relevant effects were observed following treatment with Lacprodan® BLG, neither in the presence nor absence of metabolic activation in the two experiments. In conclusion, Lacprodan® BLG did not induce gene mutations by base pair changes or frameshifts and is therefore non-mutagenic under the experimental conditions employed for this study

3.2. In vitro micronucleus assay using human lymphocytes

The results of the two experiments are shown in Table 2. No precipitation or cytotoxic effects were observed in any of the experiments with and without S9 mix. All positive controls induced statistically significant increases in micronucleus frequency, confirming the efficacy of the S9 mix and the sensitivity of the test system.

In Experiment I without S9 mix, the micronucleated cell frequency of the negative control (0.25 %) was below the historical control limits (0.29 %–1.16 %). The mean values of micronucleated cells after Lacprodan® BLG treatment were 0.50 % at 1000 μg/mL, 0.75 % at 1500 μg/mL and 0.40 % at 2000 μg/mL.

In Experiment I with S9 mix, the micronucleated cell frequency of the negative control (0.65 %) was within the historical control range (0.28 %–1.26 %). The mean values of micronucleated cells after treatment with Lacprodan® BLG were 0.65 % at 500 μg/mL, 0.70 % at 1000 and 1500 μg/mL and 0.80 % at 2000 μg/mL.

In Experiment II (without S9 mix), the micronucleated cell frequency of the negative control (0.80 %) was within the historical control limits (0.29 %–1.16 %). The mean values of micronucleated cells of the Lacprodan® BLG-treated groups were 0.80 % at 1000 μg/mL, 0.95 % at 1500 μg/mL and 0.90 % at 2000 μg/mL.

All individual numbers of micronucleated cells in Experiments I and II were within or below the historical control limits of the negative control and did not show any biologically relevant increases compared to the concurrent negative control.

No statistically significant increases of cells with micronuclei were observed after Lacprodan® BLG-treatment, except for the 1500 μg/mL test concentration in Experiment I without S9 mix. However, as there was no dose-relationship and the increase was within the range of the historical negative controls, this is not considered biologically relevant.

In conclusion, Lacprodan® BLG did not induce structural and/or numerical chromosomal damage in human lymphocytes, and is therefore non-genotoxic with respect to clastogenicity and/or aneugenicity in the *in vitro* mammalian cell micronucleus assay.

3.3. Repeated dose toxicity: Preliminary fourteen-day study in rats

No mortality or clinical signs were observed in the study. There were no treatment-related changes in body weight and food consumption during the treatment period. Treatment with the test item had no toxicologically relevant effect on hematology and clinical biochemistry parameters. There was an increase of white blood cells in all male and female dose groups (only statistically significant in medium- and high-dose males), which is considered to be related to a mild immunological reaction to the test item. The effect on the white blood cells was not seen in the subsequent 13-week study. No macroscopic findings related to treatment were observed in any male or female dose groups at necropsy.

Treatment with Lacprodan® BLG up to 1000 mg/kg bw per day was

Table 2
In vitro micronucleus results after Lacprodan® BLG exposure.

Exposure	Concentration (μg/mL)	CBPI slide 1 / slide 2	Relative cell growth (%)[a]	Micronucleated cells (%) culture 1 /culture 2	Average number of micronucleated cells (%)	Historical negative control limits
Experiment I (–S9)						
	0	1.51 / 1.40	100	0.20 / 0.30	0.25	
	1000	1.36 / 1.38	81	0.70 / 0.30	0.50	
	1500	1.48 / 1.58	116	1.10 / 0.40	0.75*	0.29 % - 1.16 %
	2000	1.44 / 1.50	102	0.40 / 0.40	0.40	
Positive controls						
MMS	65	1.38 / 1.38	83	4.90 / 1.75	3.33*	
Colchicine	0.8	1.19 / 1.15	37	3.60 / 5.80	4.70*	
Experiment I (+S9)						
	0	1.46 / 1.47	100	0.90 / 0.40	0.65	
	500	1.44 / 1.48	100	0.50 / 0.80	0.65	
	1000	1.45 / 1.44	96	0.70 / 0.70	0.70	0.28 % - 1.26 %
	1500	1.44 / 1.42	93	1.00 / 0.40	0.70	
	2000	1.40 / 1.59	108	0.60 / 1.00	0.80	
Positive control						
CPA	15	1.28 / 1.28	60	2.30 / 3.70	3.00*	
Experiment II (–S9)						
	0	1.60 / 1.71	100	0.90 / 0.70	0.80	
	1000	1.60 / 1.62	93	1.30 / 0.30	0.80	
	1500	1.65 / 1.60	96	1.00 / 0.90	0.95	0.29 % - 1.16 %
	2000	1.72 / 1.58	99	0.60 / 1.20	0.90	
Positive controls						
MMS	50	1.44 / 1.61	80	3.70 / 5.10	4.40*	
Colchicine	0.04	1.23 / 1.22	34	4.60 / 6.49	5.55*	

CBPI = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3)) / n$, where c_1, c_2 and c_x are mononucleate, binucleate and multinucleate cells, respectively, and n = total number of cells.
Relative cell growth = $100 \times ((CBPI_{test\ conc.} - 1) / (CBPI_{control} - 1))$.

* $P < 0.05$ as compared with control (Chi-square test). MMS = methylmethanesulfonate; CPA = cyclophosphamide.

[a] Relative to negative control.

Table 3
Hematology and clinical chemistry of rats administered Lacprodan® BLG for 13 weeks.

Parameter	Male dose groups (mg/kg bw)				Female dose groups (mg/kg bw)			
	Control	100	300	1000	Control	100	300	1000
Hematology								
Hematocrit (%)	48.71 ± 1.41	48.87 ± 1.80	50.61 ± 2.74	49.89 ± 2.17	45.23 ± 2.42	45.84 ± 1.57	45.91 ± 3.01	48.78 ± 2.12**
Hemoglobin (g/dL)	15.43 ± 0.47	15.57 ± 0.59	16.17 ± 0.68*	16.03 ± 0.50	14.39 ± 0.81	14.72 ± 0.37	14.74 ± 0.76	15.58 ± 0.67**
RBC (10^{12}/L)	9.20 ± 0.23	9.27 ± 0.27	9.71 ± 0.70	9.55 ± 0.39	8.09 ± 0.58	8.24 ± 0.32	8.29 ± 0.37	8.92 ± 0.38***
MCV (fL)	52.98 ± 1.64	52.70 ± 0.72	52.21 ± 1.41	52.27 ± 1.49	55.98 ± 1.45	55.68 ± 0.83	55.35 ± 1.66	54.72 ± 1.17
MCH (pg/erythrocyte)	16.77 ± 0.65	16.80 ± 0.24	16.70 ± 0.69	16.80 ± 0.53	17.80 ± 0.42	17.89 ± 0.57	17.79 ± 0.45	17.48 ± 0.23
MCHC (g/dL)	31.70 ± 0.54	31.85 ± 0.33	31.97 ± 0.63	32.17 ± 0.50	31.83 ± 0.42	32.14 ± 0.87	32.12 ± 0.65	31.93 ± 0.77
Reticulocytes (%)	1.66 ± 0.30	1.59 ± 0.22	1.48 ± 0.25	1.40 ± 0.22	2.15 ± 0.74	1.82 ± 0.19	2.07 ± 0.42	1.77 ± 0.39
Thrombocytes (10^9/L)	684.0 ± 54.7	670.8 ± 64.0	694.4 ± 53.0	649.8 ± 69.3	693.0 ± 61.3	706.2 ± 86.1	699.5 ± 109.1	737.9 ± 86.3
WBC (10^9/L)	4.29 ± 0.99	3.46 ± 1.06	5.25 ± 0.85	5.13 ± 2.23	1.98 ± 0.65	2.25 ± 0.99	1.96 ± 0.73	2.55 ± 0.86
Neutrophils (%)	24.82 ± 6.93	23.32 ± 5.37	23.80 ± 11.06	22.74 ± 12.30	29.13 ± 23.25	24.44 ± 14.90	18.82 ± 8.15	22.71 ± 17.85
Lymphocytes (%)	71.93 ± 6.32	73.32 ± 5.32	73.40 ± 11.64	73.64 ± 12.67	68.54 ± 23.48	72.59 ± 16.38	77.22 ± 10.87	74.04 ± 19.61
Monocytes (%)	2.49 ± 0.86	2.64 ± 0.83	2.02 ± 0.56	2.75 ± 0.86	1.62 ± 0.68	2.22 ± 1.11	2.42 ± 0.79	2.24 ± 1.03
Eosinophils (%)	0.389 ± 0.209	0.470 ± 0.183	0.420 ± 0.274	0.400 ± 0.306	0.390 ± 0.242	0.570 ± 0.660	1.230 ± 2.775	0.700 ± 1.026
Basophils (%)	0.078 ± 0.067	0.070 ± 0.068	0.110 ± 0.057	0.110 ± 0.088	0.110 ± 0.088	0.070 ± 0.095	0.100 ± 0.082	0.089 ± 0.078
Luc (%)	0.256 ± 0.194	0.180 ± 0.123	0.230 ± 0.134	0.330 ± 0.183	0.170 ± 0.134	0.120 ± 0.092	0.220 ± 0.092	0.233 ± 0.132
Blood coagulation								
Prothrombin time (sec)	23.76 ± 1.37	23.90 ± 1.33	23.78 ± 1.73	24.77 ± 1.78	24.54 ± 0.78	25.35 ± 1.74	24.94 ± 1.69	25.56 ± 0.79
aPTT (sec)	10.75 ± 0.92	11.32 ± 1.52	10.14 ± 1.58	9.96 ± 1.42	8.99 ± 0.73	9.02 ± 0.69	9.16 ± 0.77	9.23 ± 1.54
Clinical chemistry								
ALAT (U/L)	34.03 ± 5.62	32.26 ± 3.43	30.60 ± 4.27	32.73 ± 9.06	28.77 ± 3.90	29.12 ± 5.48	28.54 ± 7.37	27.35 ± 4.10
ASAT (U/L)	99.06 ± 9.30	86.40 ± 11.83	86.41 ± 12.88	102.94 ± 43.36	89.55 ± 10.28	87.95 ± 13.21	80.63 ± 16.25	83.28 ± 20.08
AP (U/L)	111.2 ± 26.9	104.1 ± 22.5	102.8 ± 24.6	104.1 ± 20.3	55.59 ± 17.83	48.87 ± 21.20	44.09 ± 13.38	56.15 ± 29.63
Creatinine (μmol/L)	24.30 ± 2.45	26.10 ± 13.49	22.80 ± 8.08	40.60 ± 56.26	27.30 ± 3.06	24.40 ± 3.34	24.30 ± 2.95	23.80 ± 4.21
Total protein (g/L)	55.68 ± 2.58	55.31 ± 1.70	55.99 ± 2.07	54.76 ± 1.87	60.26 ± 1.80	60.56 ± 2.72	59.99 ± 3.04	58.27 ± 3.46
Albumin (g/L)	31.18 ± 1.34	30.82 ± 0.80	30.85 ± 0.65	30.70 ± 0.95	34.73 ± 1.46	34.65 ± 1.39	34.17 ± 1.86	33.27 ± 2.38
Urea (mmol/L)	7.75 ± 1.23	7.33 ± 1.29	7.41 ± 1.00	8.22 ± 3.87	7.79 ± 0.66	7.52 ± 1.29	7.32 ± 1.19	7.26 ± 1.13
Bilirubin (μmol/L)	1.96 ± 0.18	2.25 ± 0.61	2.07 ± 0.19	2.30 ± 0.32	2.39 ± 0.24	2.59 ± 0.40	2.85 ± 0.46*	2.93 ± 0.51*
Bile acids (μmol/L)	30.79 ± 10.25	34.95 ± 21.55	26.18 ± 10.50	27.71 ± 13.04	30.02 ± 10.52	23.52 ± 10.28	44.21 ± 41.75	42.14 ± 28.09
Cholesterol (mmol/L)	1.65 ± 0.37	1.72 ± 0.34	1.50 ± 0.21	1.61 ± 0.27	1.31 ± 0.23	1.42 ± 0.25	1.62 ± 0.55	1.37 ± 0.21
LDL (mmol/L)	0.458 ± 0.164	0.492 ± 0.178	0.345 ± 0.099	0.431 ± 0.087	0.294 ± 0.072	0.336 ± 0.094	0.444 ± 0.245	0.363 ± 0.088
HDL (mmol/L)	1.09 ± 0.21	1.11 ± 0.26	1.04 ± 0.14	1.06 ± 0.19	0.95 ± 0.17	1.04 ± 0.17	1.12 ± 0.31	0.95 ± 0.14
Triglycerides (mmol/L)	0.488 ± 0.176	0.583 ± 0.165	0.577 ± 0.150	0.601 ± 0.151	0.315 ± 0.115	0.234 ± 0.072	0.280 ± 0.046	0.259 ± 0.071
Glucose (mmol/L)	11.09 ± 2.77	8.52 ± 1.25**	9.62 ± 1.25	10.41 ± 1.44	6.55 ± 1.66	5.93 ± 1.44	5.99 ± 1.87	7.60 ± 4.06
Sodium (mmol/L)	142.3 ± 2.00	141.1 ± 3.11	140.7 ± 1.83	141.2 ± 1.93	142.7 ± 3.5	143.9 ± 3.2	142.7 ± 1.8	143.5 ± 2.6
Potassium (mmol/L)	4.02 ± 0.24	4.14 ± 0.28	4.38 ± 0.88	4.48 ± 1.01	3.76 ± 0.31	3.57 ± 0.36	4.15 ± 1.48	4.31 ± 1.44
Hormones								
T3 (ng/mL)	3.35 ± 0.71	3.42 ± 0.77	3.75 ± 0.81	3.94 ± 0.53	3.33 ± 0.86	3.21 ± 1.36	3.56 ± 0.84	3.68 ± 1.32
T4 (ng/mL)	56.73 ± 4.86	55.30 ± 13.76	56.28 ± 8.02	55.97 ± 9.71	31.47 ± 6.62	31.67 ± 8.04	36.65 ± 9.37	38.01 ± 10.36
TSH (ng/mL)	2.77 ± 0.99	2.32 ± 0.78	2.03 ± 0.63	1.75 ± 0.57*	1.57 ± 1.12	1.40 ± 0.78	1.66 ± 0.76	1.36 ± 0.64
FSH (ng/mL)	5.71 ± 3.24	4.18 ± 2.02	4.39 ± 2.28	4.04 ± 2.67	2.98 ± 2.33	4.18 ± 3.76	4.16 ± 3.70	1.89 ± 1.69
LH (ng/mL)	0.46 ± 0.37	0.36 ± 0.27	0.43 ± 0.52	0.46 ± 0.31	1.60 ± 0.43	1.02 ± 0.44	1.60 ± 1.37	1.24 ± 0.66
Estradiol (ng/mL)	0.10 ± 0.07	0.12 ± 0.12	0.07 ± 0.04	0.16 ± 0.12	0.14 ± 0.13	0.07 ± 0.03	0.15 ± 0.07	0.15 ± 0.12
Testosterone (ng/mL)	0.92 ± 0.74	1.11 ± 0.56	1.27 ± 0.78	1.62 ± 1.53	0.54 ± 0.48	0.40[a]	0.73 ± 0.33	0.58 ± 0.24

Results are expressed as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 as compared with control.
RBC = red blood cell count; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WBC = total white blood cell count; Luc = large unstained cells; aPTT = activated partial thromboplastin time; ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; AP = alkaline phosphatase; LDL = low density lipoprotein; HDL = high density lipoprotein.
[a] Based on one animal.

well tolerated and was not associated with any adverse effects. Dose levels of 100, 300 and 1000 mg/kg bw per day were therefore decided to be used for the 13-week toxicity study.

3.4. Repeated dose toxicity: Thirteen-week study in rats

3.4.1. Body weight and food consumption

Treatment with Lacprodan® BLG had no effect on body weight development (Fig. 1). All animals showed body weight gain during treatment. The average body weight of control males on study day 90 was 404 g and the corresponding values of the Lacprodan® BLG -treated groups ranged from 391 to 400 g. For females, the average body weight of the control group on study day 90 was 242 g and the corresponding values of Lacprodan® BLG -treated rats were 244–256 g. The slight statistically significant increase in body weight on day 43 and 64 in the female high-dose group (up to 7 % increase) compared to control was therefore considered to be incidental. There were no significant differences in food intake between control rats and Lacprodan® BLG-treated rats (data not shown).

3.4.2. Clinical observations and mortality

No mortality occurred during the study. There were no clinical signs except for “moving the bedding” observed for all male and female high-dose group animals on 3 treatment days. “Moving the bedding” is considered a sign of local reaction after test item application and not an adverse systemic effect of the test item. There were no opthalmoscopic changes and no effects in the functional observation battery attributed to the administration of Lacprodan® BLG.

3.4.3. Fertility

There were no changes on male and female fertility parameters related to treatment with Lacprodan® BLG (data not shown). Cycle

Table 4
Absolute and relative organ weights of rats administered Lacprodan® BLG for 13 weeks.

Parameter	Dose group (mg/kg bw)			
	Control	100	300	1000
Males				
Body weight (g)	386.2 ± 27.00	384.7 ± 28.54	369.7 ± 27.90	375.5 ± 37.70
Brain (g)	2.086 ± 0.119	2.044 ± 0.101	2.022 ± 0.112	2.073 ± 0.086
Brain/body (%)	0.542 ± 0.045	0.534 ± 0.051	0.550 ± 0.052	0.556 ± 0.050
Heart (g)	1.260 ± 0.180	1.235 ± 0.104	1.096 ± 0.100*	1.117 ± 0.130
Heart/body (%)	0.326 ± 0.033	0.322 ± 0.034	0.297 ± 0.028	0.298 ± 0.018
Heart/brain (%)	60.67 ± 9.759	60.53 ± 6.042	54.39 ± 5.998	53.86 ± 5.888
Liver (g)	9.834 ± 0.861	9.228 ± 0.828	8.903 ± 0.769	9.213 ± 0.953
Liver/body (%)	2.545 ± 0.105	2.398 ± 0.102	2.408 ± 0.105	2.459 ± 0.181
Liver/brain (%)	472.8 ± 49.10	452.9 ± 51.15	441.6 ± 46.62	444.7 ± 45.61
Thymus (g)	0.328 ± 0.044	0.386 ± 0.102	0.378 ± 0.067	0.336 ± 0.082
Thymus/body (%)	0.085 ± 0.010	0.100 ± 0.022	0.103 ± 0.020	0.089 ± 0.018
Thymus/brain (%)	15.72 ± 1.901	18.98 ± 5.292	18.81 ± 3.981	16.21 ± 3.972
Kidneys (g)	2.400 ± 0.123	2.524 ± 0.194	2.303 ± 0.194	2.333 ± 0.229
Kidneys/body (%)	0.623 ± 0.037	0.658 ± 0.045	0.623 ± 0.034	0.623 ± 0.050
Kidneys/brain (%)	115.5 ± 10.15	123.8 ± 12.15	114.3 ± 12.11	112.6 ± 10.86
Adrenals (g)	0.059 ± 0.010	0.058 ± 0.007	0.059 ± 0.008	0.064 ± 0.011
Adrenals/body (%)	0.015 ± 0.002	0.015 ± 0.002	0.016 ± 0.002	0.017 ± 0.003
Adrenals/brain (%)	2.841 ± 0.514	2.814 ± 0.306	2.926 ± 0.432	3.085 ± 0.598
Spleen (g)	0.780 ± 0.144	0.736 ± 0.067	0.678 ± 0.072	0.710 ± 0.089
Spleen/body (%)	0.202 ± 0.034	0.192 ± 0.017	0.185 ± 0.026	0.190 ± 0.022
Spleen/brain (%)	37.41 ± 6.412	36.10 ± 3.760	33.67 ± 4.517	34.32 ± 4.724
Thyroid (g)	0.028 ± 0.008	0.028 ± 0.005	0.026 ± 0.003	0.026 ± 0.008
Thyroid/body (%)	0.007 ± 0.002	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.002
Thyroid/brain (%)	1.370 ± 0.389	1.371 ± 0.258	1.287 ± 0.100	1.276 ± 0.371
Pituitary (g)	0.009 ± 0.002	0.009 ± 0.002	0.008 ± 0.002	0.008 ± 0.002
Pituitary/body (%)	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
Pituitary/brain (%)	0.410 ± 0.089	0.420 ± 0.078	0.399 ± 0.108	0.367 ± 0.099
Testes (g)	3.531 ± 0.355	3.549 ± 0.247	3.522 ± 0.152	3.472 ± 0.222
Testes/body (%)	0.914 ± 0.066	0.924 ± 0.045	0.957 ± 0.072	0.932 ± 0.103
Testes/brain (%)	169.4 ± 16.12	174.0 ± 14.52	174.6 ± 10.00	167.6 ± 11.95
Epididymides (g)	1.479 ± 0.169	1.437 ± 0.148	1.456 ± 0.089	1.493 ± 0.173
Epididymides/body (%)	0.383 ± 0.035	0.376 ± 0.050	0.395 ± 0.026	0.399 ± 0.037
Epididymides/brain (%)	71.00 ± 8.050	70.19 ± 5.216	72.13 ± 4.897	72.15 ± 9.377
Prostate (g)	2.744 ± 0.355	3.000 ± 0.541	2.525 ± 0.415	2.682 ± 0.643
Prostate/body (%)	0.710 ± 0.076	0.780 ± 0.122	0.683 ± 0.101	0.722 ± 0.196
Prostate/brain (%)	132.0 ± 19.32	146.9 ± 26.08	125.7 ± 25.61	129.6 ± 31.89
Females				
Body weight (g)	228.8 ± 12.44	231.0 ± 8.498	234.1 ± 9.916	243.5 ± 16.24
Brain (g)	1.933 ± 0.107	1.905 ± 0.072	1.888 ± 0.101	1.909 ± 0.069
Brain/body (%)	0.847 ± 0.066	0.826 ± 0.049	0.807 ± 0.040	0.787 ± 0.061
Heart (g)	0.814 ± 0.064	0.847 ± 0.129	0.826 ± 0.112	0.824 ± 0.096
Heart/body (%)	0.357 ± 0.038	0.366 ± 0.048	0.352 ± 0.040	0.338 ± 0.029
Heart/brain (%)	42.12 ± 2.889	44.56 ± 7.423	43.74 ± 5.380	43.31 ± 6.071
Liver (g)	5.840 ± 0.448	5.973 ± 0.478	6.420 ± 0.980	6.442 ± 0.872
Liver/body (%)	2.555 ± 0.190	2.587 ± 0.203	2.739 ± 0.372	2.655 ± 0.340
Liver/brain (%)	303.2 ± 30.54	314.2 ± 32.12	339.9 ± 47.95	337.6 ± 46.43
Thymus (g)	0.335 ± 0.075	0.320 ± 0.053	0.329 ± 0.061	0.293 ± 0.045
Thymus/body (%)	0.146 ± 0.030	0.139 ± 0.025	0.140 ± 0.022	0.121 ± 0.021
Thymus/brain (%)	17.34 ± 3.700	16.85 ± 2.960	17.40 ± 3.151	15.35 ± 2.427
Kidneys (g)	1.582 ± 0.108	1.697 ± 0.119	1.732 ± 0.151	1.764 ± 0.131**
Kidneys/body (%)	0.693 ± 0.054	0.734 ± 0.042	0.740 ± 0.058	0.726 ± 0.054
Kidneys/brain (%)	81.94 ± 5.365	89.19 ± 7.459	91.80 ± 7.735**	92.43 ± 7.344**
Adrenals (g)	0.079 ± 0.015	0.080 ± 0.016	0.077 ± 0.022	0.070 ± 0.017
Adrenals/body (%)	0.035 ± 0.006	0.034 ± 0.007	0.033 ± 0.009	0.029 ± 0.007
Adrenals/brain (%)	4.107 ± 0.737	4.179 ± 0.854	4.094 ± 1.259	3.648 ± 0.830
Spleen (g)	0.578 ± 0.084	0.591 ± 0.096	0.622 ± 0.083	0.628 ± 0.107
Spleen/body (%)	0.253 ± 0.037	0.256 ± 0.040	0.266 ± 0.034	0.259 ± 0.043
Spleen/brain (%)	29.90 ± 3.967	31.11 ± 5.538	32.91 ± 3.857	32.99 ± 6.080
Thyroid (g)	0.023 ± 0.006	0.024 ± 0.004	0.026 ± 0.007	0.021 ± 0.007
Thyroid/body (%)	0.010 ± 0.003	0.011 ± 0.002	0.011 ± 0.003	0.008 ± 0.003
Thyroid/brain (%)	1.187 ± 0.339	1.278 ± 0.266	1.359 ± 0.358	1.072 ± 0.382
Pituitary (g)	0.012 ± 0.002	0.012 ± 0.003	0.011 ± 0.004	0.011 ± 0.004
Pituitary/body (%)	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.002	0.005 ± 0.002
Pituitary/brain (%)	0.641 ± 0.093	0.620 ± 0.142	0.592 ± 0.184	0.572 ± 0.217
Ovaries (g)	0.118 ± 0.019	0.116 ± 0.018	0.122 ± 0.025	0.134 ± 0.032
Ovaries/body (%)	0.052 ± 0.008	0.050 ± 0.007	0.052 ± 0.009	0.055 ± 0.012
Ovaries/brain (%)	6.095 ± 0.825	6.124 ± 1.003	6.493 ± 1.297	6.992 ± 1.553
Uterus (g)	0.822 ± 0.410	1.048 ± 0.455	0.870 ± 0.372	0.733 ± 0.125
Uterus/body (%)	0.363 ± 0.191	0.450 ± 0.185	0.371 ± 0.154	0.302 ± 0.054
Uterus/brain (%)	42.32 ± 20.07	55.39 ± 25.458	45.93 ± 18.87	38.39 ± 6.436

Results are expressed as mean ± SD.

* $P < 0.05$ as compared with control.
** $P < 0.01$ as compared with control. The organ weights were recorded at study day 91.

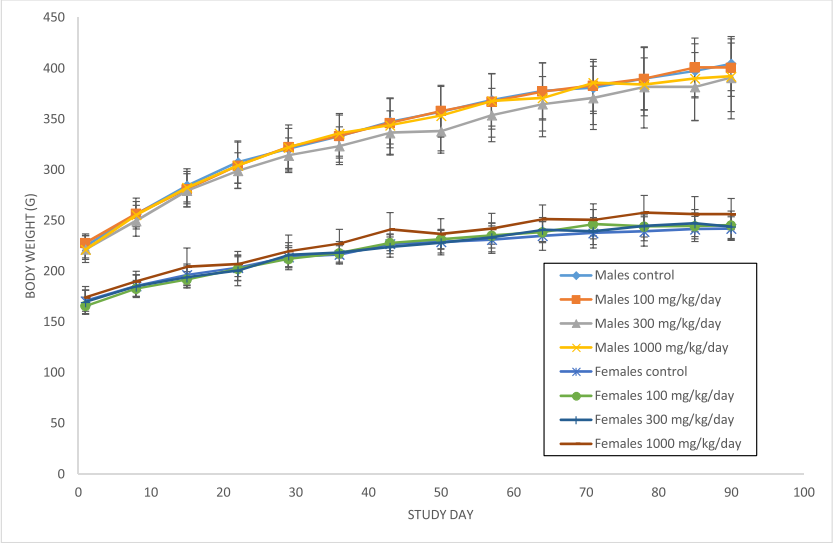

Fig. 1. Body weight development of male and female rats provided Lacprodan® BLG for 13 weeks. Values are shown as mean ± SD.

length in all female dose groups was comparable to the control group. A prolonged cycle length observed in one high-dose female animal compared to control group animals was considered to be incidental. There were no treatment-related changes of male fertility parameters, including mean sperm count, mean sperm motility, and mean sperm morphology, and all group mean values were comparable to the control group.

3.4.4. Hematology and blood coagulation

There were no treatment-related changes in the hematology or coagulation parameters (Table 3). All inter-group differences from controls were minor, lacked dose-related response or were limited to one sex and were therefore ascribed to normal biological variation. These included the slight, but statistically significant increase of hemoglobin (4.8 %) in medium-dose males and the increase of red blood cells (10 %), hemoglobin (8.3 %) and hematocrit (7.8 %) in high-dose females compared to controls. All group mean values were within the historical control range. The increase in white blood cells seen in the preliminary fourteen-day study, was not observed in the 13-week study.

No statistically significant differences were observed for blood coagulation parameters when compared to the respective control group.

3.4.5. Clinical biochemistry and hormones

There were no adverse treatment-related blood biochemistry changes (Table 3). All inter-group differences from controls were minor, lacked dose-related response or were limited to one sex and were therefore ascribed to normal biological variation. Such changes included the slight decrease of glucose (23 %) in low-dose males and the slight increase of total bilirubin (19 % and 23 %) in medium- and high-dose females, respectively, which were statistically significant when compared with the controls. The increase in creatinine (67 %) and urea (6.1 %) in high-dose males (not statistically significant) were attributed to a high individual value in one male. As there were no correlating adverse findings in the histopathological evaluation, the changes seen in blood biochemistry were considered not to be of toxicological relevance. All group mean values were within the normal range of variation.

No treatment-related effects on T3, T4, TSH, FSH, LH, estradiol, and testosterone were observed. No statistically significant changes were noted between the control and dose groups, except for a slight decrease of TSH in high-dose males. In the absence of abnormal findings for thyroid hormones, the change in TSH was not considered to be toxicologically relevant.

3.4.6. Urinalysis

The test item had no toxicologically relevant effect on urinary parameters (data not shown). As the urinary parameters were measured using qualitative indicators, no statistical analyses were performed. Bilirubin was found in a few animals in the male medium-dose and high-dose groups, in all female dose groups and in one male control animal. Additionally, ketones was found in the urine of a few male and female medium-dose and high-dose animals. Since there were no associated histopathological or clinical biochemistry observations, these findings were not considered to be toxicologically relevant.

3.4.7. Organ weight

There were no treatment-related differences in absolute or relative organ weights (Table 4). All inter-group differences were minor, lacked dose-related response or were limited to one sex and were therefore ascribed to normal biological variation. These included the slightly, but statistically significant reduced absolute heart weight (13 %) in the medium-dose males, the increased absolute kidney weight (11 %) in the high-dose females and the increased kidney relative to brain weight (12 % and 13 %) in females receiving medium- and high-dose, respectively. As there were no correlating findings at the necropsy or at the histopathological evaluation of the organs, the changes in organ weights were considered not to be toxicologically relevant.

3.4.8. Pathology

Macroscopic findings at necropsy were noted in a few animals of the male and female control or dose groups (data not shown). None of these findings were, however, considered treatment-related, as they are commonly seen in rats of the same strain and age and occurred only at random in a few animals. Macroscopic observations in males included abnormal content in the urinary bladder (one high-dose male), enlarged mesenterial lymph nodes (one high-dose male), and red axillary lymph nodes and cyst in the mandibular lymph nodes (one mid-dose male). Findings in females included enlarged and red axillary lymph nodes (one high-dose female), fluid filled uterus (one low-dose female), lung with

mass and fluid filled uterus (one control), white focus in the lung (one control), red colored thymus and enlarged and red mesenterial lymph nodes (one control).

3.4.9. Histopathology

No treatment-related microscopic findings were observed (data not shown). All findings were considered incidental or were background alterations typically seen in rats of this strain and age. Microscopic observations included lymphoid depletion in the spleen, mixed cell and mononuclear infiltrates in the lungs and lymphoid depletion and hemorrhage in the mesenteric lymph nodes. The findings were observed in both treated and control animals, except for the lymphoid depletion in the spleen, which was seen in only one high-dose female.

4. Discussion and conclusion

The safety of the whey protein Lacprodan® BLG was evaluated by two genotoxicity studies and a 13-week oral toxicity study in rats, all conducted in compliance with GLP and current OECD test guidelines.

A basic battery of *in vitro* genotoxicity tests were conducted with the whey protein Lacprodan® BLG in accordance with the tiered testing approach recommended by EFSA for the authorisation of novel foods [15,16]. The test battery consisted of a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus assay. Lacprodan® BLG did not induce gene mutations in the bacterial reverse mutation assay in any of the methods applied, *i.e.* the standard plate incorporation and the pre-incubation methods. Therefore, Lacprodan® BLG is concluded to be non-mutagenic in the bacterial reverse mutation assay.

The micronucleus assay was designed to assess the potential of Lacprodan® BLG to induce micronuclei in cultured human peripheral blood lymphocytes. Micronuclei formation may originate from both numerical and structural chromosomal aberrations, two mechanisms involved in genetic and carcinogenic effects. Treatment of human peripheral blood lymphocytes with Lacprodan® BLG did not result in any biologically relevant increases in micronucleated cells. Lacprodan® BLG is therefore concluded to be non-genotoxic with respect to clastogenicity and/or aneugenicity in the *in vitro* mammalian cell micronucleus assay.

According to EFSA, it is reasonable to conclude that a substance has no genotoxic potential if all adequately performed *in vitro* tests are clearly negative [15]. It is therefore concluded that there is no concern with respect to genotoxicity for Lacprodan® BLG and that further *in vivo* testing is not warranted.

In the 13-week toxicity study, Lacprodan® BLG was administered by gavage at dose levels up to 1000 mg/kg bw/day. In this study, Lacprodan® BLG was well-tolerated by the rats, and there were no effects of Lacprodan® BLG on food intake, general health or growth. No treatment-related changes were observed at the ophthalmological examinations.

Although statistically significant differences were observed between test and control animals for a few of the hematology and clinical chemistry parameters, these changes were ascribed to normal biological variation as they were minor, lacked dose-relationship or were limited to one sex. The changes in red blood cell parameters (hemoglobin, hematocrit, red blood cells) were within or close to 10 %, which is generally considered to be a non-adverse change [17].

There were no macroscopic or histopathological findings related to treatment with Lacprodan® BLG. All observations were considered incidental or were background alterations typically encountered in rats of this strain and age. Statistically significant changes in organ weights were generally considered to be incidental. These included the slightly reduced absolute heart weight in the medium-dose males and the increased absolute and relative kidney weight in the medium- and/or high-dose females. All changes were in the range 11–13 %. As WHO has reported the threshold of adversity of a toxicological effect on the relative heart and kidney weights in rats to be 15 %, the observed changes are considered not to be toxicologically significant [17]. Under the conditions of the present 13-week study, the NOAEL was 1000 mg/kg bw/day, the highest dose evaluated.

The lack of toxicological effects of Lacprodan® BLG in the present study are in accordance with the absence of toxicity seen with other isolated proteins from whey [18–23] and plant sources [24,25].

Lacprodan® BLG is intended for use in medical nutrition, food and sports nutrition, targeting the adult population, and will be used for example as protein-enriched drinks. The highest daily intake of Lacprodan® BLG is expected to occur in sports nutrition by athletes and consumer groups wishing to increase their muscle mass (not considering medical purposes). In these groups, the maximum mean and high (95th percentile) daily intake of Lacprodan® BLG is estimated to be 230 mg/kg bw and 1010 mg/kg bw, respectively, based on the EFSA Comprehensive Food Consumption Database. The NOAEL from the 13-week toxicity study was established to be 1000 mg/kg bw/day, which is 4 times higher than the estimated mean intake of 230 mg/kg bw/day and at the same level as the estimated maximum high (95th percentile) daily intake of Lacprodan® BLG for adults.

In conclusion, the present data generated from the bacterial reverse mutation assay and the *in vitro* micronucleus assay demonstrated a lack of genotoxic effects of whey-derived Lacprodan® BLG. In addition, Lacprodan® BLG was well tolerated by the animals in the 13-week subchronic study and no adverse effects were observed. Under the conditions of the 13-week study, a NOAEL of 1000 mg/kg bw/day, the highest dose level tested, can be established for Lacprodan® BLG in adult rats.

Author contributions

Marianne Dybdahl: Conceptualization, Project administration, Visualization, Writing – original draft

David Benjamin Selesko: Conceptualization, Funding acquisition, Resources, Writing – review and editing

Ulla Ramer Mikkelsen: Conceptualization, Funding acquisition, Resources, Writing – review and editing

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

David Benjamin Selesko and Ulla Ramer Mikkelsen are employees of Arla Foods Ingredients Group P/S.

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