2-year carcinogenicity study of rats with GM maize MON810

Multi-Site Study Plan

Study No: 632165 B/2015/GLP

Draft

Sponsor:	EU Project G-TwYST
Sponsor´s representative:	Prof. Dr. Pablo Steinberg
Test Facility:	Slovak Medical University Testing Laboratories Center Laboratory of Toxicology Limbová 14, 83303 Bratislava Slovakia
Study Director:	Dagmar Zeljenková, MVD, PhD Department of Toxicology Slovak Medical University Limbová 12 83303 Bratislava Slovakia E-mail: dagmar.zeljenkova@szu.sk

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Signatures of Approval of the Multi-Site Study Plan:

Study Director:	**Name** Dagmar Zeljenková	**Date**	**Signature**
Test Facility Management:	**Name** Martin Gajdoš	**Date**	**Signature**
Sponsor's Representative:	**Name** Pablo Steinberg	**Date**	**Signature**

Confirmation of Study plan in accordance with GLP

This study plan meets the requirements for GLP compliance

Head of QAU	**Name** Eva Němcová	**Date**	**Signature**

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NATIONAL REGULATIONS, GUIDELINES AND STANDARDS

Good Laboratory Practice

The study will be conducted in accordance with the OECD Principles of Good Laboratory Practice, as revised in 1997 (ENV/MC/CHEM(98)17), and the EU Commission Directive 2004/10/EC (adopted on the 11th of February 2004; Official Journal No L 50/44).

The test facility has received a statement of GLP compliance from the Slovak National Accreditation Service (certificate No. G-036). The National GLP Compliance Programme in the Slovak Republic is based on Act No. 67/2010 Coll. and in compliance with Government Decree No. 320/2010 Coll.

Each Principal Investigator at the histology processing test site and the histopathology examination test site will be responsible for compliance with their national GLP regulations, for any work performed at their test site and for data provided to the test facility for inclusion in the report. Any phase report or data provided by the principal investigator should include a statement of GLP compliance signed by them and a quality assurance statement signed by the test site quality assurance.

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI).

Test site 3, the biostatistics study phase, will not be claiming GLP compliance for this phase of the study. This test site does not hold a national certificate of GLP compliance, however the expertise of the Principal Investigator (Contributing Scientist) was considered by the Sponsor to be necessary for the study.

Other Guidelines

The study design is based on the procedures indicated by the following internationally accepted guidelines and recommendations:

- The OECD Test Guideline 451 for Testing of Chemicals; "Carcinogenicity studies" (adopted on the 9th of September 2009)

- The EFSA Considerations on the applicability of OECD TG 453 to whole food/feed testing (EFSA Scientific Opinion, 2013).

Animal Welfare

The study will be conducted in accordance with EU Directive 2010/63/EU of the European Parliament and the Council of 22nd September 2010 on the protection of animals used for scientific purposes. This study has been approved by the Veterinary State Administration, Slovak Republic (Statna veterinarna a potravinova sprava Slovenskej republiky). Animal care will be in compliance with SOPs of the Department of Toxicology, Slovak Medical University Bratislava and with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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The criteria described in the OECD Guidance Document on the recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation (ENV/JM/MONO[2000]7) such as changes in external physical appearance and clinical signs (described in Annex 3 of the above-mentioned OECD Guidance Document) will be taken into account to determine when an animal is in a moribund condition, is expected to become moribund or experiences pain and distress, and should therefore be euthanised.

GENERAL INFORMATION

Multi-Site Study Details

Test Sites:

Study Phase:	**Histology Processing**
Test Site 1:	Department of Pathology University of Veterinary Medicine Hannover Bischofsholer Damm 15 30173 Hannover Germany
Principal Investigator:	Prof. Dr. Wolfgang Baumgärtner wolfgang.baumgaertner@tiho-hannover.de
Test Site Quality Assurance:	Dr. Ilona Fleischhauer Fraunhofer Institut für Toxikologie und Experimentelle Medizin Leitung Qualitätssicherung Nikolai-Fuchs-Str. 1 30625 Hannover, Germany ilona.fleischhauer@item.fraunhofer.de
Study Phase:	**Histopathology**
Test Site 2:	Roger Alison Ltd., Caerfyrddin Fach, Cilcennin, Lampeter, SA48 8RN United Kingdom
Principal Investigator:	Roger Alison, BVSc., MRCVS, DiplECVP roger@rogeralison.com
Test Site Quality Assurance:	Clare Alison, BSc., MSc., PhD., MRQA, Roger Alison Ltd. clare@clarealison.com

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Study Phase:	**Biostatistics**
Test Site 3:	Stichting Dienst Landbouwkundig Onderzoek (DLO) Wageningen University and Research Centre Droevendaalsesteeg 1 6708 PB Wageningen The Netherlands
Principal Investigator:	Dr. Hilko van der Voet hilko.vandervoet@wur.nl

Additional Responsibilities

Toxicology:	Dagmar Zeljenková, VMD, PhD
Clinical Chemistry:	Prof. Spustova Viera, M.D., Ph.D.
Haematology:	Jana Tulinská, M.D., Ph.D.
Ophthalmology:	Prof. Andrej Černák, M.D., Dr.Sc.
Necropsy:	Katarina Ambrušová, VMD
Lead Quality Assurance:	Eva Němcová, Mgr.
Ethics Committee:	Ludmila Novotná, Dr.
Peer Reviewer:	To be added by amendment

Distribution List

The original signed study plan will be retained in the study file, to be archived at the completion of the study. Copies of the final study plan along with any amendments will be distributed to all relevant staff via supervisors/department heads specified as follows:

Sponsor:	pablo.steinberg@tiho-hannover.de
Study Director:	dagmar.zeljenkova@szu.sk
Deputy Study Director:	jana.tulinska@szu.sk
Clinical Chemistry:	viera.spustova@szu.sk
Haematology:	jana.tulinska@szu.sk
Ophthalmology:	andrej.cernak@pe.unb.sk
Necropsy :	katarina.ambrusova@szu.sk
Lead Quality Assurance:	eva.nemcova@szu.sk

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All Principal Investigators:	See multi-site study details
All Test Site QA:	See multi-site study details

Study Plan Amendments and Deviations

Any intended change to the study plan will result in an amendment to study plan approved by the study director and also signed by test facility management and the Sponsor. Amendments will be distributed to all recipients of the study plan.

Deviations (unplanned changes) from the study plan will be documented and acknowledged by the study director. Each principal investigator will document deviations from the study plan affecting their study phase, acknowledge and report them to the study director.

Quality Assurance

Lead quality assurance will audit and inspect study-related procedures and will report any audit and inspection results in writing to the study director and test facility management. This includes review of the study plan and any amendments, inspection of specific critical phases of the study and audit of the final report. Details of inspections will be included within the Quality Assurance Statement issued with the final report.

Test site quality assurance will audit and inspect study-related work conducted at their test site according with their SOPs and will report any audit and inspection results in writing to the principal investigator, test site management, study director, test facility management and lead quality assurance. Details of inspections will be included within the test site Quality Assurance Statement.

Reporting

A GLP compliant report will be presented. This will include the reporting requirements as described in OECD Test Guideline 451 and will be written in the English language. A draft report will be sent to the Sponsor for review and comments before issue of the final report. The pre-QA draft report and the post-QA draft report will be issued before the final report.

The report will be prepared by the study director based on the raw data / phase reports received from the responsible principal investigator/contributing scientist; the phase reports received from the principal investigator/contributing scientist will be included in the appendices of the report.

Archiving

The following documents will be archived under code number 632165B/2015/GLP at the Registry of accredited laboratories and laboratories with GLP certificate of SZU until the year 2026:

- the study plan and any amendments
- correspondence between the SD and test sites

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- QA reports of audits/inspections
- all raw data (paper and electronic)
- all original documents/primary documentation (including chain of custody records)
- samples of the test items
- copy of the histology processing records (original at the Department of Pathology, University of Veterinary Medicine Hannover, Germany)
- histological specimens (as long as the quality permits evaluation)
- the original histopathology phase report
- reports from contributing scientists

Further details of documents to be retained are included in the Appendix, Attachment 3. No data will be discarded without the Sponsor's written consent.

Proposed Time Schedule

Test feeds arrive:	April 2016
Arrival of animals:	April-May 2016
Starting of the treatment:	
- males	May - Day 1-5
- females	May - Day 7-11
Last necropsy of the animals:	Day 376
Histological processing:	1 month after Day 376
Histopathology evaluation:	2 months after Day 376
Draft Report to Sponsor:	3 months after Day 376

See Appendix, Attachment 1 for a more detailed proposed time frame.

OBJECTIVE

The purpose of this oral toxicity study is to assess the effects of GM maize MON810 when fed to rats for a period of 2 years. This carcinogenicity study is being conducted in association with the 90-day and the 12-month feeding trials (designed according to OECD Test Guidelines 408 and 453) as part of the GRACE project. These studies will provide a comparative assessment of the results of shorter term subchronic toxicity studies versus extended chronic toxicity and carcinogenicity studies.

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TEST AND CONTROL ITEMS

Test Item

GM maize crop: Variety containing the MON810 event expressing the insect-resistance trait based on expresion of the newly expressed Cry1Ab protein from *Bacillus thuringiensis.* Variety to be chosen after the analyses of the harvests.

Records including test item and reference item characterisation, batch number, purity, composition/concentrations, date of receipt, expiry date, storage conditions, quantities received and used will be maintained within the study file.

Control Item

Near-isogenic non-GM crop: Variety to be chosen after the analyses of the harvests.

TEST SYSTEM

Species and strain

Rat Wistar Rcc Han/Specific Pathogen Free (SPF)

Source

Harlan Italy, reg. No 2-2914 - 15-06-1994

Approximate weight and age

Upon arrival, the animals will weigh between 100-120 g and will be 5 weeks old. The animals will be 6-weeks old at the start of the study and will weigh between 110-140 g. Ideally, they should be born within 1-5 days of each other and be of uniform weight (± 20% of the mean).

Identification

Each rat will be marked by a code (tattoo) on the tail base or marked with a chip on the neck in accordance with SOP ŠPP/TOX/V002 to identify the animals individually. Each cage will be marked with a colored cage card.

Justification for the selection and number of animals

The animal species (*Rattus norvegicus* ssp. *alba*) and strain (Wistar Rcc Han) is recognized by international guidelines as a recommended test system for carcinogenicity studies. Females will be

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nulliparous and non-pregnant. The number of animals used in this study is planned to be 50 males and 50 females in each of the two dose groups, a total of 100 animals, as recommended by the OECD Test Guideline 451 (1998). A prospective power analysis will be performed to critically assess proposed sample sizes and meaningful effect sizes, and, if needed and practically possible, the number of animals will be adapted. Six male and six female rats more than those determined through the power analysis will be ordered and those animals not assigned to the study will be used as sentinels, which will be held in the same rooms as the rest of the animals in this study. Two animals of the same gender will be placed in one cage, and cages will be considered as experimental units.

MATERIALS AND METHODS

General Remark

Details of the materials and methods that are not specified in the subsequent sections of this study plan are contained in the appropriate standard operating procedures.

Test item preparation - Diet formulation

The test item will be supplied to the test facility as a pre-prepared complete pelleted diet. The diet formulation will be done so as to produce separate diet compositions according to the dose group requirements. The test diets will be provided as single batches (containing portions of diets packed in separate vacuum, gamma-irradiated packs). Specific details of this process and the analyses performed will be included in the accompanying „Carcinogenicity Study plan - Supplementary Information“ and records/data will be retained at JKI.

Storage conditions

The pelleted test diets will be stored in a closed storage room (cool and dry, controlled temperature and humidity) by the test facility. The temperature and humidity of the room will be recorded and the records will be kept.

Water

The rats will be supplied water *ad libitum* during the acclimatisation and study periods. Tap water with a special filter to eliminate microorganisms will be used. The bottles containing this water will be autoclaved before use. The microbiological and chemical quality of the water from the local mains will be monitored quarterly by the Waterworks Bratislava. The test facility will receive a corresponding quality certificate.

Animal housing

All animals will be housed in rooms B2 - 315 and 316 of the Specific Pathogen Free (SPF) experimental animal house equipped with a pressurized climatic system at the Department of

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Toxicology of the Slovak Medical University. The temperature and relative humidity in the animal room will be recorded every 20 minutes and every week the computer readout for the past week will be evaluated. Mean temperature will be maintained at 22 ± 2°C and relative humidity at 40-70%. The animals will be subjected to a 12-hour light/12-hour dark cycle.

Rats will be housed in Tecniplast cages Type 2145 F from Tecniplast Italy. The cages have a high-density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm^2.
The animals will be provided with environmental enrichment items: wooden chew blocks and a plastic tunnel or suitable alternatives. Certificates of analysis for the environmental enrichment items will be provided by the supplier. These enrichment items are considered not to contain any contaminants that could be expected to affect the study in any way.

We will use sterilized animal bedding (sawdust, JRS Lignocel®) from Charles River in Germany. It will be stored in the clean, dry and cold store room on the second floor in the animal facility. One lot of sawdust bedding will be purchased and used for the entire study.

The cages will be cleaned twice a week outside of the animal room. Animals will first be transferred to a clean cage. The cages will then be emptied and cleaned with water and detergent. After cleaning they will be dried and thereafter immersed in disinfectant. The cages will then be brought into the animal house and placed in an additional Tecniplast disinfectant solution. Then the cages will be placed in the SPF unit on a drying rack before use.

The cage racks will be cleaned in the SPF rooms every week manually with water and detergent.

Feed containers and any other containers or equipment being used in the SPF rooms will be cleaned in the same way as the cages are cleaned.

Bottles will be exchanged and cleaned daily according to SOP ŠPP/SPF/V005. They will be cleaned in a special automatic washing machine set aside for the bottles in this study. The cleaning solution will include detergent followed by a disinfectant.

Experimental Design

Animal receipt and acclimatisation

All animals will be purchased from Harlan and will only be a few days apart in age. Therefore, we will have the required number of test animals of uniform weight and age, and house them all under identical conditions.

Upon arrival, the animals will be placed in cages, 4 per cage. 48 hours after arrival, the animals will be weighed and kept in cages for the next 4 - 6 days prior to the start of the study to allow for acclimatisation to the laboratory conditions. These are identical to those defined for the feeding trial. During this period of time the health status of the animals will be monitored twice a day (see the section Periodical Health Status Observations below for a full description of the health status evaluation) according to SOP ŠPP/TOX/V006.

One day before the start of treatment, all animals will be housed in 2 separate rooms (1 for males, 1 for females) under standard SPF conditions. To verify the health condition of the rats, a detailed examination of all animals will be carried out on study day 1, prior to the start of the treatment (see the section Periodical Health Status Observations for a full description).

Randomization

Tables with cage numbers and the random diet assignment will be prepared by the local statisticians. We will use the Random Number Generators (RNG) of SPSS software to allocate rats to cage for male and female animals separately. All male animals will be numbered from 01 to 50. We will assign 2 animals into 1 cage, using RNG. These animals will be excluded from next option and random choice will be repeated until all animals are randomly assigned to cages. The same procedure will be done with female animals - they will be numbered 101 to 150. Six male and six female rats will be used as sentinels.

Four racks contain 5 rows of 5 cages. Each cage houses two rats. Dose groups are randomized within pairs of cages (there are 2 pairs on each row). This implies that the design is a randomised complete block design in which each row contains 2 blocks. The experiment starts in week 1 with 5 blocks on Monday (10 cages, 20 male animals). This is repeated on the other four days in week 1. On Monday-Thursday the first four vertical rows of racks 1 and 2 are used, and racks are filled from top to bottom, left to right. On Friday the last vertical row is used, with the lower cages of the two racks forming one block. The scheme of week 1 is repeated in week 2 with female rats. At the end of the feeding trial experiment the cages are handled block by block in the same order as at the start of the experiment. This design ensures that possible differences between starting and ending days, and also possible differences between the position of cages, are confounded with blocks implying that the analysis accounts for such differences.

Table 1. Randomised order of the 2 dose groups for each block in the MON810 carcinogenicity experiment. The dose group codes 1-2 are randomised by the feed supplier over the two dose groups in the study.

Start Week	Sex	Block No.	Row	Start Day	Rack 1 Male				
Week 1	Male	Block 1/2/21	Row 1	Mon / Mon / Fri	2	1	2	1	2
Week 1	Male	Block 3/4/21	Row 2	Mon / Mon / Fri	2	1	1	2	1
Week 1	Male	Block 5/6/22a	Row 3	Mon / Tues / Fri	1	2	2	1	1
Week 1	Male	Block 7/8/22b	Row 4	Tues / Tues / Fri	1	2	2	1	2
Week 1	Male	Block 9/10/25a	Row 5	Tues / Tues / Fri	1	2	1	2	2

Start Week	Sex	Block No.	Row	Start Day	Rack 2 Male				
Week 1	Male	Block 11/12/23a	Row 1	Wed / Wed / Fri	1	2	1	2	2
Week 1	Male	Block 13/14/23b	Row 2	Wed / Wed / Fri	2	1	2	1	1
Week 1	Male	Block 15/16/24a	Row 3	Wed / Thurs / Fri	2	1	1	2	1
Week 1	Male	Block 17/18/24b	Row 4	Thurs / Thurs / Fri	2	1	2	1	2
Week 1	Male	Block 19/20/25b	Row 5	Thurs / Thurs / Fri	1	2	2	1	1

Start Week	Sex	Block No.	Row	Start Day	Rack 3 Female				
Week 3	Female	Block 26/27/46a	Row 1	Mon / Mon / Fri	2	1	1	2	1
Week 3	Female	Block 28/29/46b	Row 2	Mon / Mon / Fri	1	2	1	2	2
Week 3	Female	Block 30/31/47a	Row 3	Mon / Tues / Fri	2	1	1	2	1
Week 3	Female	Block 32/33/47b	Row 4	Tues / Tues / Fri	1	2	1	2	2
Week 3	Female	Block 34/35/50a	Row 5	Tues / Tues / Fri	2	1	2	1	2

Start Week	Sex	Block No.	Row	Start Day	Rack 4 Female				
Week 3	Female	Block 36/37/48a	Row 1	Wed / Wed / Fri	1	2	1	2	2
Week 3	Female	Block 38/39/48b	Row 2	Wed / Wed / Fri	2	1	2	1	1
Week 2	Female	Block 40/41/49a	Row 3	Wed / Thurs / Fri	1	2	2	1	1
Week 3	Female	Block 42/43/49b	Row 4	Thurs / Thurs / Fri	1	2	2	1	2
Week 3	Female	Block 44/45/50b	Row 5	Thurs / Thurs / Fri	1	2	1	2	1

283 On a regular basis (every two weeks) racks will be rotated clockwise within the original room
284 configuration.

285 A skeleton analysis of variances with the appropriate degrees of freedom is given below, both for an
286 analysis with all cages including both sexes as well for an analysis for a single sex.

287 **Mon810 carcinogenicity experiment (2 dose groups, 100 cages, 200 rats)**

ANOVA for both sexes		**ANOVA for a single sex**	
Source of variation	d.f.	Source of variation	d.f.
startweeks stratum		block stratum	24
sex	1		
		block.cage stratum	
startweeks.block stratum	48	dosegroup	1
		Residual	24
startweeks.block.cage stratum			
dosegroup	1	Total	49
sex.dosegroup	1		
Residual	48		
Total	99		

Route of administration

The route of administration will be the oral route as this route is the most appropriate for the safety assessment of foods. The test item will be incorporated into the diet, since this is the way humans could be exposed to the test item. Attention will be paid that there will be no nutritional imbalances as a result of dietary incorporation of the test item.

Food will be supplied *ad libitum*. Measurement of feed consumption and food efficiency will be made once weekly for the first 13 weeks and monthly thereafter. At the beginning of each food consumption measurement, full feeders with stainless steel lids will be weighed and placed in each cage. The feeders will be weighed again on the day of the feeder change-out, the difference in weight being an estimate of the total amount consumed by two rats in one cage. Food spillage will be documented and the amount will be noted and subtracted. Feed consumption will be determined once weekly for the first 13 weeks and monthly thereafter and reported as the total amount of feed consumed by two animals in one cage per week.

General experimental design with MON810 maize, start May-June 2014

Group	Isogenic maize (% of diet)	MON810 (% of diet)	No. of Males	No. of Females
1	33	0	50	50
2	0	33	50	50
Sentinels[1]			5	5
Total animals			105	105

[1] Sentinels will be fed the standard rat diet Teklad Global Diet®.

The two diets will be randomised and labelled I and II by the supply company. The code will only be given to Ralf Wilhelm and Josefine Engel (JKI). Feed containers and scoops will be colour-coded. However, animal house staff will be “blind” with respect to the identity of the diets.

The codes will be unblinded for the histopathological evaluation of the tissues after necropsy.

Blood and urine collection, haematology, clinical chemistry and urine analyses as well as body weight, feed consumption and organ weight measurements will be performed block by block, from cages in the order of the randomisation scheme. This minimises sampling variation between dose groups within blocks.

Periodical Health Status Observations

Morbidity, mortality

Normally observations are done twice a day. However, in case of moribund animals, we will isolate them in the quarantine area to prevent cannibalism and will carefully observe them at least 4 times daily. Selection criteria are made explicit in SOP ŠPP/TOX/V004. If a study animal dies, we will subject it to necropsy as soon as possible after death. The criteria described in the OECD Guidance Document on the recognition, assessment and use of clinical signs as humane endpoints for

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experimental animals used in safety evaluation (ENV/JM/MONO[2000]7) such as changes in external physical appearance and clinical signs (described in Annex 3 of the above-mentioned OECD Guidance Document) will be taken into account to determine when an animal is in a moribund condition, is expected to become moribund or experiences pain and distress, and should therefore be euthanized. In such a case animals will be anaesthetized with ketamine/xylazine (SOP ŠPP/TOX/V005) and thereafter immediately necropsied.

Clinical signs

Cage side observations / uncovered cage

Rats will be inspected twice daily for evidence of reaction to treatment or illness, which includes the following signs: changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions as well as activity level and change in behavior in accordance with SOP ŠPP/TOX/V003.

Detailed physical examination and functional assessment

Rats will be examined out of the cage once weekly. Any deviations from normal will be recorded in terms of nature and severity, date and time of onset, duration and progress of the observed response. Signs noted will include changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, and unusual respiratory patterns as well as activity level and change in behavior.

Changes in gait, posture and response to handling as well as the occurrence of clonic or tonic movements or bizarre behavior (self-mutilation, walking backwards) will also be recorded. The outcome of this examination will be recorded for each animal in accordance with the SOP ŠPP/TOX/V003 (Origin of score system: Országh A. et al. [1985] Veterinárna ortopédia a rontgenológia, Bratislava: Príroda, 243 p.). The animals will also be assessed for gait disturbances using the *Accuplacer* treadmill equipment.

Ophthalmologic examination

The eyes of all animals will be examined in line with OECD TG 453 prior to the administration of the test feeds and at the end of the study. Pupillar dilation and ophthalmologic examination of both eyes will be performed by an experienced opthalmologist in the conscious rat during gentle manual restraint by a technician. In a first step, the eyes and the peribulbar structures will be macroscopically examined. Thereafter, direct ophthalmoscopy will be performed using an opthalmoscope. Ophthalmoscopic findings will be recorded on data sheets and transcribed into the computer system for compilation and analysis.

Body weight

Each animal will be weighed at the following times: 1) 48 hours after arrival, 2) on the first day of feeding, 3) weekly for the first 13 weeks, 4) and monthly thereafter, 5) at the end of the study, 6) in the event of an early death or sacrifice *in extremis*. The General Linear Model (GLM) for Repeated Measures will be used for the analysis of the body weight.

Procedures For Sample Collection

Samples will be collected for the following analyses: haematology, blood chemistry, urinalysis and histopathology. Samples collected will include blood, urine and tissues/organs. Blood samples will be

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divided for the haematology and clinical chemistry analyses. Tissues/organs will be removed and evaluated histopathologically.

Sample collection and tissue processing
Sufficient personal will be available:

Urine will be collected by person No. 1
Urine processing and transport to the Laboratory of Clinical and Experimental Biochemistry - person No. 2
Blood taking from the tail vein - person No. 3
Blood processing, dividing of samples - person No. 4
Blood transport to the Laboratory of Immunotoxicology (haematology) - person No. 5
Blood transport to the Laboratory of Clinical and Experimental Biochemistry (clinical chemistry) - person No. 6

Sample collection and tissue processing at the end of the study
This will be done in accordance with SOP ŠPP/TOX/V006. Sufficient personal will be available:

Animals will be anaesthetized by person No. 1
Animal transport to the autopsy room on the same floor - person No. 2
Necropsy of the thorax part of the body - person No. 3
Necropsy of the abdominal part of the body - person No. 4
Necropsy of the genital organs - person No. 5
Removal (and weighing) of tissues and organs in line with OECD guideline 451 - person No. 6
Decapitation and necropsy of the head including brain - person No. 7
All organs will be stored in formalin or Bouin´s solutions for the histological examination - person No. 8

Details will be documented by subsequent amendment.

Haematology
At the end of the study and before sacrifice, blood samples from the tail vein will be taken from all animals for haematological examination after 12 hours fasting. EDTA will be used as anticoagulant. Blood samples will be stored at room temperature (17-25°C), maximally up to 4 hours, until measurement. Haematological analysis will be performed in accordance with SOP ŠPP/IMU/M002 by using a Sysmex K-4500 automated haematology analyzer (Sysmex, Kobe, Japan).

Parameters scheduled for examination are:

- erythrocyte count (RBC)
- haematocrit (HT)
- haemoglobin (Hb) -
- leukocyte count (WBC)
- differential leukocyte count
- platelet count (PLT)

Differential leukocyte counts will be performed by using a light microscope. Blood smears will be subjected to panoptic staining by using May-Grunwald and Giemsa-Romanowski dyes. The

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percentage of lymphocytes, neutrophils, eosinophils, basophils and monocytes will be determined by examining 200 cells.

Clinical chemistry

At the end of the study and before sacrifice, blood samples from the tail vein will be taken from all anaesthetized animals for blood chemistry examination after 12 hours fasting. Samples will be analysed using an Ortho Clinical Vitros® 250 Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ, USA). Methodologies include colorimetric, potentiometric and rate tests using multi-layered Vitros Slides in accordance with SOP ŠPP/LEKB/M001. Blood samples will be stored at room temperature (17-25°C) for a maximum of 4 hours until measurement. Parameters will include:

- total protein
- albumin
- aspartate aminotransferase
- alanine aminotransferase
- alkaline phosphatase
- creatinine
- urea
- glucose
- total cholesterol
- Na
- K
- Cl

In addition:

- Ca
- P
- triglycerides
- 17β-estradiol, testosterone, T3 and T4

Urinalysis

During the last week of the study urine analyses will be performed. Urine will be collected from each individual rat in metabolic cages under the same conditions in groups of 8 animals during 5 consecutive days. For every collected group of animals, every test diet will be balanced for the number of animals submitted to urine collection. Sixteen animals will be kept in metabolic cages for 16 hours each day of urine collection. The total volume of urine excreted during the 16-hour period will be measured at the end of every 16-hour collection period, and the animals will be brought back from the metabolic cages to their respective conventional cages. Every sample collected at different time points will be identified by a unique code. Data concerning the volumes of urine collected at different time points will be recorded.

Parameters will include:

- appearance
- volume
- osmolality

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- pH
- total trotein
- glucose
- occult blood
- ketone
- urobilinogen

Necropsy and Histopathology

Gross necropsy

A complete necropsy will be performed on all animals at the end of the study. Organs/tissues will be examined macroscopically for any deviations from normal (in accordance with ŠPP/TOX/V005). A supervising toxicopathologist will be present at terminal necropsy. The results will be manually recorded and subsequently transferred and saved in the computer system.

As described in the OECD Test Guideline 451, organ weights of animals sacrificed after 2 years will not be recorded, since geriatric changes and the development of tumours will confound the usefulness of organ weight data.

Organs and tissues for histopathological examination will be formalin-fixed (neutrally buffered 10% formalin). Details will be added later by amendment.

As described in the OECD Test Guideline 451, the following tissues will be subjected to a histopathological examination after fixation:

- all gross lesions
- adrenal glands
- aorta
- brain (representative regions including cerebrum, cerebellum, medulla/pons and pituitary)
- caecum
- cervix
- coagulating gland
- epididymides
- eyes
- femur (femoro-tibial joint)
- gonads (testes, left and right; ovaries, left and right)
- Harderian gland
- heart
- kidneys (left and right)
- lacrimal gland
- large intestine
- liver
- lymph nodes: submandibular and mesenteric
- oesophagus
- ovaries
- pancreas
- parathyroid

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- peripheral nerve (sciatic) preferably in close proximity to the muscle
- prostate
- rectum
- salivary glands
- section of bone marrow and/or a fresh bone marrow aspirate
- seminal vesicles
- skeletal muscle
- skin with mammary gland area
- small intestine (including the Gut-Associated Lymphoid Tissue, GALT)
- spinal cord (cervical, mid-thoracic and lumbar regions)
- spleen
- sternum with bone marrow
- stomach
- testes
- thymus
- thyroid
- tongue
- trachea and lungs inflated with fixative and then immersed in formalin
- urinary bladder
- uterus
- vagina
- additional tissues may need to be investigated based on clinical or any other findings

Trimming will be done by the Department of Toxicology at SZU, who will ship the tissue samples to the histology processing test site at the Institute of Pathology at the University of Veterinary Medicine Hannover immediately after the tissue samples have been formalin-fixed. Tissue samples of animals that have to be prematurely necropsied (because of their moribund condition) will also be shipped immediately after having fixed the samples.

Histology processing

The trimmed tissue specimens will be transported in labelled cassettes in 10% buffered formalin at ambient temperature to the histology processing test site at the Department of Pathology, University of Veterinary Medicine, Hannover, Germany. They will be stored in neutral buffered 10% formalin at room temperature until they are further processed. The whole processing will take place under GLP conditions in room B2-317 of the Department of Pathology, and all procedures will be performed by trained technicians.

Briefly, trimmed tissue samples within the cassettes will be checked and recorded and a confirmatory dispatch note will be sent back to SZU. Then, specimens will automatically be embedded in paraffin wax according to a standardized protocol. Paraffin blocks will be made manually. Each block will be cut until the whole tissue specimen is visible on its surface. Then, a 3-5µm thick section will be taken, straightened on a warm water bath and mounted on a glass slide. The glass slides will be labelled according to the labelling on the respective cassette. Slides will be stained with haematoxylin and eosin according to a standardized protocol. The slides will then be covered with a cover glass, dried, and stored at room temperature until shipped. The slides will then be packed in a shatter-proof manner and shipped to the histopathology examination test site Roger Alison Ltd. by DHL or a comparable courier.

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The paraffin blocks will be stored at the Department of Pathology, University of Veterinary Medicine, Hannover, Germany, temporarily. Final archiving will take place at SZU.

Histopathology

The above-mentioned tissue specimens of all animals in the control group (group 1) and the high dose group (group 2) will be examined. Furthermore, all tissue specimens from animals having died or having had to be sacrificed before the actual end of the feeding trial as well as all tissues showing macroscopic abnomalities will be examined microscopically.

A histopathology phase report will be provided by the principal investigator for inclusion in the main report as an appendix. A peer review of findings will be performed and the peer review statement will be attached as a separate appendix within the final report.

All histological slide samples will be returned to the test facility for archiving.

DATA EVALUATION AND STATISTICAL ANALYSIS

Evaluation of the data and screening for any obvious errors and outliers will be performed by the local statistical team at the test facility, SZU. Outliers will be checked against the original paper records. Outliers which are not due to transcription or other obvious types of error will be retained, but noted. The statistical analysis will be performed by the Biostatistics test site, the Biometris group of partner DLO. Analyses will be performed with and without the outliers. If the conclusion depends on the presence of one or more outliers, then this will require further investigation on a case-by-case basis. If an outlier makes no difference to the conclusions, it will be retained.

The statistical analysis will be performed according to a pre-established protocol. Cages will be the experimental units. Summary statistics will be tabulated. Weight and food consumption data will be plotted over time. Data of males and females will be analysed together unless there is a prior biological argument to analyse males and females separately. Conclusions for males and females will also be reported separately if a significant interaction between treatment and sex is found in the joint analysis.

The statistical analysis will present the results as differences between the treated group and the control group on an appropriate scale with a 95% confidence interval, and compare these results with a zero difference (difference test) and pre-specified limits of concern (equivalence test). The precise statistical methods may vary depending on the nature of the data. For many quantitative endpoints an ANOVA type analysis with fixed factors group and sex will be appropriate. The results of the statistical analyses will be presented in tabular and graphical form.

The data used within the statistical analyses will be made publically available on the G-TwYST web site.

For histopathology data, statistical analyses may be performed at the discretion of the Study Pathologist if required and full details of statistical tests employed will be included in the histopathology phase report.

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APPENDIX

Attachment 1

Proposed Time schedule

				Month number																						
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	Day - 7	Day 0	Day 1																							
Quarantine	7 days																									
Randomization		males, day 0; females, day +14																								
Ophtalmology	day - 5/6																									715-720
Application males			day 1 start																							end day 730
Application females			day 1+ 14 start																							end day 730
Weighing of the feed		every 7 days	every 7 days	every 7 days	every 7 days	every 7 days	monthly																			
Weighing of animals		every 7 days	every 7 days	every 7 days	every 7 days	every 7 days	monthly																			
General clinical observations		every day twice																								
Detailed clinical observations		every 7 days	every 7 days	every 7 days	every 7 days	every 7 days	monthly																			

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Sensory reactivity		every 7 days	every 7 days	every 7 days	every 7 days	every 7 days	monthly																				
Hematology, males+females															days 360-365												days 720-730-
Clinical chemistry, males+females															days 360-365												days 720-730-
Urinalysis, males+females															days 360-365												days 720-730
Gross necropsy, males																											day 731
Gross necropsy, females																											day 731
Tissue processing																											731+1 month
Histopathology																											731+ 4 months
Draft report																											731+6 months

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Attachment 2

List of Materials and Equipment

Laboratory of Toxicology, SZU

- Electronic balance Kern ABJ 220-4M, No. WB 0850106, range: 0.01-220g, precision: 0.0000g, Kern &Sohn GmbH, Germany, room No. B2-326
- Personal computers, office

Experimental animal rooms:

- Temperature and humidity detector, PMICRO-LCD-THSYS, Dallas Semiconductor, rooms No. B2 - 315 and 316
- Personal computers, office
- Data backup system - 2 external hard drives and the eXtplorer system established by JKI
- Electronic balance Sartorius BP 1200, No. 6080646, range: 0-1000 g, Sartorius AG, Germany, the operating room of experimental animal rooms
- Pressure air conditioning system VENTO, No. RMK 01.2, REMAK LTD., Czech Republic, experimental animal rooms on the 3th floor at SZU
- Personal computers, office
- Type of animal cages in TECNIPLAST Filter top cages Type 2145 F with an H-Temp™ (PSU) durable filter cover from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940 cm^2.
- Ophthalmoscope Welch Allyn
- Apparatus for neurobehavioural testing: Accupacer treadmill

Laboratory of Immunotoxicology, SZU

- Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan, No. VČ F-1466, room B2-212
- Personal computers, office

Laboratory of Clinical and Experimental Biochemistry, SZU

- Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, USA, room B-048
- Personal computers, office, software for processing of the data
- Windows XP, program Office 2003
- Windows 2007, program Office 2010
- Software SPPS version 16.0.

Materials:

- Syringes, needles, tubes, tubes microvette, tips, gloves, gauze, racks, paper, cartridge

Department of Pathology, University of Veterinary Medicine Hannover

- Embedding apparatus, embedding solutions, paraffin wax, microtome blades, glass slides, cover glasses, staining solutions, packing materials, paraffin block storage cabinets

Equipment for Histopathology, Roger Alison Ltd.

- PathData software, Olympus microscope, personal computers, slide storage cabinet

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Attachment 3

List of records to be maintained for this study includes:

- animal receipt records and quarantine records
- randomization records
- serology reports
- feed log and analysis reports
- water analysis reports
- moribundity/mortality checks
- rack/cage rotation records
- Temperature/relative humidity/light intensity and cycle checks
- dose analysis data
- dose preparation and accountability records
- dose administration records
- necropsy and histopathological findings
- pathology specimens as specified
- histology processing records

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