Chronic toxicity (1year) study of rats with Monsanto MON 810 maize

Study Plan

Study No: 311957- C/14/ GLP

Draft Version
4 Dec 2013

Sponsor: EU Project GRACE

Sponsor´s representative:
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Test Facility:
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Study Director:
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Test Site 1:
Histopathology
Test Site PrincipalInvestigator:
[according to tender outcome]
[redacted]

Test Site 2:
Diet analysis
RIKILT – Institute of Food Safety
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Building 123, Akkermaalsbos 2
NL-6708WB Wageningen
Netherlands
[redacted]
Principal investigator: Dr G.A. Kleter (gijs.kleter@wur.nl)

Test Site 3:
Diet preparation

Mucedola s.r.l. [redacted]

[redacted]

Test Site 4:
Maize production, diet and transcriptomic analysis

Center for Research in Agricultural Genomics (CRAG)
Campus UAB – CRAG building
Bellaterra, Cerdanyola del Vallès
08193 Barcelona
Spain
[redacted]
Principal investigator: Dr M. Pla de Sola Morales(maria.pla@udg.edu)

Test Site 5:
Diet analysis, and immunological and metabolomic analyses

Laboratoire d'Immuno-Allergie Alimentaire
Service de Pharmacologie et Immunologie (SPI)
CEA Saclay / Building 136
iBiTec-S
F-91191 Gif-Sur-Yvette cedex
France
[redacted]
Principal investigator: Dr Karine Adel-Patient (karine.adel-patient@cea.fr)

Test Site 6:
Experimental procedures

Institute of Veterinary Biochemistry
Free University of Berlin
Oertzenweg 19b
14163 Berlin
Germany
[redacted]
Principal investigator: Prof Dr R. Einspanier (einspani@zedat.fu-berlin.de)

Approval of the Study plan

	Name	Date	Signature
Study Director	Dagmar Zeljenková, VMD, PhD.		
Test Facility representative	[redacted]		
Principal Investigator Test Site 1	[redacted]		
Principal Investigator Test Site 2	Dr Gijs A. Kleter		
Principal Investigator Test Site 3	[redacted]		
Principal Investigator Test Site 4	Dr Maria Pla de Sola Morales		
Principal Investigator Test Site 5	[redacted] Dr Karine Adel-Patient		
Principal Investigator Test Site 6	Ralf Einspanier, Prof., Dr.rer.nat.		

Sponsor	Name	Date	Signature
	Prof. Dr. Joachim Schiemann		

Confirmation of Study plan accordance with GLP

This study plan meets the requirements for GLP compliance

Headof QAU	**Name**	**Date**	**Signature**
Test facility	[redacted]		

Headof QAU	**Name**	**Date**	**Signature**
Test Site 1	[redacted]		

Confirmation of Study Plan accordance with ISO 17025

	Name	**Date**	**Signature**
Head of QAU	[redacted]		
Test Site 2			

Head of QAU	**Name**	**Date**	**Signature**
Test Site 3	[redacted]		

Disclaimer

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Regulatory Test Guidelines

The study will be carried out in accordance with OECD Test Guideline 452 for Testing of Chemicals, adopted September 7st, 2009

GoodLaboratoryPractices

Animal trials (SZU, Slovakia):
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 of 11th February 2004 (Official Journal No L 50/44). The national GLP compliance programme in the SlovakRepublic is based on Act No. 67/2010 Coll. and in compliance with Government Decree No. 320/2010 Coll. The Laboratory of Toxicology of the SlovakMedicalUniversity has received a statement of GLP compliance from the Slovak National Accreditation Service (certificate No. G-036) [certificates of Test Site 1 will be included based on tender outcome]. The Laboratory of Clinical and Experimental Biochemistry of the SlovakMedicalUniversity holds an accreditation certificate (M-013) from Slovak National Accreditation Service and is subject to the national quality control programme for clinical biology and is controlled by the quality assurance unit (QAU) of the SlovakMedicalUniversity. All procedures executed by the Laboratory of Toxicology and the Laboratory of Clinical and Experimental Biochemistry[Test Site 1 will be included based on tender outcome]are described in standard operating procedures (SOP), approved by the QAU.

Analysis of feed materials
Maize culture, harvesting and grain packaging will be performed in experimental and commercial fields not subjected to specific GLP. This will be supervised by CRAG-UdG. Sampling is performed according to EN ISO 24333:2009, based on "Cereals and cereal products".

Maize and diet samples collected at Mucedola srl. (Test Site 3, diet manufacturer) are to be sent to RIKILT (Test Site 2), where these samples will be registered through the sample registration system based on information provided in the Sample Information Form ("MIF") to be prepared and submitted to RIKILT's Sample Room ("Monsterkamer"). These samples will be assigned a Laboratory Information Management System (LIMS) number and divided into subsamples for dispatch towards the subcontractor Covance and the other Test Sites 2 and 4-6 for further analysis. Registration and processing of samples is done under the pertinent SOPs. The analyses of maize and diet samples for target constituents are to be carried out by various partners and a subcontractor, as follows:

- RIKILT (Test Site 2) is to analyse samples for mycotoxins, organic contaminants (dioxins, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), nitrosamines, and presence of genetically modified organisms (GMOs). Analyses will be carried out under ISO 17025:2005 on "General requirements for the competence of testing and calibration laboratories". Methods have been validated and accredited except for nitrosamines, which will be carried out under a SOP. More specifically, the following SOPs apply: dioxins, A0565; PAHs, combined A0824 / A0834, PCBs (included in aforementioned SOPs); and GMOs, A1033 and A1132. The subcontractor Covance will analyse maize and diets for key compounds according to the OECD consensus document (proximate composition, micronutrients including vitamins and minerals, fatty and amino acid profiles, anti-nutrients and other secondary metabolites) as well as heavy metals, pesticide residues, and nitrate.
- Mucedola (Test Site 3) will test maize for the presence of mycotoxins, and maize and diets for microbiological quality and proximate composition, under ISO 17025. Manufacturing of

custom feeds is done under Good Manufacturing Practice. Coding of diets and samples is carried out according to instructions received from the contractor.

- INRA (Test Site 5) will test selected samples of maize and diets for the presence of the newly expressed Cry1Ab protein (known to be present in the genetically modified MON810 maize)

Supplementary analysis of diet and animal tissues at other Test Sites

Omics analysis of animal tissues and feed materials will only be performed on candidate metabolites, transcripts and proteins if results from a previous 90-day feeding study (Study No: 311957 - A / 13/ GLP) carried out with the same feed materials and dose levels indicate relevant alterations.

Possible omics analyses on animal tissues to be performed in parts by FUB (Test Site 6) and CRAG (Test Site 4) and on feed materials to be performed by CRAG/UdG (Test Site 4):omics labs are not subjected to GLP. However, the experimental procedures will be guided by the principles of GLP when applicable. Possible analyses performed at INRA (Test Site 5) on blood and tissues will be done in respect of the quality reference system developed and used for research and experimentations at INRA in order to meet the objectives of INRA's quality policy, *i.e.* traceability of research activities and reliability of measurable results.

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 will be approved by the Veterinary State Administration, Slovak Republic (Statna veterinarna a potravinova sprava Slovenskej republiky) Ro-4372/12-221. Animal care will be in compliance with SOPs of the Department of Toxicology, Slovak Medical University Bratislava and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

1. OBJECTIVE

This study is based on the OECD Test Guideline 452 for Testing of Chemicals (chronic toxicity studies).The objective of this study is to determine the added scientific value of chronic toxicity studies with whole food/feed compared to 90-day feeding studies (e.g. Study No: 311957 - A / 13/ GLP) and comparative compositional analyses in the risk assessment of GM food/feed using Monsanto MON810 maize.

2. PROFESSIONAL AND SUPERVISORY STAFF

Test facility SZU:
CVs of all engaged scientists are deposited in Department of Toxicology, Slovak Medical University.

Toxicology:
Dagmar Zeljenková, VMD, PhD.
SZU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Veterinary and gross pathology:
[redacted]
SZU, Department of Toxicology, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Clinical chemistry:
[redacted]
SZU, Laboratory of Clinical and Experimental Biochemistry, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Haematology:
[redacted]
SZU, Laboratory of Immunotoxicology
Limbová 14, 833 03 Bratislava 37, Slovak Republic

Ophthalmology:
[redacted]
SZU, University Hospital**,** Anatolská 11, 85107 Bratislava, Slovak Republic

Quality Assurance Manager:
[redacted]SZU QA Unit, Limbová 14, 833 03 Bratislava 37, Slovak Republic

Statistical Analysis:
[redacted]
SZU, Department of Biophysics, Biostatistics and Informatics,
Limbová 14, 833 03 Bratislava 37, Slovak Republic

Ethics Committee:
[redacted]
Cancer Research Institute, Slovak Academy of Sciences, Vlárska7, 83391 Bratislava, Slovak Republic

Test Site 1:[Histological laboratory has to be assigned according to tender outcome]
Histology preparation:
[Has to be included according to tender outcome]

Histology evaluation:
[Has to be included according to tender outcome]

Quality assurance manager:
[Has to be included according to tender outcome]

Test Site 2:
Diet analysis
Dr Esther J. Kok, Dr Gijs A. Kleter

Test Site 3:
Diet preparation
[redacted]

Test Site 4:
Maize production and handling, diet analysis
Dr Maria Pla

Test Site 5:
Diet analysis, and immunological and metabolomic analyses
[redacted]
Dr Karine Adel-Patient

Test Site 6:
Experimental procedures:
Tissue analysis using transcriptomics or proteomics
[redacted]
Prof. Dr. Dr. Ralf Einspanier

3. TEST FACILITIES

Test Facility:
Testing Laboratories Center
Laboratory of Toxicology,
Slovak Medical University
Limbová 14,
83303 Bratislava 37

Test Site 1:
[Has to be included according to tender outcome]

Test Site 2:
RIKILT – Institute of Food Safety
Wageningen University and Research Center Campus
Building 123, Akkermaalsbos 2
NL-6708WB Wageningen
Netherlands

Test Site 3:
Mucedola s.r.l. (licensed by Harlan)
Via Galileo Galilei 4
20019 Settimo Milanese (MI)
Italy

Test Site 4:
Center for Research in Agricultural Genomics
Campus UAB - CRAG building
Bellaterra
Cerdanyola del Vallès
08193 Barcelona
Spain

Maize growing, harvesting and drying:
Estació Experimental Mas Badia
17134 La Tallada d'Empordà, Girona, Spain.
[redacted]

Test Site 5:
Laboratoire d'Immuno-Allergie Alimentaire
iBiTec-S, Service de Pharmacologie et Immunologie (SPI),Building 136
CEA de Saclay
F-91191 Gif-Sur-Yvette cedex
France

Test Site 6:
FreieUniversitaetBerlin
InstituteforVeterinaryBiochemistry
Oertzenweg 19b
14163 Berlin
Germany

4. TIME SCHEDULE

Test feeds arrival		Planned date: December 2013 - January 2014
Arrival of animals		January 8 or 15, 2014
Starting of the treatment	males	January, 15 or 22,2014
	females	January, 16 or 23, 2014
Necropsy animals		January, 15-19 or 22 -26 2015
Histology	Slides preparation	February, 2015
	Histology evaluation	February - March, 2015
Final report – draft to Sponsor:		May 2015

5. TEST AND CONTROL CROPS

GM crop: Variety: DKC6667-YG (containing the MON810 event with insect-resistance trait based on expression of the newly expressed Cry1Ab protein)
Non-GM near-isogenic crop: Variety: DKC6666
Conventional crop : SY-NEPAL

All crops: production during the 2013 season, all in a small area in the Empordà (NW of Catalonia, Spain) in the same conditions and according to the standard cultural practices in the region. No insecticides applied in any case. Herbicide and other treatments recorded. Monitoring of the date of sowing, flowering and harvesting, yield, grain humidity and relevant pathogen attacks, particularly corn borer incidence. Climatic data are available.
Crops are dried in a commercial dryer facility at 60ºC and sampled (EN_ISO_24333) to prepare about 4 tons of each variety for preparation of the diets. Grains are packaged in commercial big-bags of 300-350 kg, each labeled with the full name of the variety and other details.

Example of container label:

Producto / Product: MAIZE GRAIN
Variedad / Variety: DKC6666
Masa (Kg) / Mass weight (Kg): 10 Kg
Lote nº / Batch nº: DKC6666-1 (code is variable between varieties)
Proyecto o Contrato / Project or Contract: GRACE
Fecha realización / Date: 19/11/2012
Lugar realización / Location: The Mas Badia Field Station
Persona contacto / Contact person: [redacted]
Muestra para / Sample for: Rat Feed Compound

6. TEST SYSTEM

Species and strain
Rat Wistar Rcc Han /Specific Pathogen Free (SPF)

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

Number of animals
90 male and 90 female rats will be ordered. Only 80 males and 80 females will be used for the study. Females will be nulliparous and non-pregnant. Animals not assigned to the study will be deemed assentinels.

Approximate weight and age
Upon arrival, the animals will weigh between 100-120g and will be 4 weeks old. The animals will be 5 weeks old at the start of the study and will weigh between 110-140g. Ideally, they should be born within 1-5 days of each otherand be of uniform weight (± 20% of the mean).

Identification
Within the frame of treatment groups, each rat will be marked by code (marked every 2 weeks with a permanent marker) on the tail base in accordance with SOP: ŠPP/TOX/V002 to identify the animal individually. Each cage will be marked with a colored cage card.

Justification for the selection and number of animals
This species (*Rattusnorvegicus*ssp. *alba*) and strain (Wistar) of animal is generally recognized as appropriate for the conduct of sub-chronic and chronic toxicity studies. The Wistar rat is a widely used strain of rats for which significant control data are available. The toxicology laboratory of the Slovak Medical University has a record of the regular use of this strain of rats. Group size was determined by a power analysis for quantitative biomarkers. A group size of 20 rats will have a 90% chance of detecting a standardized effect size of 1.10 standard deviations assuming a 5% significance level and a two-sided test when comparing the GM diet with its isogenic control. For qualitative characters such as pathological lesions and tumours with a group size of 20 animals there is an 80% chance of detecting an incidence of 40% or more (8 animals) against a background of 5% (one animal) with a significance level of 0.05.

Animal housing
All animals will be housed in rooms N^{o} B 2 and 3 of the Specific Pathogen Free (SPF) experimental animal house equipped with a pressure climatic system at the Department of Toxicology of the Slovak Medical University. The temperature and relative humidity in the animal room will be recorded every 20 minutes by the PMICRO-LCD-THSYS, Dallas Semiconductor system and every week the computer readout for the past week will be evaluated. Mean temperature will be maintained at 22 $\pm$ 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 cagesType 2145 F from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940cm^2.Toys (plastic tubes) will be provided.

We will use sterilized bedding : Hygienic animal bedding Lignocel®, sterilized sawdust from JRS, Germany. It will be stored in the clean, dry and cold store room on the first floor in the animal facility. Two lots of sawdust bedding will be purchased in successive steps and used for the entire study.

The cages will be cleanedtwice a week outside of the animal room. The cages will be emptied and cleaned with water and detergent. After cleaning they will be dried and then immersed in disinfectant. The cages will then be brought into the animal house and placed in an additional disinfectant solution. Then the cages will be placed into 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 othercontainers or equipment being used in the SPF rooms will be cleaned the same way that the cages are cleaned.

Bottles will be exchanged and cleaned every 2 days. 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 disinfect.

Diet formulation, sampling and analysis

Diet formulation, sampling and shipping

- Maize harvested from the Catalonian production sites is shipped to the Italian production facility (Mucedola srl.) licensed by Harlan for the production of diets. Shipping will be done with the same transport company as the monthly truck service offered by the facility. Grains are to be packaged in commercial big-bags of 300-350 kg, each labelled with the full name of the variety and other details.
- Milling of maize kernels is done by this facility, as is the formulation, *i.e.* mixing with other ingredients, using a customized pelletizing processusing a pasta press without the use of steam,which aids to prevent loss of heat-labile compounds.
- Formulation is carried out according to the diet composition recommended by the Harlan Company's nutritionist so as to achieve isoproteic and isocaloric diets with 11% and 33% transgenic variety and 22% and 33% for the near-isogenic maize inclusion levels as well as 33% for the conventional variety. Hence, each diet contained 33% maize *in toto*. The composition will include plant-based ingredients (hence no animal-derived ingredients). Samples for dispatch to the analytical laboratories for nutrition and contaminants, as well as for "omics" studies, are taken after milling and after pelletizing (before and after irradiation) according to instructions from the responsible GRACE scientist(company has been instructed to take multiple, i.e. at least five samples, at different spots from the batches prepared).
- A complete battery of tests for different GMOs will be performed on a sample of each variety at the RIKILT facilities (including a broad GMO screen and a quantitative event-specific PCR assay for MON810), while INRA will test for the presence of the Cry1Ab protein expressed by transgenic maize MON810.

- Diets are coded in a "double blind" fashion by the diet-producing company (Mucedola srl.). Samples of the diets are coded with different codes than the diets themselves. The coding scheme is shared with Ralf Wilhelm (the company's contact within the GRACE consortium).It is to be kept confidential and therefore not to be distributed further among consortium members during the course of the animal experiments and analyses of samples derived from these experiments, and the code is to be broken after termination of these activities when analytical and experimental data have been recorded and stored.

Analysis of diets (diets will be provided in several batches by the diet manufactorer)

- The key parameters for the analysis of maize will include, for the first batches of diets to be prepared:
  - Macronutrients & fibre (ADF, NDF, dietary),
  - Minerals,
  - Vitamins (A, B, C, E), zeaxanthin
  - Amino acid composition (including tryptophan),
  - Fatty acid composition,
  - Antinutrients (phytic acid, trypsin inhibitor),
  - Other secondary metabolites (furfural, phenolics, sterols) and carbohydrates, e.g. raffinose, stachyose.
  - GMOs (DNA), Cry1Ab protein, pesticide residues, mycotoxins, heavy metals, other contaminants (e.g. dioxins, PAHs, PCBs, nitrate, nitrosamine),
- The key parameters of the analysis of the diets will include:
  - Same parameters as for maize, plus:
    - Isoflavones
    - Lectins

Follow-up batches will be checked for parameters that provide an indication of the consistency of diet quality, including fatty acids, amino acids, and vitamins, as well as for content of Cry1Ab.

Storage conditions

- Kernels and pellets will be keptat ambient temperature and measures will be taken to avoid build-up of moisture and fungal growth. Every variety can be considered as a single batch as it all was cultured, harvested and dried as a single batch. The size of the batches is of about 300 kg per variety. Drying prevents grains from fungal infections, while gamma-irradiation of the diets will be performed after milling and diet preparation.
- After receipt of the analytical samples, the receiving laboratories will keep them undercontrolled cool, dry and confined conditions to ensure the stability of the sample.

Spare samples from the irradiated diets after receipt at the animal testing facility will be taken and kept for later analysis.

Samples of diets will be sent to the analytical laboratories contracted for the analysis of the composition (macronutrients, micronutrients, anti-nutrients and other secondary metabolites) as well as for the presence of genetically modified organisms (GMOs; element screen and event-specific test for MON810), mycotoxins, residues of pesticides and contaminants (*e.g.* dioxins, PAHs, PCBs, nitrate, nitrosamines, heavy metals), and pathogens.

Storage of the test diet during the study: in closed rooms (cool and dry, controlled temperature and humidity), Laboratory of Toxicology, SZU, Limbová 14, Bratislava, Slovak Republic. The test diets will be provided as single batches (containing portions of diets packed in separate vacuum, gamma-irradiated packs).

Water

The rats will be supplied water *ad libitum* during the acclimation and study periods. We will use tap water with a special filter to eliminate microorganisms. The bottles containing this water will be autoclaved before use. The water from the local mains will be monitored for quality by testing for the microbiological and chemical quality by Waterworks Bratislava quarterly. We will receive a certificate of quality.

7. EXPERIMENTAL DESIGN

Animal receipt and acclimation

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 acclimation to the laboratory conditions. These are identical to those defined for the experimental part of the study. During this period the animals will be monitored for their health status twice a day (see section 8. PERIODICAL HEALTH STATUS OBSERVATIONS below for a full description of the health status evaluation).

Randomisation

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 and will be randomized using completely randomised designs (SOP: ŠPP/TOX/V001).

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 for male and female animals separately.
All male animals will be numbered from 1 to 90. 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 animals 1-80 are randomly assigned to cages. Remaining 10 animals will be used as sentinel group, e.g. 5 cages with 2 animals.
The same procedure will be done with female animals – they will be numbered 101 to 190.

All animals will be purchased from Harlan and will be only 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.

Two animals will be placed in 1 cage. Animals will be randomly allocated to cages by dose group and sex. To minimize the chance of mistakes being made, cages of the same treatment groups will be clustered in vertically arranged groups, which will be rotated on a regular basis (once per week). Each vertical row of cages (within the same dose group) will be rotated from top to bottom. Racks will be rotated clockwise every two weeks within the original room configuration.

Group allocation and dosing

Prior to the start of treatment on study day 1, a detailed examination of all animals will be carried out to verify their health condition (see section 8.PERIODICAL HEALTH STATUS OBSERVATIONS for a full description).

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 (maize) will be administered by incorporation into the diet since this mimics most human exposure to these foods. Attention will be paid that there will be no nutritional imbalance as a result of dietary incorporation of the test item.

Food will be supplied *ad libitum*. Measurement of food consumption and food efficiency will be made at least weekly for the first 13 weeks and at last every two weeks thereafter. At the beginning of each food consumption measurement, weighed full feeders with stainless steel lids will be placed in each cage. At feeder change-out (once weekly), the feeders will be weighed again, the difference in weight is an estimate of total amount consumed by the 2 occupants of the cage. Feed consumption will be recorded, and will be reported as grams/animal/day.

Feed containers and scoops will also be colour coded. However, animal house staff will be "blind" with respect to the identity of the diets.

The different feeds will be coded and labelled by Mucedola company. The code will be given only to Ralf Wilhelm. All others will be blinded to the feeds.

General experimental design with Monsanto MON810 maize, start January 2014

Group	***% (w/w) of diet***				***Number of animals***	
Unknown identity for the staff *	***Reference diet***	***GM***	***Near-Isogenic non-GM***	***Conventional***	***Males***	***Females***
x*	67	33	0	0	20	20
x*	67	11	22	0	20	20
x*	67	0	33	0	20	20
x*	67	0	0	33	20	20
Total					80	80

Group coding by Mucedola (example)	***Group/ colour coding by SZU (example)***	***Number of animals***	***Number of cages***	***Number of animals***	***Number of cages***
		Males		***Females***	
3001	1 blue	20	10	20	10
3002	2 red	20	10	20	10
3003	3 green	20	10	20	10
3004	4 yellow	20	10	20	10
	Sentinels -white	10	5	10	5
	Total	90	45	90	45

8. 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 observe them carefully at least 4 times daily. If a study animal dies, we will subject it to necropsy as soon as possible after death. Any animal whose condition makes it unlikely that it will survive to the next observation period will be euthanized by ketamine/xylazine anaesthesia (SOP No. TOX/TS/004) and immediately necropsied.

Clinical signs

Cage side observations / uncovered cage

Rats will be inspected twice daily for evidence of reaction to treatment or ill-health 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 behaviour.

Detailed physical examination and functional assessment

Prior to the first exposure (see section 7), at the end of the first week of the study, weekly during the first 13 weeks and monthly thereafter rats will be examined out of cage. 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 behaviour.
Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements or bizarre behaviour (self-mutilation, walking backwards) will also be recorded. The outcome of this examination will be recorded for each animal, in accordance with SOP: ŠPP / TOX / V003 (Origin of score system: Országh A. et al. (1985): Veterinárnaortopédia a rontgenológia, Bratislava: Príroda, 243 s. (Veterinary orthopaedy and X-ray). The animals will also be assessed for gait disturbances using theAccuPacer treadmill equipment.

Ophthalmologic examination

Using an ophthalmoscope, we will examine the eyes of all animals in line with TG 452 prior to the administration of the test feeds and 2 weeks before the termination of the study. This will be done by the chief of ophthalmology who has expertise in this area.

Body weight

Each animal will be weighed at the following times: 1) 48 hours after arrival, 2) on the day of randomization, 3) on the first day of feeding, 4) weekly during the first 13 weeks, 5) monthly thereafter and 6) at the termination of the study, or 7) in the event of an early death or sacrifice in extremis. The General Linear Model (GLM) for Repeated Measures will be used for analysisof the body weight.

9. PROCEDURES FOR SAMPLE COLLECTION

Sample collection for the following analyses will be done: haematology, clinical chemistry, urinalysis, and pathology. Samples collected will include blood, tissues, urine and organs. Blood samples will be divided for haematology and clinical chemistry. Tissues and organs will be removed and evaluated by histology

Sample collection and tissue processing:

Sample collection during the study

Personal distribution for forced progress:
Urine will be collected by person No 1
Urine processing and transport to the competent laboratory: Laboratory of Clinical and Experimental Biochemistry - person No 2
Blood taking from the tail vein by person No 3
Blood processing, dividing of samples - person No 4
Blood transport to the competent laboratory: Laboratory of Immunotoxicology (haematology) - person No 5;
Laboratory of Clinical and Experimental Biochemistry (clinical chemistry) - person No 6

Sample collection and tissue processing at the end of the study

Personal disposition for forced progress (40 animals per day will be necropsied):
- Animals will be anaesthetized by person No. 1
- Blood taking from the abdominal vesselwill be done by person No. 2
- Blood processing and transport to the Laboratory of Clinical and Experimental Biochemistry (clinical chemistry) – person No. 3
-Animal transport to the Autopsy room on the same floor by person No. 4
Removal and weighing of selected organs for „omics“ study and their preparation – person No 5
- Necropsy of thorax part of the body- person No. 6
- Necropsy of abdominal part of the body - person No. 7
- Necropsy of genital organs - person No. 8
- Removal and weighing of tissues and organs in line with OECD guideline 452 - person No. 9
- Decapitation and necropsy of the head including brain by person No. 10
- All organs will be stored into formaline for the histological examination - person No. 11

All steps are inspected continuously by QA

Haematology

At months 3 and 6 and 7 days before sacrifice, blood samples from the tail vein will be taken from 10 male and 10 females per group after 16 hours fasting using the same animals throughout for haematological (as well as clinical chemistry) examination. EDTA will be used as anticoagulant. Blood samples will be stored under room temperature (17-25°C) maximum up to 4 hours until

measurement. Haematological analysis will be performed in accordance with SOP: ŠPP/IMU/M002 using Haematological analyzer Sysmex K-4500, SYSMEX TOA Medical Electronics Co. LTD, Japan. Parameters scheduled for examination are
- Erythrocyte Count (RBC)
- Haematocrit (HT)
- Haemoglobin (Hb)
- Mean Corpuscular Haemoglobin (MCH)
- Mean Corpuscular Haemoglobin Concentration (MCHC)
- Mean Cell Volume (MCV)
- Leukocyte Count (WBC)
- Differential Leukocyte Count
- Platelet Count (PLT)
- Prothrombin time
- Activated partial thromboplastine time

Differential Leukocyte Count will be examined using light microscope. Blood smears will be stained by panoptic staining using May-Grunwald and Giemsa-Romanowski dyes. The percentage of lymphocytes, neutrophils, eosinophils, basophils and monocytes will be determined by examination of 200 cells.

Clinical chemistry

At months 3 and 6, blood samples from the tail vein will be taken. At the end of the study in anaesthesia before sacrifice, blood samples from the abdominal vessel will be taken. The same 10 males and 10 females will be used throughout the study for clinical chemistry examination after 16 hours fasting. Samples will be analysed using an Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, 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 (TP)
- albumin (ALB)
- aspartate aminotransferase (AST)
- alanine aminotransferase (ALT)
- alkaline phosphatase (ALP)
- creatinine (CREA)
- Urea (urea nitrogen) (BUN)
- fasting blood glucose (GLU)
- total bilirubin (TBIL)
- total cholesterol(CHOL)
- Triglycerides (TGL)
- Na
- K
- Ca
- Cl

- P

Urinalysis

At months 3 and 6 and at the end of the study (same intervals as specified for haematology and clinical chemistry) urinalysis determinations will be performed on 10 males and 10 females using the same animals throughout.
Urine will be collected from each individual rat in metabolic cages under the same conditions in groups of 16 animals during 5 consecutive days and one week apart for males and females. For every collected group of animals, every test diet will be balanced for the number of animals submitted to urine collection. 16 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, animals will be moved from the metabolic cages to their respective conventional cages.
Every sample collected at different time points will be completely 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
- pH
- Total protein
- Glucose
- Ketone
- Urobilinogen
- Bilirubin
- Occult blood

10. PATHOLOGY

Gross necropsy

A complete necropsy will be performed on all animals at study termination on days 366- 367. The wet-weight of organs will be recorded in line with OECD Test Guideline 452 and organs/tissues will be examined macroscopically for any deviations from normal (in accordance with ŠPP / TOX / V005).

The wet-weight of the following organs will be recorded:

- Brain
- Lungs
- Heart
- Liver,
- Kidneys
- Spleen
- Adrenal glands
- Pancreas
- Testes
- Uterus
- Ovaries
- Epididymides
- Thymus.
- Thyroid (weighed post-fixation, with parathyroid)

The following tissues will be preserved in fixative medium (neutralbuffered 10% formalin) for histopathological examination:

Tissue specimens include:

- All gross lesions
- Adrenal gland
- Aorta
- Brain (including sections of cerebrum, cerebellum, and medulla/pons)
- Caecum
- Cervix
- Coagulating gland
- Colon
- Duodenum
- Epididymis
- Eye (including retina)
- Harderian gland
- Heart
- Ileum

- Jejunum
- Kidney
- Lacrimal gland (exorbital)
- Liver
- Lymph nodes (both superficial and deep)
- Mammary gland (obligatory for females and, if visibly dissectable, from males)
- Oesophagus
- Ovary
- Pancreas
- Parathyroid gland
- Peripheral nerve
- Pituitary
- Prostate
- Rectum
- Salivary gland
- Seminal vesicle
- Skeletal muscle
- Skin
- Spinal cord (at three levels: cervical, mid-thoracic, and lumbar)
- Spleen
- Stomach (forestomach, glandular stomach)
- Testes
- Thymus
- Thyroid
- Trachea and lungs (preserved by inflation with fixative and then immersion)
- Urinary bladder
- Uterus (including cervix)
- Vagina
- A section of bone marrow and/or a fresh bone marrow aspirate
- In the case of paired organs, e.g. kidney, adrenal, both organs will be preserved
- Additional tissues may need to be investigated based on clinical or any other findings.

Histopathology

Organs and tissues preserved in neutral buffered 10% formalin will be shipped to histological laboratory (has to be included according to tender outcome) for histopathological evaluation in accordance with SOPs. Complete microscopic examination of the tissues listed above will be performed in accordance with the OECD TG 452 on all animals from the high dose and control group. Groups will be identified by Ralf Wilhelm, JKI, without informing SZU and the histological laboratory (has to be included according to tender outcome) about the identity of each group. Furthermore all tissues from animals dying or killed during the study and all tissues showing macroscopic abnormalities will be examined microscopically.

Supplementary analysis

Metabolomics, Transcriptomics and Proteomics

Omics analyses of animal tissues and feed materials will only be performed on selected candidate metabolites, transcripts and proteins if results from a previous 90 day feeding study (**Study No:** 311957 - A / 13/ GLP) carried out with the same feed material and dose levels indicate significant effects.

For animal tissues, the procedures for sample collection and tissue processing will be as followed

Blood samples:

For every animal, after collecting blood from the abdominal vessel at the end of the study in heparinised vial, samples will be kept not more than 15 min at 4°C before starting centrifugation to separate red cells from plasma. Blood will be centrifuged at 3000 g at 4°C for 15 min and all plasma collected. Aliquots of plasma (ca. 500 µL) will be used for the metabolomic and immunological analyses and kept at -80°C. Centrifuged plasma will be kept not more than 15 min at 4°C before preparation of aliquots and storage at -80°C. Plasma will be stored in 3-ml polypropylene vials with a cap or seemingly equivalent vials.

Immunological studies of plasma for INRA (Test Site 5)

For possible immunological studies, plasma collected at the end of the chronic toxicity study will be tested for total and maize/cry1Ab specific antibodies (i.e. IgG, IgM and IgE) using specific immunoassays developed and validated in Test Site 5.

Organ samples:

Selected organs and tissues will then be collected immediately after euthanasia.

Metabolomic studies of tissue samples for INRA (Test Site 5)

- After weighing, the right lateral lobe will be collected and immediately snap frozen in liquid nitrogen. Liver samples will be stored in 1.5 ml Eppendorf vials or seemingly equivalent containers.
- After weighing, the third to the half higher part of the right kidney will be cut and immediately snap-frozen in liquid nitrogen. Kidney samples will be stored in 1.5 ml Eppendorf vials. The dissection of kidneys will be done by the same person to ensure consistency and reproducibility.
- After preparation all samples will be frozen in liquid nitrogen and then stored at -80°C.

- For metabolomic studies, plasma, liver and kidney samples will be extracted with organic solvents to discard insoluble macromolecules and non-polar analytes. The resulting hydrosoluble fractions will be fingerprinted by ultra high resolution mass spectrometry (plasma, liver and kidney extracts) or by nuclear magnetic resonance (liver extracts) to quantify the distribution of detectable analytes.

Transcriptomics of tissue samples for FUB (Test Site 6)

At the end of the chronic toxicity study, the following tissues should be collected for possible transcriptomics studies depending on the scientific outcome of a previous 90-day study (Study No: 311957 - A / 13/ GLP):
Intestinal sections of mid-jejunum, mid-ileum, ascending colon (2cm each), superior mesenteric and ileocolic lymph nodes (2 ileocolic and 1 superior mesenteric lymph nodes) and spleen (identical anatomical section for each animal). For these specimens, it is important to have the same-sized pieces, weighed equivalents and thus this will be done by the same person to ensure consistency and reproducibility. Freshly sampled tissue sections must be quick-frozen in liquid nitrogen as soon as possible after necropsy to prevent degradation of RNA. If reasonable total RNA will be extracted from specifically selected frozen tissue sections and candidate mRNA expression will be studied by quantitative real-time PCR.

Transport of samples:

- Blood, plasma and tissue samples will be sent on dry ice to FUB (Test Site 6), INRA (Test Site 5), and CRAG (Test Site 4) for further immunological and “omics” analyses (according to the work plan for GRACE Work Package 2 with the list of samples.
- Every partner must receive and their identification (e.g. no of rats, type of sample, and day of collection) as well as the weight of the organs.
- Each sample must be clearly and unambiguously identified by the animal number/nature of the sample (*e.g.*liver, kidneys, plasma).
- Aliquots are to be set aside so that these can be used in case of problems with transport of samples shipped from the animal testing facility to other test sites.

11. DATA EVALUATION AND STATISTICAL ANALYSIS

The statistical analysis will be done by local statistical team, using any high-level statistical packages such as BMDP, SAS, or R.

As a first step the data will be screened for any obvious errors and outliers. 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 then be done 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.

Data from males and females will be analysed separately and together (ANOVA).

Summary statistics (e.g. "n", means, standard deviations and/or medians and quartiles, as appropriate), will be tabulated based on the cage means(as the cage is considered the experimental unit in this study). A one-way analysis with planned or *post-hoc* comparisons will be used to evaluate statistical significance of each outcome (trait). In some cases more detailed statistical analysis including correlations between characters or even a multivariate analysis may be needed, but this should be decided on a case-by-case basis. Methods of analysing longitudinal data such as growth and food consumption will be decided on a case-by-case basis.

Tables of results (means, SDs and statistical significance; raw individual data) will be prepared and in some cases additional statistical analyses and graphical methods may also be used.
The raw data will be made publically available on the GRACE web site.

12. REFERENCES

BMDP Statistics Software, Inc. (1990). BMDP Statistical Software Manual. W.J. Dixon, Chief Ed. 1990 rev. or later. University of California Press, Berkeley, CA, USA.

European Committee for Standardization (2010) EN ISO 24333:2009 Cereals and cereal products – Sampling.

European Committee for Standardization (2010) General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005).

The European Parliament and the Council (2004) Directive 2004/10/EC. Official Journal of the European Union L 50: 44-45.

The European Parliament and the Council (2010) Directive 2010/63/EU. Official Journal of the European Union L 276: 33-79.

OECD (1998) Principles of Good Laboratory Practice, as revised in 1997- ENV/MC/CHEM(98)17. Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris.

OECD (2009)Test No. 452 - Chronic Toxicity Studies. OECD Guidelines for the Testing of Chemicals

Slovak Republic, Act No 67/2010 on Conditions of Marketing of Chemical Substances and Chemical Mixtures and on amendment and supplement of other acts.

Slovak Republic , Government Decree No. 320/2010 Coll.

13. ARCHIVING

Under the Code Number: 311957 C/14/ GLP
The following will be archived until the year 2024 at the SZU, the Registry of accredited laboratories and laboratories with GLP certificate:

- Study plan
- Correspondence
- Final report
- Reports of quality inspection
- All histological samples
- All original documents/Primary documentation

14. REPORTING

The final report will include the reporting requirements as described in OECD TG 452 method:

The final report will be written in English language. The sponsor can revise the draft report for 14 days from its date of issue. Then the final report will be finalized.

The draft report will be made in two copies – one electronic copy for the sponsor and one paper copy for test facility. The study report will be made in four paper copies - two for the sponsor and two copies for the test facility and will include, but not limited to, the following:

- The name and address of the sponsor and the testing facility.
- The study schedule, the data of the start and the end of the study.
- The names of all personnel involved in the study, including the study director, other scientists and supervisory personnel.
- The item identification by code number. The appropriate properties of the item.
- The description of the test system, including species, strain, source, allocation, sex, age and method of identification.
- The description of the coded doses, dose regimen, route of administration and duration of the treatment period, the description of all methods used.
- Clinical signs and relevant raw data.
- The summary and description of all the toxic signs.
- Body weight data.
- Food consumption data.
- A description of all circumstances that may have affected the quality or integrity of the study.
- The authentification signed by study director.
- Test Facility Management Statement.
- The QAU Statement signed by QA Manager.
- The copy of the Certificate of GLP.
- The storage locations of study plan, all raw data, specimens and the reports.

15. DISTRIBUTION

This study plan will be distributed as follows:
Issue No 1 Study Director
Issue No 2 Sponsor
Issue No 3 QA Manager

16. ATTACHMENTS

Attachment 1

Chronic Toxicity Study (1 year) ACCORDING TO OECD GUIDELINE 452

Time schedule

	Day -7	**Day 0**	**Day 1**	**Day 90**	**Day 180**	**Day 360**	**Day 365**	**Day 366 - 367**	**Following 3 months**
Quarantine	5/7 days +								
Randomi-sation		Males day 0 Females day0+1							
Ophtalmology	Day – 5/6					**Day 360**			
Application males			Day 1 start				Day 365 end		
Application females			Day 1+1 Start				Day 365+1 end		
Weighingofthe feed		Every 7 days	Every 7 days	Every 14 days	Every 14 days	Every 14 days	weighing		
Weighingof animals		Every 7 days	Every 7 days	Every 1 month	Every 1 month	Every 1 month	weighing		
Generalclinical observations		Everyday –Twice or more frequently	Everyday Twice or more frequently	Everyday Twice or more frequently	Everyday Twice or more frequently	Everyday Twice or more frequently	Everyday		
Detailedclinical observations		Every 7 days	Every 7 days	Every 1 Month	Every 1 Month	Every 1 Month	Every 1 Month		
Sensory reactivity		Every 7 days	Every 7 days	Every 1 Month	Every 1 Month	Every 1 Month	Every 1 Month		
Hematology Males Females				10 animals / group	10 animals / group		Day 358-362 10 animals / group		
Clin.chemistry Males, Females				10 animals / group	10 animals / group		10 animals / group		
Urinoanalysis Males, Females				10 animals / group	10 animals / group	10 animals / group	Day 358 - 362 10 animals / group		
Gross necropsy Males								Day 366-369	
Gross necropsy Females								Day 366-369	
Slides preparation									Slides preparetion

Histology evaluation									Histology evaluation
Final report acceptance									Final report draft
Final report to sponsor									Final report

Attachment 2

LIST OF MATERIAL AND EQUIPMENT

Equipment:
Laboratory of Toxicology:

- 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. B2-2, B2- 3.
- 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: 0-1000g, 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.
- Type of animal cages in TECNIPLAST Filter top cagesType 2145 F) from the Tecniplast Company, Italy. The cages have a high density polypropylene body, measuring 480 x 265 x 210 mm - floor area 940cm^2
- Ophthalmoscope Welch Allyn
- Apparatus for neurobehavioural testing: Accupacer treadmill

Laboratory of Immunotoxicology

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

LaboratoryofClinical and ExperimentalBiochemistry

- Analyzer Vitros 250, Ortho-Clinical Diagnostics, No. 219037234, USA, room B-048.
- Personal computers, office

Software forprocessingofthedata

- Windows XP, program Office 2003
- Windows 2007,program Office 2010

- Software SPPS version 16.0.

Material

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

Equipments Histology (has to be addedd accordingto tender outcome)

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
- Temperature/relative humidity/light intensity and cycle checks
- Dose analysis data
- Dose preparation and accountability records
- Dose administration
- Necropsy and histopathological findings
- Pathology specimens as specified
- Histology processing records

Records – primary documentation -will be kept in room B 2 – 209
All records during the study will be kept in computer room B 2 – 221
External backup will be kept in room B2 – 210
Second external backup will be kept in room B – 358 (QA)

Attachment 4 – 6 (available from SZU)

4. GLP CERTIFICATE SZU
5. GLP CERTIFICATE histological laboratory [according to tender outcome]
6. ACCREDITATION CERTIFICATE Clinical chemistry lab