Article

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A 90-day toxicity and genotoxicity study with high-purity phenylcapsaicin

Torbjørn Rage Paulsen[1], Sebastian Stiller[2], Klaus Weber[3], Claudia Donath[4], Gudrun Schreiband[4], and Knut Helge Jensen[1]

Abstract

To evaluate the safety of the synthetic capsaicin analogue phenylcapsaicin (PheCap; 7-phenylhept-6-yne-acid-hydroxy-3-mathoxylbenzylamide, CAS no 848127-67-3), a 90-day repeated dose oral gavage of 0, 30, 100 or 250 mg/kg body weight (bw)/day toxicity study with a 28-day recovery period was conducted using Wistar rats. Examinations of clinical signs, body and organ weight, haematology, urinalysis, clinical chemistry, food consumption and macroscopic, as well as histopathological tissue examinations were carried out for signs of toxicity. Degenerative, but reversible changes in the liver at 250 mg/kg bw/day, and local irritating effects in the stomach at 100 and 250 mg/kg bw/day were found. These findings were associated with test item-related clinical symptoms, that is, diarrhoea, salivation and moving of bedding material. PheCap did neither cause gene mutations by base pair changes or frame shifts in the genome of the tester stains *Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 or TA 102 nor induce structural and/or numerical chromosomal damage in human lymphocytes. Therefore, it can be concluded that PheCap is not genotoxic. The No Observed Adverse Effect Level (NOAEL) of PheCap for systemic toxicity is considered to be at 100 mg/kg bw/day which is based on degenerative changes in the liver. Due to irritating effects in the stomach, the NOAEL for local effects was established at 30 mg/kg bw/day.

Keywords

Phenylcapsaicin, oral dose toxicity, genotoxicity, Ames assay, micronucleus, rats

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Introduction

There are many areas of use for the synthetic capsaicin analogue phenylcapsaicin (PheCap), including food industries, agriculture,[1–4] pharmacology[5,6] and marine antifouling paint.

In the meat and poultry industries, intestinal colonization of *Salmonella* spp. is a major concern for food safety in humans. Antibiotic growth promoters have been widely used in commercial meat production. Problems related to antibiotic resistant pathogens and the European Union's (EU) 2006 ban of antibiotic growth promoters in animal feed[7,8] have made the search for replacements urgent. Several phytobiotics and essential oils have been considered.[9–11]

Capsaicin has been found to both increase broiler body weight (bw)[3] and reduce gut *Salmonella enteritidis*, *Escherichia coli* and *Clostridium perfringens*.[1,2,4,12–14] However, due to the limited production of red peppers and the challenges of capsaicin purification, capsaicin has not been available in the volumes needed for the meat and poultry industry. This obstacle has now been removed by the commercially available *synthetic* capsaicin analogue PheCap (aXichem AB, Malmö, Sweden). PheCap is designed to have capsaicin's chemical properties (Figure 1).

[1] Department of Biological Sciences, University of Bergen, Bergen, Norway.
[2] BSL BIOSERVICE Scientific Laboratories Munich, Planegg, Germany
[3] AnaPath GmbH, Liestal, Switzerland
[4] Eurofins BioPharma Product Testing Munich GmbH, Planegg, Germany

Corresponding author:
Torbjørn Rage Paulsen, Department of Biological Sciences, University of Bergen, Postboks 7803, 5020 Bergen, Norway.
Email: torbjorn.paulsen@uib.no

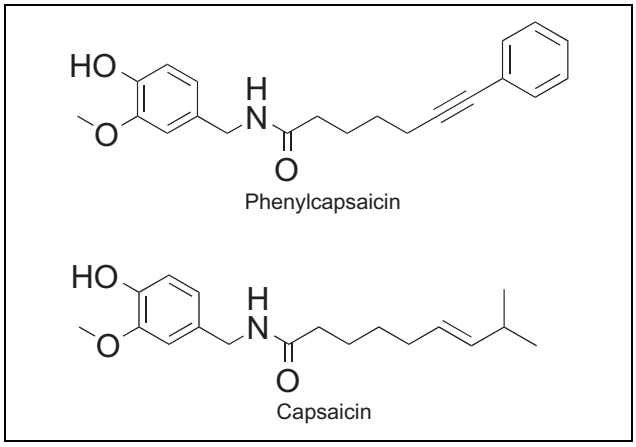

Figure 1. Chemical structure of PheCap and capsaicin. PheCap: phenylcapsaicin.

aXichem AB has reported that the addition of PheCap to feed increased broiler chicken bw[15] and reduced *Salmonella* in both broiler chickens and 10-week-old piglets[15–17] compared to controls. To the best of our knowledge, no literature is available on the mode of action, metabolism or toxicology of PheCap.

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is well known from spicy foods and is especially common in Latin America and Southeast Asia. It is a highly selective agonist for the transient receptor potential cation channel subfamily V member 1 (TrpV1).[18,19] An unpublished study by aXichem shows that PheCap is a functional capsaicin analogue and a highly potent Vanilloid TrpV1 agonist with an EC_{50} (half maximal effective concentration) of 57.8 nM. The anabolic effects, however, cannot be explained by this mechanism. Vanilloid agonists present desensitization and their effect is significantly reduced by a TrpV1-specific antagonist.[20]

Orally ingested capsaicin is quickly absorbed, some in its intact form, from the stomach and entire intestine by a non-active process.[21,22] For instance, within 3 h, 85% of an in vivo orally administered 3-mg dose was absorbed in the gastrointestinal tract, and after 60 min, an in situ administration into the stomach, jejunum and ileum, 50, 80 and 70% of the respective doses had been absorbed.[23] Metabolism of capsaicin occurs primarily in the liver.[24,25]

Capsaicin, when given orally, stimulates salivation[26] and is considered a strong irritant to gastric mucosa and might produce severe gastritis and diarrhoea.[27] In rats, after 30 days of oral administration of 50 mg/kg bw/day capsaicin, significant reductions in plasma urea nitrogen, glucose (Gluc), phospholipids, triglycerides, total cholesterol (Chol), free fatty acids, glutamic pyruvic transaminase and alkaline phosphatase (AP) were found. After 60 days, a significant reduction in growth rate but no differences in relative organ weights, rectal temperature, water intake, plasma chemistry or urine dilution and concentration was found compared to control rats.[28]

Available literature on the genotoxicity of capsaicin is inconclusive, as summarized by Chanda et al.[29] In mice, after administering doses up to 1.6 mg/kg bw/day intraperitoneally for 5 days, Muralidhara and Narasimhamurthy[30] did not find any in vivo mutagenic effects of capsaicin in either somatic or germ cells. Of eight bacterial point mutation tests, half gave positive and half gave negative responses, and with two-point mutation tests in Chinese hamster V79 cells, one was positive and one negative.[31,32] One in vivo micronucleus test in mice was positive,[33] one micronucleus and sister chromatid exchange study in human lymphocytes (Lym) is interpreted as positive[34] and DNA strand breaks were found in human neuroblastoma cells SHSY-5Y.[25] However, Chanda et al.[29] found that the genotoxic potential of pure *trans*-capsaicin was very low and argued that the inconsistency observed between previous studies was caused by differences in the sources of and the purity and impurity profiles of the capsaicin item tested, which therefore might not exhibit the same toxicological profile as pure capsaicin. For a comprehensive safety assessment of capsaicin and capsicum extracts and resins, see Cosmetic Ingredient Review Expert Panel.[21]

The purpose of the present study is to examine the potential toxicity and genotoxicity of high-purity PheCap for use in human food industries and animal feed. Toxicity was evaluated by a 90-day repeated oral dose with a 28-day recovery period toxicity study using Wistar rats. Genotoxicity was evaluated by *Salmonella typhimurium* reverse mutation assays and a human Lym micronucleus assay.

Materials and methods

The experiments were conducted by Eurofins BioPharma Product Testing Munich GmbH, BSL BIOSERVICE Scientific Laboratories Munich GmbH and AnaPath GmbH and followed the guidelines for risk assessment of food and feed additives in accordance with EU regulations[7,8,35] and Organisation for Economic Co-operation and Development (OECD) guidelines.[36–39] The animal experiments were performed at AAALAC-accredited test site BSL BIOSERVICE Scientific Laboratories Munich GmbH and were monitored by Institutional Animal Care Committee. All experiments were approved by German authorities, section Consumer Protection, Veterinary Services and Food Hygiene of the Government of Upper Bavaria (Regierung Oberbayern, Munich, Germany). The Quality Assurance Unit of Eurofins Munich assessed the study for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich and BSL Munich.

Test substance

PheCap, 7-phenylhept-6-yne-acid-hydroxy-3-mathoxyl-benzylamide (CAS no 848127-67-3), a synthetically

produced natural analogue of capsaicin (aXichem AB), was supplied as an olive brown viscous liquid with 98.8% nominal purity with pH 6.12 at room temperature. The stock purity was independently verified by HPLC-UV to be within 99.6–102.7% of nominal value by Swiss BioQuant AG (Reinach, Switzerland). Analysis of dose concentrations in weeks 1, 5, 9 and 13 shows sample homogeneity, and the treatment concentrations were (%; mean ± SD) 94.43 ± 4.20, 93.50 ± 4.02 and 97.08 ± 5.82 of the nominal values for the 30, 100 and 250 mg/kg bw/day treatment groups, respectively (Swiss BioQuant AG).

The concentrations to be used in the oral dose experiment were determined through a 28-day dose range finder experiment with doses up to 1000 mg/kg bw/day in three male and three female 7–8 weeks old Wistar rats (Crl: Wi(Han); Charles River, 97633 Sulzfeld, Germany) per group. On day 4, all animals at 500 and 1000 mg/kg bw/day were either found dead or killed due to ethical reasons. The animals developed severe clinical findings such as prone position, bradykinesia and reduced spontaneous activity partially accompanied by closed eyes. Animals administered with doses up to 250 mg/kg bw/day for 28 days developed only mild or local clinical findings such as diarrhoea, salivation and moving of bedding material with no considerable effects on bw, food consumption, clinical chemistry or haematology parameters. At necropsy on day 29 study, no macroscopic findings were observed. No histological evaluation was performed on organs from animals of the dose range finder experiment. In order to induce mild toxic effects but no death or severe suffering, the dose of 250 mg/kg bw/day was chosen for a subsequent 90-day study in rats. For the genotoxicity studies, the concentrations used were determined through pre-experiments in accordance with the corresponding OECD guidelines.[36,39]

Due to the inherent aversion towards capsaicin in mammals,[40] gavage administration was the selected application route.

Oral dose toxicity

Fifty healthy Wistar rats (Crl: Wi(Han); Charles River, 97633 Sulzfeld, Germany) per sex (nulliparous and non-pregnant) were kept in individually ventilated cages (type III, polysulphone cages) on Altromin saw fibre bedding (Altromin Spezialfutter GmbH, Lage, Germany) in a full barrier air-conditioned room at 22 ± 3°C, 55 ± 10% relative humidity and a 12:12 h light: dark artificial light cycle. Tap water, sulphur acidified to a pH of approximately 2.8 to prevent the spread of bacterial disease through drinking water and Altromin 1324 maintenance diet for rats and mice (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) were available ad libitum. Forty animals per sex (6–7 weeks old) were included in the 90-day oral dose test study. The animals were allocated into four groups, each containing 10 animals per sex. The animals were treated once daily by oral gavage administration of either 0, 30, 100 or 250 mg/kg bw/day PheCap in a 5 mL/kg bw application volume (0, 6, 20 and 50 mg PheCap/mL for the dose groups, respectively). To detect the possible persistence of, or delayed recovery from toxic effects, a separate recovery period group of 20 animals, 5 males and 5 females per control and 250 mg/kg bw/day dose groups of both sexes were dosed as described above for 90 days with observations continuing for a period of 28 days following the last capsaicin administration.

Treatment doses were prepared by adding the required volume of PEG 400 mixed (Ultraturrax, 3 min) and sonicated (5 min in water bath) without heating.

Before the first administration, all animals were weighed and assigned to an experimental group, achieving homogenous bw variation between treatment groups. Clinical observation showed no animals with pathological signs before the first administration.

Proof of exposure was confirmed in blood samples from five male and five female animals per experimental group in weeks 4 and 13. Individual bw was measured weekly, and PEG 400 was used as both a vehicle and control item.

A functional observational battery of tests[41] was performed before the first exposure and once in the last week of exposure or recovery period. Multiple detailed behavioural cage-side observations including spontaneous activity, lethargy, recumbent position, convulsions, tremors, apnoea, asphyxia, vocalization, diarrhoea, changes in skin and fur, eyes (ophthalmological examination, using an ophthalmoscope) and mucous membranes (salivation, discharge), piloerection and pupil size were made outside the home cage in a standard arena, once before the first administration and once the last weeks of administration and recovery periods. General clinical observations, morbidity and mortality were controlled once a day, and individual rat bw and food consumption per cage were measured weekly during the treatment and recovery periods.

The 15 haematological parameters, such as haematocrit value (Hct), haemoglobin content (Hb), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, reticulocytes (RE), platelet count, white blood cells (WBCs), neutrophils (Neu), Lym, monocytes (Mono), eosinophils (Eos), basophils and large unstained cells (Luc), were examined using the ADVIA 120 Hematology System (Siemens Healthcare GmbH, Erlangen, Germany). The two blood coagulation parameters, such as prothrombin time and activated partial thromboplastin time, were examined on the ACL 7000 (Instrumentation Laboratory, Munich, Germany or Kugelkoagulometer, ADW). The 13 clinical biochemistry parameters, such as alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), AP, creatinine, total protein (TP), albumin (Alb), urea, total bilirubin (TBIL), total bile acids (TBAs), Chol, Gluc, sodium (Na) and potassium (K), were examined

on the Olympus AU480 (Beckman Coulter GmbH, Krefeld, Germany). All parameters were examined at the end of the treatment and recovery period prior to or as part of killing animals. After overnight fasting, blood from the abdominal aorta of the animals was collected in EDTA-coated tubes, citrate tubes or serum separator tubes. Urinalyses on the 10 qualitative indicators, such as specific gravity, nitrite, pH, protein, Gluc, ketone bodies (Ket), urobilinogen (UBG), BIL, erythroctes (Ery) and leukocytes (Leu), were performed using the Heiland Urine Stripes URI 10SL on samples collected prior to or as part of killing animals.

On the day following the end of the treatment and recovery periods, the surviving test animals were killed (using anaesthesia ketamine and xylazin) and subjected to detailed gross necropsy including careful examination of the external body surface; all orifices and the cranial, thoracic and abdominal cavities and their contents. During necropsy, organ weights were taken as soon as possible from the adrenal glands, brain, heart, kidneys, liver, pituitary gland, spleen, thymus, thyroid/parathyroid glands, epididymides, prostate (including seminal vesicles and coagulating glands), testes, ovaries or the uterus with cervix. Paired organs were measured together. Organ weight of animals found dead or killed for animal welfare reasons was not recorded.

In addition to samples from the preceding list of organs, samples from all gross lesions, aorta, caecum, colon, duodenum, eyes with optic nerve and Harderian gland, ileum (including Peyer's patches), jejunum, lungs, lymph nodes (mesenteric and axillary), mammary gland area (male and female), oesophagus, pancreas, rectum, salivary glands (sublingual and submandibular), sciatic nerve, skeletal muscle, skin, spinal cord (cervical, thoracic and lumbar segments), sternum (with bone marrow), stomach, trachea and urinary bladder were preserved at necropsy and examined histopathologically. They were preserved in 4% neutral-buffered formaldehyde with the exceptions of eyes, testes and epididymides that were fixed in Modified Davidson's fixative for approximately 24 h before being transferred to 70% ethanol. The samples were trimmed, processed, embedded in paraffin, cut to thickness of approximately 4 μm, stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Histopathological examinations were performed on the animals from the 0 mg/kg bw/day and 250 mg/kg bw/day PheCap groups as well as on the animals found dead or were killed for animal welfare reasons. Organs and tissue showing treatment-related changes in the 250 mg/kg bw/day group were also examined in all other treatment groups (liver, spleen, adrenal glands, thymus and gastrointestinal tract). Any gross lesion macroscopically identified was examined microscopically. Histological processing of tissues to microscope slides and the histopathological evaluation were performed at the Good Laboratory Practices (GLP)-certified contract laboratories AnaPath GmbH (AnaPath Services, Liestal, Switzerland) and AnaPath GmbH (Oberbuchsiten, Switzerland), respectively. Blocking, embedding, cutting, H&E staining and scientific slide evaluation were performed according to the corresponding standard operating procedures of the test sites.

Reverse mutation assay

Standard plate incubation and preincubation assay procedures were performed, both with and without S9 activation.[42] To increase solubility, PheCap was heated to 50–60°C and dissolved in DMSO (AppliChem GmbH, Darmstadt, Germany) and diluted to treatment concentrations at 3.16, 10.0, 31.6, 100, 316, 1000, 2500 and 5000 μg. Positive controls were 4-NOPD (Fluka), NaN_3, MMS and 2-AA (Sigma-Aldrich Chemie GmbH, Munich, Germany), and distilled water and DMSO were used as negative and solvent controls, respectively. *S. typhimurium* TA 98, TA 102 and TA 1535 (Molecular Toxicology Inc., Boone, North Carolina, USA) and TA 100 and TA 1537 (Xenometrix AG, Allschwil, Switzerland) were used as tester strains and the genetic integrity of the strains was verified according to Ames et al.[43]

Samples of the five *S. typhimurium* test strains were grown in nutrient broth (0.8% nutrient broth and 0.5% sodium chloride (NaCl)) for 12 h at 37°C to c. 10^9 cells/mL. To retain the phenotypic characteristics, 125 μL ampicillin (10 mg/mL) was added to tester strains TA 98, TA 100 and TA 102. For the plate incorporation procedure, 100 μL of the overnight bacterial cultures were mixed with 100 μL test solution, 500 μL of S9 mix or S9 mix substitution buffer and 2000 μL overlay agar and poured over sterile minimal Gluc (2%) bottom agar.[42] For the preincubation procedure, the bacterial culture, test solution and either S9 mix or S9 mix substitution buffer were incubated for 60 min at 37°C prior to adding the overlay agar. All plates were incubated inverted for 48–72 h at 37°C in the dark.

Colonies were either counted using a ProtoCOL counter (Meintrup DWS Laborgeräte GmbH) or manually when precipitation precluded automatic counting. The test strains TA 1535 and TA 1537 with low spontaneous mutation frequency were also counted by hand. Assays were performed in triplicates.

Micronucleus assay

PheCap's ability to induce micronuclei in human Lym was examined using an in vitro micronucleus assay both with and without S9 activation. PheCap was dissolved in DMSO and diluted in cell culture medium to a final concentration of 1% v/v DMSO and 50, 100, 120 and 130 μg/mL PheCap treatment concentrations for the short-term exposure without metabolic activation, 50, 100, 130 and 140 μg/mL for the short-term exposure with metabolic activation and 10, 15 and 20 μg/mL for the long-time exposure without metabolic activation. For the short-time exposure,

ultrasonication for 15 min at 37°C was performed. The test item was dissolved in DMSO within 1 h prior to treatment. Cell culture medium with and without 1% DMSO was included as solvent and negative controls, respectively. EMS (900 and 1400 μg/mL) and CPA (15 μg/mL; Sigma-Aldrich Chemie GmbH) were included as clastogenic positive controls in the absence and presence of metabolic activation, respectively. Colchicine (0.04 and 0.8 μg/mL; Sigma-Aldrich Chemie GmbH) was used as aneugenic positive control in tests without metabolic activation. The controls were prepared immediately prior to the experiments and the mutagenic responses were in the expected range. Duplicate cultures were examined.

RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS), 100 U/100 μg/mL penicillin/streptomycin solution, 2 mM L-glutamine and 2.4 μg/mL phytohaemagglutinin (PHA) was used as complete culture medium. Complete culture medium without FBS was used as short-term exposure treatment medium and complete culture medium with 15% FBS, and 6 μg/mL cytochalasin B, was used as long-term exposure treatment medium.

Human peripheral blood Lym were collected from two healthy non-smoking donors following OECD guidelines.[39] Donors, one and two, were used for the short- and long-term exposures, respectively. Blood samples were drawn by venous puncture and collected in heparinized tubes and stored under sterile conditions at 4°C for a maximum of 4 h before use. Whole blood samples were precultured (44 to 48 h) in the presence of mitogen (PHA) prior to exposure to the test items. For the short-time exposure, the Lym were incubated with the test item for 4 h in presence or absence of metabolic activation. At the end of the incubation period, the treatment medium was removed and the cells were washed twice with PBS with 10% FBS. The cells were then incubated in complete culture medium with 6 μg/mL cytochalasin B from 40 h to 42 h at 37°C and 5% carbon dioxide.[44] For the long-time exposure, the test item was added to complete culture medium and 6 μg/mL cytochalasin B was added 1 h later. The cells were incubated for 43 h at 37°C. Duplicate cultures were prepared for all test item concentrations.

Following cultivation, the complete culture medium was removed and the cells were treated with cold hypotonic solution (0.075 M potassium chloride (KCl)) for 30 min and centrifuged. The pellets were resuspended in fixation solution (3:1 methanol: glacial acetic acid) with 0.9% NaCl (1+1) and centrifuged again. The resulting pellets were resuspended in fixation solution (3:1 methanol: glacial acetic acid) and this step was repeated twice. The cells were gently resuspended again, dropped onto clean glass slides and dried on a heating plate. The cells were stained with acridine orange solution and 1000 (if possible) binucleated cells per slide were analysed for micronuclei according to the criteria of Fenech.[45] Mononucleated and multinucleated cells and binucleated cells with more than six micronuclei were not considered.[44]

A cytokinesis block proliferation index (CBPI) was determined from 500 cells per culture according to $CBPI = (c_1 \times 1) + (c_2 \times 2) + (c_x \times 3)/n$ where c_1, c_2 and c_x are the number of mononucleate, binucleate and multinucleate cells, respectively, and n is the total number of cells. The CBPI was further used to calculate the % cytostasis, indicating the inhibition of cell growth of treated cultures in comparison with control cultures, according to $\%\ \text{Cytostasis} = 100 - 100 \times [(CBPIT - 1)/(CBPIC - 1)]$ where CBPIT is the CBPI of treated cultures and CBPIC is the CBPI of control cultures. The limit for discrimination between no cytotoxicity and a cytotoxic effect was set to a CBPI value of 70% compared to the negative/solvent control, corresponding to 30% relative cytostasis.

Metabolic activation. For metabolic activation, male Wistar rats were orally induced with phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) for three consecutive days. Eurofins (Munich, Germany) prepared the S9 microsomal fractions, with a final protein concentration of 30.1 mg/mL. The S9 mix was prepared according to Mortelmans and Zeiger,[42] with a final protein concentration of 15 mg/mL and a final S9 mix concentration in the cultures of 5% v/v for the in vitro micronucleus assay. For the reverse mutation assays, the final concentration of S9 fraction in the S9 mix was 5% v/v and 0.2 M phosphate buffer mixed with 0.15 M KCl solution was used as the metabolic inactive S9 mix substitution buffer.

Statistical analysis

All statistics and plots were done using R v. 3.4.3.[46] In all models described below, males and females were analysed separately. Models were first analysed for overall effect of PheCap using the analysis of variance (ANOVA) function of R. Further, when an overall effect was found, the different concentrations of PheCap were compared against the control (zero level of PheCap) using treatment contrasts from the summary output of R. A general description of the statistical methods used in this study is found in Crawley.[47]

The data sets for haematology, blood coagulation, clinical biochemistry and organ and tissue weight were all analysed using one-way ANOVA models or Welch *t*-tests. The latter was used for the recovery groups of each data set, since they only contained two levels of the predictor, that is, 0 and 250 mg/kg bw/day PheCap. For organ and tissue weight, the analyses were controlled for bw by first creating models with bw as predictor and then using the residuals of these models in the analyses of organ and tissue weight.

For the bw and daily feed consumption data sets, we used linear mixed effect models (LME). Time (week) was set as a continuous predictor. We also included the second-order polynomial predictor for time to account for the curvatures in bw increase or feed consumption change. Treatment (dose

of PheCap) was set as categorical predictor and includes the zero level (control). We allowed for interaction between the two predictors, that is, testing for differences in regression slopes depending on treatment (dose of PheCap). Each individual was repeatedly measured over several weeks. To account for the repeated measurements, the intercept of each individual rat was set as random effect factor in the models and the models included a temporal autocorrelation. The R syntax for the models was:

$$\text{lme}(\text{Response} \sim \text{poly}(\text{Time}, 2) \times \text{Treatment}, \text{random} = \sim + 1 | \text{Individual}, \text{corAR1}())$$

where 'Response' is either *bw* or *feed consumption* depending on the data set in question. For the feed consumption data, each observation represents the mean of two rats that were grouped together in a cage and repeatedly measured over several weeks. Thus, for this model, the random effect factor was the intercept of each individual cage instead of each individual rat.

The data sets for clinical observations, urinalysis, reverse mutation assay and micronucleus assay were all analysed using generalized linear models (GLMs) to account for the non-normal nature of these data, as described below. GLMs that contain predictor levels where all observations are zero will often give unreliable estimates and p values. Thus, in cases where this was a problem, we added the constant 1 or 0.001 to all response variables of models with quasi-Poisson or quasi-binomial error term, respectively.

All urinalysis data were analysed with binomial error term; Ery, BIL, protein, nitrite, Ket, Gluc and Leu were given the value 0 for all negative observations and 1 for the non-negative. UBG was given the value 0 for a normal observation and 1 for a non-normal. Specific gravity was given the value 0 for values smaller than 1.03 and 1 for values equal to or larger than 1.03. pH was given the value 0 for values from 6 to 7 and 1 when smaller or larger than this. The R syntax for the models was:

$$\text{glm}(\text{Response} \sim \text{Treatment}, \text{family} = \text{'binomial'})$$

where 'Response' represents either *Ery* or *BIL* and so on as described above. 'Treatment' is a categorical predictor that represents the concentration of PheCap, including zero concentration for the control group.

The reverse mutation assay data were analysed using a Poisson model since the response variable represents count data (the number of colonies of *S. typhimurium*). We used quasi-Poisson error term to account for overdispersion of the data. The R syntax for the model was:

$$\text{glm}(\text{Counts} \sim \text{Treatment}, \text{family} = \text{'quasipoisson'})$$

where 'Treatment' is the same as described for the urinalysis models.

The micronucleus assay data were analysed with a binomial error term. In addition to the different levels of PheCap, we included cell culture as predictor in this model. We did this to account for the variation that is caused by the two different cell cultures used in this study. Each observation represents the number of 'successes' (the number of cells with micronuclei) out of a total number of trials (the number of scored cells). We, therefore, used binary logistic regression models where the response variable was represented with two variables for cell numbers; micronuclei and normal using the following R syntax:

$$\text{glm}(\text{cbind}(\text{Micronuclei}, \text{Normal}) \sim \text{Cell\_culture} + \text{Treatment}, \text{family} = \text{'binomial'})$$

The clinical observations were analysed as the number of days the given symptoms were observed divided by the total number of days the given rat was observed. We, therefore, analysed the data with a quasi-binomial error term using the following R sytax:

$$\text{glm}(\text{Prop .days} \sim \text{Treatment}, \text{family} = \text{'quasibinomial'})$$

where 'Treatment' is the same as described for the previous models.

Results

Oral dose toxicity

A single female from each of the 30 and 100 mg/kg bw test groups died accidently or due to technical reasons during administration, whereby no gastrointestinal lesions were noted. Two females from the 250 mg/kg bw main test and recovery groups were found dead due to gavage-related issues. However, in both animals, there were also local gastrointestinal reactions to PheCap.

No biologically relevant differences between test item treated and control animals were observed in any of the parameters of the functional observation battery including body temperature over the course of the study. Diarrhoea, salivation and moving of bedding material were observed frequently (Table 1). Diarrhoea was observed in all treatment groups as well as in the controls.[48,49] In control animals, diarrhoea was observed with lower incidence and was related to the vehicle, PEG 400. The incidence was increased by PheCap for both sexes in the 250 mg/kg bw groups as the mean number of affected days was statistically significantly higher than in the respective controls (Table 1). Salivation and moving of bedding material immediately after administration showed a statistically significant dose-dependent increase (Table 1) and suggested some discomfort of the test item formulation. In some treated animals, piloerection, wasp waist, hunched posture, kyphosis and spontaneous reduction in spontaneous activity were observed for short durations or were transient, that is, the animals recovered during the treatment phase (Table 1A in Online Appendix). The single observations of alopecia, crust and nasal discharge circling, flipping and

Table 1. Statistics of main clinical observations.[a]

			Males						Females					
Parameter	PheCap	Exp	Mean	SD	*n*	df	*F* or *t*	*P*	Mean	SD	*n*	df	*F* or *t*	*P*
Diarrhoea						3, 36	22.949	**< 0.001**				3, 36	3.027	**0.042**
	0	Test	55.30	5.81	10				54.10	6.61	10			
	30	Test	60.70	3.27	10	36	3.443	**0.001**	52.50	13.16	10	36	0.134	0.894
	100	Test	57.90	1.91	10	36	1.644	0.109	54.60	16.59	10	36	0.534	0.596
	250	Test	67.30	1.16	10	36	7.821	**< 0.001**	62.00	7.75	10	36	2.641	**0.012**
	0	Recov	55.00	6.96	5				57.00	0.00	5			
	250	Recov	65.40	0.89	5	1. 8	10.952	**0.011**	66.60	11.59	5	1. 8	6.936	**0.030**
Salivation						3, 36	1033.5	**< 0.001**				3, 36	177.13	**< 0.001**
	0	Test	0.00	0.00	10				0.00	0.00	10.000			
	30	Test	4.80	0.42	10	36	7.319	**< 0.001**	6.10	2.88	10.000	36	2.393	**0.022**
	100	Test	42.30	6.55	10	36	18.415	**< 0.001**	45.10	17.08	10.000	36	3.813	**0.001**
	250	Test	59.90	1.52	10	36	21.676	**< 0.001**	58.20	7.83	10.000	36	4.156	**< 0.001**
	0	Recov	0.00	0.00	5				0.00	0.00	5.000			
	250	Recov	60.00	3.94	5	1. 8	1941.4	**< 0.001**	58.60	2.07	5.000	1. 8	601.64	**< 0.001**
Moving the bedding						3, 36	3559.3	**< 0.001**				3, 36	217.17	**< 0.001**
	0	Test	0.00	0.00	10				0.00	0.00	10.000			
	30	Test	51.80	1.93	10	36	17.020	**< 0.001**	44.80	14.08	10.000	36	4.012	**< 0.001**
	100	Test	65.70	2.79	10	36	18.640	**< 0.001**	64.40	17.54	10.000	36	4.606	**< 0.001**
	250	Test	68.90	0.74	10	36	19.080	**< 0.001**	68.00	7.75	10.000	36	4.699	**< 0.001**
	0	Recov	0.00	0.00	5				0.00	0.00	5.000			
	250	Recov	68.00	2.24	5	1. 8	7003.5	**< 0.001**	62.00	10.86	5.000	1. 8	131.72	**< 0.001**

PheCap: phenylcapsaicin; GLM: generalized linear model; SD: standard deviation. *P*-values ≤ 0.05 are highlighted in bold.
[a]Mean represents the mean number of days with the given symptom, while the statistical analyses are performed on the proportion 'number of affected days'/'total number of days' using a GLM.

paresis are considered to be incidental findings not toxicologically relevant (Table 1A).

No persistent effects of PheCap on food consumption or bw gain were found between treatment and control groups for either sex (Figure 2; see Appendix 1 for details).

Regarding haematological parameters, PheCap had small but statistically significant effects on RBC, MCV, Luc and RE in males and Hct and Mono in females at the end of the treatment period (Table 2, Table 1B and Figure 1A).

In males, RBC and RE were dose dependently reduced, MCV was dose dependently increased and for RBC, the 100 (−3.2%) and 250 (−4.7%) mg/kg bw dose groups were statistically significantly reduced compared to the control. For MCV and RE, a statistically significant increase (3.2%) and decrease (−46%) were found for the 250 mg/kg bw dose groups, respectively. For Luc, although an overall effect was found, none of the dose groups differed statistically significantly from the control. In the 250 mg/kg bw dose group at the end of the recovery period, a statistically significant decrease was found in WBC (−40%) and Mono (−56%), and a statistically significant increase was found in RE (25%).

In females at the end of the treatment period, a statistical significant decrease was found for the Hct in the 30 (−5.9%) and 250 (−8.5%) mg/kg bw dose groups and a statistically significant increase (82%) was found in the Mono for the 250 mg/kg bw dose group compared to the controls (Table 2, Table 1B and Figure 1A). At the end of the recovery period, a statistically significant increase in Eos (78%) was found in the 250 mg/kg bw dose group. A non-statistically significant dose-dependent increase and decrease was found in Neu and RE (Table 1B and Figure 1A), respectively.

A minor but statistically significant increase in the blood coagulation parameter, PT, was found for the 100 (16%) and 250 (11%) mg/kg bw male dose groups at the end of the test period and in the 250 (8%) mg/kg bw dose group at the end of the recovery period for females (Table 1C and Figure 1B).

In males, at the end of the treatment period, a reduction in the clinical biochemistry parameters, such as TBIL, TBA, Chol and Gluc, was found (Table 3, Table 1D and Figure 1C). TBIL and Chol were reduced by 20, 37 and 19% and 28, 41 and 38% or the 30, 100 and 250 mg/kg bw dose groups, respectively, while TBA and Gluc were reduced by 50 and 51% and 35 and 38% for the 100 and 250 mg/kg bw dose groups, respectively (Table 3, Table 1D). In Gluc, the reduction was dose dependent. There was also a non-statistically significant dose-dependent increase in AP at the end of the treatment period (Table 3). At the end of the recovery period, a statistically significant

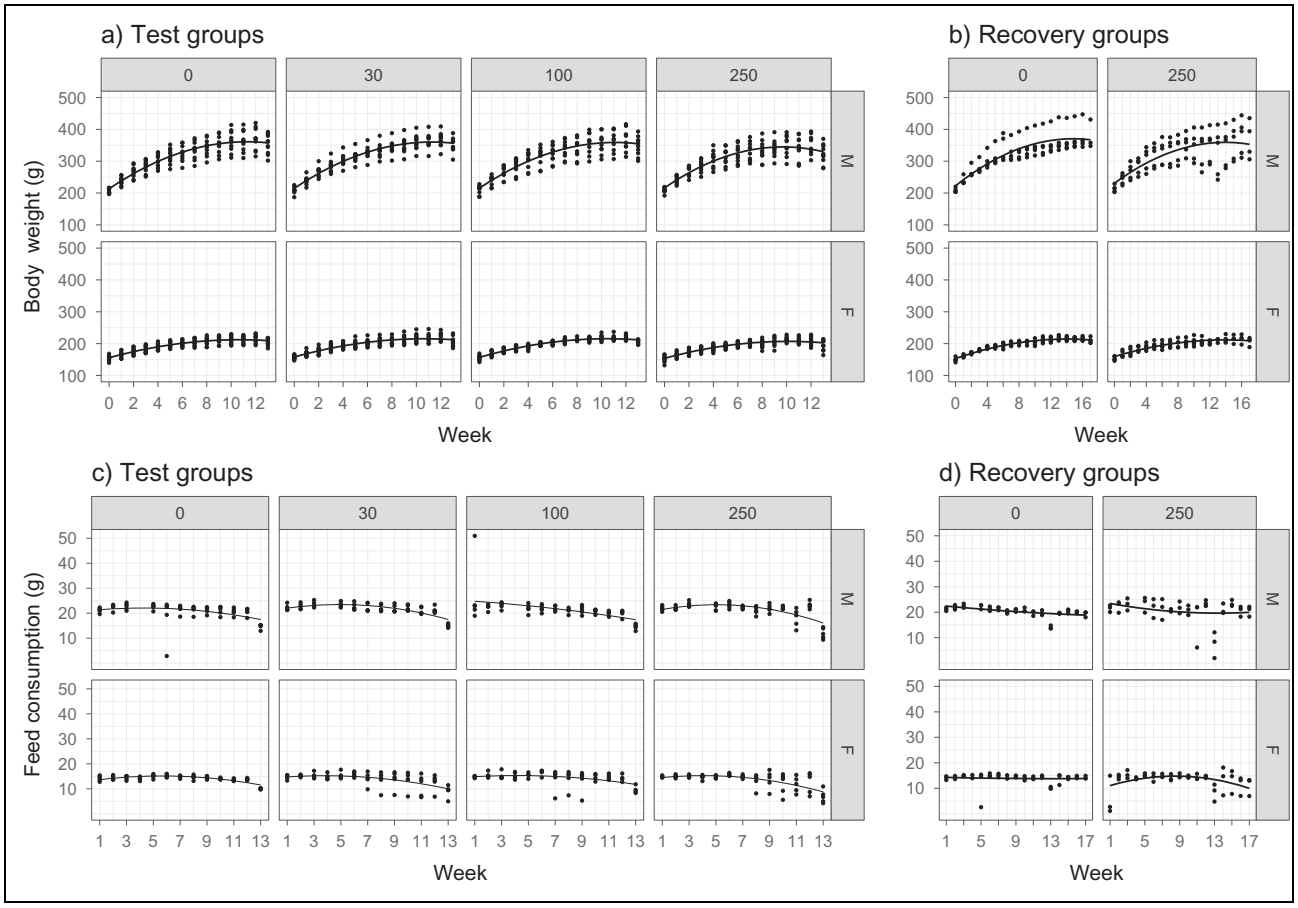

Figure 2. (a) and (b) Bw gain and (c) and (d) feed consumption. The concentrations of PheCap represent the mg/kg bw/day treatment and control groups. bw: body weight; PheCap: phenylcapsaicin; M: males; F: females.

reduction was found in ASAT (−67%), ALAT (−61%), AP (−71%), TBIL (−52%), TBA (−47%), Chol (−61%), Gluc (−57), Na (−54) and K (−58%) for the 250 mg/kg bw dose groups compared to controls (Table 1D and Figure 1C).

In females, statistically significant effects of PheCap were found in ALAT, AP and TBIL (Table 3). In ALAT (73%) and AP (84%), a statistically significantly increase was found in the 250 mg/kg bw dose group. In AP, there was a statistically significant dose-dependent increase of 32, 37 and 84% for the 30, 100 and 250 mg/kg bw dose groups, respectively (Table 3). There was a non-significant dose-dependent decrease in TP, Alb and urea at the end of the treatment period (Table 1D). No effects on the clinical biochemistry parameters were found at the end of the recovery period in the females (Table 1D and Figure 1C).

The large variation in clinical biochemistry parameters is partially explained by PEG 400 induced diarrhoea.[48,49]

No test item-derived effects were found in urinalysis (Table 1E and Figure 1D).

Few specific gross pathological changes were recorded, but the most frequent finding was a fluid-filled uterus identified as cornual dilation. This was found in four test period females (all dose groups) and four recovery period females (100 and 250 mg/kg bw dose groups) but is considered to be cyclic change normal for this strain of Wistar rats. Single incidental findings of liver herniation, red thymus with haemorrhage and red mesenteric lymph nodes with erythrophagocytosis were found in three test group males. In two test group females, single incidental findings of enlarged liver and pituitary gland and perivascular inflammation in the lung were found.

At the end of the test period, both sexes showed a statistically significant dose-dependent increase in liver weights, with an increase of 2.5, 3.7 and 33% in males and 2.1, 12.3 and 37% in females for the 30, 100 and 250 mg/kg bw dose groups, respectively (Table 4). This effect was reversible as the difference in mean liver weights was 9.33 and 4.55% for males and females at the end of the recovery period, respectively. In males at the end of the test period, there was also a dose-dependent organ weight decrease of spleen, 5.4, 16.3 and 21%, and prostate, 5, 10 and 34%, for the 30, 100 and 250 mg/kg bw dose groups, respectively (Table 4). For prostate and spleen, the decrease was statistically significantly different for the 250 and 100 and 250 mg/kg bw dose groups, respectively. Testis weight was also significantly decreased by PheCap

Table 2. Main haematological findings.

					Males						Females					
Parameter	Unit	PheCap	Exp	Statistical test	Mean	SD	*n*	df	*F* or *t*	*P*	Mean	SD	*n*	df	*F* or *t*	*P*
Haematology																
Red blood cell count	10^{12}/L			ANOVA				3.360	4.327	**0.011**				3.310	2.393	0.087
(RBC)		0	Test		9.15	0.23	10				8.26	0.44	10			
		30	Test		9.14	0.18	10	36	0.048	0.962	8.00	0.64	8			
		100	Test		8.86	0.34	10	36	2.030	**0.050**	8.08	0.50	9			
		250	Test		8.72	0.47	10	36	2.945	**0.006**	7.58	0.60	8			
		0	Recov		9.27	0.49	5				7.99	0.50	5			
		250	Recov	Welch t-test	8.71	0.58	4	5.956	1.513	0.181	7.83	0.48	4	6.689	0.481	0.646
Haematocrit value	%			GLM				3.360	0.751	0.529				3.310	3.622	**0.024**
(Hct)		0	Test		47.92	1.85	10				45.43	1.74	10			
		30	Test		47.87	1.22	10				42.76	3.41	8	31	2.179	**0.037**
		100	Test		46.71	2.04	10				43.88	2.09	9	31	1.308	0.201
		250	Test		47.13	3.06	10				41.59	2.96	8	31	3.143	**0.004**
		0	Recov		48.32	1.52	5				43.76	2.76	5			
		250	Recov	GLM	47.35	2.05	4	1.700	0.671	0.440	43.28	2.76	4	1.700	0.069	0.801
Mean corpuscular volume	fL			ANOVA				3.360	3.720	**0.020**				3.310	1.074	0.374
(MCV)		0	Test		52.36	1.52	10				55.08	2.11	10			
		30	Test		52.35	1.09	10	36	0.017	0.986	53.49	1.92	8			
		100	Test		52.73	0.94	10	36	0.635	0.530	54.39	1.75	9			
		250	Test		54.03	1.55	10	36	2.865	**0.007**	54.94	2.30	8			
		0	Recov		52.22	1.41	5				54.82	1.81	5			
		250	Recov	Welch t-test	54.45	2.22	4	4.863	1.750	0.142	55.33	0.76	4	5.608	0.565	0.594
Monocytes	%			GLM				3.360	1.006	0.401				3.310	3.625	**0.024**
(Mono)		0	Test		2.35	0.54	10				2.08	0.95	10			
		30	Test		1.89	0.87	10				1.89	0.55	8	31	0.367	0.716
		100	Test		2.75	1.40	10				2.57	1.24	9	31	0.890	0.380
		250	Test		2.22	1.45	10				3.79	2.21	8	31	2.702	**0.011**
		0	Recov		3.44	1.62	5				2.52	1.07	5			
		250	Recov	GLM	1.53	0.41	4	1.700	6.805	**0.035**	2.80	1.25	4	1.700	0.131	0.728
Large unstained cells	%			GLM				3.360	3.056	**0.041**				3.310	2.361	0.090
(Luc)		0	Test		0.16	0.10	10				0.22	0.09	10			
		30	Test		0.21	0.13	10	36	1.011	0.319	0.19	0.10	8			
		100	Test		0.09	0.06	10	36	1.703	0.097	0.10	0.09	9			
		250	Test		0.11	0.10	10	36	1.180	0.246	0.25	0.23	8			
		0	Recov		0.22	0.16	5				0.24	0.05	5			
		250	Recov	GLM	0.23	0.17	4	1.700	0.002	0.966	0.20	0.18	4	1.700	0.205	0.664
Reticulocytes	%			GLM				3, 36	4.108	**0.013**				3, 31	1.459	0.245
(Re)		0	Test		1.99	0.51	10				2.57	1.18	10			
		30	Test		1.70	0.46	10	36	0.959	0.344	2.37	0.66	8			
		100	Test		1.46	0.39	10	36	1.837	0.075	1.88	1.02	9			
		250	Test		1.07	0.81	10	36	3.309	**0.002**	1.60	1.27	8			
		0	Recov		1.35	0.21	5				1.58	0.16	5			
		250	Recov	GLM	1.70	0.20	4	1, 7	6.042	**0.044**	1.34	0.21	4	1, 7	3.740	0.094

PheCap: phenylcapsaicin; SD: standard deviation; RBC: red blood cell count; Hct: haematocrit value; GLM: generalized linear model; ANOVA: analysis of variance; MCV: mean corpuscular volume; Mono: monocytes; Luc: large unstained cells; RE: reticulocytes. *P*-values ≤ 0.05 are highlighted in bold.

for the 30 and 250 mg/kg bw dose groups at the end of the test period. The effects on prostate and testes were reversible, but the spleen weight was still statistically significantly reduced (18%) at the end of the recovery period. In males at the end of the recovery period, there was also a statistically significant increase in heart weight

Table 3. Main clinical biochemistry findings.

					Males						Females					
Parameter	Unit	PheCap	Exp	Statistical test	Mean	SD	*n*	df	*F* or *t*	*P*	Mean	SD	*n*	df	*F* or *t*	*P*
Aspartate-aminotransferase	U/L			ANOVA				3, 36	0.753	0.528				3, 32	1.488	0.2364
(ASAT)		0	Test		106.80	28.76	10				86.93	18.20	10			
		30	Test		88.50	28.42	10				79.90	17.21	9			
		100	Test		91.62	33.67	10				85.34	23.55	9			
		250	Test		91.60	29.02	10				101.85	29.23	8			
		0	Recov		90.80	27.46	5				59.30	19.80	5			
		250	Recov	Welch t-test	29.63	2.56	4	4.087	4.955	**0.007**	85.13	46.62	4	3.868	1.036	0.361
Alanine aminotransferase	U/L			ANOVA				3, 36	2.391	0.085				3, 32	4.502	**0.010**
(ALAT)		0	Test		35.54	8.88	10				34.58	10.99	10			
		30	Test		31.23	9.07	10				26.48	8.32	8	32	0.888	0.382
		100	Test		32.58	7.27	10				35.90	10.63	9	32	0.149	0.882
		250	Test		44.59	19.78	10				59.83	35.89	8	32	2.764	**0.010**
		0	Recov		39.30	7.91	5				25.84	10.99	5			
		250	Recov	Welch t-test	15.48	1.49	4	4.35	6.588	**0.002**	38.03	16.96	4	4.94	1.243	0.270
Alkaline phosphatase	U/L			ANOVA				3, 36	0.394	0.758				3, 32	2.936	**0.048**
(AP)		0	Test		103.15	28.65	10				54.68	22.09	10			
		30	Test		112.62	46.37	10				72.13	30.41	9	32	1.164	0.253
		100	Test		113.50	39.00	10				74.84	25.67	9	32	1.345	0.188
		250	Test		123.31	48.96	10				100.48	49.28	8	32	2.959	**0.006**
		0	Recov		115.31	45.51	5				57.27	10.00	5			
		250	Recov	Welch t-test	33.10	6.25	4	4.19	3.993	**0.015**	67.46	27.48	4	3.64	0.705	0.523
Total bilirubin	μmol/L			ANOVA				3, 36	4.8154	**0.006**				3, 32	3.511	**0.026**
(TBIL)		0	Test		2.21	0.52	10				2.64	0.63	10			
		30	Test		1.77	0.48	10	36	2.037	**0.049**	2.07	0.44	9	32	2.537	**0.016**
		100	Test		1.39	0.38	10	36	3.797	**0.001**	1.99	0.31	9	32	2.881	**0.007**
		250	Test		1.78	0.53	10	36	1.991	**0.054**	2.11	0.52	8	32	2.261	**0.031**
		0	Recov		2.32	0.38	5				2.36	0.47	5			
		250	Recov	Welch t-test	1.13	0.26	4	6.93	5.590	**0.001**	1.90	0.45	4	6.68	1.483	0.184
Total cholesterol	mmol/L			ANOVA				3, 33	5.383	**0.004**				3, 32	2.153	0.113
(Chol)		0	Test		1.74	0.44	10				1.27	0.31	10			
		30	Test		1.25	0.44	10	33	2.520	**0.017**	1.03	0.21	9			
		100	Test		1.02	0.43	10	33	3.682	**0.001**	1.08	0.27	9			
		250	Test		1.08	0.43	7	33	3.073	**0.004**	1.33	0.37	8			
		0	Recov		1.66	0.17	5				1.08	0.32	5			
		250	Recov	Welch t-test	0.65	0.09	4	6.08	11.331	**< 0.001**	1.32	0.38	3	3.72	0.942	0.403
Glucose	mmol/L			ANOVA				3, 36	4.615	**0.008**				3, 32	1.475	0.240
(Gluc)		0	Test		7.32	2.08	10				5.22	1.93	10			
		30	Test		6.66	2.44	10	36	0.734	0.468	4.85	1.02	9			
		100	Test		4.77	1.83	10	36	2.810	**0.008**	4.50	1.28	9			
		250	Test		4.53	1.72	10	36	3.069	**0.004**	6.48	3.36	8			
		0	Recov		11.93	2.52	5				6.71	1.50	5			
		250	Recov	Welch t-test	5.16	0.45	4	4.31	5.898	0.003	7.30	3.54	4	3.86	0.307	0.775

PheCap: phenylcapsaicin; SD: standard deviation; ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase; AP: alkaline phosphatase; TBIL: total bilirubin; Chol: total cholesterol; Gluc: glucose; ANOVA: analysis of variance. *P*-values ≤ 0.05 are highlighted in bold.

Table 4. Statistics of main organ weight findings.[a]

				Males						Females					
Organ	Unit	PheCap	Exp	Mean	SD	*n*	df	*F* or *t*	*P*	Mean	SD	*n*	df	*F* or *t*	*P*
Liver	g						3, 36	17.031	**< 0.001**				3, 32	12.740	**< 0.001**
		0	Test	8.53	0.77	10				5.34	0.60	9			
		30	Test	8.74	0.83	10	36	0.439	0.663	5.46	0.48	9	32	0.389	0.700
		100	Test	8.84	0.97	10	36	0.711	0.482	6.00	0.44	9	32	2.021	**0.052**
		250	Test	11.34	2.04	10	36	6.190	**< 0.001**	7.30	0.93	9	32	5.538	**< 0.001**
		0	Recov	9.32	1.08	5				5.59	0.40	5			
		250	Recov	9.23	1.31	5	7.809	1.159	0.281	6.04	0.44	4	6.603	2.383	**0.051**
Spleen	g						3, 36	2.949	**0.046**				3, 32	1.350	0.276
		0	Test	0.78	0.14	10				0.52	0.07	9			
		30	Test	0.74	0.09	10	36	0.877	0.386	0.50	0.05	9			
		100	Test	0.66	0.11	10	36	2.573	**0.014**	0.52	0.07	9			
		250	Test	0.62	0.09	10	36	2.307	**0.027**	0.45	0.08	9			
		0	Recov	0.65	0.04	5				0.50	0.10	5			
		250	Recov	0.73	0.06	5	7.304	2.482	**0.041**	0.50	0.02	4	5.277	0.419	0.692
Prostate[l]	g						3, 36	6.383	**0.001**						
		0	Test	2.65	0.30	10									
		30	Test	2.51	0.24	10	36	0.787	0.437						
		100	Test	2.38	0.45	10	36	1.436	0.160						
		250	Test	1.75	0.41	10	36	4.115	**<0.001**						
		0	Recov	2.45	0.35	5									
		250	Recov	2.62	0.19	5	6.369	1.820	0.116						
Testes	g						3, 36	2.894	**0.049**						
		0	Test	3.88	0.34	10									
		30	Test	3.57	0.18	10	36	2.824	**0.008**						
		100	Test	3.65	0.24	10	36	1.928	0.062						
		250	Test	3.54	0.27	10	36	2.068	**0.046**						
		0	Recov	3.42	1.01	5									
		250	Recov	3.84	0.15	5	4.193	1.035	0.357						

PheCap: phenylcapsaicin; SD: standard deviation; ANOVA: analysis of variance; bw: body weight. *P*-values ≤ 0.05 are highlighted in bold.
[a]Effects of PheCap were analysed using an ANOVA for the test period and Welch *t*-test for the recovery period. In the statistical analysis, the effect of bw has been controlled for (see 'Methods' section).
[l]prostate including seminal vesicles and coagulating glands.

(3.7%) and thymus (11.3%) and a decrease in pituitary gland weight (−13%; Table 1F and Figure 1E). In females, there was a reversible decrease in pituitary gland weight with a significant difference between the control and the 100 (−19%) and 250 (−27%) mg/kg bw dose groups at the end of the treatment period (Table 1F and Figure 1E).

Most histomorphological findings were within the range of normal background lesions for animals of this strain and age or were incidental lesions related to the dosing procedure (misgavage). However, changes related to systemic exposure of PheCap were recorded in the liver, and local irritant effects were recorded in the stomach and cecum (Table 5 and Figure 1F). In the liver, diffuse hepatocellular hypertrophy was recorded in males at 100 mg/kg bw and in both sexes at 250 mg/kg bw at the end of the treatment period. At the end of the treatment period, a slight increase in the incidence and/or group mean severity of periportal vacuolation (fatty change) was noted in one male at 100 mg/kg bw, and focal necrosis was recorded in one female at 250 mg/kg bw (Table 5 and Figure 1F). In addition, in one male at 250 mg/kg bw group, eosinophilic cytoplasmic inclusions were observed in periportal hepatocytes. The latter is considered a non-specific lesion,[50] although the cause of this inclusions remains unclear.

In the stomach at the end of the treatment period, diffuse forestomach hyperkeratosis and squamous hyperplasia were found for all but one female at 250 mg/kg bw. Submucosal oedema was present in 3 animals per sex at 250 mg/kg bw groups that was associated with submucosal inflammation in one female at 250 mg/kg bw (Table 5 and Figure 1F). In the glandular stomach, erosion/ulcer affected one female at 30 mg/kg bw, one animal per sex at 100 mg/kg bw and three males and five females at 250 mg/kg bw. The severity of these lesions increased with increasing PheCap dose (Table 5 and Figure 1F). There was an inflammation in the cecum of one male and two females at 100 mg/kg bw groups and three males and seven females at 250 mg/kg bw. The findings were more prominent in males than females at the end of the treatment period: Increased surface epithelial cell basophilia in cecum, characterized by a decrease in

Table 5. Summary of the main histopathology findings.[a]

		Dose (mg/kg bw/day)																			
		Non-recovery														Recovery					
		Male							Female							Male				Female			
		0		30		100		250	0		30		100		250	0		250		0		250	
		n=10		n=10		n=10		n=10	n=10		n=9		n=9		n=9	n=5		n=5		n=5		n=4	
Organ	Finding	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ	*N*	μ
Liver	Heptocellular hyperyrophy, diffuse	0		0		3	1.0	9	1.0	0		0[n]		0[n]		7	1.0	0		0		0		0	
	Vacuoloation (fatty change), periportal	0		0		1	1.0	4	1.5	2	1.0	1[n]	1.0	0[n]		3	1.7	0		0		1	1.0	0	1.0
	Eosinophilic cytoplasmic inclutions, periportal heptocytes	0		0		0		1	1.0	0		0[n]		0[n]		0		0		0		0		0	
	Focal necrosis	0		0		0		0		0		0[n]		0[n]		1	1.0	0		0		0		0	
Stomach	Hyperkeratosis, diffuse	0		0		0		10	1.5	0		0		0		8	1.3	0		0		0		0	
	Squamous hyperplasia	0		0		0		10	1.0	0		0		0		8	1.0	0		0		0		0	
	Edema, submucosa	0		0		0		3	2.0	0		0		0		3	1.0	0		0		0		0	
	Inlammation submucosa	0		0		0		0		0		0		0		1	1.0	0		0		0		0	
Glandular	Erosion/ulcer	0		0		1	1.0	3	1.7	0		1	1.0	1	2.0	5	2.4	0		1	1.0	0		1	1.0
stomach	Inflammation	0		0		1	1.0	3	1.7	0		0		2	2.0	7	1.6	0		1	1.0	0		0	
Cecum	Congestion, lamina propria	0		0		2	1.0	8	1.0	0		0		0		2	1.0	0		0		0		0	
	Increased basophilia, surface epithelia cells	0		0		3	1.0	9	1.0	0		0		0		5	1.0	0		0		0		0	
	Increased apoptopsis, surface epithelia cells	0		0		0		2	1.0	0		0		0		0		0		0		0		0	
Bone marrow	Decreased cellularity/cell density	0		0		0		5	2.0	0		0		0		6	1.8	0		1	1.0	0		1	1.0
Spleen	Lymphoid depletion	0		ne		ne		0		0		0		0		1	1.0	ne		ne		0		0	
Thymus	Atrophy	9	1.2	10	1.3	9	1.2	10	1.7	10	1.3	9	1.4	9	1.4	8	2.3	5	1.2	5	1.0	5	1.4	4	1.5
Lymph node	Axillary; lymphoid depletion	0		ne		ne		0		0		0		0		1	1.0	ne		ne		0		0	
	Mesenteric; lymphoid depletion	0		ne		ne		0		0		0		0		1	1.0	ne		ne		0		0	
Adrenal glands	Cortical hypertrophy, diffuse	0		0		0		2	1.0	0		0		0		5	1.0	0		0		0		0	

N: incidents of findings, μ: mean severity of the findings on a five-step value scale from 1 (least severe) to 5 (most severe); *n*: $n = 8$, ne: not examined.
[a]Data are from survivors.

eosinophilic cytoplasmic appearance and/or reduction in cell size, was observed in 3 of 10 males but none of 9 females administered with 100 mg/kg bw. In the 250 mg/kg bw group, it was observed in nine males but only five females. Further, congestion of the lamina propria in cecum was found in two males and no females dosed with 100 mg/kg bw and in eight males but only two females of the 250 mg/kg bw dose groups (Figure 1F). An increase in the surface epithelial cell apoptosis was only found in two males at 250 mg/kg bw group (Table 5). At the end of the treatment period for the 250 mg/kg bw treatment groups, decreased cellularity (decreased cell density) in bone marrow and diffuse adrenocortical hypertrophy were recorded in both sexes (Figure 1F), and lymphoid depletion in the spleen, axillary lymph node and mesenteric lymph node was recorded in one female. Thymic atrophy was noted in all dose groups of both sexes for both non-recovery and recovery groups with increased severity for the 250 mg/kg bw treatment groups (Table 5).

Reverse mutation assay

No mutagenic effect of PheCap was found in the tested *S. typhmurium* strains for all of the tested concentrations as no significant increase in revertant colony numbers was observed either with or without S9 activation, compared to the negative control dH_2O and solvent control DMSO (Tables 6 and 7; Figure 1G). For some of the strains, PheCap inhibited growth as the number of colonies was decreased, but this effect was reduced by metabolic activation (Tables 6 and 7). For the plate incorporation test, an overall statistically significant reduction in the number of colonies was found for TA 100 both with and without metabolic activation and for TA1535 and TA 102 with and without metabolic activation, respectively. Looking at the responses to individual PheCap concentrations, a statistically significant reduction in combination with a mutation factor ≤ 0.5 was found for concentration 1000 μg/plate and above for TA 100 and 316 μg/plate for TA 1535. For TA 1535, a mutation factor of 0.5 with almost statistical differences was also found for 1000 μg/plate and above (Table 7). With S9 activation, an overall statistically significant reduction in the number of colonies was found for TA 100 and TA 102, but a statistically significant reduction in the number of colonies in combination with a mutation factor ≤ 0.5 was only found for concentration 2500 μg/plate and above for TA 100 (Table 6). For the pre-incubation test without S9 activation, an overall statistically significant reduction of the number of colonies was found for all tester strains, and a statistically significant reduction in colony numbers in combination with a mutation factor ≤ 0.5 was found at 1000 μg/plate and above for TA 98 and TA 1535, at 316 μg/plate and above for TA 100 and at concentrations 31.6, 316 and 1000 μg/plate for TA 1537 (Table 7). A statistically significant reduction in colonies was also found for TA 102 at 1000 μg/plate and above, but the mutation factors were > 0.7.

With S9 activation, an overall statistically significant reduction of the number of colonies was found for the tester strains TA 98, TA 100 and TA 102 and statistically significant reduction in colony numbers in combination with a mutation factor ≤ 0.5 was only found for concentrations at 2500 μg/plate and 5000 μg/plate for TA 98 and TA 1535, respectively. A mutation factor of 0.5 without statistical significance was found for TA 98 at 2500 μg/plate and statistically significant reduction with mutation factor > 0.5 was found concentration at 1000 μg/plate and above for TA 100 and concentration at 316 μg/plate and above for TA 102 (Table 7).

Reduction in background lawn generally co-occurred with reduced growth and precipitation and was observed in all tested strains for concentration at 5000 μg/plate for both the plate incorporation and pre-incubation tests (Tables 6 and 7). The positive controls induced distinct and statistically significant increases in revertant colonies, demonstrating the validity of the assay (Tables 6 and 7).

Micronucleus assay

In the short-time exposure experiment, no increase in relative cytostasis above 30% was found for PheCap concentrations up to 100 μg/mL, either with or without metabolic activation. Without metabolic activation, relative cytostasis was 35 and 58% at 120 and 130 μg/mL PheCap, respectively. With metabolic activation, cytostasis was 36 and 57% for 130 and 140 μg/mL PheCap, respectively (Table 8 and Figure 1H). In the long-time exposure experiment without metabolic activation, no increase in relative cytostasis above 30% was found for 10 μg/mL PheCap. At 15 and 20 μg/mL PheCap, the relative cytostasis was 32 and 60%, respectively.

Following exposure to PheCap, the micronucleated cell frequencies found in all experiments and all PheCap test item concentrations were within the historical control limits of the negative and solvent controls (Table 8 and Figure 1H). Furthermore, no concentration-related trend increases in the number of micronucleated cell frequencies were found in the experiments compared to the solvent controls. No statistical differences were found between PheCap and the control groups (Table 8). The positive controls induced distinct and statistically significant increases of the micronucleus frequency, demonstrating the validity of the assay.

Discussion

There are many areas of use for PheCap, including agriculture, food industries and pharmacology, and synthetic capsaicin analogues have the potential to solve problems with regard to the quality requirements and the volumes needed by the different industries. Here, we present data on the local

Table 6. Plate-incorporation (Ames) assay with (+S9) and without (−S9) metabolic activation.[a]

			Plate-incorporation test											
			Without activation (-S9)						With activation (+S9)					
Strain	Treatment	Dose (μg/plate)	Colonies	SD	MutF	df	*F* or *t*	*P*	Colonies	SD	MutF	df	*F* or *t*	*P*
TA 98	dH2O		36.7	3.1	1.0				49.7	3.8	1.1			
	DMSO		37.0	0.0	1.0	8. 18	1.810	0.141	45.3	6.8	1.0	8. 18	1.965	0.112
	PheCap	3.16	38.0	5.6	1.0				49.7	7.0	1.1			
		10	46.0	11.0	1.2				41.3	4.7	0.9			
		31.6	38.0	5.0	1.0				44.3	7.5	1.0			
		100	33.3	6.4	0.9				50.7	4.2	1.1			
		316	36.3	7.8	1.0				48.0	1.7	1.1			
		1000	33.7	5.1	0.9				43.0	5.3	0.9			
		2500	25.7	3.5	0.7				34.3	5.0	0.8			
		5000	36.3[P]	11.7	1.0				44.3[P]	9.6	1.0			
	4-NOPD	10	297.3	18.8	8.0									
	2-AA	2.5							1916.0	118.2	42.3			
TA 100	dH2O		80.0	6.6	1.2				83.0	7.6	1.0			
	DMSO		69.0	11.5	1.0	8. 18	21.993	**< 0.001**	87.0	7.2	1.0	8. 18	8.689	**< 0.001**
	PheCap	3.16	72.0	7.6	1.0	18	0.295	0.771	86.3	4.9	1.0	18	0.071	0.944
		10	75.3	5.9	1.1	18	0.616	0.546	88.3	15.0	1.0	18	0.141	0.890
		31.6	73.3	5.5	1.1	18	0.424	0.676	85.7	2.5	1.0	18	0.142	0.889
		100	67.7	4.2	1.0	18	0.133	0.895	87.7	16.9	1.0	18	0.070	0.945
		316	48.0[B]	15.7	0.7	18	2.257	**0.037**	82.0	12.8	0.9	18	0.537	0.598
		1000	26.0[B]	4.0	0.4	18	4.957	**< 0.001**	71.7	10.8	0.8	18	1.697	0.107
		2500	16.7[B]	6.5	0.2	18	6.084	**< 0.001**	46.3[B]	3.5	0.5	18	4.838	**< 0.001**
		5000	11.3[BP]	9.3	0.2	18	6.587	**< 0.001**	46.0[BP]	12.2	0.5	18	4.882	**< 0.001**
	NaN3	10	409.0	65.8	5.9									
	2-AA	2.5							1888.7	76.5	21.7			
TA 1535	dH2O		13.3	1.2	1.1				10.3	2.1	1.2			
	DMSO		12.0	2.0	1.0	8. 18	4.544	**0.004**	8.3	4.0	1.0	8. 18	1.294	0.307
	PheCap	3.16	19.7	2.1	1.6	18	1.844	0.082	11.0	3.5	1.3			
		10	17.0	4.6	1.4	18	1.263	0.223	9.7	0.6	1.2			
		31.6	15.0	3.6	1.3	18	0.788	0.441	14.0	4.6	1.7			
		100	11.7	3.2	1.0	18	0.094	0.926	11.7	4.5	1.4			
		316	5.3	2.5	0.4	18	2.131	**0.047**	8.0	2.0	1.0			
		1000	9.3[B]	5.9	0.8	18	0.787	0.441	11.3	2.1	1.4			
		2500	6.0[B]	4.6	0.5	18	1.896	**0.074**	8.0	3.0	1.0			
		5000	5.7[BP]	3.8	0.5	18	2.013	**0.059**	8.7[P]	1.5	1.0			
	NaN3	10	719.7	142.8	60.0									
	2-AA	2.5							138.0	7.0	16.6			
TA 1537	dH2O		7.3	2.5	1.2				9.7	3.5	1.2			
	DMSO		6.3	2.1	1.0	8. 18	0.665	0.715	8.3	2.1	1.0	8. 18	1.385	0.268
	PheCap	3.16	8.3	5.1	1.3				9.3	5.7	1.1			
		10	7.0	1.0	1.1				10.7	4.0	1.3			
		31.6	6.7	0.6	1.1				7.0	2.6	0.8			
		100	9.7	2.3	1.5				13.7	2.3	1.6			
		316	5.7	1.2	0.9				9.7	1.2	1.2			
		1000	5.3[B]	1.2	0.8				9.3	1.5	1.1			
		2500	5.0[B]	2.6	0.8				12.0	2.0	1.4			
		5000	6.7[BP]	6.4	1.1				8.7[P]	1.2	1.0			
	4-NOPD	40	121.7	4.5	19.2									
	2-AA	2.5							359.0	23.4	43.1			
TA 102	dH2O		410.0	43.6	1.2				451.0	49.9	1.2			
	DMSO		334.3	6.7	1.0	8. 18	1.664	0.176	365.3	34.1	1.0	8. 18	2.938	**0.027**
	PheCap	3.16	325.3	21.5	1.0				421.0	29.3	1.2	18	1.649	0.116
		10	345.0	17.8	1.0				425.7	19.6	1.2	18	1.782	0.092

(continued)

Table 6. (continued)

			Plate-incorporation test											
			Without activation (-S9)						With activation (+S9)					
Strain	Treatment	Dose (μg/plate)	Colonies	SD	MutF	df	*F* or *t*	*P*	Colonies	SD	MutF	df	*F* or *t*	*P*
		31.6	349.7	36.4	1.0				405.7	32.5	1.1	18	1.207	0.243
		100	330.7	13.1	1.0				371.7	29.7	1.0	18	0.194	0.848
		316	340.7	5.7	1.0				327.3	47.8	0.9	18	1.200	0.246
		1000	318.0	28.7	1.0				353.3	73.2	1.0	18	0.372	0.714
		2500	319.3	21.2	1.0				359.3	25.1	1.0	18	0.185	0.855
		5000	301.7[P]	11.6	0.9				311.7[P]	35.5	0.9	18	1.713	0.104
	MMS	101	1625.7	91.6	4.9									
	2-AA	2.5							919.0	41.9	2.5			

PheCap: phenylcapsaicin; GLM: generalized linear model; B: background lawn reduced; P: precipitation; MutF: mutation factor (mean revertant test item/mean revertant DMSO). *P*-values ≤ 0.05 are highlighted in bold.
[a]Effects of PheCap were analysed using GLM (see Methods). Contrasts are between the individual PheCap concentrations and DMSO.

and systemic toxicology and genotoxicology of the commercially available synthetic capsaicin analogue PheCap.

Adverse tissue injuries such as erosion/ulcer, inflammation or increased apoptosis of mucosal epithelium in the stomach or cecum of some rats were found in the 100 and 250 mg/kg bw/day groups, respectively. In addition, simultaneous appearance of salivation, diarrhoea and moving of bedding material immediately after administration indicates dose-dependent discomfort of PheCap. Similar to our observations, capsaicin has also been suggested to increase salivary secretion by TrpV1-mediated modulation of paracellular permeability of the tight junctions in submandibular glands.[51,52] Capsaicin is known to be a strong irritant to gastric mucosa, irritating to mucous membranes and to cause severe gastritis and diarrhoea.[27] PheCap seems to have the same transient effects as diarrhoea is absent during the recovery period. However, in the present study, the high occurrence of diarrhoea could be due to a combined effect of PheCap and PEG 400, as PEG 400 has been reported to cause diarrhoea in rats.[49]

The effects found in forestomach from animals of both sexes at 250 mg/kg bw could be caused by the retention time of digesta. The rodent forestomach acts as a storage organ,[53] and hyperkeratosis, hyperplasia and inflammatory responses can be elicited in response to local irritation from ingested substances.[54]

Capsacin and capsacin analogues have been found to have a gastroprotective effect against ulcerogenic injuries at low doses but could increase rat stomach mucosal damage at high dosages.[55] The increased occurrence of forestomach lesions with increasing PheCap dosages reported here follows this pattern, and the effects on the glandular stomach are probably caused by local irritating properties of PheCap. Inflammatory changes and erosion/ulcers in the glandular stomach are also occasionally found as spontaneous lesions in control rodents.[56,57] Such lesions are more common in gavage studies due to possible trauma during dosing.[58] The occurrence in one animal per group as found for the 100 mg/kg bw male group and 30 and 100 mg/kg bw female groups is, therefore, within the range of background incidents. It should be considered, however, that a mechanically induced alteration would be enhanced by contact with an irritative test item.

The observed increase in surface epithelial cell basophilia is considered to be an adaptive cellular response to contact with PheCap, that is, the basophilic change represents regeneration (regenerative hyperplasia).[59] The cause of the lamina propria congestion remains unclear but may be indicative of a minor severity inflammatory change. In contrast, the increased mucosal cell apoptosis in two 250 mg/kg bw males is of adverse nature. Intestinal mucosal surface epithelial cells are efficiently renewed[60] and no abnormal findings were present after the 28-day recovery period. Furthermore, the cecum of rats is a large blind end sac storing ingesta and facilitating microbial fermentation. The findings reported here might be specific to ceca with such anatomical and functional characteristics. No changes in stomach mucosa, jejunum or colon have been reported in $B6C3F_1$ mice fed powdered capsaicinoids (64.5% capsaicin and 32.6% dihydrocapsaicin)[61] at doses up to 1500 mg/kg bw.[62] The increased apoptosis found is, therefore, considered to be of low general toxicological concern.

Considering the known effects in broiler production, it is unclear why no changes were noted on food consumption or bw gain between treatment and control groups. It might be speculated that the beneficial effects in poultry are mainly due to effects on the microbiota at smaller doses, but this needs to be examined in future studies. Nevertheless, the maximum effect on bw was in the range of -7% of the control, and this is not considered a toxicologically relevant effect.

The observed degenerative changes in the liver of both sexes (periportal vacuolation associated with hepatocellular hypertrophy, one case of eosinophilic inclusions and one case of possible-induced hepatocellular necrosis)

Table 7. Pre-incubation (Ames) assay with (+S9) and without (−S9) metabolic activation.[a]

			Pre-incubation test											
			Without activation (-S9)						With activation (+S9)					
Strain	Treatment	Dose (μg/plate)	Colonies	SD	MutF	df	*F* or *t*	*P*	Colonies	SD	MutF	df	*F* or *t*	*P*
TA 98	dH_2O		28.3	5.0	1.1				38.0	7.0	1.2			
	DMSO		25.0	2.6	1.0	8,18	3.881	**0.008**	31.3	8.1	1.0	8, 18	3.823	**0.009**
	PheCap	3.16	25.3	5.1	1.0	18	0.058	0.955	37.3	2.3	1.2	18	0.884	0.388
		10	30.3	13.1	1.2	18	0.880	0.391	29.3	3.5	0.9	18	0.314	0.757
		31.6	25.7	6.4	1.0	18	0.115	0.910	37.7	4.0	1.2	18	0.931	0.364
		100	23.0	7.0	0.9	18	0.355	0.727	35.7	11.1	1.1	18	0.647	0.526
		316	16.7	6.1	0.7	18	1.576	0.133	31.3	7.6	1.0	18	0.000	1.000
		1000	10.3^{B}	1.5	0.4	18	2.936	**0.009**	23.7	7.5	0.8	18	1.260	0.224
		2500	13.7^{B}	1.5	0.5	18	2.206	**0.041**	13.3^{B}	5.9	0.4	18	3.195	**0.005**
		5000	12.7^{BP}	7.6	0.5	18	2.423	**0.026**	21.0^{BP}	10.0	0.7	18	1.735	0.100
	4-NOPD	10	421.0	98.9	16.8									
	2-AA	2.5							1326.3	253.8	42.3			
TA 100	dH_2O		78.0	7.2	1.1				76.3	4.0	1.1			
	DMSO		71.0	9.8	1.0	8,18	130.650	**< 0.001**	72.0	13.1	1.0	8, 18	4.611	**0.003**
	PheCap	3.16	61.7	4.0	0.9	18	1.446	0.165	71.0	8.2	1.0	18	0.094	0.926
		10	65.7	4.5	0.9	18	0.815	0.426	77.3	9.6	1.1	18	0.489	0.631
		31.6	62.3	3.8	0.9	18	1.339	0.197	69.0	9.5	1.0	18	0.283	0.780
		100	70.0	3.5	1.0	18	0.150	0.882	73.7	5.8	1.0	18	0.155	0.879
		316	8.3^{B}	7.5	0.1	18	10.564	**< 0.001**	66.3	14.7	0.9	18	0.540	0.596
		1000	0^{B}	0.0	0.0	18	7.640	**< 0.001**	51^{B}	8.2	0.7	18	2.111	**0.049**
		2500	0^{B}	0.0	0.0	18	7.640	**< 0.001**	39.7^{B}	20.4	0.6	18	3.378	**0.003**
		5000	0^{BP}	0.0	0.0	18	7.640	**< 0.001**	40.3^{BP}	2.3	0.6	18	3.302	**0.004**
	NaN_3	10	677.3	158.5	9.5									
	2-AA	2.5							592.0	385.2	8.2			
TA 1535	dH_2O		9.7	2.1	1.1				11.7	4.9	1.5			
	DMSO		9.0	1.7	1.0	8,18	7.357	**< 0.001**	8.0	3.6	1.0	8, 18	1.812	0.141
	PheCap	3.16	9.0	8.0	1.0	18	0.000	1.000	12.7	4.7	1.6			
		10	14.3	3.8	1.6	18	1.656	0.115	8.3	3.5	1.0			
		31.6	10.0	2.6	1.1	18	0.344	0.735	7.0	2.0	0.9			
		100	7.3	2.1	0.8	18	0.612	0.548	7.0	3.6	0.9			
		316	5.3	2.1	0.6	18	1.416	0.174	7.3	2.1	0.9			
		1000	4.7	1.5	0.5	18	1.701	0.106	7.7	4.9	1.0			
		2500	0^{B}	0.0	0.0	18	3.457	**0.003**	11.7^{B}	2.1	1.5			
		5000	1.3^{BP}	1.5	0.1	18	3.152	**0.006**	4^{BP}	1.7	0.5			
	NaN_3	10	824.7	138.9	91.6									
	2-AA	2.5							824.7	138.9	91.6			
TA 1537	dH_2O		6.3	1.5	0.9				8.3	1.5	1.4			
	DMSO		6.7	2.1	1.0	8,18	2.564	**0.046**	6.0	0.0	1.0	8, 18	2.190	0.080
	PheCap	3.16	4.7	2.5	0.7	18	1.029	0.317	8.7	4.7	1.4			
		10	6.7	1.5	1.0	18	0.000	1.000	6.0	2.6	1.0			
		31.6	3.3	1.2	0.5	18	1.799	0.089	6.0	1.7	1.0			
		100	6.0	2.6	0.9	18	0.326	0.748	7.3	1.5	1.2			
		316	2^{B}	1.7	0.3	18	2.601	**0.018**	6.7	2.1	1.1			
		1000	1.7^{B}	1.2	0.3	18	2.788	**0.012**	4^{B}	1.0	0.7			
		2500	4^{B}	2.6	0.6	18	1.407	0.177	3^{B}	1.7	0.5			
		5000	4.7^{BP}	2.1	0.7	18	1.029	0.317	3.3^{BP}	2.3	0.6			
	4-NOPD	40	95.7	20.8	14.3									
	2-AA	2.5							168.3	9.0	28.1			
TA 102	dH_2O		289.7	26.8	1.1				375.0	23.3	1.1			
	DMSO		263.7	60.9	1.0	8, 18	4.922	**0.002**	345.3	27.0	1.0	8, 18	3.925	**0.008**
	PheCap	3.16	275.3	21.5	1.0	18	0.395	0.697	356.0	15.5	1.0	18	0.345	0.734
		10	312.0	21.9	1.2	18	1.582	0.131	380.3	28.3	1.1	18	1.114	0.280
		31.6	277.7	13.9	1.1	18	0.473	0.642	302.3	43.1	0.9	18	1.448	0.165

(continued)

Table 7. (continued)

Strain	Treatment	Dose (μg/plate)	Pre-incubation test											
			Without activation (-S9)						With activation (+S9)					
			Colonies	SD	MutF	df	*F* or *t*	*P*	Colonies	SD	MutF	df	*F* or *t*	*P*
		100	237.3	28.6	0.9	18	0.925	0.367	312.3	35.1	0.9	18	1.103	0.285
		316	224.3	20.2	0.9	18	1.399	0.179	266.3	48.4	0.8	18	2.732	**0.014**
		1000	191.0	23.1	0.7	18	2.669	**0.016**	272.0	49.8	0.8	18	2.525	**0.021**
		2500	183.0	46.1	0.7	18	2.985	**0.008**	274.3	26.0	0.8	18	2.441	**0.025**
		5000	207.7[P]	35.2	0.8	18	2.024	**0.058**	287.7[P]	19.8	0.8	18	1.963	**0.065**
	MMS	101	1234.3	300.2	4.7									
	2-AA	2.5							936.7	92.6	2.7			

PheCap: phenylcapsaicin; GLM: generalized linear model; B: background lawn reduced; P: precipitation; MutF: mutation factor (mean revertant test item/mean revertant DMSO). *P*-values ≤ 0.05 are highlighted in bold.
[a]Effects of PheCap were analysed using GLM (see Methods). Contrasts are between the individual PheCap concentrations and DMSO.

correlated with slightly elevated levels of ASAT, ALAT and AP only in females in the 250 mg/kg bw treatment group (Table 3, Table 1D and Figure 1C). The morphological effects, if isolated, would not be considered as adverse and they had returned to normal values during the recovery period. This is also true for the enzyme changes in treated animals that were below established adverse x-fold increases when compared to controls.[63]

The observed diffuse hepatocellular hypertrophy described corresponds to previously reported findings of hepatocellular hypertrophy/increased liver weights in capsaicin analogue studies.[57,62,64,65] It has been suggested that low incidences are within the normal range for rats[62] and that hepatocellular hypertrophy when observed alone can be an adaptive response.[66] Again, hepatocellular hypertrophy alone is not considered to be of adverse nature.

Focal hepatocellular necrosis is found in 1.2 to 1.5% of control Wistar rats in 13-week studies.[67] The focal necrosis recorded in a single female at 250 mg/kg bw is thus within normal background occurrence for this type of rats but could possibly also be a treatment-related degenerative effect because hepatocellular necrosis has previously been suspected to be a negative effect of capsaicin analogues.[64]

The slight reduction at the end of the treatment period in RBC, Hb, Hct and the appropriate secondary increase in RE, following daily doses of 100 and 250 mg/kg bw PheCap is interpreted as non-adverse mild anaemia. The reduction in RBCs was minor and assumed not to affect oxygen delivery.[68] Furthermore, the changes were reversible. The slight increase in MCV in males at 250 mg/kg bw treatment group is consistent with regenerative macrocytic anaemia likely due to gastrointestinal affection and haemorrhage; and hence, based on the erosions and ulceration noted in the gastrointestinal tract, the haematology parameters indicate anaemia by blood loss.

The minor effects on WBC, Neu, Mono and Eos are considered normal background with higher variation which might be explained by the observed gastrointestinal inflammation.

The lower levels in the clinical biochemistry parameters, Chol and Gluc, in males and TBIL in males and females following PheCap ingestion are not considered to be adverse since no clear histopathological correlation was observed.[68] The reduction in Na and K is considered within normal range, but reductions in Na and K are often found in response to severe diarrhoea.[69] The low urea values at the end of the recovery period are often found in response to liver damage, and the reduction in TBAs is considered not clinically significant. Regarding the gastrointestinal lesions, all these findings are considered to be unspecific due to malabsorption by gastrointestinal irritation[69] that applies especially to the larger variation of biochemical end points at the end of the recovery.

Changes in bone marrow, spleen, thymus, lymph nodes and adrenal glands have previously been reported in response to stressful conditions.[70] The observed changes in the 250 mg/kg bw groups are thus interpreted as a normal adaptive stress response associated with the daily administration of the high test dosage.[71] The increase in Mono could also be due to a stress response associated with daily handling and gavage dose administration. No clear effect of stress was seen in Lym.

The findings presented here correspond to the findings by Monsereenusorn.[28] The author found no differences in relative organ weights, rectal temperature, water intake, plasma chemistry, urine dilution and concentration between treatment and control rats following 60 days of oral gavage administration of 50 mg/kg bw/day capsaicin. Nevertheless, Monsereenusorn,[28] as in the present study, found a significant reduction in AP, Gluc, Chol, reddening of gastric mucosa, increase in mucus materials and slight hyperaemia without haemorrhage in the livers. Contrary to our findings, he found no difference in liver weights between treatment and control animals. Monsereenusorn[28] also found a significant reduction in blood urea nitrogen and total growth rate, which was not seen in the present study.

Table 8. Micronucleus assay.[a]

		Without activation (-S9)								With activation (+S9)							
Treatment	Dose	Cells	CWM	MCF (%)	CBPI	RCG	df	Deviance or z	*P*	Cells	CWM	MCF (%)	CBPI	RCG	df	Deviance or z	*P*
4 h treatment																	
dH2O		2000	12	0.60	1.33	90				2000	16	1.60	1.39	128			
DMSO		2000	19	0.95	1.36	100	4, 4	2.920	0.571	2000	19	0.70	1.31	100	4, 4	2.490	0.646
PheCap	50	2000	14	0.70	1.31	85				2000	17	0.35	1.31	99			
	100	2000	10	0.50	1.28	78				4000	29	0.35	1.24	78			
	120	2000	15	0.75	1.23	65											
	130	4000	28	0.70	1.16	42				2000	11	0.80	1.20	64			
	140									2000	16	1.25	1.13	43			
EMS	1400	2000	49	2.45	1.21	59	1, 1	13.938	**< 0.001**								
Colchicine	0.8	1763	60	3.40	1.22	60											
CPA	15									2000	39	1.70	1.26	85	1, 1	7.144	**0.008**
44 h treatment																	
dH2O		4000	32	0.40	1.46	119											
DMSO		2000	14	0.95	1.39	100	3, 3	4.132	0.248								
PheCap	10	2000	7	0.85	1.32	82											
	15	2000	14	1.45	1.26	68											
	20	2000	16	0.80	1.16	40											
EMS	900	1042	25	3.74	1.08	21											
Colchicine	0.04	887	34	4.40	1.05	13	1, 1	28.378	**< 0.001**								

PheCap: phenylcapsaicin; Cells: scored cells; CWM: cells with micronuclei; MCF: micronucleated cell frequency; CBPI: cytokinesis block proliferation index = $((c_1 \times 1) + (c_2 \times 2) + (c_x \times 3))/n$ where c_1 is the number of mononucleate cells, c_2 is the number of binucleate cells and c_x is multinucleate cells and n is the total number of cells; RCG: relative cell growth; GLM: generalized linear model. *P*-values ≤ 0.05 are highlighted in bold.
[a]Effects of PheCap were analysed using GLM (see Methods). Two separate cultures per test group. Doses are μg per plate.

High-purity PheCap is non-mutagenic under the conditions of the present study. In the reverse mutation (Ames) assay investigating PheCap's potential to induce gene mutations at concentrations of up to 5000 μg, no biologically relevant increase in revertant colony numbers was found for any of the tester strains *S. typhimurium* TA 98, TA 102, TA 1535, TA 100 and TA 1537, either with or without metabolic activation.

In the human Lym micronucleus assay, no biologically relevant increase in micronucleated cells was found compared to the concurrent solvent control under the conditions of the presented micronucleus assay. Furthermore, the number of micronucleated cells found in the groups treated with up to 130 μg/mL PheCap for short-term exposure without metabolic activation, up to 140 μg/mL for the short-term exposure with metabolic activation and up to 20 μg/mL for the long-time exposure without metabolic activation was all within the historical control limits of the negative and solvent controls. The results in this study correspond to the findings on synthetic high-purity capsaicin where Chanda et al.[29] concluded that capsaicin is not genotoxic in the bacterial assay nor did it induce structural or numerical chromosomal aberrations in cultured human Lym, either with or without metabolic activation.

The findings presented in this study are thus aligned with the findings by Chanda et al.[29] that the genotoxic potential of synthetic capsaicin is very low. This is also found for the synthetic capsaicin analogue PheCap. This supports the suggestion that the inconsistency observed between previous studies on the toxicology and genotoxicology of capsaicin is caused by differences in source, purity and impurity profile of the capsaicin item tested and that natural extracts may not exhibit the same toxicological profile as high-purity capsaicin and capsaicin analogues.

Due to the adverse local effects in the digestive system and systemic effects on the liver found for oral doses of 100 or 250 mg/kg bw/day PheCap, the NOAEL is considered to be either 30 mg/kg bw/day for local effects or 100 mg/kg bw/day for systemic toxicity under the conditions of the present study.

The purpose of the present study was to examine the potential toxicity and genotoxicity of high-purity PheCap for use in animal feed and human food industries, and the adverse effects found here correspond to the adverse effects described for capsaicin.[28] The addition of 10–20 ppm PheCap to animal feed was found to increase broiler chicken bw and reduced *Salmonella* in both broiler chickens and 10-week old piglets.[15–17] This corresponds to a daily dose of up to 6 and 1 mg/kg bw/day PheCap for broiler chickens and piglets, with a maximum daily feed consumption of 30 and 5% of their bws, respectively. This is well below the 100 mg/kg bw/day NOAEL dose found for system toxicity and should be safe to use in meat and poultry industries from both toxicological and animal welfare perspectives. The findings reported here, by mirroring the toxicological effects of capsaicin in rats, indicate that PheCap is a

functional capsaicin analogue and that the potential for adverse effects in humans should correspond to that of capsaicin.

Conclusions

The synthetic capsaicin analogue PheCap has low systemic toxicity although salivation, diarrhoea and moving of bedding material immediately after administration indicate discomfort for rats. The NOAEL of PheCap for systemic toxicity is considered to be at 100 mg/kg bw/day based on the degenerative changes in the liver and 30 mg/kg bw/day for local effects due to irritating effects in stomach tissue. PheCap did it neither cause gene mutations by base pair changes or frameshifts nor induce structural and/or numerical chromosomal damage in human Lym under the conditions of the study.

Author contributions

SS, KW, CD and GS performed the studies. TRP and SS drafted the manuscript and KHJ performed the data analyses. All contributed in the presentation and interpretation of the data and critical revisions of the manuscript.

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Supplementary Material

Supplementary material for this article is available online.

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