Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

[1] 9-Octadecenoic acid (Z)-, sulfonated, potassium salts;
[2] Reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts;
[3] 9(or 10)-sulphooctadecanoic acid, potassium salt

EC Number: **[1] 271-843-1; [2] -; [3] 267-966-5**

CAS Number: **[1] 68609-93-8; [2] - ; [3] 67968-63-2**

Index Number: **N/A**

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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3.1 Acute toxicity - oral route

Evaluation not performed for this substance.

3.2 Acute toxicity - dermal route

Evaluation not performed for this substance.

3.3 Acute toxicity - inhalation route

Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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3.8.1 In vitro data

3.8.1.1 [Study 1]

Study reference:

NN, 2014a

Detailed study summary and results:

Test type

An in vitro bacterial reverse mutation assay according to OECD TG 471 was performed. GLP compliance is given (certificate not provided).

A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 9-Octadecenoic acid (Z)-, sulfonated, potassium salts
- Test material form: beige-yellow powder/ flakes with lumps
- Degree of purity: 100% (analytical)
- Water content: 0.5%
- Impurity: not specified
- Lot/Batch number: 7495382
- Expiration date of Lot/Batch: December 31, 2015
- Storage condition of test material: at room temperature, in the dark

Administration/exposure

- Strains: Salmonella typhimurium TA 98, TA 100, TA 1535, and TA 1537, and E. coli WP2 uvr A
- Target gene: Histidine and Tryptophan
- Type and composition of metabolic activation system:
  - species and cell type: rat, liver microsomal enzymes from liver homogenate (S9-mix)
  - quantity: 5% or 10% (v/v)
  - induced or not induced: induced
  - chemicals used for induction: Aroclor, 500 mg/kg bw
  - co-factors used: not provided
- Test concentrations:
  - Range finding test: eight concentrations (3; 10; 33; 100; 333; 1,000; 3,330 and 5,000 µg/plate) tested with strains TA 100 and WP2uvrA, with and without 5% (v/v) metabolic activation (S9-mix)

- Mutation assay (plate incorporation method):
  - Experiment 1: five concentrations (increase by approx. half-log steps, 100; 333; 1,000; 3,300 and 5,000 µg/plate) tested with strains TA 98, TA 1535, and TA 1537, with and without 5% (v/v) metabolic activation (S9-mix)
  - Experiment 2: five concentrations (increase by approx. half-log steps, 100; 333; 1,000; 3,300 and 5,000 µg/plate) tested with strains TA 98, TA 100, TA 1535, TA 1537, and WP2uvrA, with and without 10% (v/v) metabolic activation (S9-mix)
- Number of replicates: 3

- Vehicle: Milli-Q water, used volume not given, test material is stable and completely miscible in the vehicle
- Statistical methods: A statistical evaluation according to hypothesis testing was not performed.

Results and discussion

- Tested dose levels based on range-finding test (3; 10; 33; 100; 333; 1,000; 3,330 and 5,000 µg/plate), tested up to maximum concentration according to guideline
- Cytotoxic concentrations with and without metabolic activation:
  - no toxicity was observed in all tested strains in Experiment 1 and 2
- Genotoxic effects with and without metabolic activation: negative for all tested strains with and without metabolic activation (for details see Table 1

Table 1 to Table 3)

- Concurrent negative (solvent/vehicle) and positive control data:
    - negative control: yes, valid
    - solvent control: yes, valid,
        - mean plate count of solvent control of TA 1535 without S9-mix (value = 32) was above the laboratory historical range (value = 25). Because the mean plate count was just above the historical range and all other experiments resulted in clear negative results, the deviation of the solvent control from the historical range had no effect on the outcome of the study and thus did not adversely affect the study integrity.
    - positive control: yes (sodium azide (without S9-mix, strain TA 1535), ICR-191 (without S9-mix, strain TA 1537), methyl methanesulfonate (without S9-mix, strain TA 100), 2-nitrofluorene (without S9-mix, strain TA 98), 4-nitroquinoline-N-oxide (without S9-mix, strain WP2uvrA) and 2-aminoanthracene (with S9-mix, all strains), valid
        - mean plate count of positive control of WP2uvrA without S9-mix (4-nitroquinoline-N-oxide, value = 1664) was above the laboratory historical range (value = 1479). Because the mean plate count was more than three-folds above the concurrent solvent control and showed a clear positive response, which is the purpose of a positive control, the deviation of the positive control from the historical range had no effect on the outcome of the study and thus did not adversely affect the study integrity.
- Test-specific confounding factors:
    - Effects of pH: pH = 6 at concentration of 1 g/L
    - Effects of osmolality: not provided
    - Water solubility: completely miscible
    - Precipitation: no precipitation of test substance observed
- Statistical results:
    - no statistical evaluation of results available, revertant colony numbers were not increased in all experiments

- fluctuation in number of revertant colonies was above the laboratory historical control data range in strain TA 1535 without metabolic activation at 100 and 333 µg/plate, but the increase was below the concurrent vehicle control and therefore the observed increases were not considered to be relevant

- Provide information that may be needed to adequately assess data for reliability
  - mean number of revertant colonies per plate and standard deviation: for details see Table 1

Table 1 to Table 3

- Evaluation criteria:
  - Validity/Acceptability given, if: in all tested strains the negative and positive controls are in range of historical control data (for positive control: mean plate count value should be at least three times the concurrent vehicle control group mean); selection if dose range should be based on either: a clearly toxic concentration or exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or extend to 5 mg/plate
  - Positive response given, if: total number of revertants in strain TA100 is greater than two times the concurrent control, or total number of revertants in strains TA1535, TA1537, TA98 or WP2uvrA is greater than three times the concurrent vehicle control; a positive response is observed in one of the tester strains in a repeated experiment (in at least one independent repeated experiment the positive response should be reproducible)
  - Negative response given, if: total number of revertants in strain TA100 is not greater than two times the concurrent control, or total number of revertants in strains TA1535, TA1537, TA98 or WP2uvrA is not greater than three times the concurrent vehicle control; in at least one independent repeated experiment the negative response should be reproducible

Table 1: Mutagenic response of 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the Salmonella typhimurium reverse mutation assay and in the Escherichia coli reverse mutation assay (dose range finding test)

Dose (µg/plate)	**Mean number of revertant colonies/3 replicate plates (± S.D.) with one strain of *Salmonella typhimurium* and one *Escherichia coli* strain**	
	TA100	WP2uvrA
	Without S9-mix	
Positive control	1053 ± 38	1308 ± 99
Solvent control	129 ± 7	31 ± 8
3	133 ± 10	24 ± 4
10	117 ± 15	28 ± 10
33	143 ± 10	29 ± 6
100	134 ± 23	29 ± 6
333	119 ± 13	24 ± 5
1000	120 ± 9	19 ± 1
3330	108 ± 9	12 ± 0
5000	90 ± 7 n NP	17 ± 6 n NP
	With S9-mix[1]	
Positive control	1304 ± 88	233 ± 11
Solvent control	118 ± 16	37 ± 7
3	131 ± 4	27 ± 8
10	126 ± 9	34 ± 10
33	131 ± 25	43 ± 9
100	127 ± 19	42 ± 10
333	129 ± 7	36 ± 13
1000	113 ± 6	19 ± 3

3330	104 ± 16	22 ± 8
5000	92 ± 11 [n NP]	18 ± 6 [n NP]

[1] The S9-mix contained 5% (v/v) S9 fraction

[NP] No precipitate

[n] Normal bacterial background lawn

Table 2: Mutagenic response of 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the Salmonella typhimurium reverse mutation assay (Experiment 1)

Dose (µg/plate)	**Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium***		
	TA1535	TA1537	TA98
	Without S9-mix		
Positive control	611 ± 14	622 ± 39	823 ± 59
Solvent control	22 ± 7	11 ± 3	19 ± 4
100	21 ± 7	7 ± 7	20 ± 4
333	19 ± 4	6 ± 3	17 ± 5
1000	24 ± 10	10 ± 4	18 ± 3
3330	23 ± 7	14 ± 7	21 ± 4
5000	15 ± 7 [n NP]	13 ± 4 [n NP]	15 ± 4 [n NP]
	With S9-mix[1]		
Positive control	245 ± 29	273 ± 51	659 ± 20
Solvent control	16 ± 2	7 ± 3	23 ± 6
100	13 ± 1	8 ± 4	36 ± 4
333	16 ± 6	8 ± 3	25 ± 13
1000	15 ± 1	12 ± 9	17 ± 1
3330	13 ± 4	11 ± 10	20 ± 9

5000	22 ± 4 [n NP]	5 ± 3 [n NP]	30 ± 7 [n NP]

[1] The S9-mix contained 5% (v/v) S9 fraction

[NP] No precipitate

[n] Normal bacterial background lawn

Table 3: Mutagenic response of 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the Salmonella typhimurium reverse mutation assay and in the Escherichia coli reverse mutation assay (Experiment 2)

Dose (µg/plate)	**Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain**				
	TA1535	TA1537	TA98	TA100	WP2uvrA
	Without S9-mix				
Positive control	786 ± 59	725 ± 72	719 ± 2	969 ± 27	1664 ± 35
Solvent control	32 ± 11	9 ± 2	22 ± 3	132 ± 9	26 ± 6
100	26 ± 4	13 ± 5	13 ± 5	133 ± 13	32 ± 8
333	27 ± 4	7 ± 3	18 ± 2	126 ± 6	25 ± 4
1000	19 ± 3	5 ± 5	18 ± 4	117 ± 12	16 ± 9
3330	19 ± 5	7 ± 4	14 ± 1	80 ± 7	33 ± 5
5000	14 ± 3 [n NP]	9 ± 3 [n NP]	20 ± 3 [n NP]	101 ± 19 [n NP]	18 ± 2 [n NP]
	With S9-mix[1]				
Positive control	166 ± 14	494 ± 76	273 ± 9	1115 ± 108	227 ± 27
Solvent control	14 ± 10	10 ± 2	30 ± 9	105 ± 11	38 ± 11
100	15 ± 4	9 ± 6	18 ± 6	121 ± 13	36 ± 3
333	16 ± 5	11 ± 7	30 ± 6	118 ± 15	42 ± 4
1000	12 ± 2	10 ± 5	19 ± 4	121 ± 16	39 ± 6
3330	21 ± 2	7 ± 3	21 ± 4	91 ± 7	25 ± 6
5000	15 ± 4 [n NP]	9 ± 4 [n NP]	24 ± 8 [n NP]	87 ± 6 [n NP]	27 ± 6 [n NP]

[1]	The S9-mix contained 10% (v/v) S9 fraction
[NP]	No precipitate
[n]	Normal bacterial background lawn

3.8.1.2 [Study 2]

Study reference:

NN, 2014b

Detailed study summary and results:

Test type

An in vitro mammalian chromosome aberration test according to OECD TG 473 was performed. GLP compliance is given (certificate not mentioned).

A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 9-Octadecenoic acid (Z)-, sulfonated, potassium salts
- Degree of purity: 100% (analytical)
- Water content: 0.5%
- Impurities: not specified
- Test material form: beige-yellow powder/flakes with lumps
- Storage condition of test material: in the dark at room temperature
- Batch/Lot number: 7495382
- Expiration of batch/Lot: December 31, 2015

Administration/exposure

- Strain or cell type or cell line, target gene: peripheral human lymphocytes
- Type and composition of metabolic activation system:
  - species and cell type: rat liver, S9 mix
  - quantity: 1.8% (v/v)
  - induced or not induced: not provided
  - chemicals used for induction: not provided
  - co-factors used: not provided
- Test concentrations, and reasoning for selection of doses:
  - prior to cytogenetic assays a dose range finding study was conducted
  - Assay 1, 3-hour exposure without metabolic activation, 24-hour fixation: 10; 100, and 1,000 µg/mL culture medium
  - Assay 1, 3-hour exposure with metabolic activation, 24-hour fixation: 10; 100, and 1,000 µg/mL culture medium
  - Assay 2, 24- and 48-hour exposure without metabolic activation, 24- and 48-hour fixation: 10; 30; 100; 200; 300; 400, and 500 µg/mL culture medium
  - Assay 2, 3-hour exposure with metabolic activation, 48-hour fixation: 10; 100, and 1,000 µg/mL culture medium
  - Assay 2a (repeat experiment), 24-hour exposure without metabolic activation, 24-hour fixation: 10; 100; 300; 325; 350; 375; 400, and 450 µg/mL culture medium
  - Assay 2a (repeat experiment), 48-hour exposure without metabolic activation, 48-hour fixation: 10; 100; 250; 300; 325; 350; 375, and 400 µg/mL culture medium
- Vehicle: RPMI 1640 medium was used as cell culture medium, no further information available, test substance is completely miscible in water
- Method of application: in medium
- Duration: exposure duration was 3, 24 or 48 hours, the fixation time was 24 or 48 hours

- Spindle inhibition: colchicine (0.5 µg/mL medium)
- Number of replications: duplicate cultures in two independent experiments
- Number of cells evaluated for mitotic index: 1,000 metaphases from each culture (maximum deviation of 5%)
- Determination of cytotoxicity/ scoring of cytogenetic assay: at least 3 analysable concentrations were used, chromosomes of cultures with an inhibited mitotic index of at least 50% or above whereas the mitotic index of the lowest concentration was approximately the same as the solvent control were analysed regarding chromosome aberrations, additionally cultures treated with an intermediate concentration were analysed regarding chromosome aberrations
- Other examinations: polyploidy and endoreduplication
- Statistical methods: Chi-square test was used for evaluating the incidence of aberrant cells (cells with one or more chromosomal aberrations, including or excluding gaps) for each treatment group outside the laboratory's historical control data range compared with the solvent control

Results and discussion

- Tested dose levels were based on a dose-range finding test: during this test the test substance precipitated at 1,000 µg/mL in the cell culture medium. Thus, 1,000 µg/mL was used as the highest concentration.
- Cytotoxic concentrations with and without metabolic activation:
    - Assay 1, 3-hour exposure without metabolic activation, 24-hour fixation: selection of all concentrations for scoring of chromosome aberrations; no cytotoxicity observed
    - Assay 1, 3-hour exposure with metabolic activation, 24-hour fixation: selection of all concentrations for scoring of chromosome aberrations; no cytotoxicity observed
    - Assay 2, 24- and 48-hour exposure without metabolic activation, 24- and 48-hour fixation: none of the investigated concentrations was suitable for scoring, cytotoxicity was too low at 300 µg/mL (36% after 24-hour exposure and 43% after 48-hour exposure) but too high at 400 µg/mL (69% after 24-hour exposure and 85% after 48-hour exposure), experiment was repeated in assay 2a

- Assay 2, 3-hour exposure with metabolic activation, 48-hour fixation: no cytotoxicity provided, evaluation of concentrations: 10; 100, and 1,000 µg/mL
- Assay 2a, 24-hour exposure without metabolic activation, 24-hour fixation: inhibition of mitotic index was 41% at 300 µg/mL and 70% at the next higher concentration (325 µg/mL), evaluation of concentrations: 10; 100, and 300 µg/mL
- Assay 2a, 48-hour exposure without metabolic activation, 48-hour fixation: no cytotoxicity provided, evaluation of concentrations: 10; 100; 250, and 300 µg/mL

- Genotoxic effects: negative, with and without metabolic activation; for details see Table 4 to Table 8
- Concurrent negative (solvent/vehicle) and positive control data:
  - negative control: yes, valid
  - solvent control: yes, valid
  - positive control: yes (cyclophosphamide (with metabolic activation) and mitomycin C (without metabolic activation)), valid
- Test-specific confounding factors:
  - Effects of pH: pH = 6 at concentration of 1 g/L
  - Effects of osmolality: not provided
  - Water solubility: water soluble
  - Precipitation: precipitation of test substance in cell culture medium observed at 1,000 µg/mL (= highest concentration used)
- Statistical results: no statistically significant increase was observed after test item treatment
- Provide information that may be needed to adequately assess data for reliability
  - frequency of aberrations: not statistically significant increased (for details see Table 4 to Table 8)
  - frequency of polyploidy: no biologically relevant effects observed
  - frequency of endoreduplication: no biologically relevant effects observed
  - precipitation concentration: were observed equal 1,000 µg/mL

- mitotic index: provided; should be at least 50 percent
- Evaluation criteria:
  - Validity/Acceptability given, if: number of chromosome aberrations found in cultures of the solvent control is within the historical control data range; a statistically significant increase ($p < 0.05$) in number of cells with chromosome aberrations is caused by the positive control substance; replicated cultures have a homogenous response, the scoring of chromosome aberrations is not interfered by precipitation
  - Positive response given, if: a dose-related statistically significant increase ($p < 0.05$) in number of cells with chromosome aberrations is caused by the test substance; in absence of a clear dose-response relationship the frequencies of number of cells with chromosome aberrations are statistically significant and biologically relevant increased
  - Negative response given, if: a statistically significant increase ($p < 0.05$) in number of cells with chromosome aberrations is not observed in any of the tested concentrations

Definitions of chromosome aberrations scored in metaphases:

- g': chromatid gap
- g'': chromosome gap
- b': chromatid break
- b'': chromosome break
- d': chromatid deletion
- m': minute
- m'': double minutes
- dic: dicentric chromosome
- tric: tricentric chromosome

- r: ring chromosome
- exch: exchange figure
- intra: chromosome intrachange
- p: pulverized chromosomes
- ma: multiple
- poly: polyploidy
- endo: endoreduplication

Table 4: Chromosome aberrations in human lymphocyte cultures treated with 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the absence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**MMC-C 0.5 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			107			102			72			74		
No. of Cells scored	100	100	200	100	100	200	100	100	200	100	41	141	100	100	200
No. of Cells with aberrations (+ gaps) [a)]	0	0	0	0	1	1	0	0	0	0	0	0	30	25	***) 55
No. of Cells with aberrations (- gaps)	0	0	0	0	0	0	0	0	0	0	0	0	28	23	***) 51

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**MMC-C 0.5 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
g’					1										
g”													2	2	
b’													18	7	
b”													2	3	
m’														1	
m”															
exch.													13	14	
dic															
d’															
misc.		poly		poly						3poly				p, intra	
total aberr (+ gaps)	0	0		0	1		0	0		0	0		35	29	
total aberr (- gaps)	0	0		0	0		0	0		0	0		33	27	

a) Abbreviations used for various types of aberrations are listed above.

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variation polyploidy (poly) was not counted as an aberration.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Table 5: Chromosome aberrations in human lymphocyte cultures treated with 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the presence of S9-mix in the first cytogenetic assay (3 h exposure time, 24 h fixation time)

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**CP 10 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			99			113			72			47		
No. of Cells scored	100	100	200	100	100	200	100	100	200	100	100	200	100	100	200
No. of Cells with aberrations (+ gaps) [a)]	0	0	0	0	0	0	0	0	0	1	0	1	32	37	***) 69
No. of Cells with aberrations (- gaps)	0	0	0	0	0	0	0	0	0	1	0	1	32	34	***) 66
g’													4	1	
g”														3	
b’										1			25	19	
b”													3	6	
m’													2		
m”														2	
exch.													8	10	
dic															
d’															

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**CP 10 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
misc.											2poly		p		
total aberr (+ gaps)	0	0		0	0		0	0		1	0		43	41	
total aberr (- gaps)	0	0		0	0		0	0		1	0		39	37	

[a)] Abbreviations used for various types of aberrations are listed above.

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

The numerical variation polyploidy (poly) was not counted as an aberration.

[*)] Significantly different from control group (Chi-square test), * P < 0.05, ** P < 0.01 or *** P < 0.001.

Table 6: Chromosome aberrations in human lymphocyte cultures treated with 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the absence of S9-mix in the cytogenetic assay 2A (24 h exposure time, 24 h fixation time)

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**300 µg/ml**			**MMC-C 0.2 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index (%)	100			99			99			59			75		
No. of Cells scored	100	100	200	100	100	200	100	100	200	100	100	200	50	50	100
No. of Cells with aberrations (+ gaps) [a)]	1	0	1	1	1	2	2	1	3	2	3	5	27	27	[***)] 54

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**300 µg/ml**			**MMC-C 0.2 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of Cells with aberrations (- gaps)	1	0	1	1	1	2	0	1	0	2	2	4	27	27	***) 54
g’							1				1		1		
g”							1								
b’	1			1	1			1		2	2		43	23	
b”														2	
m’															
m”															
exch.													8	10	
dic															
d’															
misc.															
total aberr (+ gaps)	1	0		1	1		2	1		2	3		52	35	
total aberr (- gaps)	1	0		1	1		0	1		2	2		51	35	

a) Abbreviations used for various types of aberrations are listed above.

misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.

*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Table 7: Chromosome aberrations in human lymphocyte cultures treated with 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the absence of S9-mix in the cytogenetic assay 2A (48 h exposure time, 48 h fixation time)

Conc g/ml	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**250 µg/ml**			**300 µg/ml**			**MMC-C 0.1 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index	100			98			94			56			43			55		
No. of Cells	100	100	200	100	100	200	100	100	200	100	100	200	100	100	200	50	100	150
No. of Cells with aberratio	3	1	4	1	1	2	1	0	1	3	1	4	3	3	6	26	34	***) 60
No. of Cells with aberratio	2	1	3	1	0	1	1	0	1	1	1	2	3	2	5	26	34	***) 60
g’	1									2				2				
g”					1											1	1	
b’	2	1		1			1			1			2	2		19	23	
b”											1		1	2		1	3	
m’																		
m”																		
exch.														1		16	15	

Conc g/ml	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**250 µg/ml**			**300 µg/ml**			**MMC-C 0.1 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
dic																		
d'																		
misc																6p	3p	
total aberr (+ gaps)	3	1		1	1		1	0		3	1		3	7		43	45	
total aberr (- gaps)	2	1		1	0		1	0		1	1		3	5		42	44	

a) Abbreviations used for various types of aberrations are listed above.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
*) Significantly different from control group (Chi-square test), * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Table 8: Chromosome aberrations in human lymphocyte cultures treated with 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the presence of S9-mix in the second cytogenetic assay (3 h exposure time, 48 h fixation time)

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**CP 10 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
Mitotic Index	100			89			101			43			- [b)]		
No. of Cells scored	100	100	200	100	100	200	100	100	200	100	100	200	100	100	200
No. of Cells with aberrations (+ gaps) a)	1	1	2	0	0	0	1	0	1	0	0	0	37	27	***) 64

Conc	**Culture medium**			**10 µg/ml**			**100 µg/ml**			**1000 µg/ml**			**CP 10 µg/ml**		
Culture	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B	A	B	A+B
No. of Cells with aberrations (- gaps)	1	1	2	0	0	0	1	0	1	0	0	0	37	27	***) 64
g’															
g”															
b’	1	1					1						33	26	
b”														2	
m’															
m”													2	1	
exch.													7	4	
dic															
d’															
misc.				poly				poly		2poly	poly		2intra		
total aberr (+ gaps)	1	1		0	0		1	0		0	0		44	33	
total aberr (- gaps)	1	1		0	0		1	0		0	0		44	33	

a) Abbreviations used for various types of aberrations are listed above.
misc. = (miscellaneous) aberrations not belonging to the ones mentioned above.
The numerical variation polyploidy (poly) was not counted as an aberration.
b) CP was fixed after 24 hours. Therefore, the mitotic index could not be calculated as percentage of control.

*) Significantly different from control group (Chi-square test), * P < 0.05, ** P < 0.01 or *** P < 0.001.

3.8.1.3 [Study 3]

Study reference:

NN, 2014c

Detailed study summary and results:

Test type

An in vitro mammalian cell gene test according to OECD TG 476 (version from 1997) was performed. GLP compliance is given (certificate not mentioned).

A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 9-Octadecenoic acid (Z)-, sulfonated, potassium salts
- Test material form: beige-yellow powder/ flakes with lumps
- Degree of purity: 100% (analytical)
- Water content: 0.5%
- Impurities: not specified
- Lot/Batch number: 7495382
- Expiration date of Lot/Batch: December 31, 2015
- Storage condition of test material: at room temperature, in the dark

Administration/exposure

- Strain or cell type or cell line, target gene: mouse lymphoma L5178Y cells, thymidine-kinase locus
- Type and composition of metabolic activation system:
  - species and cell type: rat, liver microsomal enzymes from liver homogenate (S9-mix)
  - quantity: 4 or 8% (v/v)
  - induced or not induced: not provided
  - chemicals used for induction: not provided
  - co-factors used: not provided
- Test concentrations, and reasoning for selection of doses:
  - Dose range-finding test, 3-hour treatment with (4% (v/v)) or without metabolic activation: 33; 100; 333; 666, and 1,000 µg/mL
  - Dose range-finding test, 24-hour treatment with (4% (v/v)) or without metabolic activation: 33; 100; 333; 666, and 1,000 µg/mL
  - Assay 1, 3-hour treatment without metabolic activation: 1; 3; 10; 33; 66; 100; 125; 150; 160; 170; 180, and 190 µg/mL culture medium
  - Assay 1, 3-hour treatment with 4% (v/v) metabolic activation: 10; 33; 100; 150; 170; 200; 225; 235; 250, and 265 µg/mL culture medium
  - Assay 2, 24-hour treatment without metabolic activation: 3; 10; 33; 50; 65; 85; 100; 115; 130; 150; 170, and 185 µg/mL culture medium
  - Assay 2, 3-hour treatment with 8% (v/v) metabolic activation: 10; 33; 100; 150; 200; 225; 235; 250; 265, and 280 µg/mL culture medium
- Vehicle:
  - For test substance: RPMI 1640 medium (Dutch modification), no further information available, test substance is completely miscible in water, dissolved in RPMI 1640 the test substance formed suspensions at concentrations between 4 and 12 mg/mL and 4 and 24 mg/mL.
  - For reference substances: dimethyl sulphoxide (DMSO) for methyl methanesulfonate, Hanks' balanced salt solution (HBSS) without calcium and magnesium for cyclophosphamide
- Method of application: in culture medium
- Duration: exposure duration was 3 and 24 hours, the expression time was 2 days

- Selection agent: trifluorothymidine (TFT), 5 µg/L
- Number of replication: duplicate cultures
- Determination of cytotoxicity: relative total growth
- Statistical methods: no information provided

Results and discussion

- Tested dose levels based on dose range-finding test (with or without metabolic activation: 33; 100; 333; 666, and 1,000 µg/mL), precipitation of test substance in culture medium occurred at 666 µg/mL and above
- Cytotoxic concentrations with and without metabolic activation
    - Dose range-finding test: no survival at 333; 666, and 1,000 µg/mL, at 100 µg/mL without S9-mix relative suspension growth was 64%
    - Assay1, 3-hour treatment without metabolic activation: cytotoxicity observed at 170, 180, and 190 µg/mL (relative total growth reduced by 65% compared to solvent control at 190 µg/mL), no toxicity observed from 1 to 125 µg/mL, selected concentrations for evaluation were 1; 3; 10; 33; 100; 125; 150, and 160 µg/mL
    - Assay1, 3-hour treatment with 4% (v/v) metabolic activation: no cytotoxicity from 10 to 235 µg/mL (relative total growth reduced by 90% compared to solvent control at 265 µg/mL), selected concentrations for evaluation were 10; 100; 170; 200; 225; 235; 250, and 265 µg/mL
    - Assay 2, 24-hour treatment without metabolic activation: no cytotoxicity from 3 to 130 µg/mL (relative total growth reduced by 90% compared to solvent control at 185 µg/mL), selected concentrations for evaluation were 3; 10; 33; 50; 100; 150; 170, and 185 µg/mL
    - Assay 2, 3-hour treatment with 8% (v/v) metabolic activation: no cytotoxicity from 10 to 150 µg/mL, concentrations from 200 to 265 µg/mL had equivalent cytotoxicity (relative total growth reduced by 94% compared to solvent control at 280 µg/mL), selected concentrations for evaluation were 33; 100; 150; 225; 235; 250; 265, and 280 µg/mL

Genotoxic effects: negative with and without metabolic activation; for details see Table 9 and

- Table 10
- Concurrent negative (solvent/vehicle) and positive control data:

- negative control: yes, valid
- solvent control: yes, valid
- positive control: yes (cyclophosphamide (with metabolic activation) and methyl methanesulfonate (without metabolic activation)), valid

- Indicate test-specific confounding factors:
  - Effects of pH: pH = 6 at concentration of 1 g/L
  - Effects of osmolality: not provided
  - Water solubility: soluble
  - Precipitation: precipitation of test substance in culture medium occurred at 666 µg/mL and above
- Statistical results: no statistical data provided
- Provide information that may be needed to adequately assess data for reliability

frequency of mutations: treatment with test substance with or without metabolic activation led to no significant increase in mutation frequency (for details see Table 9 and

- Table 10)

number of small and large colonies: numbers of treated cultures were comparable to solvent controls (for details see Table 9 and

- Table 10)
- evaluation criteria:
  - validity/acceptability given, if: absolute cloning efficiency of solvent controls is between 65 to 120%, spontaneous mutation frequency in solvent control is above 50 and below 170 per 10^6 surviving cells, for negative controls the growth rate over expression period (2 d) should be between 8 and 32 for 3-hour treatment and between 32 and 180 for 24-hour treatment, mutation frequency for methyl methanesulfonate should not be below 500 per 10^6 surviving cells and for cyclophosphamide not below 700 per 10^6 surviving cells

- considered positive, if: mutation frequency is dose-dependently induced more that mutation frequency (controls) + 126, in case of an increase a check for biological relevance and comparison with historical control data range needs to be performed
- considered equivocal, if: no clear conclusion of a positive or negative result possible after having conducted an additional confirmation study
- considered negative, if: tested concentrations do not reach a mutation frequency of mutation frequency (controls) + 126 and confirmation of results in an independently repeated test

Table 9: Cytotoxic and mutagenic response of 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the mouse lymphoma L5178Y test system (Experiment 1)

Dose (µg/ml)	RSG (%)	CE day2 (%)	RS day2 (%)	RTG (%)	mutation frequency per 10^6 survivors			
					total	(	small	large)
without metabolic activation								
3 hours treatment								
SC1	100	93	100	100	81	(	43	35)
SC2		95			70	(	30	37)
1	98	120	127	125	51	(	21	29)
3	98	135	143	140	54	(	20	32)
10	101	84	89	90	87	(	31	53)
33	119	101	107	128	70	(	33	34)
100	99	118	126	125	61	(	16	43)
125	108	94	100	108	61	(	17	43)
150	57	107	114	64	52	(	19	31)
160	34	97	103	35	72	(	31	38)
MMS	87	60	64	56	818	(	400	319)
with 4% (v/v) metabolic activation								
3 hours treatment								

SC1	100	107	100	100	52	(	22	28	)
SC2		118			56	(	20	34	)
10	99	110	98	96	44	(	15	27	)
100	85	101	90	77	61	(	25	34	)
170	96	98	87	84	47	(	21	25	)
200	80	90	80	64	71	(	27	42	)
225	82	98	87	72	62	(	28	32	)
235	82	95	85	69	48	(	27	20	)
250	57	89	79	45	58	(	22	35	)
265	10	111	99	10	87	(	54	30	)
CP	34	23	21	7	1851	(	546	1126	)

Note: all calculations were made without rounding off
RSG = Relative Suspension Growth; CE = Cloning Efficiency; RS = Relative Survival; RTG = Relative Total Growth;
SC = Solvent control = Exposure medium; MMS = Methylmethanesulfonate; CP = Cyclophosphamide

Table 10: Cytotoxic and mutagenic response of 9-Octadecenoic acid (Z)-, sulfonated, potassium salts in the mouse lymphoma L5178Y test system (Experiment 2)

Dose (µg/ml)	**RSG (%)**	**CE day2 (%)**	**RS day2 (%)**	**RTG (%)**	**mutation frequency per 10^6 survivors**				
					total	(	small	large	)
without metabolic activation									
24 hours treatment									
SC1	100	90	100	100	70	(	37	31	)
SC2		105			73	(	31	39	)
3	109	118	121	132	67	(	34	30	)
10	101	129	132	133	60	(	20	38	)
33	110	94	96	106	70	(	26	41	)
50	109	91	94	102	67	(	29	35	)
100	95	110	112	106	55	(	17	37	)

150	68	129	132	89	67	(	37	27	)
170	30	105	108	33	67	(	26	38	)
185	11	94	96	10	67	(	21	44	)
MMS	82	76	78	64	769	(	394	267	)
with 8% (v/v) metabolic activation									
3 hours treatment									
SC1	100	99	100	100	56	(	18	37	)
SC2		78			81	(	31	47	)
33	99	97	109	108	84	(	23	58	)
100	99	107	120	119	81	(	41	37	)
150	93	110	124	115	62	(	28	31	)
225	70	83	93	65	97	(	49	44	)
235	71	78	88	63	90	(	44	43	)
250	74	88	99	73	86	(	37	46	)
265	56	98	111	62	82	(	28	50	)
280	6	97	109	6	105	(	36	64	)
CP	57	51	58	33	1273	(	567	503	)

Note: all calculations were made without rounding off
RSG = Relative Suspension Growth; CE = Cloning Efficiency; RS = Relative Survival; RTG = Relative Total Growth;
SC = Solvent control = exposure medium; MMS = Methylmethanesulfonate; CP = Cyclophosphamide

3.8.1.4 [Study 4]

Study reference:

NN, 1993

Detailed study summary and results:

Test type

An in vitro mammalian cell gene test named Salmonella/Mammalian-Microsome Mutagenicity Test (Ames-Test) according to OECD Guideline for Testing of Chemicals, Section 4, No 471, "Salmonella typhimurium, Reverse Mutation Assay", adopted May 26, 1983 was performed. GLP compliance is given (certificate not mentioned). A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study: Sykanol Ke 2780/o1eic acid sulfonate -di-potassium-salt/ 9-octadecenoic acid, sulfo-K-salt
- Test material form: yellow-brown
- Aggregated state at room temperature: liquid
- Degree of purity: 50.8%
- Impurities: not specified
- Lot/Batch number: BV 8/90
- Expiration date of Lot/Batch: August 14, 1993
- Storage condition of test material: not specified

Administration/exposure

- Strain or cell type or cell line, target gene:
    - Salmonella typhimurium: All TA strains used are mutants derived from Salmonella typhimurium LT 2 and have the following genotypes:
        - S. typhimurium TA 1535 his G46 rfa- uvrB
        - S. typhimurium TA 1537 his C3076 rfa- uvrB
        - S. typhimurium TA 1538 his D3052 rfa- uvrB
        - S. typhimurium TA 98 his D3052 rfa- uvrB- R+
        - S. typhimurium TA 100 his G46 rfa- uvrB- R+

- Type and composition of metabolic activation system:
  - species and cell type: rat (Males, Wistar), liver microsomal enzymes from liver homogenate (S9-mix)
  - induced or not induced: induced
  - chemicals used for induction: 500 mg/kg bw/day for 5 days of Aroclor 1254 dissolved in olive oil
  - quantity: pH = 7.4
    - S9-fraction……... 0.1 ml
    - MgClz……. 8 µmol/L
    - KCl……. 33 µmol/L
    - NADP……. 4 µmol/L
    - Glucose-6-phosphate…….. 5 µmol/L
    - Sodium phosphate buffer……... 100 µmol/L
- Test concentrations, and reasoning for selection of doses:
  - Dose range-finding test: 8; 40; 200; 1000, and 5000 µg/plate
- Vehicle:
  - For test substance: Sykanol Ke 2780 was dissolved in bidist water and diluted with the same solvent to the desired concentrations just before the start of the test.
  - For reference substances: As positive controls, sodium azide was used in the tester strains TA 100 and TA 1535, 9-aminoacridine in the tester strain TA 1537, and 4-nitro-o-phenylendiamine in the tester strains TA 98 and TA 1538 without microsomal drug-metabolizing enzymes (S9-mix).
- Method of application: in culture medium
- Duration: the expression time was 48 hours
- Number of replication: duplicate cultures

- Determination of cytotoxicity: was considered positive if the plate background of non-reverted bacteria did not show any growth reduction versus the respective negative control and the spontaneous mutation rates of each tester strain per plate were withing the characteristic spontaneous mutation range.
- Statistical methods: no information provided

Results and discussion

- Tested dose levels based on dose range-finding test (with or without metabolic activation: 8; 40; 200; 1000, and 5000 µg/plate), precipitation of test substance in culture medium occurred at 200 µg/plate and above
- Cytotoxic concentrations with and without metabolic activation
    - Sykanol KE 2780 did not induce any reverse mutation in the absence or presence of S9-mix
    - Slightly toxic effects at 5000 µg/plate were noted as indicated by a slightly reduced revertant rate in the presence of S9-mix.
- Statistical results: no statistical data provided
- Conclusion:
    - During the described mutagenicity test and under the experimental conditions reported, the test article did not induce point mutation by base-pair changes of frameshifts on the genome of the strains used.
    - Sykanol Ke 2780 is not considered to be mutagenic in this *Salmonella typhimurium* reverse mutation assay.

Table 11: Group mean values of revertant colonies per treatment group after exposure to Sykanol Ke 2780 with the Ames test (Experiment 1)

Treatment groups (µg/plate)	Strains									
	TA 1535		TA 100		TA 1537		TA 1538		TA 98	
	-	+	-	+	-	+	-	+	-	+

Negative controls										
Buffer	14	11	110	165	13	11	8	19	15	31
Solvent [1]	9	14	112	154	11	9	8	15	18	32
Test substance										
8	15	15	127	134	8	10	7	20	17	28
40	11	15	119	145	8	10	13	19	16	26
200	10	13	122	146	11	7	7	14	14	31
1000	11	15	120	126	7	5	9	15	19	21
5000	10	12	103	105	9	9	8	5	16	22
Positive controls										
Without S9-mix [2]	656		881		1030		2206		1770	
With S9-mix [3]		95		1526		108		1110		1235

- : without metabolic activation
+: with metabolic activation (S9-mix)
[1] Solvent: H_2O bidist
[2] Without S9-mix:
- TA 1535, TA 100: sodium azide (2µg/plate)
- TA 1537 : 9-Aminoacridine (80 µg/plate)
- TA 1538, TA-98 : 1-Mitro-o-phenylendiamine (40 µg/plate)
[3] With S9-mix :
- TA 1535, TA 1537 : 2-Aminoanthracene (2.5 µg/plate)
- TA 100, TA 1538, TA 98 : 2-Aminoanthracene (5 µg/plate)

Table 12: Group mean values of revertant colonies per treatment group after exposure to Sykanol Ke 2780 with the Ames test (Experiment 2)

Treatment groups	**Strains**

(µg/plate)	TA 1535		TA 100		TA 1537		TA 1538		TA 98	
	-	+	-	+	-	+	-	+	-	+
Negative controls										
Buffer	12	12	114	151	12	9	8	19	16	34
Solvent [1]	12	17	130	130	14	10	8	19	20	29
Test substance										
8	9	17	123	127	8	7	10	19	17	32
40	13	16	132	123	9	10	9	23	19	28
200	10	12	131	114	11	9	11	25	18	29
1000	10	14	131	112	7	8	10	19	18	26
5000	8	9	117	101	7	5	6	10	16	18
Positive controls										
Without S9-mix [2]	572		756		1078		2450		2078	
With S9-mix [3]		105		1238		109		992		1624

- : without metabolic activation
+: with metabolic activation (S9-mix)
[1] Solvent: H_2O bidist
[2] Without S9-mix:
- TA 1535, TA 100: sodium azide (2 µg/plate)
- TA 1537 : 9-Aminoacridine (80 µg/plate)
- TA 1538, TA-98 : 1-Mitro-o-phenylendiamine (40 µg/plate)
[3] With S9-mix :
- TA 1535, TA 1537 : 2-Aminoanthracene (2.5 µg/plate)
- TA 100, TA 1538, TA 98 : 2-Aminoanthracene (5 µg/plate)

3.8.1.5 [Study 5]

Study reference:

NN, 2015a

Detailed study summary and results:

Test type

An *in vitro* CHO/HPRT assay which detects forward mutations of the X-linked hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells according to the OECD Guideline for the Testing of Chemicals No. 476, 21 Jul 1997, "In vitro Mammalian Cell Gene Mutation Test" was performed. GLP compliance is given (certificate given). A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study: Octadecanoic acid, sulfo-, potassium salt (CAS 67968-63-2)
- Test material form: brownish clear
- Aggregated state at room temperature: liquid
- Degree of purity: 100% minus water content and potassium sulphate = 51.92% test item.
- Impurities: A quantitative analysis of test item ingredients is not possible because it is a complex mixture.
- Lot/Batch number: 0012127444
- Expiration date of Lot/Batch: 22 Jun 2016
- Storage condition of test material: Room temperature

Administration/exposure

- Strain or cell type or cell line, target gene:
    - CHO (Chinese hamster ovary) cell line: a permanent cell line derived from the Chinese hamster with a high proliferation rate (doubling time of about 12 - 16 hours), a high plating efficiency (about 90%), and a karyotype with a modal number of 20 chromosomes.

- Type and composition of metabolic activation system:
  - species and cell type: 5 rat (Males, Wistar), liver microsomal enzymes from liver homogenate (S9-mix)
  - induced or not induced: induced
  - chemicals used for induction: 80 mg/kg b.w. phenobarbital i.p. and β-naphthoflavone orally (both supplied by Sigma-Aldrich, 82024 Taufkirchen, Germany) each on three consecutive days.
  - quantity: pH = 7.4
    - S9-fraction……... 10%
    - $MgCl_2$……. 8 mmol/L
    - KCl……. 33 mmol/L
    - NADP……. 4 mmol/L
    - Glucose-6-phosphate…….. 5 mmol/L
    - Sodium phosphate buffer……... 15 mmol/L (prepared by mixing a Na_2HPO_4 solution with a NaH_2PO_4 solution in a ratio of about 4:1).
- Test concentrations, and reasoning for selection of doses:
  - Pretest: Performed at a top concentration of 8510.0 µg/mL (approx. 10.0 mM). pH was stable after addition of test substance. In addition, a homogeneous suspension of the test substance in the vehicle HAM´s F12 was obtained from 85.1 mg/mL (test group: 8510.0 µg/mL) down to 2.659 mg/mL. Undissolved particles were obtained from 4255.0 µg/mL and above 4h after treatment in the absence and presence of S9 mix.

    After 4h of exposure without S9 mix, reduced relative cloning efficiency of about or below 20% relative survival was observed at 531.9 µg/mL and above. In presence of S9 mix, a clear reduced relative cloning efficiency was observed at 265.9 µg/mL and above.
  - Dose selection first experiment:
    - Without S9 mix: 21.9; 43.8; 87.5; 175, 350 and 700 µg/mL

  - With S9 mix: 10.9; 21.9; 43.8; 87.5; 175, and 350 µg/mL
- Dose selection second experiment:
  - Without S9 mix: 25.0; 50.0; 100.0; 200.0; 400.0, and 800.0 µg/mL
  - With S9 mix: 12.5; 25.0; 50.0; 100.0; 200.0, and 400.0 µg/mL

- Vehicle:
  - Treatment medium (without S9 mix): Ham's F12 medium containing stable glutamine and hypoxanthine supplemented with 10% (v/v) FCS.
  - Treatment medium (with S9 mix): Ham's F12 medium containing stable glutamine and hypoxanthine.
- Positive controls:
  - Without metabolic activation: 400 µg/mL ethyl methanesulfonate (EMS; SIGMA, M-0880). EMS (stock solution: 4 mg/mL) was dissolved in Ham's F12 medium without FCS and added in a volume of 2 mL to the cultures.
  - With metabolic activation: 1.25 µg/mL 7,12-dimethylbenz[a]anthracene (DMBA; SIGMA, D3254). DMBA was dissolved in DMSO (stock solution: 125.0 µg/mL) and added in a volume of 200.0 µL to the cultures.
- Method of application: in culture medium
- Duration: the exposure time was 4 hours
- Number of replication: duplicate cultures (referred to as A and B in the results tables)
- Determination of cytotoxicity:
  - The cloning efficiency (CE, %) was calculated for each test group as follows:
  - $CE_{absolute} = \frac{total\ number\ of\ colonies\ in\ the\ test\ group}{total\ number\ of\ seeded\ cells\ in\ the\ test\ group}\ x\ 100$
  - $CE_{relative} = \frac{CE_{absolute}\ of\ the\ test\ group}{CE_{absolute}\ of\ the\ negive\ control}\ x\ 100$

- **Cloning efficiency (CE) (pre-experiment):** The procedure for the determination of the cloning efficiency in the pre-experiment was similar to that described for the determination of the cloning efficency 1 (CE1) in the main experiments, excepting that every dose group contained only two cultures.
- **Cloning efficiency 1 (CE1; survival):** For the determination of the influence of the test substance directly after the exposure period, about 200 cells per dose group were seeded in 25 cm² flasks in duplicate using 5 mL Ham's F12 medium incl. 10% (v/v) FCS. Following cell attachment for 20 – 24 hours, cells were treated with the vehicle, test substance or positive control for 4 hours. Following exposure, cells were rinsed several times with HBSS. Finally, cells were cultured in 5 mL Ham's F12 medium incl. 10% (v/v) FCS.
- **Cloning efficiency 2 (CE2; viability):** For the determination of the mutation rate after the expression period, two aliquots of about 200 cells each were reserved from the transfer into selection medium (after 7 – 9 days) and seeded in two flasks (25 cm^2) containing 5 mL Ham's F12 medium incl. 10% (v/v) FCS. In all cases, after seeding the flasks were incubated for 5 - 8 days to form colonies. These colonies were fixed, stained and counted.
- **The absolute and relative cloning efficiencies (%)** were calculated for each test group according to the formulas given above.
- The number of colonies in every flask was counted and recorded. Using the formula above the values of absolute cloning efficiencies ($CE_{absolute}$,$CE_{1\ absolute}$ and/or $CE_{2\ absolute}$) were calculated. Based on these values the relative cloning efficiencies ($CE_{relative}$, $CE_{1\ relative}$ and/or $CE_{2\ relative}$) of the test groups were calculated and reported as a percentage of the respective $CE_{absolute}$ value of the corresponding negative control (negative control = 100%).

- Determination of mutant efficiency:
  - The uncorrected mutant frequency ($MF_{uncorr.}$) per 10^6 cells was calculated for each test group as follows:
  - $$MF_{uncorr.} = \frac{total\ number\ of\ mutant\ colonies}{number\ of\ seeded\ cells}\ x\ 10^6$$
  - The uncorrected mutant frequency was corrected with the absolute cloning efficiency 2 for each test group to get the corrected mutant frequency ($MF_{corr.}$):

- $MF_{corr.} = \frac{MF_{uncorr.}}{CE_{2\ absolute}}$

- Other parameters:
  - pH: measured for the top concentrations and for the negative controls with and without S9 mix.
  - Osmolality: measured for the top concentrations and for the negative controls with and without S9 mix.
  - Solubility: measured for the top concentrations and for the negative controls with and without S9 mix.
  - Cell morphology: all test groups were examined microscopically for cell morphology and cellular attachment at the end of the exposure period, which is a further indication for cytotoxicity.
- Acceptance criteria, the HPRT assay is considered valid if the following criteria are met::
  - The absolute cloning efficiencies of the negative controls should not be less than 50% (with and without S9 mix).
  - The background mutant frequency in the negative controls should be within the historical negative control data range of 0.00 – 16.43 mutants per 10^6 clonable cells.
  - The positive controls both with and without S9 mix have to induce distinctly increased mutant frequencies.
  - At least 4 dose levels should be tested ranging up to a toxic concentration or up to or beyond the limit of solubility under culture conditions.
- Assessment criteria:
  - A finding is assessed as positive if the following criteria are met:
    - Increase in the $MF_{corr.}$ both above the concurrent negative control values and our historical negative control data range.
    - Evidence of the reproducibility of any increase in mutant frequencies.
    - A statistically significant increase in mutant frequencies and the evidence of a dose-response relationship.
  - The test substance is considered non-mutagenic according to the following criteria:
    - The $MF_{corr.}$ in the dose groups is not statistically significantly increased above the concurrent negative control and is within the historical negative control data range.
- Statistical methods:

- Function RGP in MS EXCEL to assess dose-related increase of mutant frequencies.
- The number of mutant colonies obtained for the test substance treated groups was compared with that of the respective negative control groups.
- A trend is judged as statistically significant whenever the one-sided p-value (probability value) is below 0.05 and the slope is greater than 0.
- Both, biological and statistical significance were considered together.

Results and discussion

- Mutant frequency:
  - In this study, no relevant increase in the number of mutant colonies was observed with or without S9 mix.
  - In all experimental parts, no statistically significant dose-related increase in the mutant frequencies were observed after 4 hours of treatment in the absence and presence of S9 mix
  - The positive control substances EMS (without S9 mix; 400 µg/mL) and DMBA (with S9 mix; 1.25 µg/mL) induced a clear increase in mutation frequencies, as expected.

Table 13: Mutant frequency - 1st Experiment without S9 mix;4-hour exposure period

Test groups [µg/mL]		Number of colonies[a]						Mutant frequency (per 10^6 cells)	
								Uncorrected	Corrected[b]
Negative control	A	0	0	0	0	0	0	2.50	3.16
	B	0	2	2	3	1	1		
21.9	A	n.c.[1]							
	B								
43.8	A	1	0	0	0	0	0	0.28	0.30
	B	0	0	0	0	0	0		
87.5	A	6	2	4	1	1	3	5.00	5.66
	B	1	0	0	0	0	0		
175.0	A	0	0	0	0	0	0	1.11	1.33
	B	2	1	1	0	0	0		

350.0	A	1	1	1	0	0	0	1.39	1.82
	B	1	1	0	0	0	0		
700.0	A	n.c.[2]							
	B								
EMS 400	A	29	26	28	22	22	23	85.56	119.59
	B	27	24	35	26	25	21		

a = number of colonies 7 days after seeding about 300 000 cells/flask in selection medium
b = correction on the basis of the absolute cloning efficiency 2 at the end of the expression period
n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 14: Mutant frequency – 1st Experiment with S9 mix;4-hour exposure period

Test groups [µg/mL]		**Number of colonies[a]**						**Mutant frequency (per 10^6 cells)**	
								Uncorrected	**Corrected[b]**
Negative control	A	1	0	0	0	0	0	0.28	0.30
	B	0	0	0	0	0	0		
10.9	A	n.c.[1]							
	B								
21.9	A	0	0	1	2	0	0	1.11	1.40
	B	1	0	0	0	0	0		
43.8	A	1	1	2	1	0	0	2.50	3.15
	B	2	0	1	0	1	0		
87.5	A	0	0	0	0	0	0	0.28	0.36
	B	1	0	0	0	0	0		
175.0	A	0	0	0	0	0	0	0.00	0.00
	B	0	0	0	0	0	0		
350.0	A	n.c.[2]							
	B								
	A	68	70	58	56	61	53		

DMBA 1.25	B	51	55	86	59	66	51	203.89	334.53

a = number of colonies 7 days after seeding about 300 000 cells/flask in selection medium
b = correction on the basis of the absolute cloning efficiency 2 at the end of the expression period
n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 15: Mutant frequency – 2nd Experiment without S9 mix;4-hour exposure period

Test groups [µg/mL]		**Number of colonies[a]**						**Mutant frequency (per 10^6 cells)**	
								Uncorrected	**Corrected[b]**
Negative control	A	2	1	2	1	4	1	5.28	5.97
	B	1	0	2	2	2	1		
25.0	A	4	0	2	0	0	1	2.50	2.76
	B	0	0	1	1	0	0		
50.0	A	0	1	0	2	3	2	3.89	4.75
	B	1	0	2	2	0	1		
100.0	A	2	1	0	1	0	1	4.72	5.81
	B	0	5	3	1	2	1		
200.0	A	1	0	0	0	2	1	1.67	2.32
	B	0	0	1	0	1	0		
400.0	A	n.c.[2]							
	B								
800.0	A	n.c.[2]							
	B								
EMS 400	A	10	9	9	12	16	9	41.94	55.36
	B	15	14	18	13	14	12		

a = number of colonies 7 days after seeding about 300 000 cells/flask in selection medium
b = correction on the basis of the absolute cloning efficiency 2 at the end of the expression period
n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required

n.c.[2] = culture was not continued due to strong cytotoxicity

Table 16: Mutant frequency – 2nd Experiment with S9 mix;4-hour exposure period

Test groups [µg/mL]		Number of colonies[a]						Mutant frequency (per 10^6 cells)	
								Uncorrected	Corrected[b]
Negative control	A	0	0	0	0	0	0	3.33	3.49
	B	1	3	3	2	2	1		
12.5	A	n.c.[1]							
	B								
25.0	A	0	0	1	0	0	0	0.28	0.29
	B	0	0	0	0	0	0		
50.0	A	0	0	1	1	2	0	3.33	4.00
	B	3	2	1	1	1	0		
100.0	A	1	2	0	0	4	0	2.50	3.04
	B	0	0	0	0	0	2		
200.0	A	1	0	0	0	0	0	0.56	0.64
	B	0	1	0	0	0	0		
400.0	A	n.c.[2]							
	B								
DMBA 1.25	A	26	35	37	42	37	27	86.39	107.87
	B	23	25	13	18	17	11		

a = number of colonies 7 days after seeding about 300 000 cells/flask in selection medium
b = correction on the basis of the absolute cloning efficiency 2 at the end of the expression period
n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 17: Linear trend-test

Linear trend-test	Slope	One-sided p-value*
Corrected Mutation frequency 1st Experiment without S9 mix	-0.32773	0.6242
Corrected Mutation frequency1st Experiment with S9 mix	-1.13117	0.8546
Corrected Mutation frequency 2nd Experiment without S9 mix	-1.10207	0.8488
Corrected Mutation frequency2nd Experiment with S9 mix	-0.76248	0.7662

* The linear trend-test testing for an increased mutant frequency is significant (significance level of 5%), if the one-sided p-value is lower than 0.05 and the slope is greater than 0.

- Cytotoxicity:
  - Cytotoxic effects were observed in both experiments in the presence of S9 mix, at least at the highest applied concentrations.
  - Without S9 mix, there was a decrease in the number of colonies at 700 µg/mL ($CE_{1\ relative}$: 0.0%) in the 1st Experiment and from about 400 µg/mL ($CE_{1\ relative}$: 0.0%) in the 2nd Experiment. The cell densities were distinctly reduced.
  - In addition, with S9 mix, there was a decrease in the number of colonies at 350 µg/mL ($CE_{1\ relative}$: 0.0%) in the 1st Experiment and at 400 µg/mL ($CE_{1\ relative}$: 0.0%) in the 2nd Experiment. The cell densities were distinctly reduced in the 2nd Experiment, only.

Table 18: Cytotoxicity data - 1st Experiment without S9 mix;4-hour exposure period

Test groups [µg/mL]		Cell density (x 10^5/mL)	CE_1 (survival) (4 h after treatment; about 200 cells/flask seeded)				CE_2 (viability) (at the end of the expression period; about 200 cells/flask seeded)			
		at 1st sub-culture	Cell flask 1	Cell flask 2	Cloning efficiency [%]		Cell flask 1	Cell flask 2	Cloning efficiency [%]	
					Abs.	Rel.			Abs.	Rel.
Negative control	A	6.75	161	162	85.5	100.0	181	171	83.5	100.0
	B	7.14	191	170			157	159		
21.9	A	7.57	194	211	98.5	115.2	n.c.[1]			
	B	7.60	195	188						
43.8	A	7.62	183	184	96.0	112.3	190	177	81.5	97.6
	B	7.45	196	205			143	142		
87.5	A	7.46	201	195	96.5	112.9	175	182	82.8	99.1
	B	6.97	198	178			144	161		
175.0	A	7.14	156	165	81.0	94.7	152	145	78.9	94.5
	B	7.24	168	159			184	150		
350.0	A	7.08	93	82	52.1	61.0	149	144	77.3	92.5
	B	7.25	123	119			161	164		
700.0	A	0.24	0	0	0.0	0.0	n.c.[2]			
	B	0.32	0	0						
EMS 400	A	6.73	209	204	100.0	117.0	129	132	72.0	86.2
	B	6.09	207	180			163	152		

n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 19: Cytotoxicity data - 1st Experiment with S9 mix;4-hour exposure period

Test groups [µg/mL]		Cell density (x 10^5/mL)	CE_1 (survival) (4 h after treatment; about 200 cells/flask seeded)				CE_2 (viability) (at the end of the expression period; about 200 cells/flask seeded)			
		at 1st sub-culture	Cell flask 1	Cell flask 2	Cloning efficiency [%]		Cell flask 1	Cell flask 2	Cloning efficiency [%]	
					Abs.	Rel.			Abs.	Rel.
Negative control	A	7.30	133	168	75.9	100.0	171	194	90.9	100.0
	B	6.73	150	156			166	196		
10.9	A	7.24	156	151	75.8	99.8	n.c.[1]			
	B	7.19	135	164						
21.9	A	7.14	159	177	78.3	103.1	156	157	80.6	88.7
	B	6.79	136	154			181	151		
43.8	A	7.39	174	173	84.9	111.9	149	157	80.0	88.0
	B	7.00	167	165			174	160		
87.5	A	7.07	171	177	87.8	115.7	170	165	80.1	88.2
	B	7.46	186	168			154	152		
175.0	A	6.52	152	159	77.0	101.5	153	146	75.1	82.7
	B	6.43	138	167			165	137		
350.0	A	0.95	0	0	0.0	0.0	n.c.[2]			
	B	0.68	0	0						
DMBA 1.25	A	4.25	166	144	78.5	103.5	113	159	61.6	67.8
	B	4.75	162	156			114	107		

n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 20: Cytotoxicity data – 2nd Experiment without S9 mix;4-hour exposure period

Test groups [µg/mL]		Cell density (x 10^5/mL)	CE_1 (survival) (4 h after treatment; about 200 cells/flask seeded)				CE_2 (viability) (at the end of the expression period; about 200 cells/flask seeded)			
		at 1st sub-culture	Cell flask 1	Cell flask 2	Cloning efficiency [%]		Cell flask 1	Cell flask 2	Cloning efficiency [%]	
					Abs.	Rel.			Abs.	Rel.
Negative control	A	6.89	150	133	75.3	100.0	183	183	88.0	100.0
	B	7.34	177	142			158	180		
25.0	A	7.46	164	181	82.5	109.6	182	174	92.6	105.3
	B	7.02	140	175			175	210		
50.0	A	7.50	188	160	87.8	116.6	165	164	81.9	93.0
	B	6.47	184	170			156	170		
100.0	A	6.99	183	170	91.0	120.9	173	159	81.8	92.9
	B	6.89	181	194			163	159		
200.0	A	7.28	151	176	73.9	98.2	140	138	73.1	83.1
	B	6.81	128	136			151	156		
400.0	A	0.26	0	0	0.0	0.0	n.c.[2]			
	B	0.21	0	0						
800.0	A	0.03	0	0	0.0	0.0	n.c.[2]			
	B	0.09	0	0						
EMS 400	A	7.26	162	181	87.4	116.1	143	176	76.4	86.8
	B	6.81	176	180			154	138		

n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

Table 21: Cytotoxicity data – 2nd Experiment with S9 mix; 4-hour exposure period

Test groups [µg/mL]		Cell density (x 10^5/mL)	CE_1 (survival) (4 h after treatment; about 200 cells/flask seeded)				CE_2 (viability) (at the end of the expression period; about 200 cells/flask seeded)			
		at 1st sub-culture	Cell flask 1	Cell flask 2	Cloning efficiency [%]		Cell flask 1	Cell flask 2	Cloning efficiency [%]	
					Abs.	Rel.			Abs.	Rel.
Negative control	A	5.78	132	126	69.1	100.0	156	169	88.4	100.0
	B	4.69	152	143			196	186		
12.5	A	5.43	161	164	78.1	113.0	n.c.[1]			
	B	6.63	142	158						
25.0	A	5.30	151	169	82.3	119.0	196	188	92.1	104.2
	B	6.75	173	165			177	176		
50.0	A	6.66	158	180	86.1	124.6	171	156	83.0	93.9
	B	6.12	179	172			158	179		
100.0	A	5.64	161	159	81.5	117.9	146	176	84.5	95.6
	B	6.01	171	161			168	186		
200.0	A	5.27	73	100	44.8	64.7	186	169	87.5	99.0
	B	6.29	99	86			187	158		
400.0	A	0.40	0	0	0.0	0.0	n.c.[2]			
	B	0.34	0	0						
DMBA 1.25	A	5.16	163	173	80.5	116.5	153	167	80.1	90.7
	B	5.77	153	155			158	163		

n.c.[1] = culture was not continued since a minimum of only four analysable concentrations is required
n.c.[2] = culture was not continued due to strong cytotoxicity

- Cell morphology:
  - In the 1st and 2nd Experiment, in the absence of S9 mix, after 4 hours treatment the morphology and attachment of the cells were adversely influenced (grade > 2) in the highest applied concentrations (from 400 µg/mL onward).
  - In contrast, in the presence of S9 mix, only in the 2nd Experiment after 4 hours treatment the morphology and attachment of the cells were adversely influenced (grade > 2) in the highest applied concentration (400 µg/mL).

Table 22: Treatment conditions, cell morphology - 1st Experiment without S9 mix; 4-hour exposure period

Test groups	pH	Osmolality	Solubility				Cell attachment/ morphology
			Vehicle	Culture medium			
				0 h	3 – 4 h		
		mOsm	Ma	Ma	Ma	Mi	grade
Negative control	7.4	282	n.d.	n.d.	n.d.	n.d.	1
21.9 µg/mL	n.d.	n.d.	S	S	S	S	1
43.8 µg/mL	n.d.	n.d.	S	S	S	S	1
87.5 µg/mL	n.d.	n.d.	S	S	S	S	1
175.0 µg/mL	n.d.	n.d.	S	S	S	S	1
350.0 µg/mL	n.d.	n.d.	Su	S	S	S	2
700.0 µg/mL	7.4	294	Su	S	P	P	4

n.d. = not determined
mOsm = milliosmolar
S = solution
Su = suspension
P = precipitation
1 = complete attachment (fibroblast-like cells)
2 = slightly reduced attachment (few cells rounded)
3 = reduced attachment (most cells rounded and partly detached)
4 = complete detachment (all cells rounded)

Table 23: Treatment conditions, cell morphology - 1st Experiment with S9 mix; 4-hour exposure period

Test groups	pH	Osmolality	Solubility				Cell attachment/ morphology grade
			Vehicle	Culture medium			
				0 h	3 – 4 h		
		mOsm	Ma	Ma	Ma	Mi	
Negative control	7.2	257	n.d.	n.d.	n.d.	n.d.	1
10.9 µg/mL	n.d.	n.d.	S	S	S	S	1
21.9 µg/mL	n.d.	n.d.	S	S	S	S	1
43.8 µg/mL	n.d.	n.d.	S	S	S	S	1
87.5 µg/mL	n.d.	n.d.	S	S	S	S	1
175.0 µg/mL	n.d.	n.d.	S	S	S	S	1
350.0 µg/mL	7.2	251	Su	S	S	S	2

n.d. = not determined
mOsm = milliosmolar
S = solution
Su = suspension
P = precipitation
1 = complete attachment (fibroblast-like cells)
2 = slightly reduced attachment (few cells rounded)
3 = reduced attachment (most cells rounded and partly detached)
4 = complete detachment (all cells rounded)

Table 24: Treatment conditions, cell morphology – 2nd Experiment without S9 mix; 4-hour exposure period

Test groups	pH	Osmolality	Solubility				Cell attachment/ morphology grade
			Vehicle	Culture medium			
				0 h	3 – 4 h		
		mOsm	Ma	Ma	Ma	Mi	
Negative control	7.3	215	n.d.	n.d.	n.d.	n.d.	1

25.0 µg/mL	n.d.	n.d.	S	S	S	S	1
50.0 µg/mL	n.d.	n.d.	S	S	S	S	2
100.0 µg/mL	n.d.	n.d.	S	S	S	S	2
200.0 µg/mL	n.d.	n.d.	S	S	S	S	2
400.0 µg/mL	n.d.	n.d.	Su	S	P	P	4
800.0 µg/mL	7.3	200	Su	S	P	P	4

n.d. = not determined
mOsm = milliosmolar
S = solution
Su = suspension
P = precipitation
1 = complete attachment (fibroblast-like cells)
2 = slightly reduced attachment (few cells rounded)
3 = reduced attachment (most cells rounded and partly detached)
4 = complete detachment (all cells rounded)

Table 25: Treatment conditions, cell morphology – 2nd Experiment with S9 mix;4-hour exposure period

Test groups	**pH**	**Osmolality**	**Solubility**				**Cell attachment/ morphology grade**
			Vehicle	**Culture medium**			
				0 h	**3 – 4 h**		
		mOsm	**Ma**	**Ma**	**Ma**	**Mi**	
Negative control	7.1	183	n.d.	n.d.	n.d.	n.d.	1
12.5 µg/mL	n.d.	n.d.	S	S	S	S	1
25.0 µg/mL	n.d.	n.d.	S	S	S	S	1
50.0 µg/mL	n.d.	n.d.	S	S	S	S	1
100.0 µg/mL	n.d.	n.d.	S	S	S	S	1
200.0 µg/mL	n.d.	n.d.	S	S	S	S	2
400.0 µg/mL	7.1	177	Su	S	S	S	3

n.d. = not determined
mOsm = milliosmolar

S = solution
Su = suspension
P = precipitation
1 = complete attachment (fibroblast-like cells)
2 = slightly reduced attachment (few cells rounded)
3 = reduced attachment (most cells rounded and partly detached)
4 = complete detachment (all cells rounded)

- pH:
  - Not influenced by the test substance
- Osmolality:
  - Not influenced by the test substance
- Solubility:
  - In the absence of S9 mix, test substance precipitation was observed in culture medium at the end of treatment at 700 µg/mL in the 1st Experiment and at 400 µg/mL and above in the 2nd Experiment.
  - In the presence of S9 mix, precipitation was not observed up to the highest applied concentration in both experiments.
- Discussion:
  - Octadecanoic acid, sulfo-, potassium salt did not dose-dependently increase the number of mutant colonies, either without S9 mix or after the addition of a metabolizing system.
  - The mutation frequencies of the vehicle control groups were within the historical negative control data range including all vehicles used in the laboratory and, thus, fulfilled the acceptance criteria of this study.
  - The increase in the frequencies of mutant colonies induced by the positive control substances EMS and DMBA clearly demonstrated the sensitivity of the test method and/or of the metabolic activity of the S9 mix employed.
- Conclusion:
  - In the absence and the presence of metabolic activation, Octadecanoic acid, sulfo-, potassium salt is not a mutagenic substance in the HPRT locus assay using CHO cells under the experimental conditions chosen.

3.8.1.6 [Study 6]

Study reference:

NN, 2015b

Detailed study summary and results:

Test type

An *in vitro* micronucleus assay in V79 cells (cytokinesis block method) according to the OECD Guideline for the Testing of Chemicals No. 487, 26 Sep 2014, "In vitro Mammalian Cell Micronucleus Test" was performed. GLP compliance is given (certificate given). A reliability of 1 is given for this study in the registration dossier.

Test substance

- Test material used in the study: Octadecanoic acid, sulfo-, potassium salt (CAS 67968-63-2)
- Test material form: brownish clear
- Aggregated state at room temperature: liquid
- Degree of purity: 100% minus water content and potassium sulphate = 51.92% test item
- Impurities: A quantitative analysis of test item ingredients is not possible because it is a complex mixture.
- Lot/Batch number: 0012127444
- Expiration date of Lot/Batch: 22 Jun 2016
- Storage condition of test material: Room temperature

Administration/exposure

- Strain or cell type or cell line, target gene:
  - The V79 cell line is a permanent cell line derived from the Chinese hamster and has a high proliferation rate (doubling time of about 12 - 14 hours), high plating efficiency (≥ 90%), stable karyotype (modal number of 22 chromosomes).
  - The V79 cell line has shown its suitability to detect aneugenic effects in the Micronucleus test in vitro either in the absence and presence of CytB.
- Type and composition of metabolic activation system:
  - species and cell type: 5 rat (Males, Wistar), liver microsomal enzymes from liver homogenate (S9-mix).
  - induced or not induced: induced
  - chemicals used for induction: 80 mg/kg b.w. phenobarbital i.p. and β-naphthoflavone orally (both supplied by Sigma-Aldrich, 82024 Taufkirchen, Germany) each on three consecutive days.
  - quantity: pH = 7.4
    - S9-fraction……... 10%
    - $MgCl_2$……. 8 mmol/L
    - KCl……. 33 mmol/L
    - NADP……. 4 mmol/L
    - Glucose-6-phosphate…….. 5 mmol/L
    - Sodium phosphate buffer……... 15 mmol/L The phosphate buffer (4) was prepared by mixing a Na_2HPO_4 solution with a NaH_2PO_4 solution in a ratio of about 4:1.
- Test concentrations, and reasoning for selection of doses:
  - Pretest: Performed at a concentration of 5000 µg/mL (approx. 2600 µg/mL of the complex mixture) as the top concentration. pH was stable after addition of test substance. Homogeneous suspension was obtained from 25 mg/mL down to 6.25 mg/mL. Undissolved particles were obtained from 2500 µg/mL 4h after treatment in the absence and presence of S9 mix. Cytotoxicity indicated by reduced Relative Population

Doubling (RPD) of about or below 40 - 50% was observed at 312.5 µg/mL and above after 4 and 24 hours treatment in the absence of S9 mix and at 625.0 µg/mL and above after 4 hours treatment in the presence of S9 mix.

Table 26: Results pretest: 4 hours exposure, 24 hours harvest time, without S9 mix

Test groups	pH*	Solubility				Relative population doubling (RPD)	Cell attachment/ morphology
		Veh	Culture medium				
			0 h	3 - 4 h			
	Change	Ma	Ma	Ma	Mi	(%)	3 - 4 h
Negative control	no	n.d.	n.d.	n.d.	n.d.	100.0	1
39.1 µg/mL	no	S	S	S	S	96.0	1
78.1 µg/mL	no	S	S	S	S	95.2	1
156.3 µg/mL	no	S	S	S	S	97.2	1
312.5 µg/mL	no	S	S	S	S	-20.0	4
625.0 µg/mL	no	S	S	S	S	17.5	4
1250.0 µg/mL	no	Su	S	S	S	-16.2	4
2500.0 µg/mL	no	Su	P	P	P	-0.8	4
5000.0 µg/mL	no	Su	P	P	P	-29.0	4

* Visual examination by pH-indicator phenol red; normal range: about 6.7 – 8.3

Veh = vehicle
Ma = macroscopically
Mi = microscopically
S = solution
Su = suspension
P = precipitation
1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

Table 27: Results pretest: 4 hours exposure, 24 hours harvest time, with S9 mix

Test groups	pH*	Solubility				Relative population doubling (RPD)	Cell attachment/ morphology
		Veh	Culture medium				
			0 h	3 - 4 h			
	Change	Ma	Ma	Ma	Mi	(%)	3 - 4 h
Negative control	no	n.d.	n.d.	n.d.	n.d.	100.0	1
39.1 µg/mL	no	S	S	S	S	95.4	1
78.1 µg/mL	no	S	S	S	S	95.1	1
156.3 µg/mL	no	S	S	S	S	96.9	1
312.5 µg/mL	no	S	S	S	S	55.4	3
625.0 µg/mL	no	S	S	S	S	-52.5	4
1250.0 µg/mL	no	Su	S	S	S	10.8	4
2500.0 µg/mL	no	Su	P	P	P	-12.0	4
5000.0 µg/mL	no	Su	P	P	P	0.0	4

* Visual examination by pH-indicator phenol red; normal range: about 6.7 – 8.3

Veh = vehicle
Ma = macroscopically
Mi = microscopically
S = solution
Su = suspension
P = precipitation
1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

Table 28: Results pretest: 24 hours exposure, 24 hours harvest time, without S9 mix

Test groups	pH*	Solubility				Relative population doubling (RPD)	Cell attachment/ morphology
		Veh	Culture medium				
			0 h	22 - 24 h			
	Change	Ma	Ma	Ma	Mi	(%)	22 - 24 h
Negative control	no	n.d.	n.d.	n.d.	n.d.	100.0	1
39.1 µg/mL	no	S	S	S	S	101.8	1
78.1 µg/mL	no	S	S	S	S	104.6	1
156.3 µg/mL	no	S	S	S	S	83.8	1
312.5 µg/mL	no	S	S	S	S	-42.7	4
625.0 µg/mL	no	S	S	S	S	-53.2	4
1250.0 µg/mL	no	Su	S	S	S	-21.1	4
2500.0 µg/mL	no	Su	P	P	P	-27.2	4
5000.0 µg/mL	no	Su	P	P	P	-42.7	4

* Visual examination by pH-indicator phenol red; normal range: about 6.7 – 8.3

Veh = vehicle
Ma = macroscopically
Mi = microscopically
S = solution
Su = suspension
P = precipitation
1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

- Dose selection first experiment:
  - Without S9 mix, 4 hours exposure 24-hour preparation interval: 31.3; 62.5; 125.0; 250.0; 500.0 and 1000.0 µg/mL
  - With S9 mix, 4 hours exposure 24-hour preparation interval: 31.3; 62.5; 125.0; 250.0; 500.0 and 1000.0 µg/mL

- Dose selection second experiment:
  - Without S9 mix, 24 hours exposure 24-hour preparation interval: 7.8; 15.6; 31.3; 62.5; 125.0 and 250.0 µg/mL
  - With S9 mix, 4 hours exposure 44-hour preparation interval: 15.6; 31.3; 62.5, 125.0; 250.0 and 500.0 µg/mL

- Vehicle:
  - Exposure without S9-mix: MEM (minimal essential medium with Earle's salts) containing a L-glutamine source supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) penicillin/streptomycin (10000 IU / 10000 µg/mL), and 1% (v/v) amphotericine B (250 µg/mL).
  - Exposure with S9-mix: MEM (minimal essential medium with Earle's salts) containing a L-glutamine source supplemented with 1% (v/v) penicillin/streptomycin (10000 IU / 10000 µg/mL), and 1% (v/v) amphotericine B (250 µg/mL).
- Positive controls:
  - Without metabolic activation: 400 and 500 µg/mL ethyl methanesulfonate (EMS) dissolved in MEM without FCS.
  - With metabolic activation 0.5 and 1.0 µg/mL cyclophosphamide (CPP) dissolved in MEM without FCS.
- Method of application: in culture medium
- Duration: rats received 80 mg/kg b.w. phenobarbital i.p. and β-naphthoflavone orally each on three consecutive days.

Table 29: Study schedule

	Without S9 mix		**With S9 mix**	
	Exp.I	**Exp.II**	**Exp.I**	**Exp.II**
Exposure time	4 hrs	24 hrs	4 hrs	4 hrs
Recovery time	20 hrs	-	20 hrs	40 hrs
Harvest time	24 hrs	24 hrs	24 hrs	44 hrs

- Number of replication: duplicate cultures
- In case of 4-hour exposure period, at the end of exposure, medium was removed and the cultures were rinsed twice with 5 mL HBSS (Hanks Balanced Salt Solution). Subsequently, 5 mL MEM (incl. 10% [v/v] FCS) supplemented with polymerisation inhibitor cytochalasin B (CytB) (final

concentration: 3 µg/mL; stock: 0.6 mg/mL in DMSO) was added and the cultures were incubated at 37°C, 5% (v/v) CO_2 and ≥ 90% relative humidity for the respective recovery time. CytB easily allows to discriminate between proliferating and non-proliferating cells.

- In the case of 24-hour continuous exposure, CytB was added to the treatment medium at start of treatment, and cell preparation was started directly at the end of exposure. At 44 hours preparation interval in the presence of S9 mix CytB was added 24 hours before preparation of the cultures.
- Cell harvest and preparation of slides:
  - The cells were prepared based on the method described by Fenech.
  - The cell number per flask of each cell suspension was determined using a cell counter. Subsequently, $5x10^4$ cells per slide were centrifuged at 600 rpm for 7 minutes onto labelled slides using a Cytospin centrifuge. At least two slides per flask were prepared.
  - In the case of strongly reduced cell numbers below $10x10^4$ cells per flask no slides were prepared.
  - After drying, the slides were fixed in 90% (v/v) methanol for 10 minutes.
- Staining:
  - Before scoring, the slides were stained with a mixture of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; stock: 5 mg/mL) and propidium iodide (stock: 5 mg/mL) in Fluoroshield™ at a concentration of 0.25 µg/mL each. By the use of the combination of both fluorescence dyes it can be differentiated between DNA (DAPI; excitation: 350 nm, emission: 460 nm) and cytoplasm (PI; excitation: 488 nm, emission: 590 nm).
- Micronucleus analysis:
  - The cytospin slides were scored by fluorescence microscopy (Axio Imager.Z2, Zeiss, Göttingen, Germany).
  - In total at least 2000 binucleated cells per test group, were evaluated for the occurrence of micronuclei.
  - The analysis of micronuclei was carried out following the criteria of Countryman and Heddle:
    - The diameter of the micronucleus is less than 1/3 of the main nucleus.
    - The micronucleus and main nucleus retain the same colour.
  - The micronucleus is not linked to the main nucleus and is located within the cytoplasm of the cell.

- Only binucleated cells clearly surrounded by a membrane were scored.

- Slides were coded before microscopic analysis by MUVIKE software, BASF SE. Cultures with only few isolated cells were not analysed for micronuclei.
- Since the absolute values shown were rounded but the calculations were made using the unedited values, there may be deviations in the given relative values.

- Relative population doubling (RPD):
  - Under the experimental conditions described, RPD is an indication of cell viability mainly for the time period before addition of CytB. Before preparing the cytospin slides the cell count was determined from trypsinized cultures.
  - The relative population doubling will be calculated based on the following formula:

$$Population\ doubling\ (PD) = \frac{\log\left(Post - treatment\ cell\ number / Initial\ cell\ number\right)}{\log 2}$$

$$RPD = \frac{(No.\ of\ PD\ in\ treated\ cultures)}{(No.\ of\ PD\ in\ vehicle\ control\ cultures)}\ x\ 100$$

  - The data will be given tabulated in percentage compared with the concurrent negative control. A RPD of 45% indicates 55% cytotoxicity/cytostasis. Since the absolute values have been rounded, but further calculation is based on unrounded values, there may be deviations in the relative values given.

- Proliferation Index (CBPI):
  - The cytokinesis-block proliferation index (CBPI) is a direct measure of the proliferative activity of the cells and it was determined in at least 1000 cells per culture (at least 2000 cells per test group). This value indicates the average number of cell cycles per cell during the period of exposure to the actin polymerisation inhibitor cytochalasin B.

- The number of mononucleated, binucleated and multinucleated cells was recorded and the CBPI was calculated using the following formula:

$$CBPI = \frac{No.\ mononucleate\ cells + 2\ x\ No.\ binucleate\ cells + 3\ x\ No.\ multinucleate\ cells}{Total\ number\ of\ cells}$$

- The CBPI can be used to calculate the % cytostasis (relative inhibition of cell growth compared to the respective vehicle control group) - a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis:

$$\%\ Cytostasis = 100 - 100\ x \frac{CBPI_T - 1}{CBPI_C - 1}$$

T = test substance treated culture C = vehicle control culture

- The data are given tabulated. Since the absolute values have been rounded, but further calculation is based on unrounded values, there may be deviations in the relative values given.

- Cell morphology:
  - At the end of the treatment period, all test groups were examined microscopically with regard to cell morphology, which is a further indication for cytotoxicity.
- Assessment of the slides:
  - Dose selection for scoring for cytogenetic damage was based on the results of a previous check on:
    - slide and/or cell quality
    - number of analyzable cells
    - nuclear fragmentation.
- pH value:

- Changes in the pH were apparent by a colour change of the indicator in the culture medium (phenol red normal range: about pH 6.7 - 8.3). The pH was measured for the top concentration and for the vehicle control with and without S9 mix.

- Osmolarity:
  - Osmolarity was measured for the top concentration and for the vehicle control with and without S9 mix.
- Solubility:
  - Test substance precipitation was checked immediately after start of treatment of the test cultures (macroscopically) and at the end of treatment (macroscopically / microscopically).
- Acceptance criteria:
  - The in vitro micronucleus assay is considered valid if the following criteria are met:
    - The quality of the slides allowed the evaluation of a sufficient number of analyzable cells both in the control groups (vehicle/positive) and in at least three exposed test groups.
    - Sufficient cell proliferation was demonstrated in the vehicle control.
    - The number of cells containing micronuclei in the vehicle control was within the range of the laboratory's historical negative control data (95% control limit; see Appendix 6). Weak outliers can be judged acceptable if there is no evidence that the test system is not "under control".
    - The positive control substances both with and without S9 mix induced a distinct, statistically significant increase in the number of micronucleated cells in the expected range.
- Assessment criteria
  - A test substance is considered to be clearly positive if the following criteria are met:
    - A statistically significant increase in the number of micronucleated cells was obtained.
    - A dose-related increase in the number of cells containing micronuclei was observed.

- The number of micronucleated cells exceeded both the value of the concurrent vehicle control and the range of the laboratory's historical negative control data (95% control limit).

- A test substance is considered to be clearly negative if the following criteria are met:
  - Neither a statistically significant nor dose-related increase in the number of cells containing micronuclei was observed under any experimental condition.
  - The number of micronucleated cells in all treated test groups was close to the concurrent vehicle control value and within the range of the laboratory's historical negative control data (95% control limit).

- Statistics
  - The proportion of cells containing micronuclei was calculated for each test group.
  - A comparison of the micronucleus rates of each test group with the concurrent negative control group was carried out for the hypothesis of equal proportions (i.e. one-sided Fisher's exact test, MUVIKE software, BASF SE).
  - If the results of this test were statistically significant compared with the respective vehicle control, labels (* $p \leq 0.05$, ** $p \leq 0.01$ or ^{S}) have been printed in the tables.

Results and discussion

- Micronucleus analysis
  - No biologically relevant increase in the number of micronucleated cells was observed either without S9 mix or after the addition of a metabolizing system.
  - The positive control substances EMS (without S9 mix; 400 µg/mL) and CPP (with S9 mix; 0.5 µg/mL) induced statistically significant increased micronucleus frequencies in both independently performed experiments.

Table 30: Treatment conditions, micronucleus assay - 1st Experiment without S9 mix; 4-hour exposure period, 24-h harvest time

Dose µg/mL	Cells n	Cells incl. n	Micronuclei %
Negative control	2000	11	0.6
31.3	2000	10	0.5
62.5	2000	4	0.2
125.0	2000	13	0.7
EMS 400	2000	42	2.1**

Fisher's exact test (one-sided) with Bonferroni-Holm correction *: p <= 0.05, **: p <= 0.01
A pairwise comparison of each dose group with the vehicle control, Bonferroni-Holm corrected for each time

Table 31: Treatment conditions, micronucleus assay – 1st Experiment with S9 mix; 4-hour exposure period, 24-h harvest time

Dose µg/mL	Cells n	Cells incl. n	Micronuclei %
Negative control	2000	9	0.5
31.3	2000	14	0.7
62.5	2000	9	0.5
125.0	2000	12	0.6
CPP 0.5	2000	56	2.8**

Fisher's exact test (one-sided) with Bonferroni-Holm correction *: p <= 0.05, **: p <= 0.01
A pairwise comparison of each dose group with the vehicle control, Bonferroni-Holm corrected for each time

Table 32: Treatment conditions, micronucleus assay – 2nd Experiment without S9 mix; 24-hour exposure period, 24-h harvest time

Dose µg/mL	Cells n	Cells incl. n	Micronuclei %
Negative control	2000	8	0.4
62.5	2000	6	0.3
125.0	2000	7	0.4

250.0	2000	6	0.3
EMS 400	2000	53	2.7**

Fisher's exact test (one-sided) with Bonferroni-Holm correction *: p <= 0.05, **: p <= 0.01
A pairwise comparison of each dose group with the vehicle control, Bonferroni-Holm corrected for each time

Table 33: Treatment conditions, micronucleus assay – 2nd Experiment with S9 mix; 4-hour exposure period, 44-h harvest time

Dose **µg/mL**	**Cells** **n**	**Cells incl.** **n**	**Micronuclei** **%**
Negative control	2000	7	0.4
62.5	2000	7	0.4
125.0	2000	12	0.6
250.0	2000	7	0.4
CPP 0.5	2000	83	4.2**

Fisher's exact test (one-sided) with Bonferroni-Holm correction *: p <= 0.05, **: p <= 0.01
A pairwise comparison of each dose group with the vehicle control, Bonferroni-Holm corrected for each time

- Relative population doubling (RPD)
  - In the absence of S9 mix reduced RPD was obtained from 500 µg/mL onward (-102.6%) after 4 hours exposure in the 1st Experiment and at 250 µg/mL (-0.7%) after 24 hours exposure in the 2nd Experiment.
  - In the presence of S9 mix RPD was decreased after 4 hours exposure from 500 µg/mL onward (-143.9%) at 24 hours preparation interval in the 1st Experiment and at 500 µg/mL (-258.0%) at 44 hours preparation interval in the 2nd Experiment.

Table 34: Population doubling, cell morphology and attachment - 1st Experiment; 4 hours exposure, 24 hours harvest time, without S9 mix

Test groups	**Population doubling**				**Cell morphology/ attachment**
	Culture A absolute	**Culture B absolute**	**Mean absolute**	**Mean relative [%]**	

Negative control	1.57	1.59	1.58	100.0	1
31.3 µg/mL	1.54	1.50	1.52	95.9	1
62.5 µg/mL	1.43	1.61	1.52	96.3	1
125.0 µg/mL	1.70	1.58	1.64	104.0	1
250.0 µg/mL	1.83	1.87	1.85	117.1	1
500.0 µg/mL	-1.64	-1.60	-1.62	-102.6	4
1000.0 µg/mL	-2.40	-2.64	-2.52	-159.5	4
EMS 400.0 µg/mL	1.44	1.67	1.55	98.3	1
EMS 500.0 µg/mL	1.39	1.84	1.61	102.1	1

1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

Table 35: Population doubling, cell morphology and attachment - 1st Experiment; 4 hours exposure, 24 hours harvest time, with S9 mix

Test groups	Population doubling				Cell morphology/ attachment
	Culture A absolute	Culture B absolute	Mean absolute	Mean relative [%]	
Negative control	1.45	1.34	1.40	100.0	1
31.3 µg/mL	1.49	1.27	1.38	98.6	1
62.5 µg/mL	1.27	1.24	1.26	90.0	1
125.0 µg/mL	1.29	1.07	1.18	84.6	1
250.0 µg/mL	1.45	1.53	1.49	106.5	1
500.0 µg/mL	-1.84	-2.18	-2.01	-143.9	4
1000.0 µg/mL	-3.24	-3.64	-3.44	-246.4	4
CPP 0.5 µg/mL	1.13	1.37	1.25	89.4	1
CPP 1.0 µg/mL	1.32	1.80	1.56	111.9	1

1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded

4 = complete detachment, i.e. all cells rounded

Table 36: Population doubling, cell morphology and attachment – 2nd Experiment; 24 hours exposure, 24 hours harvest time, without S9 mix

Test groups	Population doubling				Cell morphology/ attachment
	Culture A absolute	Culture B absolute	Mean absolute	Mean relative [%]	
Negative control	1.49	1.38	1.43	100.0	1
7.8 µg/mL	1.17	1.15	1.16	80.9	1
15.6 µg/mL	1.18	0.76	0.97	67.4	1
31.3 µg/mL	0.96	1.10	1.03	71.8	1
62.5 µg/mL	1.10	1.08	1.09	76.0	1
125.0 µg/mL	1.12	1.25	1.19	82.8	2
250.0 µg/mL	-0.23	0.21	-0.01	-0.7	3
EMS 400.0 µg/mL	1.36	1.29	1.32	92.3	1
EMS 500.0 µg/mL	1.34	1.29	1.32	91.7	1

1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

Table 37: Population doubling, cell morphology and attachment – 2nd Experiment; 4 hours exposure, 44 hours harvest time, with S9 mix

Test groups	Population doubling				Cell morphology/ attachment
	Culture A absolute	Culture B absolute	Mean absolute	Mean relative [%]	
Negative control	1.64	0.53	1.08	100.0	1
15.6 µg/mL	1.51	1.13	1.32	122.1	1
31.3 µg/mL	1.61	1.17	1.39	128.7	1
62.5 µg/mL	1.61	1.33	1.47	136.2	1

125.0 µg/mL	1.61	1.30	1.46	134.7	1
250.0 µg/mL	1.49	1.28	1.38	127.9	1
500.0 µg/mL	-3.18	-2.40	-2.79	-258.0	4
CPP 0.5 µg/mL	1.14	1.09	1.11	103.0	1
CPP 1.0 µg/mL	1.08	1.06	1.07	99.0	1

1 = complete attachment, i.e. fibroblast-like cells
2 = slightly reduced attachment, i.e. few cells rounded
3 = reduced attachment, i.e. most cells rounded
4 = complete detachment, i.e. all cells rounded

- Proliferation index (CBPI)
  - In the 1st Experiment in the absence of metabolic activation the highest scored concentration had no adverse effect on cell proliferation indicated by 3.3% cytostasis.

    Due to strong cytotoxicity indicated by distinctly reduced cell proliferation or severe cell loss no slides could be scored or prepared at the higher applied concentrations of 250 µg/mL and above.
  - In the 2nd Experiment in the absence of S9 mix the highest applied concentration of 250 µg/mL led to a cytostasis of 25.7%.
  - In the presence of metabolic activation in the 1st Experiment at 24 hours preparation interval the highest scored concentration had no adverse effect on cell proliferation indicated by 4.2% cytostasis.

    Due to strong cytotoxicity indicated by severe cell loss no slides could be prepared at the higher applied concentrations of 500 µg/mL and above.
  - In the 2nd Experiment, in the presence of S9 mix at 44 hours preparation interval, the highest scorable concentration of 250 µg/mL led to no cytostasis (3.4%). But, due to strong cytotoxicity the slides of the highest applied concentration of 500 µg/mL was not scorable.

Table 38: Proliferation Index (CBPI) - 1st Experiment; 4 hours exposure, 24 hours harvest time, without S9 mix

Test groups	Mononucleated cells	Binucleated cells	Multinucleated cells	CBPI absolute	CBPI cyto-stasis [%]
Negative control	44 36	947 959	9 5	1.97	0.0
31.3 µg/mL	54 55	937 935	9 10	1.96	1.2
62.5 µg/mL	45 70	949 923	6 7	1.95	1.9
125.0 µg/mL	72 75	926 909	2 16	1.94	3.3
250.0 µg/mL	n.s.	n.s.	n.s.	n.s.	n.s.
500.0 µg/mL	n.p.	n.p.	n.p.	n.p.	n.p.
1000.0 µg/mL	n.p.	n.p.	n.p.	n.p.	n.p.
EMS 400.0 µg/mL	121 155	879 844	0 1	1.86	10.8
EMS 500.0 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not determined
n.p. = no cytospin slides prepared due to strong cytotoxicity
n.s. = not scorable due to strong cytotoxicity or technical problems (i.e. test substance precipitation interferes with scoring)

Table 39: Proliferation Index (CBPI) - 1st Experiment; 4 hours exposure, 24 hours harvest time, with S9 mix

Test groups	Mononucleated cells	Binucleated cells	Multinucleated cells	CBPI absolute	CBPI cyto-stasis [%]
Negative control	87 35	902 954	11 11	1.95	0.0

31.3	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.
62.5	**µg/mL**	58 53	933 945	9 2	1.95	0.0
125.0	**µg/mL**	51 39	944 955	5 6	1.96	-1.1
250.0	**µg/mL**	110 74	887 924	3 2	1.91	4.2
500.0	**µg/mL**	n.p.	n.p.	n.p.	n.p.	n.p.
1000.0	**µg/mL**	n.p.	n.p.	n.p.	n.p.	n.p.
CPP 0.5	**µg/mL**	318 195	682 803	0 2	1.74	21.6
CPP 1.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not determined
n.p. = no cytospin slides prepared due to strong cytotoxicity
n.s. = not scorable due to strong cytotoxicity or technical problems (i.e. test substance precipitation interferes with scoring)

Table 40: Proliferation Index (CBPI) - 2nd Experiment; 24 hours exposure, 24 hours harvest time, without S9 mix

Test groups	**Mononucleated cells**	**Binucleated cells**	**Multinucleated cells**	**CBPI absolute**	**CBPI cyto-stasis [%]**
Negative control	9 2	904 869	87 129	2.10	0.0
7.8 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.

15.6	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.
31.3	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.
62.5	**µg/mL**	9 9	887 895	104 96	2.09	1.0
125.0	**µg/mL**	7 28	894 910	99 62	2.06	3.6
250.0	**µg/mL**	189 189	808 798	3 13	1.82	25.7
EMS 400.0	**µg/mL**	133 95	866 901	1 4	1.89	19.4
EMS 500.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not determined
n.p. = no cytospin slides prepared due to strong cytotoxicity
n.s. = not scorable due to strong cytotoxicity or technical problems (i.e. test substance precipitation interferes with scoring)

Table 41: Proliferation Index (CBPI) - 2nd Experiment; 4 hours exposure, 44 hours harvest time, with S9 mix

Test groups	**Mononucleated cells**	**Binucleated cells**	**Multinucleated cells**	**CBPI absolute**	**CBPI cyto-stasis [%]**
Negative control	0 0	780 738	220 262	2.24	0.0
15.6 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.
31.3 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.
62.5 µg/mL	0	715	285	2.26	1.2

		3	767	230		
125.0	**µg/mL**	11 2	784 762	205 236	2.21	2.2
250.0	**µg/mL**	1 1	760 839	239 160	2.20	3.4
500.0	**µg/mL**	n.s.	n.s.	n.s.	n.s.	n.s.
CPP 0.5	**µg/mL**	6 1	638 597	356 402	2.38	-10.8
CPP 1.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not determined
n.p. = no cytospin slides prepared due to strong cytotoxicity
n.s. = not scorable due to strong cytotoxicity or technical problems (i.e. test substance precipitation interferes with scoring)

- Cell morphology
  - In this study, in the absence of metabolic activation cell attachment/morphology was adversely influenced (grade > 2) at 500 µg/mL and above in the 1st Experiment and at 250 µg/mL in the 2nd Experiment.
  - In the presence of metabolic activation cell attachment/morphology was adversely influenced at 500 µg/mL and above in the 1st Experiment and at 500 µg/mL in the 2nd Experiment.
  - No slides were prepared due to strongly reduced cell numbers at 500 µg/mL and above in the 1st Experiment in the absence and presence of metabolic activation.
- Osmolarity:
  - Not influenced by test substance treatment
- pH:
  - Not influenced by test substance treatment

Table 42: Treatment conditions – 1st Experiment; 4 hours exposure, 24 hours harvest time, without S9 mix

Test groups		pH	Osmolarity mOsm	Solubility			
				Vehicle	Culture medium		
					0 h	3 - 4 h	
				Ma	Ma	Ma	Mi
Negative control		7.5	291	n.d.	n.d.	n.d.	n.d.
31.3	**µg/mL**	n.d.	n.d.	S	S	S	S
62.5	**µg/mL**	n.d.	n.d.	S	S	S	S
125.0	**µg/mL**	n.d.	n.d.	S	S	S	S
250.0	**µg/mL**	n.d.	n.d.	S	S	S	S
500.0	**µg/mL**	n.d.	n.d.	S	S	S	S
1000.0	**µg/mL**	7.6	298	S	S	S	S
EMS 400.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EMS 500.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

mOsm = milliosmolar
Ma = macroscopically
Mi = microscopically
S = solution
n.d. = not determined

Table 43: Treatment conditions – 1st Experiment; 4 hours exposure, 24 hours harvest time, with S9 mix

Test groups	pH	Osmolarity mOsm	Solubility			
			Vehicle	Culture medium		
				0 h	3 - 4 h	
			Ma	Ma	Ma	Mi
Negative control	7.3	257	n.d.	n.d.	n.d.	n.d.

31.3 µg/mL	n.d.	n.d.	S	S	S	S
62.5 µg/mL	n.d.	n.d.	S	S	S	S
125.0 µg/mL	n.d.	n.d.	S	S	S	S
250.0 µg/mL	n.d.	n.d.	S	S	S	S
500.0 µg/mL	n.d.	n.d.	S	S	S	S
1000.0 µg/mL	7.4	256	S	S	S	S
CPP 0.5 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CPP 1.0 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

mOsm = milliosmolar
Ma = macroscopically
Mi = microscopically
S = solution
n.d. = not determined

Table 44: Treatment conditions – 2nd Experiment; 24 hours exposure, 24 hours harvest time, without S9 mix

Test groups	pH	Osmolarity mOsm	Solubility			
			Vehicle	Culture medium		
				0 h	22 - 24 h	
			Ma	Ma	Ma	Mi
Negative control	7.6	352	n.d.	n.d.	n.d.	n.d.
7.8 µg/mL	n.d.	n.d.	S	S	S	S
15.6 µg/mL	n.d.	n.d.	S	S	S	S
31.3 µg/mL	n.d.	n.d.	S	S	S	S
62.5 µg/mL	n.d.	n.d.	S	S	S	S
125.0 µg/mL	n.d.	n.d.	S	S	S	S
250.0 µg/mL	7.7	381	S	S	S	S
EMS 400.0 µg/mL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

EMS 500.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

mOsm = milliosmolar
Ma = macroscopically
Mi = microscopically
S = solution
n.d. = not determined

Table 45: Treatment conditions – 2nd Experiment; 4 hours exposure, 44 hours harvest time, with S9 mix

Test groups		pH	Osmolarity mOsm	Solubility			
				Vehicle	Culture medium		
					0 h	3 - 4 h	
				Ma	Ma	Ma	Mi
Negative control		7.3	255	n.d.	n.d.	n.d.	n.d.
15.6	**µg/mL**	n.d.	n.d.	S	S	S	S
31.3	**µg/mL**	n.d.	n.d.	S	S	S	S
62.5	**µg/mL**	n.d.	n.d.	S	S	S	S
125.0	**µg/mL**	n.d.	n.d.	S	S	S	S
250.0	**µg/mL**	n.d.	n.d.	S	S	S	S
500.0	**µg/mL**	7.4	256	S	S	S	S
CPP 0.5	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CPP 1.0	**µg/mL**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

mOsm = milliosmolar
Ma = macroscopically
Mi = microscopically
S = solution
n.d. = not determined

Conclusion

- Under the experimental conditions chosen here, the conclusion is drawn that Octadecanoic acid, sulfo-, potassium salt has not the potential to induce micronuclei (clastogenic and/or aneugenic activity) under in vitro conditions in V79 cells in the absence and the presence of metabolic activation.

3.8.2 Animal data

No studies available.

3.8.3 Human data

No studies available.

3.8.4 Other data

No studies available.

3.9 Carcinogenicity

No studies available.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 [Study 1]

Study reference:

NN, 2017b

Detailed study summary and results:

Test type

In a prenatal developmental toxicity study (according to OECD TG 414), pregnant rats were exposed to doses of the test substance (0, 100, 300, or 1,000 mg/kg bw/d) by gavage from day 6 through 20 post-coitum and effects on fertility and sexual function of female rats and malformations (external, visceral, or skeletal) and variations of foetuses were evaluated. GLP compliance is given (certificate not mentioned).

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 9-Octadecenoic acid (Z)-, sulfonated, potassium salts
- Test material form: orange-brown crystalline powder with lumps
- Degree of purity: 100%, UVCB
- Impurities: no information available
- Batch number: 200516GT41

Test animals

- Species/strain/sex: rat/ Wistar Crl:WI(Han) /female
- No. of animals per sex per dose: 22 females/dose group
- Age and weight at the study initiation: 10 - 14 weeks, no information available on weight at study initiation

Administration/exposure

- Route of administration – oral (gavage)
- Duration and frequency of test/exposure period: only females, days 6 to 20 post-coitum, once daily, 7 d/week

- Doses/concentration levels: 0; 100; 300, or 1,000 mg/kg bw/d
- Rationale for dose level selection: based on the results of a dose-ranger finding study with 0, 100, 300 and 1,000 mg/kg bw/d, which did not reveal any relevant adverse effects
- Control group and treatment: yes, treated similar to treatment groups with vehicle
- Historical control data: provided for foetal assessments/examinations, based on a total of 35 studies with Rat: Crl:WI(Han) (outbred, SPF-Quality) from the same source (performed at the same testing facility during 2014-2016)
- Vehicle: water (Elix, Millipore S.A.S., Molsheim, France), 5 mL/kg bw, trial formulations justified choice of vehicle, formulations (w/w) were prepared daily within 5 hours prior to application and homogenized
- Test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation:
    - application volume was 5 mL/kg bw, calculated and adjusted according to the latest body weight
    - analytical verification: performed by a validated method (no further information available), accuracy of prepared concentrations (for formulations: 100, 300 and 1,000 mg/kg bw/d) was shown (i.e. mean accuracies between 90% and 110%), homogeneity of lowest and highest dose was analysed and verified (i.e. coefficient of variation ≤ 10%)
- Statistical treatment of results:
    - for comparison of treated versus control group under assuming variables being distributed normally the Dunnett-test based on a pooled variance estimate was performed; if data does not follow normal distribution the Steel-test was applied
    - frequency data was analysed by Fisher Exact test
    - for comparison of mean litter proportions (percent of litter) of the number of viable and dead foetuses, early and late resorptions, total resorptions, pre- and post-implantation loss, and sex distribution the Mann Whitney test was applied
    - intergroup differences in mean litter proportions (percent per litter) of total foetal malformations and developmental variations (external, visceral and skeletal), and each particular external, visceral and skeletal malformation or variation were determined by Kruskal-Wallis

nonparametric ANOVA test; if a statistically significant ($p < 0.05$) intergroup variance was obtained performing the ANOVA test, subsequently Dunn's test was applied for comparison of treated versus control group

Description of test design:

- Details on mating procedure: females were mated with males (no further information available), observed vaginal plug was regarded as day 0 post-coitum
- Premating exposure period for males and females (P and F1): none
- Dosing schedules: one day after receipt at laboratory presumed pregnant females were randomly (computer-generated random-number sequence adjusted for body weight (varied between ± 20% to ± 35% of the mean per subgroup) assigned to 4 treatment groups, administered from days 6 to 20 post-coitum, once daily
- Standardization of litters: no
- Parameters assessed for P:
    - cage side observations: yes, twice daily
    - detailed clinical observations: yes, daily
    - body weight: yes, days 2, 6, 9, 12, 15, 18, and 21 post-coitum
    - food consumption and compound intake: yes, recorded for days 2-6, 6-9, 9-12, 12-15, 15-18 and 18-21 post-coitum
    - water consumption: yes, subjective appraisal, but no quantitative investigation
    - post-mortem examinations: yes, macroscopic abnormalities were recorded, collected and fixed in 10% buffered formalin (neutral phosphate buffered 4% formaldehyde solution)
    - day 21 post-coitum: ovaries and uterine examinations of all animals: number of corpora lutea, weight of (gravid) uterus, number of implantations (if not macroscopically visible, staining of uterus according to Salewski technique was performed), number of foetuses (alive/dead), number of early/late resorptions (embryo-foetal deaths)

- Parameters assessed for F1:
    - body weight: yes
    - sex: yes, from the ano-genital distance and gonadal inspections (during further foetal examination)
    - external examinations: all alive foetuses per litter
    - soft tissue and skeletal examinations: half of the foetuses (live and dead) in each litter of all groups
    - head examinations: yes, for a half of foetuses (only live foetuses)

Results and discussion

For P:

- Mortality: one female of the high dose group was sacrificed in extremis on day 14 post-coitum; observations revealed: signs of ill health (piloerection, hunched posture, rales and diarrhoea) from day 11 onwards; declined health status, marked body weight loss and reduced food intake from day 9 to 12 post-coitum; day 14 post-coitum further body weight loss, yellow discoloration of the urine, and emaciation. The cause of its bad health status could not be established in this study. However, the yellow discoloration of the urine and tan discoloured pasty caecum-contents may indicate that treatment with the test item was involved. Therefore, a relation to treatment was considered.
- Clinical Signs:
    - single or temporary observations, which were regarded as incidental finding and thus not toxicologically relevant in the registration dossier, included: piloerection and/or hunched posture in females at 100 and 300 mg/kg bw/d, salivation after dosing, scabs on flews and rales in females at 1,000 mg/kg bw/d
- Body weight and weight changes:

  - Body weight gain in females at 1,000 mg/kg bw/d over days 18-21 post-coitum (corrected body weight gain: 6.4% in high dose females in comparison to 11.3% in control females) and body weights (see Tables 3 and 4) in these females on day 21 (92% of controls) were statistically significant decreased

- Food consumption:
  - in females at 1,000 mg/kg bw/d food consumption was statistically significant decreased over days 6 - 9, 9 - 12, and 18 - 21 post-coitum, in the last period was the largest decrease observed (see Tables 5 and 6)
- Gross pathology: no effects observed, apart from
  - one female sacrificed in extremis on day 14 post-coitum showed tan coloured, pasty content in the caecum and emaciation, which noticed in-life as diarrhoea and lean appearance
- Oestrous cycle: no information available
- Duration of gestation: scheduled necropsy at day 21 post-coitum
- Number of implantations, corpora lutea, litter size:
  - two females at 100 mg/kg bw/d and one female at 1,000 mg/kg bw/d were not pregnant
- Number of pre- and post-implantation loss: effects observed, non-treatment-related
  - one female at 1,000 mg/kg bw/d had nine corpora lutea and only three implantation sites, which led to a relatively high mean value and corresponding large standard deviation. However, implantation occurred before the treatment was started, thus the high pre-implantation loss was a random observation.
- Number of dams with abortions, early deliveries, stillbirths, resorptions and/or dead foetuses: no effects observed
- Weight of uterus: mean gravid uterus weight of high dose females was slightly lower (approx. 15%), but not statistically significant; finding can be related to lower foetal body weights
- Number of live births: no effects observed
- Effect levels (given in dissemination database):

- Maternal NOAEL: 300 mg/kg bw/day based on mortality, body weight (gain), and food consumption

The results for maternal animals are presented in Tables 13-16; see also Table 53 for foetal data at necropsy for details on implantations and corpora lutea.

Table 46: Body weights (gram), summary females (F0-generation)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
POST COITUM					
DAYS 2	MEAN	216	217	211	215
	ST.DEV.	15.7	14.1	17.5	20.7
	N	22	20	22	21
DAYS 6	MEAN	233	234	228	233
	ST.DEV.	15.9	15.8	18.2	22.1
	N	22	20	22	21
DAYS 9	MEAN	242	241	236	238
	ST.DEV.	16.4	15.9	21.6	21.2
	N	22	20	22	21
DAYS 12	MEAN	255	256	253	250
	ST.DEV.	16.5	18.0	23.8	24.1
	N	22	20	22	21
DAYS 15	MEAN	270	270	265	265
	ST.DEV.	18.8	18.9	23.6	22.2
	N	22	20	22	20
DAYS 18	MEAN	302	301	295	294
	ST.DEV.	21.5	21.2	27.5	26.1
	N	22	20	22	20
DAYS 21	MEAN	342	339	334	315**
	ST.DEV.	25.0	26.5	31.1	29.9
	N	22	20	22	20

*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

Table 47: Body weight gain (%), summary females (F0-generation)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
POST COITUM					
DAYS 2	MEAN	-7	-7	-8	-7
	ST.DEV.	2.1	1.7	1.5	1.5
	N	22	20	22	21
DAYS 6	MEAN	0	0	0	0
	ST.DEV.	0.0	0.0	0.0	0.0
	N	22	20	22	21
DAYS 9	MEAN	4	3	3	3
	ST.DEV.	1.2	1.3	2.8	3.0
	N	22	20	22	21
DAYS 12	MEAN	9	9	11	8
	ST.DEV.	1.9	2.5	3.8	7.4
	N	22	20	22	21
DAYS 15	MEAN	16	16	16	15
	ST.DEV.	2.4	3.1	2.4	4.4
	N	22	20	22	20
DAYS 18	MEAN	29	29	29	27
	ST.DEV.	3.1	4.4	3.4	5.5
	N	22	20	22	20
DAYS 21	MEAN	47	45	46	36**
	ST.DEV.	5.3	7.3	4.5	7.6
	N	22	20	22	20
DAYS 6-21 (corrected for gravid uterine weight)#	MEAN	11.3	11.6	10.9	6.4**
	ST.DEV.	3.1	4.3	3.4	6.4
	N	22	20	22	20

*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level
Corrected weight gain in percent of weight on Day 6 P.C.

Table 48: Food consumption (g/animal/day), summary females (F0-generation)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
POST COITUM					
DAYS 2-6	MEAN	21	21	21	21
	ST.DEV.	1.7	2.4	1.9	2.2
	N	22	20	22	21
DAYS 6-9	MEAN	20	21	20	18*
	ST.DEV.	1.8	2.2	2.8	2.0
	N	22	20	22	21
DAYS 9-12	MEAN	21	22	21	19*
	ST.DEV.	1.8	2.3	2.6	4.8
	N	22	20	22	21
DAYS 12-15	MEAN	23	23	22	22
	ST.DEV.	1.9	2.4	2.5	2.4
	N	22	20	22	20
DAYS 15-18	MEAN	24	24	23	22
	ST.DEV.	2.3	3.6	2.4	2.1
	N	22	20	22	20
DAYS 18-21	MEAN	24	24	23	20**
	ST.DEV.	2.2	3.1	2.8	3.1
	N	22	20	22	20
MEAN OF MEANS		22	22	22	20

*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

Table 49: Relative food consumption (g/kg body weight/day), summary females (F0-generation)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
POST COITUM					
DAYS 2-6	MEAN	89	89	91	88
	ST.DEV.	7.0	8.0	6.7	7.6
	N	22	20	22	21

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
DAYS 6-9	MEAN	84	87	86	78*
	ST.DEV.	6.2	7.1	7.3	8.5
	N	22	20	22	21
DAYS 9-12	MEAN	83	84	84	75*
	ST.DEV.	5.3	5.5	4.8	18.7
	N	22	20	22	21
DAYS 12-15	MEAN	84	84	83	81
	ST.DEV.	4.7	6.1	6.4	8.0
	N	22	20	22	20
DAYS 15-18	MEAN	78	80	79	75
	ST.DEV.	4.8	7.9	3.7	4.6
	N	22	20	22	20
DAYS 18-21	MEAN	70	70	70	64**
	ST.DEV.	3.8	4.6	4.3	8.5
	N	22	20	22	20
MEAN OF MEANS		81	82	82	77

*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

For F1

- Viability: average number of live and dead foetuses or litter size is not affected by test substance
  - mean litter sizes: 11.6; 11.0; 11.5, and 11.1 viable foetuses/litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively
- Body weight: male, female, and combined foetal body weights were statistically significant decreased at 1,000 mg/kg bw/d
  - mean male foetal body weights: 5.4; 5.5; 5.3, and 4.7 g at 0; 100; 300 and 1,000 mg/kg bw/d, respectively
  - mean female foetal body weights: 5.2; 5.2; 5.1, and 4.4 g at 0; 100; 300 and 1,000 mg/kg bw/d, respectively
- Sex ratio: not effects observed
  - mean sex ratios (males:females): 48:52; 49:51; 50:50, and 57:43 at 0; 100; 300 and 1,000 mg/kg bw/d, respectively

- Variations/malformations (for details see the Tables 7 - 10):
  - external malformations: effects observed, treatment-related
    - one foetus with malrotated limbs at 1,000 mg/kg bw/d observed, which correlated to the skeletal observation of bent limb bones in this foetus. All foetuses skeletally examined of the high dose group had bent limb bones, therefore the single finding of malrotated limbs was considered to be treatment-related
    - single malformations (cleft palate and anasarca) were often observed in this rat strain and thus regarded as incidental finding and not toxicologically relevant
    - foetuses in control group had a smaller jaw (skeletally confirmed)
  - external variations: not effects observed
  - skeletal malformations: effects observed, treatment-related
    - dose related increase in foetuses with bent limb bones, statistically significant at 300 and 1,000 mg/kg bw/d
    - 41 of 128 foetuses at 300 mg/kg bw/d and 111 of 112 foetuses at 1,000 mg/kg bw/d were affected with this skeletal malformation, whereas no such malformations occurred in any of the foetuses at 0; and 100 mg/kg bw/d; mean litter proportions for the skeletal variation of bent limb bones were 0%; 0%; 32.1% and 99.3% per litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively (historical control data: mean: 0.8% ± 1.03 per litter basis, min-max: 0.0% – 4.5%)
    - three foetuses at 1,000 mg/kg bw/d had bent pelvic girdle bones (iliaca), this malformation was not previously seen in historical controls (0% per litter basis)
    - all foetuses with bent limb bones also had bent ribs; dose-related increase in litter incidence for bent ribs is coincidental with bent limb bones; statistical significant at 300 and 1,000 mg/kg bw/d; mean litter proportions for the skeletal variation of bent ribs were 7.7%; 12.2%; 60.7% and 99.3% per litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively (historical control data: mean: 14.3% ± 7.11 per litter basis, min-max: 0.8% – 27.4%)

- dose-related increase in litter incidences with 14th full ribs (historical control data: mean: 6.5% ± 3.87 per litter basis, min-max: 0.0% – 13.1%) and caudal shift of pelvic girdle (historical control data: mean: 6.2% ± 2.98 per litter basis, min-max: 1.7%-13.0%); incidences at 300 and 1,000 mg/kg bw/d were nearby or above their historical control maximum value and considered to be related to treatment; mean litter proportions of these respective variations were 11.3%; 3.3%; 16.4%; 21.2% and 6.1%, 3.8%, 12.0%, 18.9% per litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively
- statistical significant increase in malaligned sternebrae at 1,000 mg/kg bw/d observed; mean litter proportions of slight to moderate malaligned sternebrae were 22.4%; 20.2%; 21.7%; 40.0% per litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively (historical control data: mean: 18% ± 9.11 per litter basis; min-max: 4.4%-43.8%)
- further skeletal malformations were a vertebral centra anomaly in foetus at 1,000 mg/kg bw/d and a rib anomaly, sternoschisis and costal cartilage anomaly in three of control foetuses, but regarded as incidental findings and not toxicologically relevant

- skeletal variations: effects observed, but not dose-related
- visceral malformations: effects observed, not treatment-related
  - visceral variations were observed in 14.0%; 10.2%; 10.0% and 8.2% of foetuses per litter at 0; 100; 300 and 1,000 mg/kg bw/d, respectively
  - all variations occurred in absence of a dose-related trend, infrequently or within the historical control data, due to incidental findings and they were not considered toxicologically relevant
  - two viscerally malformed foetuses were observed; one in each at 300 and 1,000 mg/kg bw/d, respectively
  - at 100 mg/kg bw/d one foetus had an interrupted aortic arch that was accompanied with an atrial septum defect
  - at 200 mg/kg bw/d one foetus had a diaphragmatic hernia and malpositioned right subclavian

- Effect levels:
  - Developmental NOAEL: 100 mg/kg bw/d (based on retardation of male and female foetal body weight at 1,000 mg/kg and dose related increases in the incidence of several skeletal malformations and variations at 300 and 1,000 mg/kg)

Table 50: Summary of litter proportions of malformations % per litter

		0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
NUMBER OF LITTERS EXAMINED		22	20	22	20
TOTAL MALFORMATIONS					
PERCENT PER LITTER WITH EXTERNAL MALFORMATIONS	MEAN S.D.	0.4 1.78	0.0 0.00	0.0 0.00	3.3 11.26
PERCENT PER LITTER WITH SOFT TISSUE MALFORMATIONS	MEAN S.D.	0.0 0.00	0.8 3.73	0.8 3.55	0.0 0.00
PERCENT PER LITTER WITH SKELETAL MALFORMATIONS	MEAN S.D.	2.6 6.66	0.0 0.00	32.1** 35.06	100.0** 0.00
TOTAL PERCENT PER LITTER WITH MALFORMATIONS	MEAN S.D.	3.0 6.74	0.8 3.73	32.9** 34.51	96.9** 11.20
DETAILS ON SKELETAL MALFORMATIONS					
BENT LIMB BONE(S)	MEAN S.D.	0.0 0.00 (0/129)#	0.0 0.00 (0/112)	32.1** 35.06 (41/128)	99.3** 3.19 (111/112)

** = Significantly different from the control group at 0.01

results presented in parentheses: (Foetuses affected / total foetuses examined)

Table 51: Summary of litter proportions of variations % per litter

		0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
NUMBER OF LITTERS EXAMINED		22	20	22	20
TOTAL VARIATIONS					
PERCENT PER LITTER WITH EXTERNAL VARIATIONS	MEAN S.D.	0.0 0.00	0.0 0.00	0.0 0.00	0.0 0.00
PERCENT PER LITTER WITH SOFT TISSUE VARIATIONS	MEAN S.D.	14.0 13.95	10.2 14.81	10.0 12.13	8.2 22.58
PERCENT PER LITTER WITH SKELETAL VARIATIONS	MEAN S.D.	74.9 21.34	77.7 21.86	92.5** 10.71	100.0** 0.00
TOTAL PERCENT PER LITTER WITH	MEAN	88.3	87.9	102.5	108.2*

		0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
VARIATIONS	S.D.	26.02	26.53	13.28	22.58

* = Significantly different from the control group at 0.05
** = Significantly different from the control group at 0.01

Table 52: Summary of variations number of litters affected (number of foetuses affected/number of foetuses examined)

	0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
NUMBER OF LITTERS EXAMINED	22	20	22	20
DETAILS ON SKELETAL VARIATIONS				
14TH RUDIMENTARY RIB(S)	21 (62/129)	20 (70/112)	21 (75/128)	19 (64/112)
STERNEBRA(E) MALALIGNED (SLIGHT OR MODERATE)	18 (27/129)	15 (23/112)	16 (27/128)	18 (41/112)
BENT RIB(S)	7 (10/129)	9 (15/112)	21 (76/128)	20 (111/112)
14TH FULL RIB(S)	8 (14/129)	4 (4/112)	11 (21/128)	11 (25/112)
PELVIC GIRDLE- CAUDAL SHIFT	4 (7/129)	3 (5/112)	9 (15/128)	12 (22/112)

* = Significantly different from the control group at 0.05
** = Significantly different from the control group at 0.01

Table 53: Summary of foetal data at scheduled necropsy

mg/kg bw/d		Sex		Viable Foetuses	Dead Foetuses	Resorptions		Post Implantation Loss	Implantation Sites	Corpora Lutea	Pre Implantation Loss	Foetal Weights in Grams	No. of Gravid Females
		M	F			EARLY	LATE						
0	TOTAL	125	130	255	0	9	1	10	265	277	12	NA	
	MEAN	5.7	5.9	11.6	0.0	0.4	0.0	0.5	12.0	12.6	0.5	5.3	22
	S.D.	2.21	1.77	2.11	0.00	0.59	0.21	0.67	1.94	1.71	0.96	0.29	

mg/kg bw/d		Sex		Viable Foetuses	Dead Foetuses	Resorptions		Post Implantation Loss	Implantation Sites	Corpora Lutea	Pre Implantation Loss	Foetal Weights in Grams	No. of Gravid Females
		M	F			EARLY	LATE						
100	TOTAL	110	110	220	0	22	0	22	242	253	11	NA	
	MEAN	5.5	5.5	11.0	0.0	1.1	0.0	1.1	12.1	12.7	0.6	5.3	20
	S.D.	1.91	1.54	1.81	0.00	1.21	0.00	1.21	1.41	1.18	0.76	0.26	
300	TOTAL	126	128	254	0	15	0	15	269	277	8	NA	
	MEAN	5.7	5.8	11.5	0.0	0.7	0.0	0.7	12.2	12.6	0.4	5.2	22
	S.D.	1.64	2.24	1.74	0.00	0.95	0.00	0.95	1.6	1.74	0.58	0.23	
1,000	TOTAL	127	95	222	0	8	1	9	231	254	23	NA	
	MEAN	6.4	4.8	11.1	0.0	0.4	0.1	0.5	11.6	12.7	1.1	4.6**	20
	S.D.	2.08	2.10	2.83	0.00	0.68	0.22	0.69	2.82	1.75	1.50	0.32	

** = Significantly different from the control group at 0.01
NA = NOT APPLICABLE

3.10.1.2 [Study 2]

Study reference:

NN, 2015c

Detailed study summary and results:

Test type

In a reproduction/developmental toxicity screening study (according to OECD TG 422), males and females rats were exposed to doses of the test substance (0, 96, 289 and 963 mg/kg bw/d corresponding to 0, 50, 150, and 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt) by gavage. The duration of treatment covered a 2 week premating period and mating in both sexes (mating pairs were from the same dose group) as well as entire gestation and 4 days of lactation period in females up to one day prior to the day of schedule sacrifice of the animals. Parental animals were examined for their mating and reproductive performances, and effects on fertility and variations of foetuses were evaluated. GLP compliance is given (certificate mentioned).

Test substance

- Test material used in the study: Octadecanoic acid, sulfo-, potassium salt (CAS 67968-63-2);
  Synonyms as presented in study report: Agnique® OAS 50 K; Ölsäuresulfonat,KSalz; Octadecanoic acid, sulfo-, potassium salt; 9(or 10)-Sulphooctadecanoic acid, potassium salt; Disponil® OSS 50 KS
- Test material form: liquid, brownish clear
- Degree of purity: 100% minus water content and potassium sulphate = 51.92%
- Impurities: no information available
- Batch number: 0012127444
- Expiration date: 22 Jun 2016

Test animals

- Species/strain/sex: rat/ Wistar Crl:WI(Han) males/females
- No. of animals per sex per dose: 10/sex/dose group
- Age and weight at the study initiation: 11 - 12 weeks. At study initiation (Day 0) males weight from 296.8 to 323.2g and females from 198.9 to 237.11g.

Administration/exposure

- Route of administration – oral (gavage)
- Duration and frequency of test/exposure period: The duration of treatment covered a 2 week premating period and mating in both sexes (mating pairs were from the same dose group) as well as entire gestation and 4 days of lactation period in females up to one day prior to the day of schedule sacrifice of the animals, 7 d/week

- Doses/concentration levels: 0, 96, 289 and 963 mg/kg bw/d of the test item corresponding to 0, 50, 150, and 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt
- Rationale for dose level selection: range finding experiment at 1000 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt in male and female Wistar rat by oral exposure caused moribund state to the animals within 4 days.
- Control group and treatment: yes, treated similar to treatment groups with vehicle
- Historical control data: for clinical pathology testing and for reproduction toxicity data
- Vehicle: “drinking water”, 10 mL/kg bw, trial formulations justified choice of vehicle and homogeneity, formulations were prepared once daily up to study Day 15 and afterwards once a week.
- Test substance formulation, achieved concentration, stability and homogeneity of the preparation:
    - application volume was 10 mL/kg bw, calculated and adjusted according to the latest body weight
- Analytical verification: performed by a validated method (no further information available), accuracy of prepared concentrations (for formulations: 96 and 963 mg/kg bw/d) was shown (no further information available) as well as homogeneity of lowest and highest dose was analysed and verified (no further information available)
- Statistical treatment of results:
    - The food consumption (parental animals), body weight and body weight change (parental animals and pups; for the pup weights, the litter means were used), gestation days were analysed by a simultaneous comparison of all dose groups with the control group using the DUNNETT test (two-sided) for the hypothesis of equal means.
    - The male and female mating indices, male and female fertility indices, gestation index, females delivering, females with liveborn pups, females with stillborn pups, females with all stillborn pups, female pregnant were analysed by a pair-wise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions.

- The Mating days until day 0 pc, % post-implantation loss, pups stillborn, %perinatal loss were analysed by a pair-wise comparison of the dose group with the control group using the WILCOXON test (one-sided+) with BONFERRONI-HOLM adjustment for the hypothesis of equal medians.
- The implantation sites, pups delivered, pups liveborn, live pups day x, viability Index were analysed by a pair-wise comparison of the dose group with the control group using the WILCOXON test (one-sided-) with BONFERRONIHOLM adjustment for the hypothesis of equal medians.
- The % live male day x, % live female day x were analysed by a comparison of the dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
- The rearing, grip strength of forelimbs and hindlimbs, landing foot-splay test, motor activity were analysed by a non-parametric one-way analysis using KRUSKALWALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair-wise comparison of each dose group with the control group was performed using WILCOXON test (two-sided) for the hypothesis of equal medians.
- The blood parameters were analysed as followed:
  - For parameters with bidirectional changes: Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians.
  - For parameters with unidirectional changes: Pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) with Bonferroni-Holm adjustment for the hypothesis of equal medians.
- Urinalysis parameters (apart from pH, urine volume, specific gravity, colour and turbidity) were analysed by a pairwise comparison of each dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians.
- Urine pH, volume, specific gravity, colour and turbidity were analysed by Non-parametric one-way analysis using KRUSKAL-WALLIS test. If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the hypothesis of equal medians. Urine colour and turbidity are not evaluated statistically.

- In pathology, the weight parameters were analysed by a Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON-test (two-sided) for the equal medians.

Description of test design:

- Details on mating procedure: each of the male and female animals was mated overnight in a 1:1 ratio for a maximum of 2 weeks. Throughout the mating period, each female animal was paired with a predetermined male animal from the same test group. Observed vaginal plug with sperm was regarded as day 0 post-coitum.
- Premating exposure period for males and females (P and F1): the test substance was administered orally via gavage to the F0 generation parental animals, daily at the same time in the morning. The treatment lasted up to one day prior to sacrifice.
- Dosing schedules: Prior to the first detailed clinical observation, the animals were distributed according to weight among the individual test groups, separated by sex with a computer-generated random-number sequence adjusted for body weight (variation did not exceed 20%). Oral administration daily at the same time in the morning up to one day prior sacrifice.
- Standardization of litters: no
- Parameters assessed for P:
  - cage side observations: yes, twice daily
  - detailed clinical observations: Weekly starting on Day 1
  - body weight: weekly in the morning. During gestation and lactation, F0 females were weighed on gestation days (GD) 0, 7, 14 and 20, and on postnatal days (PND) 0 and 4.
  - food consumption and compound intake: yes, recorded weekly; males during 2 weeks of premating and females before and after the mating period as well as in dams during gestation and lactation.
  - water consumption: yes, no quantitative investigation

- Functional observational battery (FOB): first five surviving male animals per test group and the first five surviving females with litter (in order of delivery) of all test groups at the end of the administration period. Home cage observation, open field observation, and sensory motor tests/reflexes.
- Motor activity assessment: on the same day as the FOB was performed in the first five parental males and the first five surviving females with litter (in order of delivery) per group. The examinations were performed using the TSE Labmaster System supplied by TSE Systems GmbH, Bad Homburg, Germany
- Male/female reproduction data and female delivery data: male/female mating indexes and male/female fertility indexes were calculated. Gestation index, live birth index were calculated for the pregnant females.

- Parameters assessed for F1:
  - Pups number: determine the total number of pups, the sex and the number of liveborn and stillborn pups in each litter. Pups, which died before this initial examination, were defined as stillborn pups.
  - Body weight: yes, pups were weighed on PND 1 and on PND 4.
  - Sex: yes, on PND 0.
  - Sex ratio: sex ratio was calculated at Day 0 and Day 4 after birth.
  - External examinations: all foetuses per litter at birth
  - Mortality: twice daily except on weekends and public holidays.
  - Viability: viability index was calculated on day of birth and lactation Day 4.
  - Soft tissue and skeletal examinations: macroscopic evaluation of the organs. If notable abnormalities, animals were evaluated on a case-by-case basis.
- Clinical pathology:

- Methods: blood was taken from the retro-bulbar venous plexus from fasted animals. The animals were anaesthetized using isoflurane. Randomized sequence. For urinalysis, metabolic cages were used overnight (withdrawal of food and water). Evaluation of urine samples in a randomized sequence. Examination performed on the first 5 surviving parental males and the first 5 surviving females with litter (in order of delivery) per group.
- Haematology: Leukocyte count (WBC), Erythrocyte count (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Platelet count (PLT), Differential blood count, and Reticulocytes (RET).
- Coagulation: Prothrombin time (Hepato Quick's test) (HQT)
- Clinical chemistry: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), γ-Glutamyltransferase (GGT), Sodium (NA), Potassium (K), Chloride (CL), Inorganic phosphate (INP), Calcium (CA), Urea (UREA), Creatinine (CREA), Glucose (GLUC), Total bilirubin (TBIL), Total protein (TPROT), Albumin (ALB), Globulins (GLOB), Triglycerides (TRIG), Cholesterol (CHOL), and Bile acids (TBA).
- Urinalysis: pH, Protein (PRO), Glucose (GLU), Urobilinogen (UBG), Bilirubin (BIL), Specific gravity (SP.GR.), Sediment, Colour/turbidity (COL, TURB), and Volume (VOL).

- Pathology:
  - Methods: all parental animals were sacrificed by decapitation under isoflurane anaesthesia. Necropsy was performed after exsanguination.
  - Organ weights:
    - All males animals: epididymides and testes
    - First 5 animals/sex/group: adrenal glands, brain, heart, kidneys, liver, spleen, and thymus
  - Organ/tissue fixation: all other organs/tissues and gross lesion were fixated.
- Histopathology
  - All gross lesions were examined in all groups

- Fixated organs were examined in group 0 and 3, depending on the organ either all animals of the first 5 animals/sex/group

Results and discussion

- The term "significant" implies that the inter-group differences have attained statistical significance ($p \leq 0.05$) when compared with the control group.

Octadecanoic acid, sulfo-, potassium formulation in drinking water:

- Homogeneity: demonstrated
- Concentration: were in range of 97 to 100% of nominal concentration.
- Determination of potassium in samples:

Table 54: Determination of potassium in samples

Sample No.	Date of sample preparation	Date of sampling	Content of K (g/100 g)	Content of K[1] (g/100 mL)	Calculated content of test item[1,2] (g/100 mL)	Expected content of test item (g/100 mL)	Recovery (%)
1 (test item)	-	-	7.1	-	-	-	-
2 (vehicle)	-	-	<0.001	-	-	-	-
3	Feb 09, 2015	Feb 09, 2015	0.070	0.070	0.98	1.0	98
4	Feb 09, 2015	Feb 09, 2015	0.070	0.070	0.98	1.0	98
5	Feb 09, 2015	Feb 09, 2015	0.071	0.071	0.99	1.0	99
6	Feb 09, 2015	Feb 09, 2015	0.21	0.21	2.99	3.0	100
7	Feb 09, 2015	Feb 09, 2015	0.69	0.69	9.75	10.0	97
8	Feb 09, 2015	Feb 09, 2015	0.69	0.69	9.74	10.0	97
9	Feb 09, 2015	Feb 09, 2015	0.69	0.69	9.70	10.0	97

[1] Calculated with the given sample weights and the g yen volume of 3 ml.
[2] Calculations based on the determined K content of the samples and the pure test item (exact content of K = 7.128 g/100 g), calculated with not rounded values

Clinical observations for P:

- Mortality: no mortality
- Clinical observations
  - Group 3 (963 mg/kg bw/d), males and females, except gestation and lactation periods (see further below):
    - Slight to severe salivation after treatment had been observed intermittent during mating and post-mating in 8 males and during mating in 2 females. Finding considered to be related to treatment, most likely caused by bad taste of the test compound, and assessed as being not adverse.
    - Piloerection had been seen in one male during mating on a single day (day 3 of mating). This isolated finding was assessed as spontaneous in nature and not treatment related.
    - On one or two days of post-mating (day 2 and/or 3 of post-mating) soft faeces were seen in 7 males of the test groups 3 (963 mg/kg bw/d). This soft faeces was regarded to be test substance related but not as adverse because of its short duration.
  - Females during gestation
    - Group 1 (96 mg/kg bw/day):
      - One female (No. 117) did not deliver pups until the day of necropsy (Day 43). On days 38 to 41 this female showed a distended abdomen, piloerection, and vaginal discharge.
    - Group 2 (289 mg/kg bw/day):
      - One female (No. 127) did not deliver pups until the day of necropsy (Day 43). No abnormal findings in clinical observations.
    - Group 3 (963 mg/kg bw/d):
      - One female (No. 132) did not deliver pups until the day of necropsy (Day 43). No abnormal findings in clinical observations. Because no dose dependency had been determined for the females being unable to deliver with or without further clinical signs, these findings were considered to be incidental and not treatment related.
      - One female (No. 136) did show piloerection and a palpable mass in the abdomen, on day 23 the last day before giving birth.

- One female (No. 137) showed soft faeces on one study day (day 16 of gestation). This soft faeces was regarded to be test substance related but not as adverse because of its short duration.
- Slight to severe intermittent salivation was observed in 9 animals. The findings were considered to be related to treatment, most likely caused by bad taste of the test compound, and therefore assessed as being not adverse.

- Females during lactation:
  - Group 3 (963 mg/kg bw/d):
    - One female (No. 136) did show piloerection on the first day and a palpable mass in the abdomen over the first 4 days. No corresponding macroscopic finding was observed in necropsy to confirm this observation. Further two females showed piloerection on a single day (lactation day 1). The piloerection was considered to be treatment related.
    - Slight to severe intermittent salivation was observed in 8 animals. This finding was considered to be related to treatment, most likely caused by bad taste of the test compound, and assessed as being not adverse.

Table 55: Clinical observation, summary males and females (F0-generation), Pre-mating phase

			Test Group 0 0 mg/kg bw/d		**Test Group 1 96 mg/kg bw/d**		**Test Group 2 289 mg/kg bw/d**		**Test Group 3 963 mg/kg bw/d**	
			Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
day 0 -> day 13	Animal examined	N	10	10	10	10	10	10	10	10
	Normal NAD[a]	N	10	10	10	10	10	10	10	10

[a] Nothing abnormal detected.

Table 56: Clinical observation, summary males and females (F0-generation), Mating phase

			Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
			Males	Females	Males	Females	Males	Females	Males	Females
day 1 -> day 14	Animal examined	N	10	10	10	10	10	10	10	10
	Animals with signs	N	0	0	0	0	0	0	8	2
	Head Salivation	N	0	0	0	0	0	0	8	2
	Fur piloerection	N	0	-	0	-	0	-	1	-
	Normal NAD[a]	N	10	10	10	10	10	10	10	10

[a] Nothing abnormal detected.

Table 57: Clinical observation, summary males (F0-generation), Post-mating phase

			Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
day 1 -> day 16	Animal examined	N	10	10	10	10
	Animals with signs	N	0	0	0	10
	Dead Sacrificed scheduled	N	10	10	10	10
	Faeces softs faeces	N	0	0	0	7

	Head salivation	N	0	0	0	8
	Normal NAD[a]	N	10	10	10	10

[a] Nothing abnormal detected.

Table 58: Clinical observation, summary females (F0-generation), Gestation

			Test Group 0 0 mg/kg bw/d	**Test Group 1 96 mg/kg bw/d**	**Test Group 2 289 mg/kg bw/d**	**Test Group 3 963 mg/kg bw/d**
day 0 -> day 43	Animal examined	N	9	10	10	10
	Animals with signs	N	0	1	0	9
	Animal body Abdomen distended	N	0	1	0	0
	Dead Sacrificed scheduled	N	0	1	1	1
	Faeces Soft faeces	N	0	0	0	1
	Genitals Vaginal discharge	N	0	1	0	0
	Head Salivation	N	0	0	0	9
	Mass Palpable in abdomen	N	0	0	0	1
	Fur Piloerection	N	0	1	0	1
	Normal NAD[a]	N	9	10	10	10

[a] Nothing abnormal detected.

Table 59: Clinical observation, summary females (F0-generation), Lactation

			Test Group 0 0 mg/kg bw/d	**Test Group 1 96 mg/kg bw/d**	**Test Group 2 289 mg/kg bw/d**	**Test Group 3 963 mg/kg bw/d**
day 0 -> day 22	Animal examined	N	10	9	9	9
	Animals with signs	N	0	0	0	9
	Dead Sacrificed scheduled	N	10	9	9	9
	Head Salivation	N	0	0	0	8
	Mass Palpable in abdomen	N	0	0	0	1
	Fur Piloerection	N	0	0	0	3
	Normal NAD[a]	N	10	9	9	9

[a] Nothing abnormal detected.

Food consumption:

- No significant changes in males during pre-mating
- Significant decrease in females of test group 3 (963 mg/kg bw/d, 9%) during pre-mating between Day 0 and 7.
- Significant decrease in females of test group 3 (963 mg/kg bw/d, 20%) during gestation between Day 14 and 20.
- Decrease of food consumption in test group 3 (963 mg/kg bw/d, 10.5%) during lactation but it was not significant.

- The observed significant decreased food consumption in females was assessed to be treatment related.

Table 60: Food consumption (gram), summary males and females (F0-generation), Pre-mating phase

		Test Group 0/ M 0 mg/kg bw/d		Test Group 1/ M 96 mg/kg bw/d		Test Group 2/ M 289 mg/kg bw/d		Test Group 3/ M 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
d 0 -> 7	**Mean [g]**	21.5 n	15.8 n	22.4	16.1	20.6	15.6	21.3	14.3 *
	S.d.	1.2	1.2	1.3	1.3	1.8	0.7	1.3	1.0
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			4.1	2.4	-4.4	-1.2	-0.9	-9.3
d 7 -> 13	**Mean [g]**	22.0 n	16.5 n	22.6	17.0	21.4	16.5	22.1	15.5
	S.d.	1.0	0.9	1.4	1.1	1.3	1.1	1.4	1.3
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			2.8	2.8	-2.8	0.0	0.6	-6.1
d 0 -> 13	**Mean [g]**	21.7 n	16.1 n	22.5	16.6	21.0	16.0	21.7	14.9 *
	S.d.	1.0	1.0	1.3	1.2	1.3	0.8	1.1	1.0
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			3.4	2.9	-3.6	-0.5	-0.2	-7.8

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics d = day; n=DUNNETT

Water consumption:

- No test substance-related changes were observed.

Body weight:

- No statistical difference in males and females during pre-mating, mating, post-mating and gestation.

- Significant lower body weight of males in test group 2 (289 mg/kg bw/d; 4% on day 7 of post-mating) was an isolated finding and was assessed as incidental and not treatment related.
- Significant decrease of body weight of females in test group 3 (963 mg/kg bw/d) on lactation day 0 and 4 (10% and 8%, respectively). This finding was assessed as treatment related and adverse.

Table 61: Body weights (gram), summary males and females (F0-generation), Pre-mating phase

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
day 0	Mean	308.9 n	217.3 n	306.2	215.1	305.4	218.2	307.9	215.1
	S.d.	7.9	9.5	9.6	10.5	6.0	7.3	7.7	8.3
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			-0.9	-1.0	-1.1	0.4	-0.3	-1.0
day 7	Mean	326.9 n	221.1 n	327.6	217.9	319.3	218.2	323.8	215.6
	S.d.	9.7	7.8	10.1	13.4	10.6	9.8	10.2	8.9
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			0.2	-1.4	-2.3	-1.3	-0.9	-2.5
day 13	Mean	342.2 n	227.8 n	343.1	227.3	332.9	225.3	338.4	221.6
	S.d.	10.4	7.5	11.1	10.3	10.6	7.8	10.3	10.0
	N	10	10	10	10	10	10	10	10
	Deviation Vs Control			0.3	-0.2	-2.7	-1.1	-1.1	-2.7

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Table 62: Body weights (gram), summary males (F0-generation), Post-mating phase

		Test Group 0/ M 0 mg/kg bw/d	Test Group 1/ M 96 mg/kg bw/d	Test Group 2/ M 289 mg/kg bw/d	Test Group 3/ M 963 mg/kg bw/d
day 0	**Mean**	363.7 n	368.5	352.0	362.1
	S.d.	10.6	11.4	12.4	14.5
	N	10	10	10	10
	Deviation Vs Control		1.3	-3.2	-0.4
day 7	**Mean**	373.8 n	375.9	358.0 *	365.9
	S.d.	10.7	12.8	14.8	17.3
	N	10	10	10	10
	Deviation Vs Control		0.5	-4.2	-2.1
day 14	**Mean**	379.2 n	382.0	365.0	373.6
	S.d.	10.1	13.5	17.2	19.9
	N	10	10	10	10
	Deviation Vs Control		0.8	-3.7	-1.5

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Table 63: Body weights (gram), summary females (F0-generation), Gestation phase

		Test Group 0/ F 0 mg/kg bw/d	Test Group 1/ F 96 mg/kg bw/d	Test Group 2/ F 289 mg/kg bw/d	Test Group 3/ F 963 mg/kg bw/d
day 0	**Mean**	231.9 n	227.0	227.0	228.4
	S.d.	14.8	13.2	7.0	13.7
	N	9	10	10	10
	Deviation Vs Control		-2.1	-2.1	-1.5
day 7	**Mean**	261.6 n	258.7	257.7	256.6
	S.d.	13.8	17.5	10.5	9.7
	N	9	10	10	10
	Deviation Vs Control		-1.1	-1.5	-1.9
day 14	**Mean**	287.9 n	285.6	283.2	284.0

	S.d.	10.0	25.0	10.5	10.8
	N	9	10	10	10
	Deviation Vs Control		-0.8	-1.6	-1.4
day 20	**Mean**	345.3 n	347.1	338.8	322.9
	S.d.	21.5	38.1	29.9	28.0
	N	9	10	10	10
	Deviation Vs Control		0.5	-1.9	-6.5

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Table 64: Body weights (gram), summary females (F0-generation), Lactation phase

		Test Group 0/ F 0 mg/kg bw/d	**Test Group 1/ F 96 mg/kg bw/d**	**Test Group 2/ F 289 mg/kg bw/d**	**Test Group 3/ F 963 mg/kg bw/d**
day 0	**Mean**	269.3 n	266.0	256.5	243.8 *
	S.d.	14.6	24.9	13.4	19.2
	N	10	9	9	9
	Deviation Vs Control		-1.2	-4.7	-9.5
day 4	**Mean**	275.2 n	278.4	270.2	252.9 *
	S.d.	13.3	17.7	14.0	19.0
	N	10	9	9	9
	Deviation Vs Control		1.1	-1.8	-8.1

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Detailed clinical observation:

- Performed on Days 0, 7, 14, 21, 28, 35, and 42 in males and females and on Days 49 and 56. No abnormalities were found.

Table 65: Detailed clinical observation, summary males and females (F0-generation), in-life

	Test Group 0	**Test Group 1**	**Test Group 2**	**Test Group 3**

			0 mg/kg bw/d		**96 mg/kg bw/d**		**289 mg/kg bw/d**		**963 mg/kg bw/d**	
			Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
day 0 -> day 42	Animal examined	N	10	10	10	10	10	10	10	10
	Normal NAD	N	10	10	10	10	10	10	10	10

Functional observational battery:

- Deviations from “zero values” were obtained in several rats. As no dose-response was observed and the occurrence was in single rats, this was considered incidental.

Open field observations:

- Soft faeces were seen in 1 males of the test groups 3 (963 mg/kg bw/d), a finding known form general clinical observations. This soft faeces was regarded to be test substance related but not adverse. No further test substance-related effects were observed neither in males nor in females.

Sensorimotor tests/reflexes

- No test substance-related effects were observed.

Quantitative Parameters (rearing, GS F, GS H, and FST)

- No test substance-related effects were observed.

Table 66: Functional observational battery, summary of results males and females

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
HOME CAGE OBSERVATION									
posture									
animal is sitting or laying	0	1	0	2	1	1	1	1	2
animal is standing and moving	1	4	5	3	4	4	4	4	3
squatting posture	2	0	0	0	0	0	0	0	0
abdominal position	3	0	0	0	0	0	0	0	0
abdominal position with splayed limbs	4	0	0	0	0	0	0	0	0
lateral position	5	0	0	0	0	0	0	0	0
oblique head posture	6	0	0	0	0	0	0	0	0
opisthotonus	7	0	0	0	0	0	0	0	0
tremors									
no tremors	0	5	5	5	5	5	5	5	5
slight tremors	1	0	0	0	0	0	0	0	0
moderate tremors	2	0	0	0	0	0	0	0	0
severe tremors	3	0	0	0	0	0	0	0	0
convulsions									
no convulsions	0	5	5	5	5	5	5	5	5
slight convulsions	1	0	0	0	0	0	0	0	0
moderate convulsions	2	0	0	0	0	0	0	0	0
severe convulsions	3	0	0	0	0	0	0	0	0
abnormal movements									

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
no abnormalities	0	5	5	5	5	5	5	5	5
manege movements	1	0	0	0	0	0	0	0	0
head shaking	2	0	0	0	0	0	0	0	0
excessive cleaning	3	0	0	0	0	0	0	0	0
frequent chewing	4	0	0	0	0	0	0	0	0
gait									
animal is not walking during observation	0	1	0	2	1	3	3	3	4
no impairment of gait	1	4	5	3	4	2	2	2	1
stiff gait	2	0	0	0	0	0	0	0	0
slight impairment of coordination, unsteady gait	3	0	0	0	0	0	0	0	0
moderate impairment of coordination, shuffling gait	4	0	0	0	0	0	0	0	0
severe impairment of coordination, dragging of the hindlimbs	5	0	0	0	0	0	0	0	0
severe impairment of coordination, with splayed limbs	6	0	0	0	0	0	0	0	0
animal is unable to walk (abdominal or lateral position)	7	0	0	0	0	0	0	0	0
other findings	0	5	5	5	5	5	5	5	5
OPEN FIELD OBSERVATIONS									
behaviour on removal from the cage									
animal is tense, but it shows no resistance against handling	0	5	5	5	5	5	5	5	5
animal shows a slight resistance against the handling	1	0	0	0	0	0	0	0	0
animal shows no resistance against the handling but appears indifferent	2	0	0	0	0	0	0	0	0
animal is difficult to handle, it shows aggressiveness	3	0	0	0	0	0	0	0	0
animal is very difficult to handle, it shows severe aggressiveness	4	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
fur									
nothing abnormal detected	0	5	5	5	5	5	5	5	5
discolored fur	1	0	0	0	0	0	0	0	0
urine staining of anogenital region	2	0	0	0	0	0	0	0	0
piloerection	3	0	0	0	0	0	0	0	0
alopecia	4	0	0	0	0	0	0	0	0
reduced care on fur	5	0	0	0	0	0	0	0	0
skin									
nothing abnormal detected	0	5	5	5	5	5	5	5	5
discolored skin	1	0	0	0	0	0	0	0	0
reddening	2	0	0	0	0	0	0	0	0
paleness	3	0	0	0	0	0	0	0	0
dehydration (exsiccosis)	4	0	0	0	0	0	0	0	0
hypothermia (skin is cold during handling)	5	0	0	0	0	0	0	0	0
lesion(s)	6	0	0	0	0	0	0	0	0
crust(s)	7	0	0	0	0	0	0	0	0
salivation									
no salivation	0	5	5	5	5	5	5	5	5
slight salivation (area around the mouth is moist)	1	0	0	0	0	0	0	0	0
moderate salivation (wet mouth)	2	0	0	0	0	0	0	0	0
severe salivation (mouth very wet, wet paws)	3	0	0	0	0	0	0	0	0
nasal discharge									

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
no discharge, dry nose	0	5	5	5	5	5	5	5	5
clear discharge	1	0	0	0	0	0	0	0	0
reddish discharge	2	0	0	0	0	0	0	0	0
lacrimation									
no lacrimation	0	5	5	5	5	5	5	5	5
slight lacrimation	1	0	0	0	0	0	0	0	0
moderate lacrimation	2	0	0	0	0	0	0	0	0
severe lacrimation	3	0	0	0	0	0	0	0	0
eyes/pupil size									
nothing abnormal detected, pupils contracted at room light	0	5	5	5	5	5	5	5	5
chromodacryorrhea	1	0	0	0	0	0	0	0	0
exophthalmos	2	0	0	0	0	0	0	0	0
pupils dilated	3	0	0	0	0	0	0	0	0
abnormal shape of pupils	4	0	0	0	0	0	0	0	0
oblique eye posture	5	0	0	0	0	0	0	0	0
opacity	6	0	0	0	0	0	0	0	0
cataract	7	0	0	0	0	0	0	0	0
posture									
animal is sitting or laying	0	0	0	0	0	0	0	0	0
animal is standing and moving	1	5	5	5	5	5	5	5	5
squatting posture	2	0	0	0	0	0	0	0	0
abdominal position	3	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
abdominal position with splayed limbs	4	0	0	0	0	0	0	0	0
lateral position	5	0	0	0	0	0	0	0	0
oblique head posture	6	0	0	0	0	0	0	0	0
opisthotonus	7	0	0	0	0	0	0	0	0
palpebral closure									
nothing abnormal detected	0	5	5	5	5	5	5	5	5
eyelid(s) slight closure	1	0	0	0	0	0	0	0	0
eyelid(s) half closure	2	0	0	0	0	0	0	0	0
eyelid(s) permanent closure	3	0	0	0	0	0	0	0	0
respiration									
nothing abnormal detected	0	5	5	5	5	5	5	5	5
respiration labored	1	0	0	0	0	0	0	0	0
gasping/respiratory sounds	2	0	0	0	0	0	0	0	0
respiration accelerated	3	0	0	0	0	0	0	0	0
respiration irregular	4	0	0	0	0	0	0	0	0
tremors									
no tremors	0	5	5	5	5	5	5	5	5
slight tremors	1	0	0	0	0	0	0	0	0
moderate tremors	2	0	0	0	0	0	0	0	0
severe tremors	3	0	0	0	0	0	0	0	0
convulsions									
no convulsions	0	5	5	5	5	5	5	5	5
slight convulsions	1	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
moderate convulsions	2	0	0	0	0	0	0	0	0
severe convulsions	3	0	0	0	0	0	0	0	0
abnormal movements/stereotypics									
no abnormalities	0	5	5	5	5	5	5	5	5
manege movements	1	0	0	0	0	0	0	0	0
head shaking	2	0	0	0	0	0	0	0	0
excessive cleaning	3	0	0	0	0	0	0	0	0
frequent chewing	4	0	0	0	0	0	0	0	0
gait									
animal is not walking during observation	0	0	0	0	0	0	0	0	0
no impairment of gait	1	5	5	5	5	5	5	5	5
stiff gait	2	0	0	0	0	0	0	0	0
slight impairment of coordination, unsteady gait	3	0	0	0	0	0	0	0	0
moderate impairment of coordination, shuffling gait	4	0	0	0	0	0	0	0	0
severe impairment of coordination, dragging of the hindlimbs	5	0	0	0	0	0	0	0	0
severe impairment of coordination, with splayed limbs	6	0	0	0	0	0	0	0	0
animal is unable to walk (abdominal or lateral position)	7	0	0	0	0	0	0	0	0
activity/arousal level									
normal exploration of the area	0	5	5	5	5	5	5	5	5
reduced exploration of the area	1	0	0	0	0	0	0	0	0
severe reduced exploration of the area, animal apathetic	2	0	0	0	0	0	0	0	0
increased exploration of the area, sudden or jerky movements	3	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
hyperactivity	4	0	0	0	0	0	0	0	0
faeces									
no defecation during observation period	0	5	5	5	4	5	5	5	5
feces without abnormalities	1	0	0	0	0	0	0	0	0
discolored feces	2	0	0	0	0	0	0	0	0
crumbly feces	3	0	0	0	0	0	0	0	0
soft feces	4	0	0	0	1	0	0	0	0
muicid feces	5	0	0	0	0	0	0	0	0
diarrhea	6	0	0	0	0	0	0	0	0
urine									
no urination during observation period	0	4	3	1	3	4	4	3	3
urine without abnormalities (some wet areas on the filter paper)	1	1	2	4	2	1	1	2	2
discoloration of urine	2	0	0	0	0	0	0	0	0
polyuria (great wet areas on the filter paper)	3	0	0	0	0	0	0	0	0
other findings	0	0	0	0	0	0	0	0	0
SENSORIMOTOR TESTS/REFLEXES									
approach response									
no reaction	0	5	5	5	5	2	3	4	2
approaching to object	1	0	0	0	0	3	2	1	3
escape reaction	2	0	0	0	0	0	0	0	0
aggressive reaction and attacking of object	3	0	0	0	0	0	0	0	0
touch response									

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
no reaction	0	5	5	5	5	5	5	5	4
orientation to the stimulus	1	0	0	0	0	0	0	0	1
escape after touch	2	0	0	0	0	0	0	0	0
aggressive reaction and attacking of object	3	0	0	0	0	0	0	0	0
reaction to the stimulus but no ability to localize (e.g. turning to wrong side)	4	0	0	0	0	0	0	0	0
vision									
nothing abnormal detected (grasping with forelimbs)	0	5	5	5	5	5	5	5	5
no grasping	1	0	0	0	0	0	0	0	0
pupillary reflex									
nothing abnormal detected, physiological adaptation of the pupil to light	0	5	5	5	5	5	5	5	5
retarded adaptation of the pupil to light	1	0	0	0	0	0	0	0	0
no adaptation of the pupil to light, pupils permanently contracted	2	0	0	0	0	0	0	0	0
no adaptation of the pupil to light, pupils permanently dilated	3	0	0	0	0	0	0	0	0
pinna reflex									
immediate response to the stimulus	0	5	5	5	5	5	5	5	5
no response to the stimulus	1	0	0	0	0	0	0	0	0
audition									
nothing abnormal detected, immediate normal response to the stimulus	0	5	5	5	5	5	5	5	5
no response	1	0	0	0	0	0	0	0	0
increased response	2	0	0	0	0	0	0	0	0
hyperreaction	3	0	0	0	0	0	0	0	0
coordination of movements									

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
nothing abnormal detected, immediate righting response	0	5	5	5	5	5	5	5	5
retarded righting response	1	0	0	0	0	0	0	0	0
fails to turn into upright position, animal stays in lateral position	2	0	0	0	0	0	0	0	0
no righting response, animal stays in dorsal position	3	0	0	0	0	0	0	0	0
behavior during handling									
normal behavior, easy to handle, animal is tense, but it shows no resistance	0	5	5	5	5	5	5	5	5
very easy to handle, animal is limply hanging in the hand	1	0	0	0	0	0	0	0	0
slightly difficult to handle, animal shows a slight resistance against handling	2	0	0	0	0	0	0	0	0
difficult to handle, animal shows a severe resistance against handling	3	0	0	0	0	0	0	0	0
vocalization									
no or only sporadic vocalizations when touched	0	5	5	5	5	5	5	5	5
very frequent vocalizations when touched	1	0	0	0	0	0	0	0	0
vocalizations always when touched	2	0	0	0	0	0	0	0	0
vocalization without touching	3	0	0	0	0	0	0	0	0
pain perception									
nothing abnormal detected, immediate response to the stimulus	0	5	5	5	5	5	5	5	5
weak or retarded reaction to the stimulus	1	0	0	0	0	0	0	0	0
no response to the stimulus	2	0	0	0	0	0	0	0	0
hyperreaction to the stimulus	3	0	0	0	0	0	0	0	0
other findings	0	5	5	5	5	5	5	5	5

Motor activity measurement

- There were no significant deviations concerning the overall motor activity (summation of all intervals) in male and female animals of all test groups in comparison to the concurrent control group.
- The statistically significant decreased number of interrupted beams in interval 3 of females in test group 1 (96 mg/kg bw/d), 2 (289 mg/kg bw/d), and 3 (963 mg/kg bw/d) showed no dose response. Therefore, it was considered spontaneous in nature and not treatment-related.

Table 67: Motor activity Day 30, summary of results males and females (1/2)

		Interv. 1 Beam Interr.		Interv. 2 Beam Interr.		Interv. 3 Beam Interr.		Interv. 4 Beam Interr.		Interv. 5 Beam Interr.		Interv. 6 Beam Interr.		Interv. 7 Beam Interr.	
		Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
0 mg/kg	M	1176.6	1460.6	653.2	828.2	362.0	644.4	165.2	339.6	25.2	343.2	25.6	171.8	26.8	199.6
	SD	203.9	200.6	121.6	172.7	85.6	97.0	126.8	215.0	24.8	168.4	35.6	69.5	24.6	221.4
	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5
96 mg/kg	M	1340.4	1223.2	958.8	616.2	400.0	306.8*	191.8	265.8	112.6	208.4	25.2	174	19.4	54.6
	SD	489.4	538.1	180.7	291.8	177.5	164.1	139.3	127.0	92.3	120.2	18.8	151.9	24.3	46.2
	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5
289 mg/kg	M	1474.2	1403.0	805.2	730.8	470.8	346.4*	200.4	284.2	206	191.4	52.4	105.4	10.4	95.6
	SD	230.9	378.8	213.4	227.1	43	180.9	113.5	138.0	259.8	226.3	47.7	127.7	22.7	47.6
	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5
963 mg/kg	M	1389.4	1152.4	748.8	555.6	605.0	368.4*	202.2	164.8	41.0	243.0	22.4	181.8	37.0	82.0
	SD	289.2	273.6	124.8	156.9	252.0	126.1	77.7	200.4	38.8	166.0	28.1	177.2	26.4	86.9
	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Kruskal-Wallis + Wilcoxon-tests (two-sided): * p<=0.05; ** p<=0.01 (Statistical unit = Animal)

Table 68: Motor activity Day 30, summary of results males and females (2/2)

		Interv. 8 Beam Interr.		Interv. 9 Beam Interr.		Interv. 10 Beam Interr.		Interv. 11 Beam Interr.		Interv. 12 Beam Interr.		Interv. 1-12 Sum Interr.	
		Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
0 mg/kg	M	2.0	159.6	5.4	176.4	18.0	48.4	23.6	35.0	18.8	19.2	2502.4	4426.0
	SD	0.7	190.1	7.6	198.4	19.5	36.8	19.8	37.7	9.9	16.5	361.1	1169.4
	N	5	5	5	5	5	5	5	5	5	5	5	5
96 mg/kg	M	23.0	24.2	9.6	10.0	29.0	60.6	7.4	153.6	8.4	75.8	3125.6	3173.2
	SD	20.3	26	18.3	12.4	50.5	31.7	10.1	176.8	14.5	93.6	794.7	964.0
	N	5	5	5	5	5	5	5	5	5	5	5	5
289 mg/kg	M	38.0	42.2	35.4	26.6	8.6	28.8	6.2	64.2	4.8	92	3312.4	3410.6
	SD	61.9	38.2	32.4	39.9	7.2	40.2	7.7	73.2	4.1	98.3	648.5	660.9
	N	5	5	5	5	5	5	5	5	5	5	5	5
963 mg/kg	M	46.2	79.6	18.8	82.0	31.8	61.4	68.2	34.2	20.4	50.4	3231.2	3055.6
	SD	59.7	134.1	27.1	142.6	39.2	82.9	67.2	31.5	27.3	55.9	378.6	941.9
	N	5	5	5	5	5	5	5	5	5	5	5	5

Kruskal-Wallis + Wilcoxon-tests (two-sided): * p<=0.05; ** p<=0.01 (Statistical unit = Animal)

Male mating index

- The male mating index was 100% in all test groups.

Male fertility index

- Fertility was proven for at least 9 of 10 of the F0 parental males.

- One male of test group 2 (No. 27 mated with female No. 127) and one male in test group 3 (No. 32 mated with female No. 132) did not generate implants in utero.
- The male fertility index was 100% in test groups 0 and 1 and 90% in test groups 1 and 2.

Female mating index

- The female mating index was 100% in all test groups.
- The mean duration until sperm was detected (GD 0) was 3.6, 2.2, 1.9, 4.3 days in test groups 0 - 3. This finding reflected the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within the range of the historical control data.

Female fertility index

- All sperm positive rats got pregnant with one exception in test group 2 and one exception in test group 3.
- The female fertility index was 100% in test groups 0 and 1 and 90% in test groups 2 and 3.
- The mean duration of gestation was similar in all test groups (22.3 days in test group 0; 22.1 days in test groups 1, 22.2 days in test group 2, and 22.6 days in test group 3). These findings reflected the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within or nearby the range of the historical control data.

Gestation index

- The gestation index was 90% in test group 0, and 1 (96 mg/kg bw/d) as well as 100% in test group 2 (289 mg/kg bw/d) and 3 (963 mg/kg bw/d). The historical control data ranges from 89% to 100%. Therefore, the finding did reflect the normal range of biological variation inherent in the strain of rats used for this study.

Live birth indices

- The rate live birth indices were 99% in test group 0, 99.1% in test group 1 (96 mg/kg bw/d) and 100% in test group 2 (289 mg/kg bw/d). These values reflected the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within the range of the historical control data.

- In test group 3 (963 mg/kg bw/d) the live birth index was reduced to 64.2%. A significantly increased number of 43 pups in test group 3 were found stillborn in 8 out 9 litters (35.8% pups stillborn, 36.7% perinatal loss).

Postimplantation loss

- It was 13.9% in test group 0, 15.3% in test group 1 (96 mg/kg bw/d), 6.6% in test group 2 (289 mg/kg bw/d) and 6.9% in test group 3 (963 mg/kg bw/d). These findings reflected the normal range of biological variation inherent in the strain of rats used for this study as all respective values were within the range (0.7% - 18.3%) of the historical control data.

Table 69: Mating report, summary of results males and females

		Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
No. of females mated	N	10	10	10	10
- Inseminated	N	10 f-	10	10	10
Female mating index	%	100.0	100.0	100.0	100.0
-- Pregnant	N	10 f-	10	9	9
Female fertility index	%	100.0	100.0	90.0	90.0
No. of males mated	N	10	10	10	10
- With inseminated females	N	10 f-	10	10	10
Male mating index	%	100.0	100.0	100.0	100.0
- With pregnant females	N	10 f-	10	9	9
Male fertility index	%	100.0	100.0	90.0	90.0
Females with defined Day 0 pc	N	9	10	10	10
Mating days until Day 0 pc	Mean	3.6 x+	2.2	1.9	4.3
	S.d.	4.2	1.0	1.2	5.2
Days 0 To 4	N	8	10	10	8
	%	88.9	100.0	100.0	80.0
Days 5 To 9	N	0	0	0	0
	%	0.0	0.0	0.0	0.0
Days 10 To 14	N	1	0	0	2

	%	11.1	0.0	0.0	20.0

Statistic Profile = Fisher's exact test (one-sided-), Wilcoxon with Bonferroni-Holm (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics f=FISHER-EXACT; x=WILCOX

Table 70: Summary Pregnancy Status Report – Reproduction

		Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
No. of females at start	N	10	10	10	10
No. of females mated	N	10	10	10	10
Without evidence of mating	N	1	0	0	0
- Pregnant	N	1	0	0	0
- Not pregnant	N	0	0	0	0
Females with defined Day 0 pc	N	9	10	10	10
Pregnant	N	10	10	9	9
- sacrificed scheduled	N	10	10	9	9
Not pregnant	N	0	0	1	1
- sacrificed scheduled	N	0	0	1	1
Pregnant, not delivering	N	0	1	0	0
Delivering	N	10	9	9	9
- with liveborn pups	N	9	9	9	9
	%	90.0	100.0	100.0	100.0
- with all pups stillborn	N	1	0	0	0
	%	10.0	0.0	0.0	0.0

Statistic Profile = Fisher's exact test (one-sided-), Dunnett test (two-sided), Fisher's exact test (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics f=FISHER-EXACT; n=DUNNETT

Table 71: Summary Delivery Report

		Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
No. of females at start	N	10	10	10	10
No. of females mated	N	10 f-	10	10	10
	%	100.0	100.0	100.0	100.0
Pregnant	N	10 f-	10	9	9
	%	100.0	100.0	90.0	90.0
Dead	N	0	0	0	0
Without delivery	N	0	1	1	1
- Pregnant	N	0	1	0	0
- Not pregnant	N	0	0	1	1
- Delivering	N	10 f-	9	9	9
	%	100.0	90.0	100.0	100.0
- with liveborn pups	N	9 f-	9	9	9
Gestation index	%	90.0	90.0	100.0	100.0
Gestation days	Mean	22.3 n	22.1	22.2	22.6
	S.d.	1.1	0.6	0.4	0.7
	N	9	9	9	9
- with stillborn pups	N	1 f+	1	0	8 **
	%	10.0	11.1	0.0	88.9
- with all pups stillborn	N	1 f+	0	0	0
	%	10.0	0.0	0.0	0.0

Statistic Profile = Fisher's exact test (one-sided-), Dunnett test (two-sided), Fisher's exact test (one-sided+), * p<=0.05, ** p <=0.01, X = Group excluded from statistics f=FISHER-EXACT; n=DUNNETT

F1 generation litter/pups

Pup number and status at delivery

- The mean number of delivered F1 pups per dam was evenly distributed about test groups 0, 1, 2 and 3 and in the range of the historical control data.
- One stillborn pup was found in test groups 0 and 1 (96 mg/kg bw/d). This incidence were within the normal range of the historical control data. The number of stillborn pups in test group 3 (963 mg/kg bw/d) was significantly increased (43 stillborn pups found in 8 out 9 litters, 35.8% pups stillborn, 36.7% perinatal loss).

Pup viability/mortality

- One pup of test group 1 (96 mg/kg bw/d) was found dead. Two pups of test group 0 and one pup of test group 2 (289 mg/kg bw/d) were missed (cannibalized). These findings reflected the normal range of biological variation inherent in the strain of rats used for this study which is reflected in the corresponding viability index being within the range of the historical control data.
- In test group 3 (963 mg/kg bw/d) the number of pups found dead (4 pups) and the number of pups missing (cannibalized, 13 pups) were increased.
- The viability index indicating pup mortality during lactation (PND 0 - 4) was 97.9% in test group 0, 99.3% in test group 1 (96 mg/kg bw/d) and test group 2 (289 mg/kg bw/d). These findings reflected the normal range of biological variation inherent in the strain of rats used for this study.
- The viability index was decreased in test group 3 without statistically significance (73%, 963 mg/kg bw/d). This value was outside the range of the historical control data (83 – 100%).
- The increased number of pups with empty stomach (7 out of 120) is more likely a direct treatment-related effect than an indirect effect of reduced maternal care because an increased number of 43 pups, out of 120 pups delivered, were already stillborn indicating fetal alterations during prenatal development. No sex differences had been observed in findings of pups.

Table 72: Pup status, Summary litter report

		Test Group 0/ F 0 mg/kg bw/d		Test Group 1/ F 96 mg/kg bw/d	Test Group 2/ F 289 mg/kg bw/d	Test Group 3/ F 963 mg/kg bw/d	
Pups liveborn	N	97		112	113	77	
	%	99.0		99.1	100.0	64.2	
	Mean	9.7	x-	12.4	12.6	8.6	
	S.d.	4.9		2.7	1.9	3.6	
	N	10		9	9	9	
Pups stillborn	N	1		1	0	43	
	%	1.0		0.9	0.0	35.8	
	Mean	0.1	x+	0.1	0.0	4.8	**
	S.d.	0.3		0.3	0.0	3.1	
	N	10		9	9	9	
Perinatal Loss	Mean%	10.0	x+	0.6	0.0	36.7	**
	S.d.	31.6		1.9	0.0	25.5	
	N	10		9	9	9	
Viability Index	Mean %	97.9	x-	99.3	99.3	73.0	
	S.d.	4.2		2.0	2.1	31.8	
	N	9		9	9	9	

Sex ratio

- The sex distribution and sex ratios of live F1 pups show significant differences between the control and the test substance-treated groups 2 and 3; slight differences were regarded to be spontaneous in nature. In test group 1 (96 mg/kg bw/d) the sex ratio was statistically significant different from control but within the normal range of the used strain (going up to 60% live female F1 pups). This finding was considered to be incidental and not treatment related.

Table 73: Sex ratio of live F1 pups

	Test group 0 (0 mg/kg bw/d)	Test group 1 (96 mg/kg bw/d)	Test group 2 (289 mg/kg bw/d)	Test group 3 (963 mg/kg bw/d)
PND 0				
Live males [%]	56.5	43.0*	53.9	46.9
Live females [%]	43.5	57.0*	46.1	53.1
PND 4				
Live males [%]	56.9	42.6*	53.6	44.3
Live females [%]	43.1	57.4*	46.4	55.7

* $p \leq 0.05$; ** $p \leq 0.01$

Pup clinical observation

- The surviving F1 pups of any test group did not show adverse clinical signs up to scheduled sacrifice on PND 4. However, in test group 3 (963 mg/kg bw/d) 31 out of 61 male and 27 out of 59 female pups could not be assessed because they were found dead or were missing (cannibalized).

Table 74: Clinical observation, summary pups (F1)

			Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
			Males	Females	Males	Females	Males	Females	Males	Females
day 0 -> day 4	Animals examined N	N	56	42	48	65	60	53	61	59
	Animals with signs	N	2	1	1	1	1	0	31	27
	reproduction not assessed (died)	N	2	1	1	1	1	0	31	27
	normal NAD	N	55	42	48	64	60	53	38	39

Pup body weight data

- Mean pup body weights/pup body weight changes of in test groups 1, and 2 (96, mg/kg bw/d, and 289 mg/kg bw/d) were comparable to the control group. The pup weights of both sexes were significantly decreased at postnatal day 1 and 4 (-23.0% and -24.8%, respectively) in group 3 (963 mg/kg bw/d). No sex differences had been observed.
- One male and one female runt were seen in test group 1 (96 mg/kg bw/d). One female runt was seen in test group 2 (289 mg/kg bw/d). Ten males and 12 females runts were seen in test group 3 (963 mg/kg bw/d).

Table 75: Body weight report, summary pups (F1), Day 1

		Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
day 1 Runt	**Males**	0	1	0	10
	Females	0	1	1	12
day 1 Males	**Mean**	6.9 n	6.9	6.7	5.2 **
	S.d.	0.9	0.9	0.6	0.7
	N	9	9	9	7
	Deviation Vs Control		-0.8	-3.4	-24.6
day 1 Females	**Mean**	6.7 n	6.7	6.4	5.2 **
	S.d.	1.0	0.9	0.5	0.7
	N	9	9	9	8
	Deviation Vs Control		-0.2	-4.4	-22.1
day 1 Males + Females	**Mean**	6.8 n	6.7	6.5	5.2 **
	S.d.	1.0	0.9	0.6	0.7
	N	9	9	9	9
	Deviation Vs Control		-0.8	-4.0	-23.0

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Table 76: Body weight report, summary pups (F1), Day 4

		Test Group 0 0 mg/kg bw/d	Test Group 1 96 mg/kg bw/d	Test Group 2 289 mg/kg bw/d	Test Group 3 963 mg/kg bw/d
day 4 Males	**Mean**	10.9 n	10.6	10.6	8.2 **
	S.d.	1.7	1.5	1.1	1.4
	N	9	9	9	7
	Deviation Vs Control		-2.2	-2.8	-24.3
day 4 Females	**Mean**	10.6 n	10.4	10.2	8.2 *
	S.d.	1.9	1.6	1.1	1.5
	N	9	9	9	8
	Deviation Vs Control		-1.8	-3.6	-22.8
day 4 Males + Females	**Mean**	10.8 n	10.5	10.4	8.1 **

	S.d.	1.8	1.6	1.1	1.4
	N	9	9	9	9
	Deviation Vs Control		-2.4	-3.7	-24.8

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics n=DUNNETT

Pup necropsy observations

- In test group 1 (96 mg/kg bw/d) one male pup showed post mortem autolysis and one female pup had multiple soft tissue findings.
- In test group 2 (289 mg/kg bw/d) one male pup was missing (cannibalized) and one female pup showed a discolored liver.
- In test group 3 (963 mg/kg bw/d) one male pup had a hydroureter and hydronephrosis. One female pup had a thorax filled with fluid. These findings were assessed as being signs occurred post mortem or spontaneous in nature and thereby not related to treatment. An increased number of male and female pups showed post mortem autolysis, discolored liver, an empty stomach or were missing (cannibalized). The specific absolute numbers of pups with these findings were presented in the following table (table 39). Findings considered treatment-related were presented bold.

Table 77: Lactation pups with treatment related signs

			Test Group 0 0 mg/kg bw/d		**Test Group 1 96 mg/kg bw/d**		**Test Group 2 289 mg/kg bw/d**		**Test Group 3 963 mg/kg bw/d**	
			Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
day 0 -> day 4	Animals examined	N	56	42	48	65	60	53	61	59
	Animals with signs	N	2	1	1	1	1	1	25	30
	General	N	2	1	1	1	1	0	21	16
	Post mortem autolysis	N	0	0	1	0	0	0	13	11
	Not assessed	N	2	1	0	0	1	0	8	5
	Pup with multiple soft tissue findings	N	-	0	-	1	-	0	-	0
	Liver	N	0	0	0	0	0	1	2	7

	discoloured									
	Thorax fluid-filled	N	-	0	-	0	-	0	-	1
	Stomach empty	N	0	0	0	0	0	0	1	6
	Ureter Hydroureter	N	0	-	0	-	0	-	1	-
	Renal pelvis hydronephrosis	N	0	-	0	-	0	-	1	-
	normal NAD	N	54	41	47	64	59	52	36	29

Clinical pathology

Haematology:

- No treatment-related changes among hematological parameters were observed.
- At the end of the administration period, in females of test group 3 (963 mg/kg bw/d) platelet counts were increased. However the mean was within the historical control range (platelet counts 701-903 Giga/L).
- In males of test group 2 (289 mg/kg bw/d) relative eosinophil cell counts were higher compared to controls. Both mentioned alterations were regarded as incidental and not treatment-related.

Table 78: Haematology parameters, summary of results males and females

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
RBC [tera/L] day 44	Mean	8.72 k	8.01 k	8.67	8.24	8.52	8.08	8.52	7.71
	S.d.	0.44	0.16	0.29	0.15	0.37	0.46	0.22	0.48
	N	5	5	5	5	5	5	5	5
	Median	8.53	8.00	8.65	8.24	8.49	7.97	8.65	7.89
HGB [mmol/L] day 44	Mean	9.4 k	9.2 k	9.2	9.4	9.3	9.0	9.1	8.9
	S.d.	0.3	0.3	0.1	0.2	0.2	0.4	0.2	0.4
	N	5	5	5	5	5	5	5	5
	Median	9.3	9.1	9.2	9.4	9.3	8.9	9.2	8.8
HCT [L/L] day 44	Mean	0.438 k	0.422 k	0.434	0.438	0.435	0.422	0.429	0.425
	S.d.	0.017	0.013	0.005	0.008	0.011	0.020	0.009	0.017
	N	5	5	5	5	5	5	5	5
	Median	0.431	0.417	0.433	0.441	0.433	0.415	0.432	0.419
MCV [fL] day 44	Mean	50.2 k	52.7 k	50.1	53.2	51.1	52.3	50.4	55.2
	S.d.	1.3	1.3	1.4	0.9	2.1	0.9	0.7	3.0
	N	5	5	5	5	5	5	5	5
	Median	50.2	52.5	50.1	53.3	51.7	52.1	50.1	55.8
MCH [fmol] day 44	Mean	1.08 k	1.14 k	1.07	1.14	1.09	1.11	1.07	1.16
	S.d.	0.04	0.04	0.04	0.03	0.05	0.02	0.02	0.05
	N	5	5	5	5	5	5	5	5
	Median	1.09	1.14	1.07	1.14	1.10	1.11	1.07	1.19
MCHC [mmol/L] day 44	Mean	21.48 k	21.69 k	21.31	21.34	21.41	21.24	21.28	21.07
	S.d.	0.49	0.34	0.13	0.24	0.29	0.20	0.16	0.25
	N	5	5	5	5	5	5	5	5
	Median	21.38	21.79	21.32	21.33	21.30	21.16	21.23	21.08
RET	Mean	1.4 k	0.9 k	1.5	0.6	1.4	0.9	1.6	0.9

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
[%] day 44	S.d.	0.1	0.2	0.2	0.1	0.3	0.4	0.3	0.5
	N	5	5	5	5	5	5	5	5
	Median	1.4	0.9	1.4	0.6	1.6	0.8	1.7	1.1
PLT [giga/L] day 44	Mean	693 k	666 v	715	757	689	624	670	812 *
	S.d.	93	50	103	81	97	77	48	95
	N	5	5	5	5	5	5	5	5
	Median	718	653	685	771	633	608	660	763
HQT [sec] day 44	Mean	40.0 k	35.3 k	39.3	37.3	39.8	36.9	38.5	35.7
	S.d.	1.9	1.4	1.8	1.5	1.1	1.0	1.7	1.9
	N	5	5	5	5	5	5	5	5
	Median	40.4	35.0	38.9	37.8	39.3	36.8	38.8	35.7
WBC [giga/L] day 44	Mean	4.74 k	3.09 k	5.28	2.93	4.01	3.49	4.42	3.40
	S.d.	0.96	0.48	1.05	0.56	0.48	0.35	1.23	1.24
	N	5	5	5	5	5	5	5	5
	Median	4.51	3.20	5.12	3.01	4.11	3.49	4.28	2.81
NEUTA [giga/L] day 44	Mean	0.99 k	0.55 k	1.10	0.63	0.99	0.77	1.12	0.75
	S.d.	0.16	0.07	0.31	0.24	0.20	0.20	0.47	0.48
	N	5	5	5	5	5	5	5	5
	Median	0.94	0.56	1.00	0.60	0.95	0.72	0.97	0.69
LYMPHA [giga/L] day 44	Mean	3.52 k	2.36 k	3.92	2.10	2.78	2.50	3.11	2.48
	S.d.	0.82	0.47	0.85	0.36	0.55	0.22	0.98	0.93
	N	5	5	5	5	5	5	5	5
	Median	3.24	2.41	3.98	2.01	2.65	2.53	3.10	2.39
MONOA [giga/L] day 44	Mean	0.11 k	0.06 k	0.13	0.07	0.10	0.08	0.10	0.08
	S.d.	0.05	0.02	0.05	0.04	0.02	0.02	0.05	0.04
	N	5	5	5	5	5	5	5	5
	Median	0.10	0.05	0.11	0.06	0.09	0.08	0.08	0.07

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
EOSA [giga/L] day 44	Mean	0.09 k	0.09 k	0.10	0.11	0.12	0.11	0.06	0.05
	S.d.	0.03	0.02	0.05	0.05	0.04	0.06	0.03	0.04
	N	5	5	5	5	5	5	5	5
	Median	0.09	0.08	0.08	0.12	0.10	0.09	0.06	0.05
BASOA [giga/L] day 44	Mean	0.01 k	0.02 k	0.02	0.02	0.01	0.02	0.01	0.02
	S.d.	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01
	N	5	5	5	5	5	5	5	5
	Median	0.01	0.02	0.02	0.02	0.01	0.02	0.01	0.02
LUCA [giga/L] day 44	Mean	0.02 k	0.01 k	0.02	0.01	0.03	0.01	0.02	0.01
	S.d.	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01
	N	5	5	5	5	5	5	5	5
	Median	0.02	0.01	0.02	0.01	0.03	0.01	0.02	0.01
NEUT [%] day 44	Mean	21.3 k	18.3 k	20.9	20.8	24.9	21.8	25.5	21.6
	S.d.	4.2	4.2	4.2	5.1	6.2	4.4	9.3	9.0
	N	5	5	5	5	5	5	5	5
	Median	19.4	20.2	21.3	17.3	22.9	20.6	22.7	20.3
LYMPH [%] day 44	Mean	73.9 k	76.1 k	74.1	72.0	68.7	72.0	70.2	73.3
	S.d.	3.4	3.9	4.1	6.7	6.9	5.8	9.5	10.1
	N	5	5	5	5	5	5	5	5
	Median	75.5	75.4	73.6	74.5	71.6	72.4	72.3	76.3
MONO [%] day 44	Mean	2.2 k	1.8 k	2.3	2.4	2.5	2.1	2.1	2.3
	S.d.	0.7	0.4	0.6	1.2	0.9	0.5	0.7	1.5
	N	5	5	5	5	5	5	5	5
	Median	2.3	1.8	2.3	1.8	2.1	2.1	2.0	1.7
EOS [%]	Mean	1.9 v	2.8 k	1.9	3.7	3.0 *	3.1	1.5	1.7
	S.d.	0.2	0.6	0.5	1.3	0.9	1.4	0.4	1.2
	N	5	5	5	5	5	5	5	5

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
day 44	Median	1.9	2.5	2.1	3.4	3.1	2.6	1.5	1.7
BASO [%] day 44	Mean	0.3 k	0.6 k	0.3	0.7	0.3	0.7	0.3	0.7
	S.d.	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.2
	N	5	5	5	5	5	5	5	5
	Median	0.3	0.5	0.3	0.6	0.3	0.5	0.3	0.7
LUC [%] day 44	Mean	0.4 k	0.4 k	0.4	0.3	0.6	0.4	0.5	0.4
	S.d.	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.2
	N	5	5	5	5	5	5	5	5
	Median	0.3	0.5	0.3	0.2	0.6	0.3	0.5	0.5

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics k=KRUSKAL-WALLIS

Clinical chemistry:

- No treatment-related changes among clinical chemistry parameters were observed.
- At the end of the administration period in males of test group 3 (963 mg/kg bw/d), total bilirubin levels were increased and in females of the same test groups alanine aminotransferase (ALT) activities were increased. Both parameter values were within historical control ranges (males total bilirubin 0.85-2.24 µmol/L; females ALT 0.48-0.71 µkat/L).
- In males of test group 1 (96 mg/kg bw/d) total bile acids (TBA) levels were increased and in females of the same test group aspartate aminotransferase (AST) activities and potassium levels were increased. However, these parameters were not dose-dependently changed.

  Therefore, all mentioned alterations among clinical chemistry parameters were regarded as incidental and not treatment-related.

Table 79: Clinical chemistry parameters, summary of results males and females

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
ALT [µkat/L] day 44	Mean	0.59 k	0.45 v	0.57	0.57	0.66	0.42	0.77	0.68 *
	S.d.	0.08	0.09	0.02	0.08	0.16	0.06	0.16	0.14
	N	5	5	5	5	5	5	5	5
	Median	0.61	0.47	0.57	0.54	0.62	0.42	0.69	0.68
AST [µkat/L] day 44	Mean	1.39 k	1.23 v	1.61	1.53 *	1.62	1.32	1.63	1.34
	S.d.	0.22	0.14	0.34	0.10	0.40	0.10	0.21	0.19
	N	5	5	5	5	5	5	5	5
	Median	1.44	1.24	1.63	1.56	1.71	1.33	1.61	1.39
ALP [µkat/L] day 44	Mean	1.22 k	0.56 k	1.22	0.65	1.33	0.69	1.08	0.59
	S.d.	0.29	0.09	0.19	0.06	0.27	0.12	0.09	0.17
	N	5	5	5	5	5	5	5	5
	Median	1.10	0.52	1.14	0.65	1.34	0.64	1.06	0.50
GGT_C [nkat/L] day 44	Mean	4 k	3 k	5	4	6	8	5	3
	S.d.	4	4	3	5	6	8	4	4
	N	5	5	5	5	5	5	5	5
	Median	3	1	5	3	2	7	3	1
UREA [mmol/L] day 44	Mean	4.96 k	6.10 k	5.29	5.88	4.92	5.77	4.82	5.81
	S.d.	0.38	0.51	0.74	0.49	0.32	0.59	0.44	0.7
	N	5	5	5	5	5	5	5	5
	Median	5.04	6.05	4.99	5.72	4.94	5.67	4.81	5.66
CREA [µmol/L] day 44	Mean	22.4 k	30.2 k	24.8	28.9	22.6	31.1	24.5	26.6
	S.d.	1.5	2	1.8	2.7	1.9	1.3	2.6	4.5
	N	5	5	5	5	5	5	5	5
	Median	21.8	30.2	25.6	27.7	22.1	30.4	25.3	28
GLUC	Mean	6.83 k	5.35 k	5.78	4.89	6.36	5.39	6.44	5.01

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
[mmol/L] day 44	S.d.	0.84	0.22	0.39	0.23	0.54	0.7	1.01	0.48
	N	5	5	5	5	5	5	5	5
	Median	6.75	5.28	5.9	4.81	6.46	4.99	6.71	4.9
TBIL_C [µmol/L] day 44	Mean	0.90 v	1.18 k	1.29	1.29	1.57	1.53	2.20 **	1.44
	S.d.	0.23	0.26	0.24	0.18	0.42	0.23	0.14	0.2
	N	5	5	5	5	5	5	5	5
	Median	1.02	1.15	1.3	1.27	1.52	1.5	2.25	1.43
TBA [µmol/L] day 44	Mean	15.6 v	21.5 k	32.6 *	28.6	22.2	28.8	19.7	25.2
	S.d.	6.6	15.5	8.6	13.1	6.8	12	5.7	9.7
	N	5	5	5	5	5	5	5	5
	Median	17.1	16.8	37.5	25.8	22	30.3	17	28.9
TPROT [g/L] day 44	Mean	63.46 k	63.92 k	61.13	62.26	60.9	61.65	62.14	62.23
	S.d.	0.97	1.08	2.33	1.96	1.94	2.49	0.49	2.35
	N	5	5	5	5	5	5	5	5
	Median	63.47	63.8	61.67	61.46	60.41	61.63	61.9	62.13
ALB [g/L] day 44	Mean	36.14 k	37.90 k	35.06	37.92	35.45	37.37	35.79	36.82
	S.d.	0.71	0.8	0.82	1.57	0.61	1.71	0.49	1.34
	N	5	5	5	5	5	5	5	5
	Median	36	38.22	35.43	38.09	35.39	37.24	35.66	36.47
GLOB [g/L] day 44	Mean	27.32 k	26.02 k	26.07	24.34	25.45	24.28	26.36	25.41
	S.d.	0.9	0.96	1.61	0.87	1.55	0.85	0.7	1.44
	N	5	5	5	5	5	5	5	5
	Median	27.53	25.61	26.24	24.71	24.78	23.94	26.24	25.66
CHOL [mmol/L] day 44	Mean	1.81 k	1.30 k	1.93	1.24	1.57	1.35	1.79	1.3
	S.d.	0.19	0.35	0.29	0.27	0.26	0.13	0.24	0.23
	N	5	5	5	5	5	5	5	5
	Median	1.73	1.25	2	1.2	1.49	1.34	1.74	1.22

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
TRIG [mmol/L] day 44	Mean	0.60 k	0.40 k	0.6	0.42	0.56	0.38	0.56	0.46
	S.d.	0.15	0.03	0.1	0.05	0.19	0.05	0.2	0.14
	N	5	5	5	5	5	5	5	5
	Median	0.64	0.41	0.61	0.42	0.58	0.36	0.66	0.5
NA [mmol/L] day 44	Mean	143.1 k	143.7 k	143.7	144.3	143.9	143.0	143.6	143.8
	S.d.	1.0	1.6	0.5	1.3	1.0	0.4	0.9	1.4
	N	5	5	5	5	5	5	5	5
	Median	143.1	143.6	143.8	143.6	143.5	142.8	143.7	144.1
K [mmol/L] day 44	Mean	4.78 k	4.22 v	4.62	4.63 *	4.77	4.12	4.79	4.38
	S.d.	0.18	0.18	0.16	0.19	0.27	0.23	0.21	0.32
	N	5	5	5	5	5	5	5	5
	Median	4.75	4.25	4.62	4.61	4.72	4.10	4.69	4.40
CL [mmol/L] day 44	Mean	99.7 k	101.2 k	99.4	101.6	100.7	100.2	99.3	100.0
	S.d.	0.7	1.6	1.4	1.3	1.1	0.9	1.5	1.0
	N	5	5	5	5	5	5	5	5
	Median	99.7	101.2	99.8	101.4	100.8	100.4	99.1	100.2
INP [mmol/L] day 44	Mean	1.69 k	1.28 k	1.89	1.38	1.65	1.32	1.62	1.60
	S.d.	0.22	0.14	0.14	0.28	0.14	0.18	0.22	0.21
	N	5	5	5	5	5	5	5	5
	Median	1.74	1.24	1.86	1.36	1.69	1.41	1.62	1.46
CA [mmol/L] day 44	Mean	2.58 k	2.56 k	2.52	2.58	2.52	2.53	2.52	2.57
	S.d.	0.09	0.06	0.08	0.06	0.07	0.05	0.04	0.07
	N	5	5	5	5	5	5	5	5
	Median	2.57	2.54	2.52	2.58	2.50	2.53	2.53	2.56

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics k=KRUSKAL-WALLIS; v=KRUSKAL-WALLIS-WILCOX

Urinalysis:

- No treatment-related changes among urinalysis parameters were observed.

Table 80: Urinalysis parameters, summary of results males and females

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
PH_C [---] day 39	Mean	6.3 k	6.0 k	6.2	6.3	6.1	6.1	5.7	6.5
	S.d.	0.4	0.6	0.3	0.3	0.7	0.7	0.7	0.0
	N	5	5	5	5	5	5	5	5
	Median	6.0	6.0	6.0	6.5	6.0	6.5	6.0	6.5
PRO_C [---] day 39	Mean	1 x+	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	1	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
GLU_C [---] day 39	Mean	0	0	0	0	0	0	0	0
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	0	0	0	0	0	0	0	0
KET_C [---] day 39	Mean	1 x+	1	1	1	1	1	1	1
	S.d.	1	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
UBG_C [---] day 39	Mean	1 x+	1 x+	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
BIL_C [---] day 39	Mean	1	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
BLOOD_C [---] day 39	Mean	1 x+	1 x+	1	1	1	1	1	2
	S.d.	0	0	0	0	0	0	0	1
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
VOL [ml] day 39	Mean	3.4 k	4.0 k	3.6	3.3	3.5	2.7	2.2	3.0
	S.d.	1.7	1.1	1.5	1.1	1.1	1.8	0.9	1.3
	N	5	5	5	5	5	5	5	5
	Median	2.9	4.0	4.0	3.2	3.5	2.0	2.5	3.4
SP.GR._C [g/L] day 39	Mean	1,077 k	1,042 k	1,068	1,057	1,062	1,067	1,097	1,059
	S.d.	26	6	28	12	22	26	15	23
	N	5	5	5	5	5	5	5	5
	Median	1,086	1,045	1,051	1,058	1,049	1,074	1,090	1,049
CRYST_C [---] day 39	Mean	2 x+	2 x+	2	2	2	2	2	2
	S.d.	1	0	0	0	0	0	0	1
	N	5	5	5	5	5	5	5	5
	Median	2	2	2	2	2	2	2	2
RENAL EC_C [---] day 39	Mean	1	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
TRANS EC_C [---]	Mean	1	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
day 39	Median	1	1	1	1	1	1	1	1
SQUAM EC_C [---] day 39	Mean	1	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
CASTS_C [---] day 39	Mean	0 x+	0	0	0	0	0	0	0
	S.d.	0	0	0	0	1	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	0	0	0	0	0	0	0	0
ERY_C [---] day 39	Mean	1	1 x+	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1
LEUCO_C [---] day 39	Mean	1	1	1	1	1	1	1	1
	S.d.	0	0	0	0	0	0	0	0
	N	5	5	5	5	5	5	5	5
	Median	1	1	1	1	1	1	1	1

Statistic Profile = Wilcoxon test (one-sided+), Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics

x=WILCOX

Pathology

Absolute organ weights

- When compared to control group 0 (set to 100%), the mean absolute weights of liver were significantly changed (statistically significant changes printed in bold).

- All other mean absolute weight parameters did not show significant differences when compared to the control group 0.

Relative organ weights

- When compared to control group 0 (set to 100%), the mean relative weights of brain, epididymides, and testes were significantly changed.
- All other mean relative weight parameters did not show significant differences when compared to the control group 0.
- All above mentioned significant weight changes were regarded to be incidental and not related to treatment due to the following reasons. In case of the liver the relative organ weight was not significantly changed, in case of the brain, epididymides, and testes the absolute organ weights were not significantly changed, animals of test group 3 (963 mg/kg bw/d) revealed no histopathologic findings in these organs.

Table 81: Absolute weight, summary of results males and females (F1)

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Terminal body weight (g)	M	360.56	246.06	363.97	247.09	346.58	244.61	349.49	229.44
	% dev	100	100	101	100	96	99	97	93
	SD	9.467	8.635	13.027	23.287	19.785	10.403	16.805	36.905
	n	10	10	10	10	10	10	10	10
Adrenal glands (mg)	M	68.0	75.4	69.0	75.4	62.0	74.6	63.2	82.0
	% dev	100	100	101	100	91	99	93	109
	SD	4.848	10.55	7.842	10.431	4.472	11.305	7.085	12.124
	n	5	5	5	5	5	5	5	5
Brain (g)	M	2.072	1.972	2.07	1.948	2.092	1.98	2.03	2.0
	% dev	100	100	100	99	101	100	98	101
	SD	0.063	0.094	0.08	0.089	0.063	0.071	0.047	0.095
	n	5	5	5	5	5	5	5	5
Epididymides (g)	M	1.111	-	1.143	-	1.191	-	1.133	-
	% dev	100	-	103	-	107	-	102	-

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
	SD	0.091	-	0.124	-	0.075	-	0.1	-
	n	10	-	10	-	10	-	10	-
Heart (g)	M	1.098	0.834	1.08	0.798	1.072	0.86	1.024	0.82
	% dev	100	100	98	96	98	103	93	98
	SD	0.119	0.052	0.047	0.038	0.058	0.089	0.052	0.08
	n	5	5	5	5	5	5	5	5
Kidneys (g)	M	2.324	1.618	2.434	1.582	2.29	1.706	2.292	1.756
	% dev	100	100	105	98	99	105	99	109
	SD	0.152	0.1	0.13	0. 096	0.101	0.162	0.155	0.08
	n	5	5	5	5	5	5	5	5
Liver (g)	M	8.186	5.792	8.142	5.736	7. 462*	5.654	7.506*	6.114
	% dev	100	100	99	99	91	98	92	106
	SD	0.235	0.279	0.245	0.672	0.389	0.656	0.529	0.368
	n	5	5	5	5	5	5	5	5
Spleen (g)	M	0. 596	0.458	0.628	0.442	0.604	0.478	0.526	0.446
	% dev	100	100	105	97	101	104	88	97
	SD	0.088	0.036	0.108	0.107	0.089	0.067	0.106	0.072
	n	5	5	5	5	5	5	5	5
Testes (g)	M	3.539	-	3.628	-	3.824	-	3.723	-
	% dev	100	-	103	-	108	-	105	-
	SD	0.289	-	0.341	-	0.313	-	0.218	-
	n	10	-	10	-	10	-	10	-
Thymus (g)	M	0.271	0.352	0.345	0.318	0.271	0.344	0.258	0.324
	% dev	100	100	127	90	100	98	95	92
	SD	0.04	0.098	0.052	0.053	0.024	0.044	0.072	0.042
	n	5	5	5	5	5	5	5	5

*: P <= 0.05, **: P <= 0.01 Kruskal-Wallis Hand Wilcoxon test, two sided

Gross lesions

- All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Histopathology

- All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.
- In the testis of males of test group 0 (0 mg/kg bw/d) and test group 3 (963 mg/kg bw/d) no deviation in stages of spermatogenesis were observed and all stages were present.

Conclusion

- Under the conditions of this Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, the oral administration by gavage of octadecanoic acid, sulfo-, potassium salt to Wistar rats revealed no signs of general systemic toxicity in male parental animals up to 963 mg/kg bw/d test item, corresponding to 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt.
- Signs of systemic toxicity had been observed in female parental animals at 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt. . Thus, the no observed adverse effect level (NOAEL) for general systemic toxicity was 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt. in male parental animals and 150 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt. in female parental animals.
- The no observed adverse effect level (NOAEL) for reproductive performance and fertility was 500 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt in male and female Wistar rats. The NOAEL for developmental toxicity in the F1 progeny was 150 mg/kg bw/d octadecanoic acid, sulfo-, potassium salt. .

3.10.2 Human data

No studies available.

3.10.3 Other data (e.g. studies on mechanism of action)

No studies available.

3.11 Specific target organ toxicity – single exposure

Evaluation not performed for this substance.

3.12 Specific target organ toxicity – repeated exposure

Not evaluated in this dossier.

3.12.1 Animal data

3.12.1.1 [Study 1]

Study reference:

NN, 2017a

Detailed study summary and results:

Test type

In a repeated-dose toxicity study (according to OECD TG 408), rats were exposed to doses of the test substance (0, 100, 300, or 1,000 mg/kg bw/d) by gavage for 90 days and effects on clinical signs, functional observations, clinical chemistry, haematology and histopathology were evaluated. GLP compliance is given (certificate not mentioned).

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 9-Octadecenoic acid (Z)-, sulfonated, potassium salts
- Test material form: orange-brown crystalline powder with lumps
- Degree of purity: 100%, UVCB
- Impurities: no information available
- Batch number: 200516GT41

Test animals

- Species/strain/sex: rat/ Wistar Crl:WI(Han) /male and female
- No. of animals per sex per dose: 10
- Age and weight at the study initiation: Approximately 6 weeks, mean weights of the animals of the different dose groups on day 1: males: 163-169; females: 132-134

Administration/exposure

- Route of administration – oral (gavage)
- Duration and frequency of test/exposure period: 90 days, once daily, 7 d/week
- Doses/concentration levels: 0; 100; 300, or 1,000 mg/kg bw/d

- Rationale for dose level selection: based on the results of a 14-day dose-ranger finding study with 500 and 1,000 mg/kg bw/d, which did not reveal any toxic effects
- Post exposure observation period: no
- Vehicle: water (5 ml/kg bw)
- Control group and treatment: yes, treated similar to treatment groups with vehicle
- Test substance formulation/diet preparation, achieved concentration by sex and dose level, stability and homogeneity of the preparation
    - application volume was 5 mL/kg bw, calculated and adjusted weekly according to the latest body weight
    - analytical verification: performed by a validated method (no further information available), accuracy of prepared concentrations (for formulations: 100, 300 and 1,000 mg/kg bw/d) was shown (i.e. mean accuracies between 90% and 110%), homogeneity of lowest and highest dose was analysed and verified (i.e. coefficient of variation ≤ 10%)
- Statistical methods
    - if the variables could be assumed to follow a normal distribution, the Dunnett-test based on a pooled variance estimate was applied for the comparison of the treated groups and the control groups for each sex
    - Steel-test was applied if the data could not be assumed to follow a normal distribution
    - Fisher Exact test was applied to frequency data
    - Kruskal-Wallis nonparametric ANOVA test was applied to motor activity data to determine intergroup differences
    - all tests were two-sided and in all cases $p < 0.05$ was accepted as the lowest level of significance. Group means were calculated for continuous data and medians were calculated for discrete data (scores) in the summary tables. Test statistics were calculated on the basis of exact values for means and pooled variances.

Results and discussion

- Mortality and time to death: two dead males at 1,000 mg/kg bw/d

  - one male found dead on test Day 74, displayed hunched posture on the two days leading up to its death; reduced size of thymus and spleen (microscopic correlate: lymphoid depletion in both organs and decreased red pulp in the spleen), changes in the kidney (marked tubular degeneration);
  - one male killed in extremis on test Day 36, findings in the lung and/or stomach indicated a procedure-related accident
- Body weight and body weight changes: no effects observed (slight - not statistically significant - trends towards a lower body weight and body weight gain were seen in 1,000 mg/kg treated animals)
- Food consumption: no effects observed
- Clinical signs: dose-related increase in salivation after dosing; diarrhoea was observed on single occasions in seven 1,000 mg/kg animals towards the end of the treatment period; swelling/gas distention of the abdomen noted in three 1,000 mg/kg and two 300 mg/kg animals (only single short and transient bouts); single short and transient rales were observed in five 1,000 mg/kg animals and two 100 mg/kg animals; no findings recorded during the arena observations
- Sensory activity, grip strength and motor activity assessments:
  - Foregrip strength reduced in 300 and 1,000 mg/kg males and 1,000 mg/kg females in a statistically significant manner; slight (not statistically significant) reduction in total movement and ambulatory behaviour in 1,000 mg/kg females. Further observations, hearing ability, pupillary reflex, static righting reflex, hindgrip strength and motor activity: no effects observed
- Ophthalmologic findings: effects observed, non-treatment-related (focal corneal edema in 100 mg/kg females at week 13)
- Haematological findings: effects observed, treatment-related (for details see Table 82)
  - statistically significant changes observed in treated animals: white blood cell count increased in 1,000 mg/kg bw/d females,
  - lymphocyte levels reduced in 1,000 mg/kg males and 1,000 mg/kg bw/d females,
  - neutrophil levels increased in 300 mg/kg males and 1,000 mg/kg bw/d males and females (males not statistically significant).
  - red blood cell distribution width (%RDW) increased in 1,000 mg/kg bw/d females,

- haemoglobin levels, mean red blood cell volume (MCV), and mean cell haemoglobin concentration (MCH) reduced in 1,000 mg/kg bw/d males and females.)

- Clinical biochemistry findings: effects observed, treatment-related (for details see Table 83)
  - alanine aminotransferase (ALAT) levels significantly increased in both male and female at 1,000 mg/kg bw/d;
  - aspartate transaminase (ASAT) levels increased in 1,000 mg/kg bw/d females;
  - total protein and albumin levels increased in 1,000 mg/kg bw/d, and 100/300/1,000 mg/kg bw/d males, respectively;
  - total bilirubin levels increased in 100, 300 and 1,000 mg/kg bw/d males;
  - urea levels increased in 1,000 mg/kg bw/d males and females,
  - creatinine levels increased in 100 and 1,000 mg/kg bw/d males and 1,000 mg/kg bw/d females;
  - glucose and cholesterol levels both reduced in 1,000 mg/kg bw/d males and females;
  - inorganic phosphate levels increased in 1,000 mg/kg bw/d males and females;
  - sodium levels increased in 100 and 300 mg/kg bw/d males and females (no dose-response).
- Oestrous cycle length (determined Day 70 up to and including Day 91): no effects observed
- Spermatogenesis: no effects observed (evaluation of the testes on HE-stained and PAS-stained testes slides)
- Organ weights: treatment-related effects observed
  - increased liver weights (relative to body weights) in the 1,000 mg/kg bw/d group females (mean percent liver weight differences relative to body weight: approx. 0, -5, -4, 9 and 0, 2, -2 and 22 % in control, 100, 300 and 1,000 mg/kg bw/d males and females, respectively) and increased kidney weights (absolute and/or relative to body weights) in 1,000 mg/kg bw/d group males and females (mean percent kidney weight differences relative to body weight: 0, -2, 2, 19 and 0, 0, 3 and 21 % in control, 100, 300 and 1,000 mg/kg bw/d males and females, respectively.)
  - increased kidney weight was related to microscopic findings of tubular vacuolation with pigmented material.
- Gross pathology findings: no effects observed

- Neuropathological findings: no effects observed
- Histopathology findings: treatment-related effects observed at doses ≥ 300 mg/kg bw/d (for details see Table 84)
    - kidney findings: increased incidence and severity of tubular vacuolation, with pigmented material in the cortex - in males and females starting at 300 mg/kg bw/d; tubular degeneration and an increased severity and/or incidence in tubular basophilia at 1,000 mg/kg;
    - increased incidence and severity of macrophage foci in mesenteric lymph nodes starting at 300 mg/kg bw/d in both sexes;
    - adrenal gland: vacuolation of the zona glomerulosa at an increased incidence and/or severity in both sexes at 1,000 mg/kg:
    - pancreas: increased apoptosis of acinar cells in males treated at 1,000 mg/kg bw/d; one female at 1,000 mg/kg with minimal increased apoptosis was considered to be within background levels.
    - spleen: increased incidence and severity of extramedullary haematopoiesis and increased severity of pigmentation in females treated at 1,000 mg/kg bw/d.
    - no effects on morphology of gonads and accessory reproductive organs.

Table 82: Haematology Summary (end of treatment; only parameters with statistically significant changes in comparison to control reported)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
Males					
Neutrophils	MEAN	14.6	18.6	20.2++	20.8
%WBC	ST.DEV.	3.1	4.2	2.9	7.5
	N	10	10	10	8
Lymphocytes	MEAN	82.4	77.5	76.5++	75.7++
%WBC	ST.DEV.	3.3	5.1	2.7	8.2
	N	10	10	10	8
Haemoglobin	MEAN	10.0	10.1	9.9	9.5*
Mmol/L	ST.DEV.	0.4	0.2	0.5	0.3

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
	N	10	10	10	8
Eosinophils	MEAN	1.1	1.6+	1.3	1.3
%WBC	ST.DEV.	0.3	0.4	0.7	0.6
	N	10	10	10	8
MCV	MEAN	53.4	53.3	53.9	50.9**
fL	ST.DEV.	1.9	1.1	1.8	1.0
	N	10	10	10	8
MCH	MEAN	1.10	1.09	1.10	1.03*
fmol	ST.DEV.	0.06	0.03	0.07	0.02
	N	10	10	10	8
PT	MEAN	16.7	15.6*	15.2**	16.3
	ST.DEV.	0.6	0.5	0.6	1.4
	N	10	10	9	8
Females					
WBC	MEAN	3.9	3.6	4.6	5.9**
10E9/L	ST.DEV.	0.9	0.8	0.8	1.3
	N	10	10	10	10
Neutrophils	MEAN	13.8	21.3	17.6	21.1++
%WBC	ST.DEV.	2.4	13.8	8.9	4.9
	N	10	10	10	10
Lymphocytes	MEAN	82.4	73.1	78.9	75.7++
%WBC	ST.DEV.	2.6	16.4	9.3	4.4
	N	10	10	10	10

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
Eosinophils	MEAN	1.4	3.3+	1.2	0.9
%WBC	ST.DEV.	0.6	3.2	0.5	0.2
	N	10	10	10	10
RDW	MEAN	11.4	11.6	11.2	12.8**
%	ST.DEV.	0.6	0.4	0.4	1.0
	N	10	10	10	10
Haemoglobin	MEAN	9.3	9.1	9.3	9.0*
Mmol/L	ST.DEV.	0.3	0.2	0.2	0.4
	N	10	10	10	10
MCV	MEAN	55.6	55.7	55.9	53.1**
fL	ST.DEV.	1.3	1.5	1.5	1.7
	N	10	10	10	10
MCH	MEAN	1.14	1.12	1.14	1.07**
fmol	ST.DEV.	0.04	0.03	0.03	0.04
	N	10	10	10	10

+/++ Steel-test significant at 5% (+) or 1% (++) level
*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

Table 83: Clinical biochemistry (end of treatment; only parameters with statistically significant changes in comparison to control reported)

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
Males					
ALAT	MEAN	43.0	45.1	46.6	83.6**

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
U/L	ST.DEV.	7.1	13.5	11.8	17.2
	N	10	10	10	8
Total protein	MEAN	65.7	67.9	67.9	69.7**
g/L	ST.DEV.	1.4	2.6	3.0	3.0
	N	10	10	10	8
Albumin	MEAN	32.0	33.3*	33.6**	34.5**
g/L	ST.DEV.	1.0	0.8	1.2	0.9
	N	10	10	10	8
Total bilirubin	MEAN	1.9	2.4*	2.8**	2.8**
µmol/L	ST.DEV.	0.3	0.3	0.6	0.6
	N	10	10	10	8
Creatinine	MEAN	41.5	45.2**	42.2	45.8**
µmol/L	ST.DEV.	2.9	2.6	1.7	2.5
	N	10	10	10	8
Glucose	MEAN	10.42	10.39	9.32	9.03*
mmol/L	ST.DEV.	1.10	1.16	1.04	1.27
	N	10	10	10	8
Cholesterol	MEAN	2.37	2.04	2.06	1.67**
mmol/L	ST.DEV.	0.42	0.34	0.35	0.17
	N	10	10	10	8
Bile acids	MEAN	19.8	34.4**	23.7	11.3
µmol/L	ST.DEV.	9.8	14.8	8.0	2.7
	N	10	10	10	8

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
Sodium	MEAN	138.8	141.0**	142.6**	138.7
mmol/L	ST.DEV.	1.5	1.4	1.0	1.5
	N	10	10	10	8
Inorg. Phos	MEAN	1.49	1.39	1.60	1.99**
mmol/L	ST.DEV.	0.16	0.15	0.12	0.15
	N	10	10	10	8
Females					
ALAT	MEAN	31.3	29.0	32.7	94.2**
U/L	ST.DEV.	7.9	6.1	5.2	24.1
	N	10	10	10	10
ASAT	MEAN	73.0	69.4	70.0	88.2*
U/L	ST.DEV.	11.0	12.7	15.5	11.9
	N	10	10	10	10
Total bilirubin	MEAN	2.2	2.5	3.0	3.3**
µmol/L	ST.DEV.	0.3	0.5	0.7	1.3
	N	10	10	10	10
Urea	MEAN	7.3	6.1	6.9	10.0**
mmol/L	ST.DEV.	1.1	0.9	0.9	1.5
	N	10	10	10	10
Creatinine	MEAN	45.1	45.8	46.6	52.0**
µmol/L	ST.DEV.	2.3	5.3	2.5	4.8
	N	10	10	10	10
Sodium	MEAN	140.0	141.5**	141.8**	139.8

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
mmol/L	ST.DEV.	0.7	0.9	0.9	1.1
	N	10	10	10	10
Inorg. Phos	MEAN	1.37	1.30	1.35	1.79**
mmol/L	ST.DEV.	0.15	0.12	0.18	0.13
	N	10	10	10	10

*/** Dunnett-test based on pooled variance significant at 5% (*) or 1% (**) level

Table 84: Microscopic findings of selected organs

	Males				Females			
	0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d	0 mg/kg bw/d	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg bw/d
KIDNEYS [a]	10	10	10	10	10	10	10	10
Vacuolation, with pigmented material								
Minimal	-	-	3	1	-	-	4	-
Slight	-	-	-	3	-	-	-	2
Moderate	-	-	-	4	-	-	-	7
Marked	-	-	-	1	-	-	-	1
Degeneration, tubular								
Minimal	-	-	-	3	-	-	-	2

	Males				Females			
	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
Slight	-	-	-	1	-	-	-	3
Moderate	-	-	-	4	-	-	-	-
Marked	-	-	-	1	-	-	-	-
Basophilia, tubular								
Minimal	3	-	3	1	-	-	1	3
Slight	-	-	1	1	1	-	-	3
Moderate	-	-	-	4	-	-	-	-
Marked	-	-	-	1	-	-	-	-
MESENTRIC LYMPH NODE [a]	10	10	10	9	10	10	10	10
Increased macrophage foci								
Minimal	-	-	4	2	1	1	3	2
Slight	-	-	-	1	-	-	1	6
Moderate	-	-	-	-	-	-	-	1
ADRENAL GLANDS [a]	10	10	10	10	10	10	10	10
Vacuolation, zona glomerulosa								

	Males				Females			
	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
Minimal	-	-	-	4	1	-	-	8
Slight	-	-	-	6	-	-	-	-
PANCREAS [a]	10	10	10	10	10	10	10	10
Increased apoptosis								
Minimal	-	-	-	3	-	-	-	1
Slight	-	-	-	2	-	-	-	-
Moderate	-	-	-	2	-	-	-	-
SPLEEN [a]	10	-	1	10	10	10	10	10
Haematopoiesis								
Minimal	3	-	-	5	-	3	5	-
Slight	1	-	-	3	2	-	-	5
Moderate	-	-	-	-	-	-	-	2
Pigmentation								
Minimal	5	-	-	5	2	9	8	-
Slight	5	-	-	4	8	1	1	6
Moderate	-	-	-	-	-	-	-	4

[a] = Number of tissues examined from each group

Table 85: Microscopic findings of male reproductive organs

Sex	**Males**			
Dose Group	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
No. Animals	**10**	**10**	**10**	**10**
Prostate Gland	10	-	-	10
N.A.D	7	-	-	10
Infiltrate inflamm.	3	-	-	-
Grade 1	2	-	-	-
Grade 3	1	-	-	-
Testes	10	-	-	10
N.A.D.	10	-	-	6
Atrophy, tubular	-	-	-	1
Grade 1	-	-	-	1
Disorganization	-	-	-	1
Sperm stasis	-	-	-	1

Sex	**Males**			
Dose Group	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
No. Animals	**10**	**10**	**10**	**10**
Grade 1	-	-	-	1
Degenerat. germ cell	-	-	-	1
Grade 1	-	-	-	1
Epididymides	10	1	-	9
N.A.D	9	-	-	9
Infiltrate inflamm.	1	-	-	-
Grade 1	1	-	-	-
Sperm granuloma	-	1	-	-
Grade 4	-	1	-	-
Testes, pas staging	10	-	-	10
All stages normal	10	-	-	10
Stages abnormal	-	-	-	1

Table 86: Microscopic findings of female reproductive organs

Sex	**Females**			
Dose Group	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
No. Animals	**10**	**10**	**10**	**10**
Ovaries	10	-	-	10
N.A.D	10	-	-	9
Cyst.	-	-	-	1
Grade 2	-	-	-	1
Uterus	10	7	2	10
N.A.D.	7	-	-	7
Dilatation, liminal	3	7	2	3
Cervix	10	-	-	10
N.A.D	10	-	-	10
Vagina	10	-	-	10
Cycle: Proestrus	2	-	-	-

Sex	**Females**			
Dose Group	**0 mg/kg bw/d**	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1,000 mg/kg bw/d**
No. Animals	**10**	**10**	**10**	**10**
Cycle: Estrus	2	-	-	3
Cycle: Metestrus	4	-	-	5
Cycle: Diestrus	2	-	-	2
Clitorial Glands	1	1	2	-
N.A.D.	1	-	-	-
Dilation	-	1	2	-
Grade 2	-	1	1	-
Grade 3	-	-	1	-

Table 87: Oestrous cycle determination Females

Animal	**Individual Cycle Length**	**Cycle Classification**
Group 1 (Control)		
41	4,5,5	R
42	4, 4, 4, 4, 4	R
43	4, 4, 4, 4	R
44	4, 4, 4, 4	R
45	4, 4, 4, 5	R

Animal	Individual Cycle Length	Cycle Classification
46	4, 4, 4, 4	R
47	4, 4, 4, 4	R
48	4, 4, 4, 4	R
49	4, 4, 4, 4, 4	R
50	4, 4, 4, 4	R
Group 2 (100mg/kg bw/day)		
51	4, 4, 4, 4	R
52	4, 4, 4, 4	R
53	4, 4, 4, 4	R
54	4, 4, 4, 4, 4	R
55	4, 4, 4, 4, 4	R
56	4, 4, 4, 4, 4	R
57	4, 4, 4, 4	R
58	4, 4, 4, 4	R
59	4, 4, 4, 4	R
60	4, 4, 4, 4, 4	R
Group 3 (300mg/kg bw/day)		
61	4, 4, 4, 4	R
62	4, 4, 4, 4	R
63	4, 4, 4, 4	R
64	4, 4, 4, 5	R
65	4, 4, 4, 4, 4	R
66	4, 4, 4, 4	R
67	4, 4, 4, 4	R
68	4, 4, 4, 4	R
69	4, 4, 4, 4, 4	R
70	5, 4, 4, 4	R
Group 4 (1000 mg/kg bw/day)		

Animal	Individual Cycle Length	Cycle Classification
71	4, 4, 4, 4	R
72	4, 4, 4, 4	R
73	4, 4, 4, 4	R
74	4, 4, 4, 4	R
75	4, 4, 4, 4	R
76	4, 4, 4, 4	R
77	5, 5, 4	R
78	4, 4, 4, 5	R
79	4, 4, 4, 4	R
80	4, 4, 4, 4	R

CYCLE CLASSIFICATION CODE: R = REGULAR (all cycles either 4 or 5 days); IR = IRREGULAR (at least one cycle of 2, 3 or 6-10 days, irrespective of the number of 4-5 day cycles); A = ACYCLIC (at least 10 days without estrus irrespective of the number of 4-5 day cycles); EE = EXTENDED ESTRUS (at least 4 consecutive days of estrus).
Cycle classifications for individual animals are based on the length and stage of each estrous cycle.

Table 88: Organ weights of reproductive organs

		CONTROL	100 mg/kg bw/d	300 mg/kg bw/d	1,000 mg/kg
Males					
TESTES	MEAN	3.71	3.71	3.60	3.55
(GRAM)	ST.DEV	0.19	0.28	0.19	0.23
	N	10	10	10	8
PROSTATE GLAND	MEAN	0.870	0.779	0.844	0.865
(GRAM)	ST.DEV	0.158	0.132	0.192	0.123
	N	10	10	10	8
EPIDIDYMIDES	MEAN	1.279	1.303	1.243	1.226
(GRAM)	ST.DEV	0.128	0.284	0.099	0.181
	N	10	10	10	8

SEMINAL VESICLES (GRAM)	MEAN	1.338	1.338	1.344	1.321
	ST.DEV	0.219	0.294	0.209	0.179
	N	10	10	10	8
Females					
OVARIES (GRAM)	MEAN	0.139	0.147	0.134	0.128
	ST.DEV	0.017	0.017	0.019	0.026
	N	10	10	10	10
UTERUS (GRAM)	MEAN	0.684	0.822	0.611	0.607
	ST.DEV	0.244	0.287	0.293	0.282
	N	10	10	10	10

3.12.1.2 [Study 2]

Study reference:

NN, 2020

Detailed study summary and results:

Test type

In a repeated-dose toxicity study (according to OECD TG 408; adopted 25 Jun 2018), rats were exposed to doses of the test substance (0, 150, 450, or 1400 mg/kg bw/d; corresponding to 0, 79, 236, or 735 mg/kg bw/d of reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts by gavage for 90 days and effects on clinical signs, functional observations, clinical chemistry, haematology and histopathology were evaluated. GLP compliance is given (certificate mentioned).

Test substance

- Test material according to study report: Reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts (CAS 67968-63-2, EC -)*.
- Test material form: liquid/ brownish, clear
- Degree of purity: 52.5 g/100 g (100 g/100 g minus water content)
- Impurities: no information available
- Batch number: 0018983927

** The Dossier submitter notes that, according to ECHA, CAS 67968-63-2/EC 267-966-5 corresponds to "9(or 10)-sulphooctadecanoic acid, potassium salt" and CAS - /EC - corresponds to "Reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts"*

Test animals

- Species/strain/sex: rat/ Wistar Crl:WI(Han) /male and female
- No. of animals per sex per dose: 10
- Age and weight at the study initiation: 42 ±1 days, mean weights of the animals of the different dose groups on day 1: males: 181.8-182.5; females: 139.4-142.5

Administration/exposure

- Route of administration – oral (gavage)
- Duration and frequency of test/exposure period: 90 days, once daily, 7 d/week
- Doses/concentration levels: 0, 150, 450, or 1400 mg/kg bw/d test item; corresponding to 0, 79, 236, or 735 mg/kg bw/d of Reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts
- Rationale for dose level selection: no rationale found
- Post exposure observation period: no

- Vehicle: deionized water
- Control group and treatment: yes, treated similar to treatment groups with vehicle
- Application volume was 10 mL/kg bw, calculated and adjusted weekly according to the latest body weight
- Test substance formulation achieved concentration by dose level, stability and homogeneity of the preparation
    - analytical verification: performed by a validated method (on a comparable batch), accuracy of prepared concentrations (for formulations: 150, 450 and 1400 mg/kg bw/d) was shown (i.e. mean accuracies between 100 and 106%), homogeneity of lowest and highest dose was analysed and verified (i.e. coefficient of variation ≤ 10%).
- Statistical methods
    - Body weight, body weight change: Comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means.
    - Rearing, grip strength of forehand hindlimbs, landing foot-splay test, motor activity: Non-parametric one-way analysis using KRUSKALWALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using WILCOXON test (two-sided) for the equal medians.

Results and discussion

- Mortality: one male was found dead on Day 66, the cause of death was a malignant lymphoma based on microscopic findings. This finding was considered incidental and not related to treatment.
- Clinical observation
    - Group 3 (1400 mg/kg bw/day):
        - Slight salivation within 2h after treatment was seen in all males and females intermittently over the entire administration period (first seen on Day 8). One male animal (No. 34) still showed slight salivation 3 to 5 hours after administration on study day 91 which was the last day of administration.
        - The soft faeces were considered as treatment-related but not adverse.

- One male animal (No. 32) showed severe salivation, clear, red nasal discharge and semi-closed eyelids within 2 hours after administration and respiration sounds after 2 to 5 hours after administration on Day 27 only.
- In female animal No. 71 respiration sounds and semi-closed eyelids were observed as well on study day 19, 0 to 2 hours post dosing.
- Respiration sounds were also observed in male animals Nos. 36 (Day 33, 2 to 5 hours post dosing) and 37 (Day 43, 2 to 5 hours and Day 51, 0 to 2 hours post dosing) and female animals Nos. 76 (Day 54, 0 to 2 hours post dosing) and 80 (Day 90, 0 to 2 hours post dosing).
- Semi-closed eyelids were additionally seen in one more female (No. 75) on Day 24, 0 to 2 hours after administration.
- Soft faeces were observed in all males from Day 66 onwards.
- One female animal had an injury of the right eye (first seen on Day 48) resulting in the loss of this eyeball on Day 52. This finding was considered as accidental and not treatment-related.

o Group 2 (450 mg/kg bw/day):

- Salivation: as well as in one male (No. 27) on Day 60.

o Group 0 (0 mg/kg bw/day):

- One male animal (No. 2) in control group had an injury in the neck region from Day 17 to 31. This was considered as related to handling during gavage

o Conclusions:

- The salivation observed after administration, but not later, was considered to be related to a bad taste or slight irritant properties of the test substance preparation administrated by gavage.
- The soft faeces were considered as treatment-related but not adverse.
- The other findings (respiration sounds, and semiclosed eyelids) were considered to be caused by treatment and adverse.

Table 89: Summary males signs pre- and/or post-dosing – Clinical observation

			TG 0 / M 0 mg/kg bw/d	TG 1 / M 150 mg/kg bw/d	TG 2 / M 450 mg/kg bw/d	TG 3 / M 1400 mg/kg bw/d
Day 0 → 91 (-1h;0h)	Animal examined	N	10	10	10	10
	Animals with signs	N	1	0	0	10
	Head	N	1	0	0	0
	Salivation	N	0	0	0	0
	Injury	N	1	0	0	0
	Normal *NAD*	N	10	10	10	10
	Eye *Semi-closed eyelid*	N	0	0	0	0
	Nose *Discharge*	N	0	0	0	0
	Respiration *Sounds*	N	0	0	0	0
	Faeces *Soft faeces*	N	0	0	0	10

Table 90: Summary males signs pre- and/or post-dosing – Clinical observation

			TG 0 / M 0 mg/kg bw/d	TG 1 / M 150 mg/kg bw/d	TG 2 / M 450 mg/kg bw/d	TG 3 / M 1400 mg/kg bw/d
Day 0 → 91 (0h;2h)	Animal examined	N	10	10	10	10
	Animals with signs	N	1	0	1	10
	Head	N	1	0	1	10

	Salivation	**N**	0	0	1	10
	Injury	**N**	1	0	0	0
	Normal ***NAD***	**N**	10	10	10	10
	Eye ***Semi-closed eyelid***	**N**	0	0	0	1
	Nose ***Discharge***	**N**	0	0	0	1
	Respiration ***Sounds***	**N**	0	0	0	1
	Faeces ***Soft faeces***	**N**	0	0	0	10

Table 91: Summary males signs pre- and/or post-dosing – Clinical observation

			TG 0 / M **0 mg/kg bw/d**	**TG 1 / M** **150 mg/kg bw/d**	**TG 2 / M** **450 mg/kg bw/d**	**TG 3 / M** **1400 mg/kg bw/d**
Day 0 → 91 **(0h;5h)**	**Animal examined**	**N**	10	10	10	10
	Animals with signs	**N**	1	0	0	10
	Head	**N**	1	0	0	1
	Salivation	**N**	0	0	0	1
	Injury	**N**	1	0	0	0
	Normal ***NAD***	**N**	10	10	10	10
	Eye ***Semi-closed eyelid***	**N**	0	0	0	0
	Nose ***Discharge***	**N**	0	0	0	0

	Respiration ***Sounds***	**N**	0	0	0	2
	Faeces ***Soft faeces***	**N**	0	0	0	10

Table 92: Summary females signs pre- and/or post-dosing – Clinical observation

			TG 0 / M **0 mg/kg bw/d**	**TG 1 / M** **150 mg/kg bw/d**	**TG 2 / M** **450 mg/kg bw/d**	**TG 3 / M** **1400 mg/kg bw/d**
Day 0 → 92 **(-1h;0h)**	**Animal examined**	**N**	10	10	10	10
	Animals with signs	**N**	0	0	0	1
	Head ***Salivation***	**N**	0	0	0	0
	Normal ***NAD***	**N**	10	10	10	10
	Eye	**N**	0	0	0	1
	Semi-closed eyelid	**N**	0	0	0	0
	Injury	**N**	0	0	0	1
	Loss of eyeball	**N**	0	0	0	1
	Respiration ***Sounds***	**N**	0	0	0	0

Table 93: Summary females signs pre- and/or post-dosing – Clinical observation

	TG 0 / M **0 mg/kg bw/d**	**TG 1 / M** **150 mg/kg bw/d**	**TG 2 / M** **450 mg/kg bw/d**	**TG 3 / M** **1400 mg/kg bw/d**

Day 0 → 92 (0h;2h)	**Animal examined**	**N**	10	10	10	10
	Animals with signs	**N**	0	0	0	10
	Head ***Salivation***	**N**	0	0	0	10
	Normal ***NAD***	**N**	10	10	10	10
	Eye	**N**	0	0	0	3
	Semi-closed eyelid	**N**	0	0	0	2
	Injury	**N**	0	0	0	1
	Loss of eyeball	**N**	0	0	0	1
	Respiration ***Sounds***	**N**	0	0	0	3

Table 94: Summary females signs pre- and/or post-dosing – Clinical observation

			TG 0 / M 0 mg/kg bw/d	**TG 1 / M 150 mg/kg bw/d**	**TG 2 / M 450 mg/kg bw/d**	**TG 3 / M 1400 mg/kg bw/d**
Day 0 → 92 (2h;5h)	**Animal examined**	**N**	10	10	10	10
	Animals with signs	**N**	0	0	0	2
	Head ***Salivation***	**N**	0	0	0	1
	Normal ***NAD***	**N**	10	10	10	10
	Eye	**N**	0	0	0	1
	Semi-closed eyelid	**N**	0	0	0	0
	Injury	**N**	0	0	0	1

	Loss of eyeball	**N**	0	0	0	1
	Respiration *Sounds*	**N**	0	0	0	1

- Food consumption:
  - Group 3 (1400 mg/kg bw/d)
    - Slight deviations from control in males from Day 35 to 49 (increase of 12%, day 35 to 42 and day 42 to 49) .
    - Towards the end of the administration period a slight decreases of food consumption were seen in females (-10.7%, study day 77 to 84). It cannot be excluded that these findings were treatment-related. However, based on the degree of decrease and the short temporary occurrence, these findings were considered as non-adverse.
  - Group 2 (450 mg/kg bw/d)
    - Towards the end of the administration period a slight decreases of food consumption were seen in females (-10.9%, Day 77 to 84 and -11%, day 84 to 91). It cannot be excluded that these findings were treatment-related. However, based on the degree of decrease and the short temporary occurrence, these findings were considered as non-adverse.
  - Group 1 (150 mg/kg bw/day)
    - Slight deviations in females from day 42 to 49 (increase of 16.8%) were considered as incidental and not treatment-related.

Table 95: Summary food consumption per animal per day - Males

		TG 0 / M 0 mg/kg bw/d	**TG 1 / M 150 mg/kg bw/d**	**TG 2 / M 450 mg/kg bw/d**	**TG 3 / M 1400 mg/kg bw/d**
d -4 → 0	**Mean (g)**	19.7	19.9	19.4	20.4
	S.D.	0.7	0.4	0.5	1.1
	N	2	2	2	2
	Deviation vs Control (%)		0.8	-1.8	3.4
d 0 → 7	**Mean (g)**	21.3	21.0	20.4	21.8
	S.D.	0.6	0.9	0.1	1.8

	N	2	2	2	2
	Deviation vs Control (%)		-1.0	-4.1	2.7
d 7 → 14	**Mean (g)**	22.8	22.4	22.3	23.4
	S.D.	0.6	1.1	0.7	2.0
	N	2	2	2	2
	Deviation vs Control (%)		-1.8	-2.1	2.4
d 14 → 21	**Mean (g)**	23.7	23.4	22.6	24.4
	S.D.	0.7	0.8	0.8	1.2
	N	2	2	2	2
	Deviation vs Control (%)		-1.3	-4.6	3.1
d 21 → 28	**Mean (g)**	23.8	23.0	24.0	24.7
	S.D.	1.6	0.8	0.6	0.6
	N	2	2	2	2
	Deviation vs Control (%)		-3.1	0.9	4.0
d 28 → 35	**Mean (g)**	23.1	22.3	23.4	24.7
	S.D.	0.2	0.5	1.5	3.8
	N	2	2	2	2
	Deviation vs Control (%)		-3.6	1.1	6.8
d 35 → 42	**Mean (g)**	22.6	22.1	22.7	25.4
	S.D.	0.7	0.2	1.2	2.4
	N	2	2	2	2
	Deviation vs Control (%)		-2.5	0.1	12.0
d 42 → 49	**Mean (g)**	22.6	22.1	21.7	25.4
	S.D.	0.4		0.5	2.3
	N	2	1	2	2
	Deviation vs Control (%)		-2.5	-4.2	12.0
d 49 → 56	**Mean (g)**	22.2	21.5	21.3	23.9
	S.D.	0.1	0.9	0.1	0.4
	N	2	2	2	2
	Deviation vs Control (%)		-3.2	-4.1	7.5
d 56 → 63	**Mean (g)**	21.4	21.2	21.8	23.7
	S.D.	0.4	1.4	1.3	0.6

	N	2	2	2	2
	Deviation vs Control (%)		-0.9	1.6	10.5
d 63 → 70	**Mean (g)**	22.3	20.4	21.5	23.9
	S.D.	0.7	1.2	0.7	0.3
	N	2	2	2	2
	Deviation vs Control (%)		-8.2	-3.4	7.5
d 70 → 77	**Mean (g)**	22.6	21.5	20.9	24.3
	S.D.	0.5	0.1	0.2	1.3
	N	2	2	2	2
	Deviation vs Control (%)		-4.8	-7.5	7.2
d 77 → 84	**Mean (g)**	22.3	21.6	22.3	22.1
	S.D.	0.9	0.1	1.8	0.6
	N	2	2	2	2
	Deviation vs Control (%)		-4.8	0.1	-1.1
d 84 → 91	**Mean (g)**	19.9	21.6	19.7	20.1
	S.D.	1.1	0.0	1.2	0.6
	N	2	2	2	2
	Deviation vs Control (%)		-3.0	-1.3	0.9

d = day

Table 96: Summary food consumption per animal per day - Females

		TG 0 / F **0 mg/kg bw/d**	**TG 1 / F** **150 mg/kg bw/d**	**TG 2 / F** **450 mg/kg bw/d**	**TG 3 / F** **1400 mg/kg bw/d**
d -4 → 0	**Mean (g)**	14.4	14.6	14.8	14.4
	S.D.	0.3	0.1	0.8	0.2
	N	2	2	2	2
	Deviation vs Control (%)		1.9	2.9	0.0
d 0 → 7	**Mean (g)**	14.8	14.6	14.9	15.0
	S.D.	0.2	0.3	0.2	0.0
	N	2	2	2	2
	Deviation vs Control (%)		-1.6	0.5	1.3
d 7 → 14	**Mean (g)**	16.0	15.6	15.9	16.0

	S.D.	0.2	0.8	0.5	0.0
	N	2	2	2	2
	Deviation vs Control (%)		-2.4	-0.5	0.5
d 14 → 21	**Mean (g)**	16.1	16.1	16.0	16.7
	S.D.	0.3	0.2	0.5	0.0
	N	2	2	2	2
	Deviation vs Control (%)		0.5	-0.2	3.9
d 21 → 28	**Mean (g)**	17.0	16.9	16.5	16.5
	S.D.	0.4	0.3	0.5	0.2
	N	2	2	2	2
	Deviation vs Control (%)		-0.6	-3.2	-3.1
d 28 → 35	**Mean (g)**	17.1	16.7	16.5	17.1
	S.D.	0.4	0.3	0.3	1.1
	N	2	2	2	2
	Deviation vs Control (%)		-2.7	-3.4	-0.4
d 35 → 42	**Mean (g)**	17.3	16.4	16.3	16.9
	S.D.	0.1	0.7	0.4	0.6
	N	2	2	2	2
	Deviation vs Control (%)		-5.1	-6.1	-2.3
d 42 → 49	**Mean (g)**	17.1	19.9	16.4	16.8
	S.D.	1.0	3.6	0.1	0.5
	N	2	2	2	2
	Deviation vs Control (%)		16.8	-4.2	-1.4
d 49 → 56	**Mean (g)**	17.3	16.8	17.1	17.0
	S.D.	0.1	0.2	0.5	1.1
	N	2	2	2	2
	Deviation vs Control (%)		-3.1	-1.4	-1.7
d 56 → 63	**Mean (g)**	16.8	15.9	15.9	16.0
	S.D.	0.4	0.5	0.6	1.2
	N	2	2	2	2
	Deviation vs Control (%)		-5.6	-5.7	-5.2
d 63 → 70	**Mean (g)**	17.3	16.9	16.4	16.3

	S.D.	0.2	1.1	0.2	0.4
	N	2	2	2	2
	Deviation vs Control (%)		-2.5	-5.3	-5.9
d 70 → 77	**Mean (g)**	17.8	16.2	16.7	16.7
	S.D.	0.6	0.3	0.6	0.4
	N	2	2	2	2
	Deviation vs Control (%)		-8.8	-6.2	-6.3
d 77 → 84	**Mean (g)**	18.0	16.8	16.0	16.0
	S.D.	0.2	0.0	0.3	0.7
	N	2	2	2	2
	Deviation vs Control (%)		-6.8	-10.9	-10.7
d 84 → 91	**Mean (g)**	15.7	14.4	13.9	14.1
	S.D.	0.4	0.2	0.1	0.8
	N	2	2	2	2
	Deviation vs Control (%)		-8.1	-11.0	-9.7

d = day

- Water consumption:
  - No test substance-related findings were observed.
- Body weight:
  - No significant alteration of the body weight was observed in male and female animals of any test group.
  - Group 3 (1400 mg/kg bw/d):
    - The body weight change in males from administration day 0 to 91 was -9.7% lower than in the current control resulting in a non-significant decrease of the corresponding body weight of -5.5%. These findings were considered as treatment-related but not adverse.

Table 97: Summary Body weights per animal per day - Males

		TG 0 / M 0 mg/kg bw/d		**TG 1 / M 150 mg/kg bw/d**	**TG 2 / M 450 mg/kg bw/d**	**TG 3 / M 1400 mg/kg bw/d**
Day 0	**Mean (g)**	182.5	n	182.3	181.8	182.4
	S.D.	7.8		8.0	9.2	10.0

	N	10		10	10	10
	Deviation vs Control (%)			-0.1	-0.4	-0.1
Day 7	**Mean (g)**	225.5	n	224.2	220.5	223.5
	S.D.	8.3		8.7	8.5	17.8
	N	10		10	10	10
	Deviation vs Control (%)			-0.6	-2.2	-0.9
Day 14	**Mean (g)**	264.6	n	263.0	256.4	264.4
	S.D.	11.2		10.8	10.4	18.2
	N	10		10	10	10
	Deviation vs Control (%)			-0.6	-3.1	0.0
Day 21	**Mean (g)**	294.8	n	295.3	288.7	296.7
	S.D.	15.3		14.3	12.6	17.4
	N	10		10	10	10
	Deviation vs Control (%)			0.2	-2.1	0.6
Day 28	**Mean (g)**	315.8	n	317.5	315.8	317.0
	S.D.	16.7		18.2	13.4	19.9
	N	10		10	10	10
	Deviation vs Control (%)			0.6	0.0	0.4
Day 35	**Mean (g)**	336.7	n	337.0	33.8	333.4
	S.D.	20.3		19.9	13.3	24.9
	N	10		10	10	10
	Deviation vs Control (%)			0.1	-0.9	-1.0
Day 42	**Mean (g)**	352.6	n	355.5	350.3	348.8
	S.D.	22.3		23.8	14.3	25.0
	N	10		10	10	10
	Deviation vs Control (%)			0.8	-0.6	-1.1
Day 49	**Mean (g)**	367.0	n	368.8	360.7	362.6
	S.D.	24.2		24.5	14.5	22.0
	N	10		10	10	10
	Deviation vs Control (%)			0.5	-1.7	-1.2
Day 56	**Mean (g)**	379.5	n	380.8	371.4	31.1
	S.D.	25.3		22.6	16.1	21.5

	N	10		10	10	10
	Deviation vs Control (%)			0.3	-2.1	-2.2
Day 63	**Mean (g)**	390.0	n	390.6	381.2	380.0
	S.D.	27.9		26.5	17.8	21.3
	N	10		10	10	10
	Deviation vs Control (%)			0.2	2.2	-2.6
Day 70	**Mean (g)**	402.2	n	403.9	392.0	389.3
	S.D.	30.5		25.9	20.8	22.2
	N	10		9	10	10
	Deviation vs Control (%)			0.4	-2.6	-3.2
Day 77	**Mean (g)**	412.4	n	412.4	399.0	395.8
	S.D.	32.0		28.7	22.5	20.5
	N	10		9	10	10
	Deviation vs Control (%)			0.0	-3.2	-4.0
Day 84	**Mean (g)**	418.4	n	418.2	403.2	398.8
	S.D.	31.9		30.1	21.1	20.1
	N	10		9	10	10
	Deviation vs Control (%)			0.0	-3.6	-4.6
Day 91	**Mean (g)**	417.7	n	420.3	404.6	394.7
	S.D.	32.5		30.3	21.9	18.6
	N	10		9	10	10
	Deviation vs Control (%)			0.6	-3.1	-5.5

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics, n=DUNNETT

Table 98: Summary Body weights per animal per day - Females

		TG 0 / F **0 mg/kg bw/d**		**TG 1 / F** **150 mg/kg bw/d**	**TG 2 / F** **450 mg/kg bw/d**	**TG 3 / F** **1400 mg/kg bw/d**
Day 0	**Mean (g)**	139.4	n	142.1	141.3	142.5
	S.D.	6.2		5.5	8.5	8.9
	N	10		10	10	10
	Deviation vs Control (%)			2.0	1.4	2.2
Day 7	**Mean (g)**	155.4	n	155.2	155.9	156.5

	S.D.	9.4		5.6	7.5	11.0
	N	10		10	10	10
	Deviation vs Control (%)			-0.1	0.3	0.7
Day 14	**Mean (g)**	173.5	n	175.9	175.6	176.4
	S.D.	8.8		8.9	5.4	16.3
	N	10		10	10	10
	Deviation vs Control (%)			1.4	1.2	1.7
Day 21	**Mean (g)**	188.6	n	189.4	187.6	192.6
	S.D.	12.2		10.2	6.6	16.0
	N	10		10	10	10
	Deviation vs Control (%)			0.4	-0.5	2.1
Day 28	**Mean (g)**	198.2	n	204.3	201.1	206.4
	S.D.	13.3		14.0	5.5	16.6
	N	10		10	10	10
	Deviation vs Control (%)			3.1	1.5	4.1
Day 35	**Mean (g)**	205.9	n	210.1	207.7	210.9
	S.D.	15.0		13.1	7.5	16.7
	N	10		10	10	10
	Deviation vs Control (%)			2.0	0.9	2.4
Day 42	**Mean (g)**	215.7	n	219.3	215.7	221.2
	S.D.	14.5		14.0	7.3	21.5
	N	10		10	10	10
	Deviation vs Control (%)			1.7	0.0	2.5
Day 49	**Mean (g)**	224.6	n	227.4	222.2	229.4
	S.D.	16.8		14.2	6.0	22.2
	N	10		10	10	10
	Deviation vs Control (%)			1.2	-1.1	2.2
Day 56	**Mean (g)**	226.5	n	232.6	226.3	234.5
	S.D.	17.2		14.0	6.1	21.2
	N	10		10	10	10
	Deviation vs Control (%)			2.7	-0.1	3.6
Day 63	**Mean (g)**	227.6	n	232.7	227.0	233.2

	S.D.	17.5		13.7	10.0	19.9
	N	10		10	10	10
	Deviation vs Control (%)			2.3	-0.3	2.5
Day 70	**Mean (g)**	234.9	n	238.7	234.1	240.0
	S.D.	17.1		14.2	7.5	22.0
	N	10		10	10	10
	Deviation vs Control (%)			1.6	-0.4	2.1
Day 77	**Mean (g)**	238.6	n	241.1	236.3	245.1
	S.D.	18.7		13.9	7.3	21.7
	N	10		10	10	10
	Deviation vs Control (%)			1.1	-1.0	2.7
Day 84	**Mean (g)**	240.4	n	243.9	238.4	247.0
	S.D.	19.5		13.3	8.5	21.3
	N	10		10	10	10
	Deviation vs Control (%)			1.4	-0.8	2.7
Day 91	**Mean (g)**	240.0	n	241.8	237.4	244.5
	S.D.	16.7		16.3	11.7	20.4
	N	10		10	10	10
	Deviation vs Control (%)			0.8	-1.1	1.9

Statistic Profile = Dunnett test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics, n=DUNNETT

- Functional observational battery
  - Deviations from "zero values" were obtained in several animals. However, as most findings were equally distributed between test-substance treated groups and controls, were without a dose-response relationship or occurred in single animals only, these observations were considered to have been incidental. The following examinations were performed during FOB and have to be assessed individually:
    - Home cage observations:
      - No test substance-related adverse effects were observed.
    - Open field observations:

- No test substance related adverse effects were observed in females.
- Group 3 (1400 mg/kg bw/d):
  - Soft faeces were observed in 4 males, which represented all males of the test group with defecation during the observation period.

– Sensory-motoric test/ reflexes:

- No test substance-related effects were observed.

– Quantitative parameters:

- No test substance-related adverse effects were observed.

Table 99: Functional observational battery, summary of results males and females

		Males				Females			
	Rank	Group 0 N=5	Group 1 N=5	Group 2 N=5	Group 3 N=5	Group 0 N=5	Group 1 N=5	Group 2 N=5	Group 3 N=5
HOME CAGE OBSERVATION									
posture									
animal is sitting or laying	0	5	5	4	7	3	3	2	3
animal is standing and moving	1	5	4	6	3	7	7	8	7
squatting posture	2	0	0	0	0	0	0	0	0
abdominal position	3	0	0	0	0	0	0	0	0
abdominal position with splayed limbs	4	0	0	0	0	0	0	0	0
lateral position	5	0	0	0	0	0	0	0	0
oblique head posture	6	0	0	0	0	0	0	0	0
opisthotonus	7	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
tremors									
no tremors	0	10	9	10	10	10	10	10	10
slight tremors	1	0	0	0	0	0	0	0	0
moderate tremors	2	0	0	0	0	0	0	0	0
severe tremors	3	0	0	0	0	0	0	0	0
convulsions									
no convulsions	0	10	9	10	10	10	10	10	10
slight convulsions	1	0	0	0	0	0	0	0	0
moderate convulsions	2	0	0	0	0	0	0	0	0
severe convulsions	3	0	0	0	0	0	0	0	0
abnormal movements									
no abnormalities	0	10	9	10	10	10	10	10	10
manege movements	1	0	0	0	0	0	0	0	0
head shaking	2	0	0	0	0	0	0	0	0
excessive cleaning	3	0	0	0	0	0	0	0	0
frequent chewing	4	0	0	0	0	0	0	0	0
gait									
animal is not walking during observation	0	5	5	4	7	5	8	7	4
no impairment of gait	1	5	4	6	3	5	2	3	6
stiff gait	2	0	0	0	0	0	0	0	0
slight impairment of coordination, unsteady gait	3	0	0	0	0	0	0	0	0
moderate impairment of coordination, shuffling gait	4	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
severe impairment of coordination, dragging of the hindlimbs	5	0	0	0	0	0	0	0	0
severe impairment of coordination, with splayed limbs	6	0	0	0	0	0	0	0	0
animal is unable to walk (abdominal or lateral position)	7	0	0	0	0	0	0	0	0
other findings	0	10	9	10	10	10	10	10	9
OPEN FIELD OBSERVATIONS									
behaviour on removal from the cage									
animal is tense, but it shows no resistance against handling	0	10	9	10	10	10	10	10	10
animal shows a slight resistance against the handling	1	0	0	0	0	0	0	0	0
animal shows no resistance against the handling but appears indifferent	2	0	0	0	0	0	0	0	0
animal is difficult to handle, it shows aggressiveness	3	0	0	0	0	0	0	0	0
animal is very difficult to handle, it shows severe aggressiveness	4	0	0	0	0	0	0	0	0
fur									
nothing abnormal detected	0	10	9	10	10	10	10	10	10
discolored fur	1	0	0	0	0	0	0	0	0
urine staining of anogenital region	2	0	0	0	0	0	0	0	0
piloerection	3	0	0	0	0	0	0	0	0
alopecia	4	0	0	0	0	0	0	0	0
reduced care on fur	5	0	0	0	0	0	0	0	0
skin									
nothing abnormal detected	0	10	9	10	10	10	10	10	10
discolored skin	1	0	0	0	0	0	0	0	0
reddening	2	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
paleness	3	0	0	0	0	0	0	0	0
dehydration (exsiccosis)	4	0	0	0	0	0	0	0	0
hypothermia (skin is cold during handling)	5	0	0	0	0	0	0	0	0
lesion(s)	6	0	0	0	0	0	0	0	0
crust(s)	7	0	0	0	0	0	0	0	0
salivation									
no salivation	0	10	9	10	10	10	10	10	10
slight salivation (area around the mouth is moist)	1	0	0	0	0	0	0	0	0
moderate salivation (wet mouth)	2	0	0	0	0	0	0	0	0
severe salivation (mouth very wet, wet paws)	3	0	0	0	0	0	0	0	0
nasal discharge									
no discharge, dry nose	0	10	9	10	10	10	10	10	10
clear discharge	1	0	0	0	0	0	0	0	0
reddish discharge	2	0	0	0	0	0	0	0	0
lacrimation									
no lacrimation	0	10	9	10	10	10	10	10	10
slight lacrimation	1	0	0	0	0	0	0	0	0
moderate lacrimation	2	0	0	0	0	0	0	0	0
severe lacrimation	3	0	0	0	0	0	0	0	0
eyes/pupil size									
nothing abnormal detected, pupils contracted at room light	0	10	9	10	10	10	10	10	10
chromodacryorrhea	1	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
exophthalmos	2	0	0	0	0	0	0	0	0
pupils dilated	3	0	0	0	0	0	0	0	0
abnormal shape of pupils	4	0	0	0	0	0	0	0	0
oblique eye posture	5	0	0	0	0	0	0	0	0
opacity	6	0	0	0	0	0	0	0	0
cataract	7	0	0	0	0	0	0	0	0
posture									
animal is sitting or laying	0	0	0	0	0	0	0	0	0
animal is standing and moving	1	10	9	10	10	10	10	10	10
squatting posture	2	0	0	0	0	0	0	0	0
abdominal position	3	0	0	0	0	0	0	0	0
abdominal position with splayed limbs	4	0	0	0	0	0	0	0	0
lateral position	5	0	0	0	0	0	0	0	0
oblique head posture	6	0	0	0	0	0	0	0	0
opisthotonus	7	0	0	0	0	0	0	0	0
palpebral closure									
nothing abnormal detected	0	10	9	10	10	10	10	10	10
eyelid(s) slight closure	1	0	0	0	0	0	0	0	0
eyelid(s) half closure	2	0	0	0	0	0	0	0	0
eyelid(s) permanent closure	3	0	0	0	0	0	0	0	0
respiration									
nothing abnormal detected	0	10	9	10	10	10	10	10	10
respiration labored	1	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
gasping/respiratory sounds	2	0	0	0	0	0	0	0	0
respiration accelerated	3	0	0	0	0	0	0	0	0
respiration irregular	4	0	0	0	0	0	0	0	0
tremors									
no tremors	0	10	9	10	10	10	10	10	10
slight tremors	1	0	0	0	0	0	0	0	0
moderate tremors	2	0	0	0	0	0	0	0	0
severe tremors	3	0	0	0	0	0	0	0	0
convulsions									
no convulsions	0	10	9	10	10	10	10	10	10
slight convulsions	1	0	0	0	0	0	0	0	0
moderate convulsions	2	0	0	0	0	0	0	0	0
severe convulsions	3	0	0	0	0	0	0	0	0
abnormal movements/stereotypics									
no abnormalities	0	10	9	10	10	10	10	10	10
manege movements	1	0	0	0	0	0	0	0	0
head shaking	2	0	0	0	0	0	0	0	0
excessive cleaning	3	0	0	0	0	0	0	0	0
frequent chewing	4	0	0	0	0	0	0	0	0
gait									
animal is not walking during observation	0	0	0	0	0	0	0	0	0
no impairment of gait	1	10	9	10	10	10	10	10	10

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
stiff gait	2	0	0	0	0	0	0	0	0
slight impairment of coordination, unsteady gait	3	0	0	0	0	0	0	0	0
moderate impairment of coordination, shuffling gait	4	0	0	0	0	0	0	0	0
severe impairment of coordination, dragging of the hindlimbs	5	0	0	0	0	0	0	0	0
severe impairment of coordination, with splayed limbs	6	0	0	0	0	0	0	0	0
animal is unable to walk (abdominal or lateral position)	7	0	0	0	0	0	0	0	0
activity/arousal level									
normal exploration of the area	0	10	9	10	10	10	10	10	10
reduced exploration of the area	1	0	0	0	0	0	0	0	0
severe reduced exploration of the area, animal apathetic	2	0	0	0	0	0	0	0	0
increased exploration of the area, sudden or jerky movements	3	0	0	0	0	0	0	0	0
hyperactivity	4	0	0	0	0	0	0	0	0
faeces									
no defecation during observation period	0	10	9	8	6	10	10	10	10
feces without abnormalities	1	0	0	2	0	0	0	0	0
discolored feces	2	0	0	0	0	0	0	0	0
crumbly feces	3	0	0	0	0	0	0	0	0
soft feces	4	0	0	0	4	0	0	0	0
muicid feces	5	0	0	0	0	0	0	0	0
diarrhea	6	0	0	0	0	0	0	0	0
urine									
no urination during observation period	0	6	4	5	8	7	6	6	8

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
urine without abnormalities (some wet areas on the filter paper)	1	4	5	5	2	3	4	4	2
discoloration of urine	2	0	0	0	0	0	0	0	0
polyuria (great wet areas on the filter paper)	3	0	0	0	0	0	0	0	0
other findings	0	10	9	10	10	10	10	10	9
SENSORIMOTOR TESTS/REFLEXES									
approach response									
no reaction	0	4	5	4	2	3	3	6	6
approaching to object	1	6	4	6	8	7	7	4	4
escape reaction	2	0	0	0	0	0	0	0	0
aggressive reaction and attacking of object	3	0	0	0	0	0	0	0	0
touch response									
no reaction	0	4	2	1	2	10	9	10	10
orientation to the stimulus	1	6	7	9	8	0	1	0	0
escape after touch	2	0	0	0	0	0	0	0	0
aggressive reaction and attacking of object	3	0	0	0	0	0	0	0	0
reaction to the stimulus but no ability to localize (e.g. turning to wrong side)	4	0	0	0	0	0	0	0	0
vision									
nothing abnormal detected (grasping with forelimbs)	0	10	9	10	10	10	10	10	10
no grasping	1	0	0	0	0	0	0	0	0
pupillary reflex									
nothing abnormal detected, physiological adaptation of the pupil to light	0	10	9	10	10	10	10	10	10
retarded adaptation of the pupil to light	1	0	0	0	0	0	0	0	0

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
no adaptation of the pupil to light, pupils permanently contracted	2	0	0	0	0	0	0	0	0
no adaptation of the pupil to light, pupils permanently dilated	3	0	0	0	0	0	0	0	0
pinna reflex									
immediate response to the stimulus	0	10	9	10	10	10	10	10	10
no response to the stimulus	1	0	0	0	0	0	0	0	0
audition									
nothing abnormal detected, immediate normal response to the stimulus	0	10	9	10	10	10	10	10	10
no response	1	0	0	0	0	0	0	0	0
increased response	2	0	0	0	0	0	0	0	0
hyperreaction	3	0	0	0	0	0	0	0	0
coordination of movements									
nothing abnormal detected, immediate righting response	0	10	9	10	10	10	10	10	10
retarded righting response	1	0	0	0	0	0	0	0	0
fails to turn into upright position, animal stays in lateral position	2	0	0	0	0	0	0	0	0
no righting response, animal stays in dorsal position	3	0	0	0	0	0	0	0	0
behavior during handling									
normal behavior, easy to handle, animal is tense, but it shows no resistance	0	10	9	10	10	10	10	10	10
very easy to handle, animal is limply hanging in the hand	1	0	0	0	0	0	0	0	0
slightly difficult to handle, animal shows a slight resistance against handling	2	0	0	0	0	0	0	0	0
difficult to handle, animal shows a severe resistance against handling	3	0	0	0	0	0	0	0	0
vocalization									

		Males				Females			
	Rank	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**	**Group 0 N=5**	**Group 1 N=5**	**Group 2 N=5**	**Group 3 N=5**
no or only sporadic vocalizations when touched	0	10	9	10	10	10	10	10	10
very frequent vocalizations when touched	1	0	0	0	0	0	0	0	0
vocalizations always when touched	2	0	0	0	0	0	0	0	0
vocalization without touching	3	0	0	0	0	0	0	0	0
pain perception									
nothing abnormal detected, immediate response to the stimulus	0	10	9	10	10	10	10	10	10
weak or retarded reaction to the stimulus	1	0	0	0	0	0	0	0	0
no response to the stimulus	2	0	0	0	0	0	0	0	0
hyperreaction to the stimulus	3	0	0	0	0	0	0	0	0
other findings	0	10	9	10	10	10	10	10	9

- Motor activity measurement
  - Group 3 (1400 mg/kg bw/d):
    - The females had a decreased motor activity in interval 1, 2 and in the overall interval.
    - The findings in females were considered as treatment-related and adverse.
  - Group 2 (450 mg/kg bw/d):
    - Females showed a decreased overall interval, but no significant alteration in any single interval.
    - For the finding in females it could not be excluded that this finding was treatment-related but since there was no single interval showing a significant difference to the control, this alteration was assessed as non-adverse.
  - Group 1 (150 mg/kg bw/d):

- Single interval 2 was decreased in females.
- The alteration of motor activity in females observed in one interval only was considered as incidental and not related to treatment based on its isolated occurrence and minor deviation from the current control having no impact on the motor activity over all intervals.

Table 100: Motor activity Day 30, summary of results males and females (1/2)

		Interv. 1 Beam Interr.		Interv. 2 Beam Interr.		Interv. 3 Beam Interr.		Interv. 4 Beam Interr.		Interv. 5 Beam Interr.		Interv. 6 Beam Interr.		Interv. 7 Beam Interr.	
		Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
0 mg/kg	M	714.8	1366.0	571.1	902.9	345.0	468.7	185.7	300.1	50.9	149.4	42.3	114.5	55.9	95.7
	SD	156.2	282.7	102.7	191.3	178.1	202.6	172.0	155.1	51.1	152.5	74.2	151.6	46.2	98.2
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10
150 mg/kg	M	825.2	119.0	591.3	701.7*	425.9	471.8	178.4	240.8	91.1	196.8	72.1	118.8	69.9	91.4
	SD	207.1	249.0	206.9	173.8	269.9	163.3	164.4	152.9	104.3	223.1	117.6	146.7	86.0	156.4
	N	9	10	9	10	9	10	9	10	9	10	9	10	9	10
450 mg/kg	M	798.3	1163.4	574.3	737.9	426.4	306.6	220.6	179.4	124.9	146.6	63.1	70.7	59.9	42.9
	SD	243.1	305.1	169.4	251.7	182.6	169.0	144.0	151.3	124.7	186.3	70.6	95.4	145.7	77.7
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1400 mg/kg	M	675.5	815.6**	454.1	487.2**	368.7	325.0	202.5	285.1	91.0	186.6	61.9	114.5	70.6	87.2
	SD	215.6	216	125.1	172.3	148.2	135.4	99.4	132.5	87.0	118.5	67.3	152.0	114.6	138.0
	N	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Kruskal-Wallis + Wilcoxon-tests (two-sided): * p<=0.05; ** p<=0.01 (Statistical unit = Animal)

Table 101: Motor activity Day 30, summary of results males and females (2/2)

		Interv. 8 Beam Interr.		Interv. 9 Beam Interr.		Interv. 10 Beam Interr.		Interv. 11 Beam Interr.		Interv. 12 Beam Interr.		Interv. 1-12 Sum Interr.	
		Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
0 mg/kg	M	40.8	75.1	58.6	68.1	61.8	25.4	13.9	26.0	17.9	11.4	2158.7	3603.3
	SD	38.9	97.0	64.3	103.4	89.8	56.9	21.7	26.8	17.9	23.0	658.0	838.9
	N	10	10	10	10	10	10	10	10	10	10	10	10
150 mg/kg	M	40.9	42.1	38.4	54.3	58.4	61.3	22.1	5.4	32.8	11.8	2446.7	3115.2
	SD	58.9	50.5	55.4	105.0	60.0	111.4	34.6	7.5	31.1	10.5	831.6	819.0
	N	9	10	9	10	9	10	9	10	9	10	9	10
450 mg/kg	M	34.9	58.5	61.4	48.8	22.3	18.5	29.9	16.8	15.0	50.1	2431.0	2840.2*
	SD	56.4	92.4	100.3	84.6	35.6	14.1	25.0	20.6	25.2	99.0	673.5	903.9
	N	10	10	10	10	10	10	10	10	10	10	10	10
1400 mg/kg	M	43.5	35.7	26.9	53.6	45.3	50.7	22.1	35.4	17.4	25.5	2079.5	2502.1**
	SD	95.8	65.0	56.6	79.0	85.3	56.5	31.0	51.8	27.0	35.4	554.9	752.1
	N	10	10	10	10	10	10	10	10	10	10	10	10

Kruskal-Wallis + Wilcoxon-tests (two-sided): * p<=0.05; ** p<=0.01 (Statistical unit = Animal)

- Ophthalmological examinations
  - There were no treatment-related findings.
  - One female animal (No. 74) showed an injury of the right eye with a closed eyelid on study day 91 which is also mentioned before in the clinical observations (loss of eyeball). It was assessed as not treatment-related.

Table 102: Ophthalmological examination

	Study day -4								Studay day 91			
	TG 0 0 mg/kg bw/d		TG 1 150 mg/kg bw/d		TG 2 450 mg/kg bw/d		TG 3 1400 mg/kg bw/d		TG 0 0 mg/kg bw/d		TG 3 1400 mg/kg bw/d	
	M	F	M	F	M	F	M	F	M	F	M	F
Number of animals examined	10	10	10	10	10	10	10	10	10	10	10	10
Animals without ophthalmological findings	3	2	4	4	2	2	5	3	9	9	9	9
Corneal stipplings	3	2	1	2	3	2	0	2	1	1	1	0
Remainders of the pupillary membrane	6	8	5	5	7	8	5	6	0	0	0	0
Injury of the eye, eyelid closed	0	0	0	0	0	0	0	0	0	0	0	1

- Oestrous cycle determination

Table 103: Oestrous cycle determination

Dose Group	Animal Number	Day 93
TG 0/F 0 mg/kg bw/day	41	/P
	42	M
	43	M
	44	/E
	45	/P
	46	/E
	47	/E
	48	M
	49	M
	50	M
TG 1/F 150 mg/kg bw/day	51	D
	52	/E
	53	/P
	54	D

	55	/E
	56	D
	57	/E
	58	D
	59	D
	60	D
TG 2/F **450 mg/kg bw/day**	61	/E
	62	D
	63	M
	64	/E
	65	/E
	66	M
	67	D
	68	D
	69	D
	70	D
TG 3/F **1400 mg/kg bw/day**	71	M
	72	/P
	73	/P
	74	D
	75	/E
	76	D
	77	D
	78	D
	79	D
	80	/E

/ = Start of Cycle; E = Estrous; D = Diestrous; M = Metestrous; P = Proestrous

- Clinical pathology
  - Haematology
    - Group 3 (1400 mg/kg bw/d):

- At the end of the administration period in male and female rats haemoglobin and haematocrit values were significantly decreased. These alterations were regarded as treatment-related and adverse.
- In females of this test group red blood cell (RBC) counts were significantly decreased and absolute neutrophil cell counts were significantly increased. A dose response is observed for the females on the relative neutrophil level. Non-significant increase of absolute and relative neutrophils levels were observed in males. These alterations were regarded as treatment-related and adverse.
- In males absolute reticulocyte counts were significantly lower compared to controls. These alterations were regarded as treatment-related and adverse.
- Slight increase in the WBC levels in the females.
- In males mean corpuscular volume (MCV) and absolute basophil counts were significantly decreased. However, all values were within historical control ranges (males, MCV 47.8-51.3 fL; absolute basophils 0.01-0.04 Giga/L). The mentioned changes were regarded as incidental and not treatment-related.

– Group 2 (450 mg/kg bw/d):

- Absolute reticulocyte counts in males were significantly decreased. However, these values for the mid dose were within historical control ranges (males, absolute reticulocytes 116.2-159.1 Giga/L). The mentioned changes were regarded as incidental and not treatment-related.
- Absolute neutrophils levels are increased in males and females.

– Group 1 (150 mg/kg bw/d):

- In females absolute basophil counts were significantly increased, but the change was not dose-dependent. Therefore, the mentioned changes were regarded as incidental and not treatment-related.
- Slight increase in the WBC levels in the females.

Table 104: Haematology parameters, summary of results males (day 92) and females (day 93)

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
RBC [tera/L]	Mean	8.72k	7.78v	8.78	7.79	8.79	7.84	8.76	7.43*
	S.d.	0.45	0.34	0.30	0.26	0.33	0.37	0.35	0.23
	N	10	10	9	10	10	10	10	10
	Median	8.68	7.78	8.79	7.77	8.82	7.88	8.86	7.36
	Deviation vs. Control (%)			0.69	0.13	0.78	0.80	0.41	-4.54
HGB [mmol/L]	Mean	9.1v	8.4v	9.0	8.4	9.0	8.4	8.7**	7.9**
	S.d.	0.1	0.3	0.2	0.2	0.3	0.4	0.3	0.2
	N	10	10	9	10	10	10	10	10
	Median	9.1	8.5	9.0	8.3	9.1	8.4	8.6	7.9
	Deviation vs. Control (%)			-1.5	-0.7	-0.9	-0.7	-4.6	-6.7
HCT [L/L]	Mean	0.444v	0.401v	0.433	0.402	0.436	0.403	0.421**	0.377**
	S.d.	0.015	0.012	0.010	0.010	0.011	0.018	0.015	0.013
	N	10	10	9	10	10	10	10	10
	Median	0.444	0.405	0.431	0.400	0.435	0.408	0.417	0.378
	Deviation vs. Control (%)			-2.383	0.199	-1.780	0.548	-5.047	-5.932
MCV [fL]	Mean	50.9v	51.6k	49.4	51.6	49.6	51.4	48.1**	50.8
	S.d.	1.6	1.2	1.8	0.8	1.6	1.5	1.2	1.6
	N	10	10	9	10	10	10	10	10
	Median	50.8	51.2	49.3	51.8	49.3	51.2	48.2	50.8
	Deviation vs. Control (%)			-3.1	0.0	-2.5	-0.3	-5.5	-1.5
MCH [fmol]	Mean	1.05k	1.08k	1.02	1.08	1.03	1.07	1.00	1.06
	S.d.	0.05	0.03	0.04	0.02	0.04	0.04	0.03	0.03
	N	10	10	9	10	10	10	10	10
	Median	1.06	1.08	1.01	1.08	1.02	1.07	1.00	1.06
	Deviation vs. Control (%)			-2.43	-0.65	-2.00	-1.29	-5.05	-2.22

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
MCHC [mmol/L]	Mean	20.6k	20.97k	20.74	20.82	20.77	20.72	20.67	20.86
	S.d.	0.45	0.38	0.20	0.24	0.33	0.22	0.17	0.34
	N	10	10	9	10	10	10	10	10
	Median	20.51	21.00	141.9	20.85	20.78	20.72	20.70	20.84
	Deviation vs. Control (%)			-8.9	-0.74	0.84	-1.19	0.34	-0.53
RET [giga/L]	Mean	154.6v	159.9k	140.9	163.4	132.0*	137.0	105.4**	135.0
	S.d.	22.9	31.9	24.6	36.7	14.5	35.2	21.4	25.7
	N	10	10	9	10	10	10	10	10
	Median	159.4	154.2	141.9	162.4	130.7	120.8	102.5	131.6
	Deviation vs. Control (%)			-8.9	2.1	-14.6	-14.3	-31.8	-15.6
PLT [giga/L]	Mean	728k	696k	724	751	805	757	710	681
	S.d.	82	51	140	82	90	71	81	100
	N	10	10	9	10	9	10	10	10
	Median	703	715	666	732	818	728	700	708
	Deviation vs. Control (%)			-1	8	11	9	-2	-2
HQT [sec]	Mean	36.9k	34.7k	37.1	34.9	37.4	35.1	35.6	33.7
	S.d.	1.4	1.3	2.2	1.5	2.3	1.4	1.7	1.4
	N	10	10	9	10	9	10	10	10
	Median	36.8	35	36.8	35.2	37.2	35.2	35.8	33.8
	Deviation vs. Control (%)			0.4	0.8	1.1	1.2	-3.6	-2.7
WBC [giga/L]	Mean	5.16k	2.96k	5.35	3.85	4.79	3.03	5.07	3.55
	S.d.	0.65	1.24	1.12	1.57	0.77	0.78	0.91	1.05
	N	10	10	9	10	10	10	10	10
	Median	5.39	2.92	5.45	3.42	4.81	2.91	4.86	3.66
	Deviation vs. Control (%)			3.72	30.20	-7.12	2.33	-1.61	19.83
NEUTA	Mean	1.02k	0.54v	1.13	1.09	1.02	0.79	1.19	1.03**
	S.d.	0.30	0.20	0.28	1.02	0.29	0.34	0.28	0.45

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
[giga/L]	N	10	10	9	10	10	10	10	10
	Median	1.02	0.50	1.18	0.68	1.00	0.74	1.17	0.99
	Deviation vs. Control (%)			10.6	100.92	-0.10	46.58	16.86	90.39
LYMPHA [giga/L]	Mean	3.89k	2.26k	3.94	2.53	3.55	2.08	3.65	2.34
	S.d.	0.57	1.05	1.02	0.72	0.66	0.53	0.84	0.82
	N	10	10	9	10	10	10	10	10
	Median	3.89	2.34	3.84	2.26	3.48	2.05	3.46	2.29
	Deviation vs. Control (%)			1.37	11.8	-8.71	-8.09	-6.09	3.67
MONOA [giga/L]	Mean	0.11k	0.06k	0.12	0.10	0.09	0.06	0.1	0.07
	S.d.	0.03	0.03	0.05	0.09	0.03	0.02	0.03	0.03
	N	10	0	9	10	10	10	10	10
	Median	0.12	0.06	0.11	0.08	0.08	0.06	0.10	0.08
	Deviation vs. Control (%)			13.21	54.69	-17.92	-9.37	-3.77	15.62
EOSA [giga/L]	Mean	0.11k	0.07k	0.13	0.11	0.1	0.08	0.10	0.08
	S.d.	0.03	0.04	0.05	0.04	0.02	0.01	0.03	0.03
	N	10	10	9	10	10	10	10	10
	Median	0.11	0.05	0.12	0.11	0.10	0.08	0.09	0.08
	Deviation vs. Control (%)			11.11	49.32	-11.4	4.11	-11.4	5.48
BASOA [giga/L]	Mean	0.02v	0.01v	0.01	0.02*	0.01	0.02	0.01*	0.01
	S.d.	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01
	N	10	10	9	10	10	10	10	10
	Median	0.02	0.01	0.01	0.02	0.01	0.02	0.01	0.01
	Deviation vs. Control (%)			-29.82	64.29	-31.58	21.43	-47.37	-14.29
LUCA [giga/L]	Mean	0.01k	0.01k	0.01	0.01	0.01	0.01	0.01	0.01
	S.d.	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01
	N	10	10	9	10	10	10	10	10
	Median	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.01

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
	Deviation vs. Control (%)			1.85	83.33	0.00	0.00	16.67	50.00
NEUT [%]	Mean	19.8k	20.0k	21.5	25.2	21.4	25.7	23.8	29.2
	S.d.	5.5	8.9	5.5	11.8	5.1	6.6	5.5	9.0
	N	10	10	9	10	10	10	10	10
	Median	19.1	17.8	20.4	22.0	20.9	27.0	23.2	30.1
	Deviation vs. Control (%)			8.7	26.2	8.0	28.7	20.4	46.2
LYMPH [%]	Mean	75.4k	74.7k	73.2	68.6	74.1	68.9	71.8	65.9
	S.d.	5.4	8.9	7.0	12.2	5.4	6.8	6.0	8.9
	N	10	10	9	10	10	10	10	10
	Median	76.6	77.0	74.0	72.2	75.2	68.4	72.6	65.0
	Deviation vs. Control (%)			-2.9	-8.2	-1.7	-7.8	-4.8	-11.8
MONO [%]	Mean	2.1k	2.2k	2.4	2.4	1.9	2.0	2.0	2.1
	S.d.	0.6	0.5	1.3	0.9	0.5	0.8	0.6	0.6
	N	10	10	9	10	10	10	10	10
	Median	2.2	2.0	1.9	2.3	1.8	1.8	2.0	1.8
	Deviation vs. Control (%)			16.5	9.3	-10.1	-7.4	-2.9	-3.3
EOS [%]	Mean	2.2	2.5k	2.4	3.0	2.2	2.6	2.0	2.2
	S.d.	0.7	0.9	0.9	1.2	0.5	0.6	0.6	0.7
	N	10	10	9	10	10	10	10	10
	Median	2.0	2.4	2.6	2.5	2.2	2.6	1.8	2.2
	Deviation vs. Control (%)			8.1	18.3	-1.4	3.2	-8.2	-11.6
BASO [%]	Mean	0.3k	0.4k	0.2	0.6	0.3	0.6	0.2	0.4
	S.d.	0.1	0.2	0.1	0.2	0.1	0.3	0.1	0.2
	N	10	10	9	10	10	10	10	10
	Median	0.3	0.4	0.2	0.6	0.3	0.5	0.2	0.4
	Deviation vs. Control (%)			-25.9	47.7	-15.2	27.3	-33.3	-9.1
LUC	Mean	0.2k	0.2k	0.2	0.2	0.2	0.2	0.2	0.2

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
[%]	S.d.	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1
	N	10	10	9	10	10	10	10	10
	Median	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	Deviation vs. Control (%)			-2.8	14.3	-4.2	19.0	-16.7	0.0

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics k=KRUSKAL-WALLIS

- Clinical chemistry
  - Group 3 (1400 mg/kg bw/d):
    - At the end of the administration period, in male and female rats LDL-cholesterol and total bilirubin values were increased (total bilirubin in females not statistically significantly). These alterations were regarded as treatment-related and adverse.
    - In males HDL-cholesterol levels were significantly decreased and inorganic phosphate values were significantly increased. These alterations were regarded as treatment-related and adverse.
    - Significant increased creatinine and significant decreased glucose values in males. These changes were regarded as incidental and not treatment-related, because the values were within historical control ranges (males, creatinine 23.4-34.2 µmol/L; glucose 5.46-6.98 mmol/L).
    - Significant increased triglyceride and urea values and significant decreased chloride values in females. These changes were regarded by the study author as incidental and not treatment-related, because the values were within historical control ranges (females, triglycerides 0.44-0.82 mmol/L; urea 4.42-8.10 mmol/L; chloride 97.0- 104.2 mmol/L). The Dossier submitter however notices that at the high dose females, the value of 1.00 mmol/L for triglycerides is *outside* the range of the historical control levels.
  - Group 2 (450 mg/kg bw/d):

- In males total bilirubin levels were already significantly higher compared to controls and they were above the historical control range (males, total bilirubin 1.03-1.92 µmol/L). However, this was the only changed clinical pathology parameter among these individuals and therefore, it was regarded as treatment-related but non-adverse.
- Significant decreased glucose values and significant increased inorganic phosphate levels in males. These changes were regarded as incidental and not treatment-related, because the values were within historical control ranges (males, glucose 5.46-6.98 mmol/L; inorganic phosphate 1.42- 1.82 mmol/L).

- Thyroid hormones
  - No treatment related alterations of T3, T4 and TSH levels were observed.

Table 105: Clinical chemistry parameters, summary of results males and females

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
ALT [µkat/L]	Mean	0.63k	0.54k	0.76	0.52	0.71	0.62	0.88	0.72
	S.d.	0.11	0.08	0.20	0.08	0.15	0.20	0.33	0.24
	N	10	10	9	10	10	10	10	10
	Median	0.60	0.54	0.70	0.51	0.70	0.58	0.78	0.67
	Deviation vs control (%)			20.68	-2.79	13.88	15.08	40.99	33.71
AST [µkat/L]	Mean	1.73k	1.58k	1.92	1.55	1.91	1.72	1.99	1.59
	S.d.	0.25	0.23	0.44	0.21	0.32	0.21	0.41	0.20
	N	10	10	9	10	10	10	10	10
	Median	1.81	1.55	1.74	1.54	2.00	1.69	2.08	1.56
	Deviation vs control (%)			11.05	-1.65	10.78	8.93	15.35	0.63
ALP [µkat/L]	Mean	1.26k	0.54k	1.32	0.54	1.18	0.51	1.12	0.51
	S.d.	0.17	0.10	0.17	0.15	0.23	0.14	0.21	0.16
	N	10	10	9	10	10	10	10	10

	Median	1.27	0.57	1.31	0.51	1.20	0.50	1.10	0.52
	Deviation vs control (%)			5.02	-1.29	-6.44	-5.72	-11.29	-5.35
GGT_C [nkat/L]	Mean	25NA	25NA	25	25	25	25	25	25
	S.d.	0	0	0	0	0	0	0	0
	N	10	10	9	10	10	10	10	10
	Median	25	25	25	25	25	25	25	25
	Deviation vs control (%)			0	0	0	0	0	0
UREA [mmol/L]	Mean	4.64k	6.10v	4.68	6.14	5.01	5.99	4.72	7.00**
	S.d.	0.42	0.60	0.33	0.65	0.37	0.80	0.78	0.68
	N	10	10	9	10	10	10	20	10
	Median	4.69	6.24	4.59	5.94	5.11	6.04	4.49	6.84
	Deviation vs control (%)			0.86	0.61	7.99	-1.77	1.64	14.84
CREA [µmol/L]	Mean	28.4v	35.0k	28.2	36.0	29.0	35.7	32.2*	38.9
	S.d.	2.	3.0	2.5	3.0	2.9	4.8	3.0	3.5
	N	10	10	9	10	10	10	10	10
	Median	28.6	35.0	28.1	35.8	28.8	34.2	31.8	38.2
	Deviation vs control (%)			-0.9	3.0	1.8	2.0	13.2	11.3
GLUC [mmol/L]	Mean	6.60v	5.28k	6.05	5.58	5.74**	5.00	5.47**	5.28
	S.d.	0.57	0.37	0.62	0.71	0.47	0.42	0.5	0.65
	N	10	10	9	10	10	10	10	10
	Median	6.46	5.22	1.77	5.72	5.62	5.10	5.54	5.30
	Deviation vs control (%)			17.14	5.53	-13.06	-5.30	-17.12	-0.08
TBIL_C [µmol/L]	Mean	1.52v	2.01k	1.78	2.07	2.12**	2.10	2.40**	2.61
	S.d.	0.30	0.38	0.13	0.50	0.30	0.46	0.61	0.54
	N	10	10	9	10	10	10	10	10
	Median	1.62	1.96	1.77	2.12	2.12	2.06	2.39	2.64
	Deviation vs control (%)			17.14	2.61	39.41	4.12	57.79	29.53
TBA [µmol/L]	Mean	15.8k	26.5k	10.4	36.5	14.0	28.5	13.0	21.7
	S.d.	11.0	16.6	5.3	23.5	11.0	34.6	6.0	10.9
	N	10	10	9	10	10	10	10	10

	Median	14.2	19.4	7.7	40.2	9.2	17.0	10.7	21.6
	Deviation vs control (%)			-34.5	37.4	-11.3	7.4	-17.6	-18.3
TPROT [g/L]	Mean	64.18k	64.09k	64.41	65.13	64.72	63.16	63.09	65.17
	S.d.	1.39	2.48	1.26	3.19	1.00	2.68	2.07	1.63
	N	10	10	9	10	10	10	10	10
	Median	64.46	40.17	63.98	64.41	64.76	62.96	63.75	64.75
	Deviation vs control (%)			0.36	1.61	0.84	-1.46	-1.7	1.69
ALB [g/L]	Mean	38.80k	40.19k	38.54	40.70	39.02	39.76	38.14	40.34
	S.d.	0.99	1.77	1.00	2.08	0.77	1.38	0.79	0.79
	N	10	10	9	10	10	10	10	10
	Median	38.84	40.17	38.07	40.64	39.00	39.51	38.28	40.36
	Deviation vs control (%)			-0.66	1.27	0.57	-1.07	-1.68	0.36
GLOB [g/L]	Mean	25.38k	23.90k	25.87	24.42	25.7	23.39	24.94	24.84
	S.d.	0.85	1.90	0.71	1.35	0.91	1.49	1.57	1.65
	N	10	10	9	10	10	10	10	10
	Median	25.36	24.00	25.69	24.05	25.87	23.39	25.59	24.64
	Deviation vs control (%)			1.92	2.19	1.26	-2.12	-1.72	3.92
CHOL [mmol/L]	Mean	1.78k	1.40k	1.77	1.36	1.73	1.16	1.64	1.33
	S.d.	0.28	0.36	0.31	0.24	0.35	0.23	0.30	0.28
	N	10	10	9	10	10	10	10	10
	Median	1.75	1.45	1.84	1.37	1.83	1.17	1.66	1.26
	Deviation vs control (%)			-0.21	-2.64	-2.81	-16.94	-7.54	-5.08
HDL-CHOL [mmol/L]	Mean	1.35v	1.22v	1.32	1.18	1.25	0.97	1.04**	0.99
	S.d.	0.20	0.33	0.24	0.20	0.23	0.20	0.20	0.27
	N	10	10	9	10	10	10	10	10
	Median	1.34	1.26	1.34	1.16	1.33	0.96	1.00	1.03
	Deviation vs control (%)			-2.18	-2.95	-7.9	-20.72	-23.41	-19.16
LDL-CHOL [mmol/L]	Mean	0.23v	0.12v	0.23	0.11	0.30	0.12	0.42*	0.18**
	S.d.	0.08	0.03	0.05	0.02	0.11	0.02	0.16	0.03
	N	10	10	9	10	10	10	10	10

	Median	0.22	0.11	0.22	0.10	0.32	0.11	0.48	0.19
	Deviation vs control (%)			0.62	-8.94	30.47	-5.69	82.40	46.34
TRIG [mmol/L]	Mean	1.35v	0.71v	1.27	0.74	1.13	0.67	1.02	1.00*
	S.d.	0.20	0.25	0.45	0.18	0.45	0.22	0.18	0.30
	N	10	10	9	10	10	10	10	10
	Median	1.34	0.62	1.27	0.72	1.00	0.62	1.10	1.00
	Deviation vs control (%)			7.90	3.66	-4.24	-6.33	-13.24	40.23
NA [mmol/L]	Mean	145.2k	142.9k	145.7	143.2	145.2	143.6	145.3	141.8
	S.d.	0.7	1.0	1.1	1.0	0.9	1.2	1.0	1.7
	N	10	10	9	10	10	10	10	10
	Median	145.1	143.0	145.6	143.2	145.0	143.5	145.3	141.6
	Deviation vs control (%)			0.3	0.2	0.0	0.5	0.1	-0.7
K [mmol/L]	Mean	4.86k	3.96k	4.93	4.03	4.99	4.03	4.94	4.16
	S.d.	0.29	0.16	0.21	0.24	0.39	0.30	0.31	0.21
	N	10	10	9	10	10	10	10	10
	Median	4.72	3.97	4.96	4.01	4.97	4.10	4.98	4.14
	Deviation vs control (%)			1.55	1.82	2.74	1.95	1.67	5.26
CL [mmol/L]	Mean	101.8	101.1v	102.1	101.3	101.1	101.3	101.5	99.4**
	S.d.	0.9	1.2	0.9	1.1	1.2	1.5	0.7	1.4
	N	10	10	9	10	10	10	10	10
	Median	102.0	100.8	101.9	101.2	101.0	101.2	101.6	99.2
	Deviation vs control (%)			0.2	0.2	-0.7	0.2	-0.4	-1.7
INP [mmol/L]	Mean	1.62v	1.31k	1.71	1.33	1.75*	1.31	1.98**	1.5
	S.d.	0.09	0.23	0.11	0.23	0.14	0.27	0.18	0.23
	N	10	10	9	10	10	10	10	10
	Median	1.64	1.36	1.75	1.45	1.74	1.30	1.92	1.55
	Deviation vs control (%)			5.22	1.37	8.08	-0.61	22.26	14.46
CA [mmol/L]	Mean	2.44k	2.42k	2.46	2.43	2.44	2.41	2.40	2.42
	S.d.	0.04	0.06	0.05	0.08	0.05	0.04	0.05	0.06
	N	10	10	9	10	10	10	10	10

	Median	2.45	2.42	2.46	2.43	2.46	2.41	2.42	2.42
	Deviation vs control (%)			0.74	0.54	0.08	-0.45	-1.68	0.25
T3 [nmol/L]	Mean	1.13k	1.12k	1.16	1.18	1.13	1.18	1.20	1.24
	S.d.	0.12	0.06	0.15	0.09	0.08	0.12	0.09	0.10
	N	10	10	9	10	10	10	10	10
	Median	1.14	1.13	1.12	1.17	1.12	1.19	1.16	1.22
	Deviation vs control (%)			2.94	4.91	0.00	5.63	5.93	10.27
T4 [nmol/L]	Mean	54.91k	30.84k	57.00	30.28	58.81	32.21	61.38	36.00
	S.d.	6.94	5.63	6.24	6.52	10.01	8.63	7.21	6.15
	N	10	10	9	10	10	10	10	10
	Median	56.38	30.62	56.44	29.31	57.67	30.12	61.16	39.24
	Deviation vs control (%)			3.81	-1.82	7.10	4.45	11.79	16.74
TSH [µg/L]	Mean	5.46k	3.94k	5.89	4.02	6.29	3.67	8.34	3.72
	S.d.	1.55	0.56	2.77	0.60	2.03	0.93	5.61	0.78
	N	10	10	9	10	10	10	10	10
	Median	5.28	3.86	4.73	3.90	6.46	3.51	5.62	3.59
	Deviation vs control (%)			7.86	2.06	15.09	-6.76	52.76	-5.54

Statistic Profile = Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics k=KRUSKAL-WALLIS; v=KRUSKAL-WALLIS-WILCOX; NA=No Test Applicable

- Urinalyses
  - Group 3 (1400 mg/kg bw/d):
    - At the end of the administration period in females blood (as well as erythrocyte counts in the urine sediment, although not statistically significantly) and increased incidences of transitional epithelial cells in combination with increased pH values were found in the urine. These findings were regarded as treatment-related and adverse.
    - In males and females significantly decreased urine volume was measured. In males of this test group urine specific gravity was significantly increased and urine pH values were significantly lower compared to controls. The mentioned changes in this

- paragraph were most probably due to a decreased fluid income in the kidneys and the excretion of compound metabolites in the urine, but they reflect the normal adaptation of the renal function. Therefore, they were regarded as treatment-related, but non-adverse.

– Group 2 (450 mg/kg bw/d):

- In females pH values were already significantly higher compared to controls. However, this was the only changed clinical pathology parameter among these individuals and therefore, it was regarded as maybe treatment-related but non-adverse.

Table 106: Urinalysis parameters, summary of results males (Day 88) and females (Day 89)

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
PH_C [---]	Mean	6.8v	5.6v	6.9	6.0	7.0	6.4*	5.8**	6.4*
	S.d.	0.3	0.6	0.2	0.6	0.6	0.4	0.8	0.6
	N	10	10	9	10	10	10	10	10
	Median	7.0	6.0	7.0	6.0	7.0	6.2	6.0	6.5
PRO_C [---]	Mean	1.0 NA	1.0NA	1.0	1.0	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
GLU_C [---]	Mean	1.0NA	1.0NA	1.0	1.0	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
KET_C [---]	Mean	1.0NA	1.0NA	1.0	1.0	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
UBG_C [---]	Mean	1.0 x+	1.0x+	1.0	1.0	1.0	1.0	1.1	1.1
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
BIL_C [---]	Mean	1.0NA	1.0NA	1.0	1.0	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
BLOOD_C [---]	Mean	1.0x+	1.0x+	1.0	1.0	1.0	1.0	1.3	1.6**
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0
VOL [ml]	Mean	7.0v	4.4v	7.3	5.0	6.2	4.8	2.2**	2.8*
	S.d.	2.0	1.5	1.8	1.0	1.5	1.1	0.8	1.5
	N	10	10	9	10	10	10	10	10
	Median	7.5	4.2	7.5	5.0	5.8	5.2	2.0	2.2
SP.GR._C [g/L]	Mean	1,037v	1,041k	1,035	1,035	1,036	1,037	1,078	1,046
	S.d.	8	10	5	6	6	6	14	16
	N	10	10	9	10	10	10	10	10
	Median	1,034	1,040	1,036	1,034	1,036	1,034	1,079	1,042
CRYST_C [---]	Mean	2.2x+	2.0NA	2.2	2.0	2.3	2.0	2.0	2.0
	S.d.	0.4	0.0	0.4	0.0	0.5	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
RENAL EC_C [---]	Mean	1.0NA	1.0NA	1.0	1.0	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TRANS EC_C [---]	Mean	1.1x+	1.0x+	1.0	1.1	1.0	1.0	1.0	1.4*
	S.d.	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.5
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SQUAM EC_C [---]	Mean	1.0NA	1.0x+	1.0	1.1	1.0	1.0	1.0	1.0
	S.d.	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CASTS_C [---]	Mean	0.1x+	0.0x+	0.3	0.0	0.0	0.0	0.2	0.4
	S.d.	0.3	0.0	0.5	0.0	0.0	0.0	0.4	0.7
	N	10	10	9	10	10	10	10	10
	Median	0.0	0.0	0.0	0.0	1.0	1.0	0.0	0.0
ERY_C [---]	Mean	1.0NA	1.0x+	1.0	1.1	1.0	1.0	1.0	1.2
	S.d.	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.4
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
LEUCO_C [---]	Mean	1.2x+	1.0x+	1.0	1.0	1.0	1.0	1.0	1.2
	S.d.	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.4
	N	10	10	9	10	10	10	10	10
	Median	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Statistic Profile = Wilcoxon test (one-sided+), Kruskal-Wallis + Wilcoxon test (two-sided), * p<=0.05, ** p <=0.01, X = Group excluded from statistics

x=WILCOX

- Pathology
  - Weight parameters
    - Group 3 (1400 mg/kg bw/d):
      - The terminal body weight in males was decreased and within historical control data and therefore not regarded to be adverse. Nevertheless, it was regarded to be treatment-related decreased.
      - The increase of kidney weight in males and females reflected the findings in microscopy and was regarded to be treatment related.
      - The decrease in thymus weight in males did not have a microscopic finding that could explain the weight decrease. It was therefore most likely regarded to be a consequence to the body weight decrease.
      - The relative liver weight increase in females did not have a correlation in absolute liver weight, did not have a microscopic correlate and was within historical control values. It was therefore regarded to be incidental.
      - The changes in relative weights of epididymides and seminal vesicle were within historical control values and did not show any microscopic findings that could explain the weight increase and were therefore regarded to be incidental and/or related to the body weight reduction in test group animals.
    - Group 2 (450 mg/kg bw/d):
      - The decrease in thymus weight in males did not have a microscopic finding that could explain the weight decrease. It was within historical control values and not related to treatment.
    - Group 1 (150 mg/kg bw/d):
      - The statistically significant decrease of thyroid gland weight in females was regarded to be incidental for the following reasons: there was no dose-response relationship and no histopathologic findings that could explain the weight increase were observed in the thyroid glands examined.

Table 107: Absolute weight, summary of results males and females

		Test Group 0 0 mg/kg bw/d		Test Group 1 96 mg/kg bw/d		Test Group 2 289 mg/kg bw/d		Test Group 3 963 mg/kg bw/d	
		Males	Females	Males	Females	Males	Females	Males	Females
Terminal body weight (g)	M	395.52	226.18	398.367	228.25	382.64	223.48	366.72*	226.62
	% dev	100	100	101	101	97	99	93	100
	SD	30.367	17.367	30.355	15.04	21.655	7.627	19.332	18.371
	n	10	10	9	10	10	10	10	10
Adrenal glands (mg)	M	64.9	74.9	61.667	76.1	56.6	76.2	63.2	73.0
	% dev	100	100	95	102	87	102	97	97
	SD	6.1	9.231	7.81	10.049	4.926	9.09	11.084	11.518
	n	10	10	9	10	10	10	10	10
Brain (g)	M	2.151	2.017	2.172	1.996	2.165	2.0	2.107	1.992
	% dev	100	100	101	99	101	99	98	99
	SD	0.075	0.046	0.089	0.095	0.068	0.098	0.068	0.055
	n	100	10	9	10	10	10	10	10
Epididymides (g)	M	1.154	-	1.236	-	1.143	-	1.193	-
	% dev	100	-	107	-	99	-	103	-
	SD	0.064	-	0.1	-	0.092	-	0.112	-
	n	10	-	9	-	10	-	10	-
Heart (g)	M	1.069	0.78	1.071	0.789	1.037	0.747	1.007	0.798
	% dev	100	100	100	101	97	96	94	102
	SD	0.066	0.098	0.063	0.077	0.058	0.074	0.068	0.049
	n	10	10	9	10	10	10	10	10
Kidneys (g)	M	2.343	1.491	2.307	1.544	2.345	1.469	2.562*	1.792**
	% dev	100	100	98	104	100	99	109	120
	SD	0.163	0.137	0.217	0.142	0.15	0.133	0.194	0.132
	n	10	10	9	10	10	10	10	10
Liver (g)	M	8.775	5.206	8.778	5.285	8.373	5.287	8.179	5.679

	% dev	100	100	100	102	95	102	93	109
	SD	0.875	0.572	1.153	0.607	0.659	0.338	0.861	0.499
	n	10	10	9	10	10	10	10	10
Ovaries (mg)	M	-	104.1	-	103.7	-	113.7	-	103.6
	% dev	-	100	-	100	-	109	-	100
	SD	-	21.997	-	16.727	-	17.423	-	11.974
	n	-	10	-	10	-	10	-	10
Pituitary gland (mg)	M	10.0	12.7	9.444	13.8	10.0	11.3	9.0	13.5
	% dev	100	100	94	109	100	89	90	106
	SD	2.16	2.214	1.333	1.398	1.247	2.214	1.633	1.354
	n	10	10	9	10	10	10	10	10
Prostate (g)	M	0.919	-	1.033	-	0.987	-	1.022	-
	% dev	100	-	112	-	109	-	111	-
	SD	0.118	-	0.203	-	0.137	-	0.134	-
	n	10	-	9	-	10	-	10	-
Seminal vesicle (g)	M	1.164	-	1.219	-	1.258	-	1.277	-
	% dev	100	-	105	-	108	-	110	-
	SD	0.114	-	0.183	-	0.177	-	0.211	-
	n	10	-	9	-	10	-	10	-
Spleen (g)	M	0.562	0.499	0.6	0.457	0.572	0.407	0.552	0.483
	% dev	100	100	107	102	102	91	98	108
	SD	0.061	0.089	0.088	0.081	0.063	0.047	0.067	0.062
	n	10	10	9	10	10	10	10	10
Testes (g)	M	3.751	-	3.802	-	3.635	-	3.743	-
	% dev	100	-	101	-	97	-	100	-
	SD	0.144	-	0.26	-	0.345	-	0.215	-
	n	10	-	9	-	10	-	10	-
Thymus (g)	M	358.0	290.7	328.0	285.9	310.0*	306.6	208.8**	289.3
	% dev	100	100	92	98	87	105	58	100
	SD	64.833	57.689	67.261	37.507	75.622	45.802	50.277	66.858

	n	10	10	9	10	10	10	10	10
Thyroid glands (mg)	M	25.0	16.1	23.667	14.2	22.9	17.6	21.7	16.2
	% dev	100	100	95	88	92	109	87	101
	SD	3.091	1.792	3.536	2.251	4.886	2.797	5.122	3.12
	n	10	10	9	10	10	10	10	10
Uterus (g)	M	-	0.676	-	0.774	-	0.847	-	0.746
	% dev	-	100	-	114	-	125	-	110
	SD	-	0.265	-	0.305	-	0.451	-	0.345
	n	-	10	-	10	-	10	-	10

*: P <= 0.05, **: P <= 0.01 Kruskal-Wallis Hand Wilcoxon test, two sided

- Gross lesions
  - Decedent:
    - The animal that died revealed an enlarged liver and spleen as well as a red discoloration of skeletal muscle on the skull. This correlated to findings in microscopy.
  - All findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Table 108: Incidence of gross lesions

Sacrifice	**F1**							
Sex	**M**				**F**			
Group	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Animals in selected group	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
No abnormalities	10	6	8	8	9	9	8	8
Eyes with opt. nerve								
Organ not detectable/missing								1

Forestomach								
Focus								1
Thickening of wall								1
Glandular stomach								
Focus		1	1	2				
Kidneys								
Cyst						1		
Discoloration							1	
Retraction		1						
Liver								
Adhesion					1			
Enlarged		1						
Focus		1						
Torsion							1	
Mediastinal lymph n.								
Discoloration								1
Enlarged			1					
Renal lymph nodes								
Discoloration		1						
Skeletal muscle								
Discoloration		1						
Spleen								
Enlarged		1						
Thymus								
Cyst								1
Discoloration		1						

- Histopathology
  - Treatment-related findings were observed in kidneys, lungs and nasal cavity with incidences and grading according to the table below:

Table 109: Incidence of histopathological findings in kidneys

Kidneys	Male animals				Female animals			
Test group (mg/mg bw/d)	0 (0)	1 (150)	2 (450)	3 (1400)	0 (0)	1 (150)	2 (450)	3 (1400)
No. of animals	10	10	10	10	10	10	10	10
Degeneration/regeneration, tubular	0	0	0	9	0	0	0	8
• **Grade 1**				2				6
• **Grade 2**				4				1
• **Grade 3**				2				1
• **Grade 4**				1				
Pigment storage, tubular	0	0	1	10	0	0	8	10
• **Grade 1**			1				7	2
• **Grade 2**				4			1	1
• **Grade 3**				6				6
• **Grade 4**								1

  - In the kidneys in the cortical area, mainly affecting the proximal tubules, there was degeneration/regeneration and pigment storage. The pigment was located in the cytoplasm within proximal tubular cells and had a black to gold-brownish finely granular appearance. In the vicinity of the pigment storage, there was an increase in eosinophilia of tubular cells, loss of nuclei and occasional detritus within the lumen (degeneration) and an increase in basophilia of the tubular cells with enlarged, vesicular nuclei (regeneration). In females there was predominantly degeneration rather than regeneration. These findings were regarded to be treatment related.

Table 110: Incidence of histopathological findings in mesenteric lymph nodes

Mesenteric lymph nodes	Male animals	Female animals

Test group (mg/mg bw/d)	0 (0)	1 (150)	2 (450)	3 (1400)	0 (0)	1 (150)	2 (450)	3 (1400)
No. of animals	10	10	10	10	10	10	10	10
Aggregates increased macrophage (m)f	0	0	1	8	0	1	3	9
• **Grade 1**			1	4			3	4
• **Grade 2**				4				4
• **Grade 3**						1		1

– In contrast to regularly observed numbers of macrophages in control animals (homogeneous eosinophilic cytoplasm, sometimes pigment storage, quiescence nuclei) in treated animals there were increased macrophage aggregates that differed: active nuclei, often elongated, light eosinophilic cytoplasm, sometimes vacuolated. These findings were regarded to be treatment related.

Table 111: Incidence of histopathological findings in nasal cavity

Nasal cavity	**Male animals**				**Female animals**			
Test group (mg/mg bw/d)	0 (0)	1 (150)	2 (450)	3 (1400)	0 (0)	1 (150)	2 (450)	3 (1400)
No. of animals	10	10	10	10	10	10	10	10
Inflammation (m)f	0	3	3	2	0	4	4	4
• **Grade 1**		1				1		
• **Grade 2**			2			2		2
• **Grade 3**		2	1	2		1	3	1
• **Grade 4**							1	1
Regeneration (m)f	0	1	2	4	0	2	3	4
• **Grade 1**			1	1		2	1	1
• **Grade 2**			1	2			1	1
• **Grade 3**		1		1			1	2

– In the nasal cavity two findings were observed, which were regarded to represent one continuous lesion at different stages with regard to time. The term “Inflammation” was used, if serous exudate was intermingled with neutrophils. Almost exclusively the ventral half

of nasal cavity was affected and also in paranasal sinus exudate was observed. Mainly the olfactory epithelium was affected to a lower degree the respiratory epithelium showed also signs of inflammatory stress. In some animals plant particles interpreted as food particles were observed within the exudate.

- In case of the finding "Regeneration" mainly olfactory epithelium was affected, respiratory epithelium only in few animals. The typical findings were loss of epithelial height, no regular cellular orientation, infiltrates of neutrophils, occasional vacuole formation in respiratory epithelium, no exudate anymore. These findings were regarded to be treatment related.

Table 112: Incidence of histopathological findings in lungs

Lungs	**Male animals**				**Female animals**			
Test group (mg/mg bw/d)	0 (0)	1 (150)	2 (450)	3 (1400)	0 (0)	1 (150)	2 (450)	3 (1400)
No. of animals	10	10	10	10	10	10	10	10
Macrophage aggregates	0	0	0	2	0	1	0	1
• **Grade 1**						1		
• **Grade 2**				2				1

- In contrast to the loosely arranged intra-alveolar macrophages observed regularly in the lungs, few animals revealed closely packed aggregates of macrophages filling the complete alveolus. This finding was regarded to be treatment related.
- Decedent:
  - The male animal No. 13 of test group 1 (150 mg/kg bw/d) that died revealed a malignant lymphoma which was the cause of death in this animal. Infiltration of the lymphoma led to the observed macroscopic findings (e.g. enlarged liver and spleen, discoloration of muscle at the skull) but was regarded to be incidental and unrelated to treatment.
- All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Discussion:

- Clinical examination
  - The signs of general systemic toxicity were observed in following findings:
    - In males and females of test group 3 (1400 mg/kg bw/d) the manifestations were in semi-closed eyelid, respiration sounds, and nose discharge. No significant alteration of the body weight was observed. However, the body weight change in males of test group 3 (1400 mg/kg bw/d) from administration day 0 to 91 was -9.7% lower than in the current control resulting in a non-significant decrease of the corresponding body weight of -5.5%. This finding is in line with the decreased terminal body weight in this test group.
    - However, the later finding was within the historical control range and assessed as non-adverse.
- FOB examination
  - Decreased motor activity in females of test group 3 (1400 mg/kg bw/d) described a further manifestation of the systemic toxicity.
- Clinical pathology
  - A normocytic, normochromic anemia was observed in male and female rats of test group 3 (1400 mg/kg bw/d).
    - Because of higher total bilirubin values in these individuals a hemolysis of red blood cells occurred. The regeneration rate of red blood cells seemed to be poor at least in males of test group 3 having lower absolute reticulocyte counts in the blood.
    - Higher absolute neutrophil counts in the blood of females in this test group may indicate an acute-phase reaction.
    - Increased LDL-cholesterol levels in both sexes of test group 3 (1400 mg/kg bw/d) and lower HDL-cholesterol values in females of this test group were most probably due to a changed lipid metabolism.
    - In the urine of females of test group 3 (1400 mg/kg bw/d) blood and higher counts of erythrocytes as well as transitional epithelial cells were observed. This may indicate a bleeding in the renal tract.
- Pathology
  - The kidneys, nasal cavity and mesenteric lymph nodes were the target organs.

- In the kidneys of test group 3 (1400 mg/kg bw/d) male and female animals degeneration/regeneration of tubules and pigment storage in proximal tubules was seen. These findings were regarded to have led to the weight increase observed in male and female animals of this test group. The pigment might be stored compound or compound metabolites which would also fit to the finding in clinical pathology ("excretion of compound metabolites in the urine"). These findings in the kidneys were regarded to be treatment-related and adverse.
- In test group 2 (450 mg/kg bw/d) there was still pigment found in the proximal tubules of one male and eight female animals. There was no other finding observed and the severity was lower compared to the high dose group, therefore it was regarded to be treatment related but not adverse.
- The macrophage aggregates observed in mesenteric lymph nodes were regarded to be treatment related. During macrophagic phagocytosis, an uncomplete degradation of foreign material might lead to aggregation of macrophages. As there was no dose-response relationship and no other findings were observed, it was regarded to be part of the degradation process of the compound, therefore treatment related but not adverse.
- In the nasal cavity of all test groups acute inflammation (with serous exudate and neutrophils) or regeneration of the mainly olfactory epithelium were observed. This was assumed to have be caused e.g. by reflux after gavage for the following reasons: no clear dose response relationship was present, there was plant material interpreted as food particles in the inflammatory exudate of some animals, also the paranasal sinus were affected, only the ventral part of the nasal cavity showed findings. According to Damsch et al. (2011) these are all indications for a gavage-related reflux. It was regarded to be treatment-related and locally adverse.
- Also, the increased numbers of alveolar macrophage aggregates in the lung were considered to be indicative for reflux (Damsch et al., 2011). They were therefore regarded to be treatment related and locally adverse.
- These findings in the nasal cavity and lungs were regarded as a treatment related effect, but only caused by the way of administration (oral gavage) which led in some animals to a gastroesophageal reflux. Secondary to that, the findings developed but would not have been observed without the local contact with the reflux. That's why these findings were considered as treatment-related and adverse but no test substance related effect per se.

- The local toxicity has been observed in all test groups without a clear dose response in the nasal cavity. Therefore, the no observed adverse effect level (NOAEL) for local toxicity was below 150 mg/kg bw/d corresponding to 79 mg/kg bw/d of the active ingredient.
- All other findings occurred either individually or were biologically equally distributed over control and treatment groups. They were considered to be incidental or spontaneous in origin and without any relation to treatment.

Conclusion

- The administration of the test substance “Reaction products of fatty acids, C18 (unsaturated) alkyl with sulfur trioxide, potassium salts” by gavage to male and female Wistar rats for 3 months caused signs of systemic and local toxicity.
- The findings of systemic toxicity were observed in males and females of test group 3 (1400 mg/kg bw/d corresponding to 735 mg/kg bw/d of the active ingredient). Therefore, under the conditions of the present study the no observed adverse effect level (NOAEL) for systemic toxicity was 450 mg/kg bw/d for male and female rats corresponding to 236 mg/kg bw/d of the active ingredient. In addition, local toxicity was observed with a NOAEL of <150 mg/kg bw/day.

3.13 Aspiration hazard

Evaluation not performed for this substance.

4 ENVIRONMENTAL HAZARDS

Evaluation not performed for this substance.

5 REFERENCES

See confidential Annex for a detailed list of study reports.