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Report

Chronic toxicity study of pharmaceutical substance Killevir-16 at peroral administration in rats during 90 days

(Contract #ZT-22/2011)

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SUMMARY

Report: 24 pages, 18 tables, 6 annexes

Key words: *Killevir-16, substance, general toxic effect, chronic toxicity, peroral administration*

Killevir-16, a new pharmaceutical substance, was produced by ZAO "Intelpharm" by the original technology.

The study objective is assessment of the level and character of the substance damaging effect on laboratory animals and safety assessment at repeated exposure.

The task is determination of tolerable and toxic doses of the substance, identification of the most sensitive organs and systems, assessment of the level and character of the substance-caused adverse effects, and study of reversibility of the detected damages.

Chronic toxicity of the test substance was studied on outbred white rats at i/g administration during 90 days in doses 0.57, 5.7, and 57 mg/kg/day (1-, 10- and 100fold therapeutic doses for humans). Control animals received a vehicle - 1% starch gel in the volumes equal to maximal doses of the test substance.

Clinical observations of health status of animals, hematological analysis of peripheral blood, biochemical analysis of blood serum, urine analysis, and pathohistological examination of internal organs after 90-day administration in 1-, 10- and 100fold clinical doses and 14 days of recovery period have not revealed major adverse effects caused by the test substance. The only change detected was a minor reversible increase in the total number of segmented neutrophils and decrease in total number of lymphocytes at 90-day exposure in dose 57 mg/kg/day.

The test substance does not have local irritating effect on mucous membranes of gastrointestinal tract at repeated exposure.

Results of the studies conducted suggest that Killevir-16 has a high therapeutic index and is harmless at a long-term treatment.

CONTENTS

INTRODUCTION

Killevir-16, a new pharmaceutical substance, was produced by ZAO “Intelpharm” by the original technology.

The study objective is assessment of the level and character of the substance damaging effect on laboratory animals and safety assessment at repeated exposure.

The task is determination of tolerable and toxic doses of the substance, identification of the most sensitive organs and systems, assessment of the level and character of the substance-caused adverse effects, and study of reversibility of the detected damages.

The study was conducted in compliance with “Methodical guidance on general toxicity studies of pharmaceuticals” /Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev – 2nd edition, corrected and amended; ad. – M.: OAO “Izdatel’stvo Medicina”, 2005. – P.41-54.

1. MATERIALS AND METHODS

For the studies an experimental batch of Killevir-16 substance produced by ZAO "Intelpharm" was supplied.

Test substance characteristics:

1. **Molecular formula**: $\mathbf{C_{60}(NH(CH2)_5COOH)_n}$, where n=4-6

Molecular mass 1500

2. **Description**: amorphous powder, brown or dark brown

3. **Physical properties**: decomposition temperature without melting 400-450 °C

4. **Solubility**:

- freely soluble in DMSO;
- soluble in dimethyl formamide (10 mg of substance in 10 ml of DMF);
- soluble in ice acetic acid (1 mg/10 ml);
- almost insoluble in water, 95% ethyl alcohol, and 1,2-dichlorbenzene

5. **pH**: 5.2

6. **Amino-caproic acid**: 3-3.5 %

7. **Chlorides**: ≤0.2%

8. **Total ash**: ≤0.5%

9. **Hard metals**: ≤0.001%

10. **Residual vehicles**: 1, 2- dichlorbenzene ≤0.032%

11. **Nitrogen**: 5.0-5.4%

12. **Elemental analysis**:

%C 69.52

%H 4.82

%N 5.20

Batch #54, produced December 22, 2009

Shelf life – 5 years

Storage conditions: dry, light-protected, temperature ≤30°C

The experiments were conducted on outbred rats received from Animal Nursery at Research Center for Bio-Medical Technologies, Russian Academy of Medical Sciences (SE RCBMT RAMS). Animals were housed and handled in compliance with sanitary rules approved by the USSR Ministry of Health of 06.07.73 on construction, equipping and maintenance of experimental-biological clinics (vivariums). Animals were fed *ad libitum* by extruded combined feed PK-120-1 prepared in accordance with GOST P 50258-92. All animals were kept under

quarantine and acclimated to laboratory conditions for a minimum of 10 days prior to the start of dosing.

Experimental animals were randomly assigned to study groups based on body weight.

Suspensions of test substance in 1% starch gel were prepared in aceptic conditions *ex tempora* and poured in labeled vials. Before treatment of animals (no longer than 3 hours) they were kept at room temperature.

Test substance was i/g administered in rats in doses 0.57, 5.7, and 57 mg/kg/day. Control animals were dosed with 1% starch gel in the volumes equal to maximal doses of the test substance. The volumes to be administered were adjusted based on individual body weights of animals. Each dose was tested on 12 per sex animals.

During the observation period physical status of animals was evaluated by motor activity, consumption of feed and water, state of hair and mucous membranes, and body weight.

In 24 hours after completion of dosing and after 14 days of recovery period 5 animals from each group were sacrificed to perform hematological, biochemical and pathohistological analysis.

Hematological analysis was performed on semi-automatic 2-channel Hema-screen-13 hematological analyzer (Hospitex Diagnostics, Italy). The following parameters were evaluated: number of erythrocytes, leukocytes and thrombocytes, concentration of hemoglobin, hematocrit, mean corpuscular volume (MCV). The number of reticulocytes and leukocyte formula were calculated under microscope in dyed blood smears.

Biochemical parameters of blood serum were evaluated on semiautomatic analyzer Stat Fax 3300 (Awareness Technology, USA) using the following unified methods:

- total protein – by Biuret test;
- glucose and total cholesterol – by enzymatic colorimetric method,
- urea– urease/phenol-hypochlorite method;
- creatinie – by Yaffe method with deproteinization;
- total bilirubin – by Jendrassik & Grof method;
- AlAT and AsAT activity – by Raitman-Frenkel method;
- alkaline phosphatase – by unified kinetic method.

In urine samples on semiautomatic analyzer Urisys 1100 (Roche Diagnostics, Hungary) with the use of test stripes the following parameters were evaluated: relative density, pH, number of leukocytes and erhythrocytes, concentrations of protein, bilirubin, glucose, ketones, nitrites and urobilinogen.

Morphological analysis of internal organs was performed visually at necropsy and by examination of histological preparations of organs - cerebrum, lungs, liver, spleen, kidney, heart,

thymus, stomach, empty intestine and testicles. Samples of organs were fixed in 10% formalin, dehydrated, embedded in paraffin blocks. Then slices of thickness 5-6 μm were done, dyed with hematoxilin and eosin and embedded in Canada balsam. Analysis of histological preparations was performed under light microscope.

For all quantitative data obtained in the studies descriptive statistics was applied; average randomized values and standard deviations (M±SD) were calculated and presented in the summarized tables with n values (number of variants in a group). Reliability of differences between groups was confirmed by variation statistics methods using Student's criterion. The differences are evaluated at confidence probability 95%. Analysis was performed separately for males and females.

2. STUDY RESULTS

2.1. Clinical observation

During 90 days of dosing and 14 days of recovery period there were no clinical symptoms of intoxication or deaths of animals; no differences in physical status between experimental and control animals were noted. At almost all terms of weighing there were no statistically reliable differences between average group weights of treated and control animals except decrease in body weights of male rats in dose group 5.7 mg/kg/day on day 49 and decrease in body weights of female rats in dose group 0.57 mg/kg/day on day 42, 84, and 91 (Table 1-2).

Table 1

Body weights of male rats during the dosing and in recovery period, g (M±SD)

Substance dose, mg/kg/day	Day of experiment							
	0	7	14	21	28	35	42	49
0 (vehicle)	169.1±7.6	229.9±13.6	282±20.9	319.9±25	347.1±26	368.6±28.4	394±30.8	409.6±29.5
n	10	10	10	10	10	10	10	10
0.57	170±8.1	227.9±10.6	278.3±18	315.4±23.8	338±27.1	357.3±34.2	381.5±34.6	397.2±39.4
n	10	10	10	10	10	10	10	10
5.7	170.8±12.4	225.7±15.8	276.6±14.1	309.5±13.6	329.6±14.4	352.9±20.3	375.8±25	382.5±25.5*
n	10	10	10	10	10	10	10	10
57	173.8±13.1	232.8±17.6	291±23.3	324.7±28	348.8±26.1	367±29.7	390.2±31.5	381.1±46.5
n	10	10	10	10	10	10	10	10

Note: «*» – statistically reliable difference from control by Student's t-criterion at $p \leq 0.05$

Table 1 (continued)

Substance dose, mg/kg/day	Day of experiment							
	56	63	70	77	84	91	98	105
0 (vehicle)	417.6±30.4	431.7±33.4	443.1±34.6	455±33.6	474.5±37.5	483.7±37.8	493.2±56.1	501.4±55.6
n	10	10	10	10	10	10	5	5
0.57	408.2±40	416.9±40.4	431.1±39.4	446.8±39.6	464.7±45	476±48.4	470±26.7	475±26.4
n	10	10	10	10	10	10	5	5
5.7	395.1±26.9	408.6±27.3	421.8±29.9	433.5±31.6	447.2±33	454.7±35.3	438.4±38	444.2±41.7
n	10	10	10	10	10	10	5	5
57	399.6±43.9	428.5±37.8	443.2±37.8	454.7±37	474.8±40.3	483.6±43.7	487.8±25	491.6±25.6
n	10	10	10	10	10	10	5	5

Table 2

Body weights of female rats during the dosing and in recovery period, g (M±SD)

Substance dose, mg/kg/day	Day of experiment							
	0	7	14	21	28	35	42	49
0 (vehicle)	164.2±11.2	201.4±13.9	224.6±16.8	237.5±15.9	249±13.6	259.3±19.1	275.7±20.3	282.2±19.4
n	10	10	10	10	10	10	10	10
0.57	169±10.4	198.4±11.1	216.2±10.5	227.9±12.2	239.1±9.9	250.6±10.6	259.6±12.9*	265.4±16.2
n	10	10	10	10	10	10	10	10
5.7	168.2±10.3	204.6±13	225.7±15.3	239.4±19.4	252.1±20.3	260.2±22.6	271.6±27.5	278.8±28.1
n	10	10	10	10	10	10	10	10
57	167.2±6.7	201.8±11.3	223.9±10	237.5±12.2	250±12.9	256.7±15.1	268±18.1	277.4±28.4
n	10	10	10	10	10	10	10	10

Note: «*» – statistically reliable difference from control by Student's t-criterion at p ≤0.05

Table 2 (continued)

Substance dose, mg/kg/day	Day of experiment							
	56	63	70	77	84	91	98	105
0 (vehicle)	286.5±18	294.1±24.5	302.9±24.3	305.8±24.1	311.1±23.3	314.2±24.8	313.6±25.1	316.4±23
n	10	10	10	10	10	10	5	5
0.57	270.9±16.6	275.7±15.4	283.4±17.9	286±19.3	290.8±16.2*	294.6±15.4*	305.8±12.4	308.6±11.7
n	10	10	10	10	10	10	5	5
5.7	283.4±28.9	285.9±30.8	292.5±28.9	299.1±30.2	306.2±30.4	314.2±33.1	338.4±13.2	340±14
n	10	10	10	10	10	10	5	5
57	281.5±15.1	283±18.2	294.2±13.6	298.8±15.6	306.2±16.7	307±20.5	326±16.5	333.2±20.7
n	10	10	10	10	10	10	5	5

Note: «*» – statistically reliable difference from control by Student's t-criterion at p ≤0.05

The detected sporadic changes in body weighs compared to control are not dose-dependent and are not caused by the test substance.

Clinical observation demonstrated that pharmaceutical substance Killevir-16 at i/g administration in rats in daily doses 0.57, 5.7 and 57 mg/kg during 90 days did not affect their physical status.

2.2. Hematological analysis of peripheral blood

After 90 days of exposure, in experimental groups statistically reliable differences compared to control - increase in total number of segmented neutrophils and decrease in total number of lymphocytes - were noted only in male rats at dose 57 mg/kg/day (Tables 3-4).

Table 3

Hematological indices of male rats after exposure period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Hemoglobin, mmol/dm^3	7.8±1.1	8.5±0.8	7.3±0.4	8.0±0.5
Erythrocytes, mln/mm^3	6.9±1.4	7.3±0.8	6.0±0.6	6.9±0.4
Hematocrit, %	34.7±5.6	35.5±4.0	31.5±2.3	34.5±2.0
Mean corpuscular volume, μm^3	50.6±4.8	49.0±1.4	52.4±1.8	50.0±2.4
Reticulocytes, %	4.4±0.2	4.2±0.5	4.1±0.5	4.7±0.9
Thrombocytes, thousand	467.0±90.2	424.6±34.4	399.0±56.2	440.6±87.1
Leukocytes, thousand/mm^3, including:	16.7±4.7	18.3±2.4	11.6±5.1	16.9±3.8
Basophiles, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Eosinophiles, %	2.8±2.3	2.0±1.4	1.6±0.9	3.2±1.8
Young, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Stab, %	1.6±1.7	1.6±1.7	2.0±1.4	1.6±2.6
Segmented, %	20.8±3.0	20.4±3.3	21.2±1.8	36.0±8.1*
Lymphocytes, %	68.0±5.3	69.6±3.8	69.2±3.3	53.6±8.7*
Monocytes, %	6.8±1.1	6.4±0.9	6.0±1.4	5.6±1.7

Note: «*» – statistically reliable difference from control by Student's t-criterion at $p \leq 0.05$

The revealed changes - increase in total number of segmented neutrophils and decrease in total number of lymphocytes - are within normal physiological limits; however, there is a tendency to dose-dependence that is likely a response of the organism to the test substance.

Table 4

Hematological indices of female rats after exposure period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Hemoglobin, mmol/dm^3	7.8±0.6	7.5±0.6	7.3±0.4	7.7±0.5
Erythrocytes, mln/mm^3	6.3±0.3	5.8±0.5	6.0±0.6	6.3±0.4
Hematocrit, %	33.1±1.5	31.6±2.7	31.5±2.3	33.8±3.7
Mean corpuscular volume, μm^3	52.8±2.5	54.0±1.2	52.4±1.8	51.6±0.5
Reticulocytes, %	4.9±0.9	4.3±0.6	4.1±0.5	4.5±0.4
Thrombocytes, thousand	413.8±107.4	343.0±28.8	379.0±76.9	352.2±90.4
Leukocytes, thousand/mm^3, including:	15.2±3.8	16.8±3.5	14.0±5.0	13.5±3.5
Basophiles, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Eosinophiles, %	3.2±2.7	2.4±0.9	2.4±2.2	3.2±2.3
Young, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Stab, %	1.2±1.8	2.0±0.0	2.4±1.7	1.6±0.9
Segmented, %	24.8±6.4	22.4±5.5	23.2±2.3	25.2±8.8
Lymphocytes, %	66.0±4.0	67.2±4.1	66.4±3.3	65.2±6.6
Monocytes, %	4.8±1.1	6.0±1.4	5.6±1.7	4.8±2.3

After the recovery period no reliable differences in indices of experimental and control animals were found (Tables 5-6).

Table 5

Hematological indices of male rats after recovery period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Hemoglobin, mmol/dm^3	7.9±0.8	7.9±0.2	7.7±0.7	7.9±0.5
Erythrocytes, mln/mm^3	6.8±0.7	6.9±0.4	7.0±1.1	6.9±0.5
Hematocrit, %	35.9±4.3	36.1±2.6	34.9±2.0	36.6±2.6
Mean corpuscular volume, μm^3	52.4±1.7	51.8±2.5	51.8±1.8	51.8±1.5
Reticulocytes, %	4.5±0.6	4.7±0.9	4.4±0.5	3.8±0.5
Thrombocytes, thousand	500.0±106.8	455.4±75.3	490.0±121.9	472.0±85.5
Leukocytes, thousand/mm^3, including:	16.0±4.1	16.2±4.6	15.4±3.7	17.6±2.0
Basophiles, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Eosinophiles, %	2.4±1.7	2.8±1.1	2.0±2.4	2.8±1.8
Young, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Stab, %	2.0±1.4	1.4±1.3	1.2±1.1	1.8±1.8

Segmented, %	21.2±2.3	21.4±3.4	21.2±2.3	20.8±2.3
Lymphocytes, %	68.4±3.6	68.4±2.9	70.4±3.0	68.8±3.9
Monocytes, %	6.0±1.4	6.0±1.4	5.2±1.1	5.8±0.4

Table 6

Hematological indices of female rats after recovery period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Hemoglobin, mmol/dm^3	7.4±0.6	7.8±0.6	7.7±0.7	7.6±0.8
Erythrocytes, mln/mm^3	6.3±0.4	6.6±0.6	6.4±0.4	6.6±0.5
Hematocrit, %	34.1±2.4	34.2±2.4	35.1±2.4	34.8±2.5
Mean corpuscular volume, μm^3	53.8±2.3	54.2±2.5	55.4±2.1	53.8±1.8
Reticulocytes, %	4.4±0.6	4.1±0.8	4.3±0.7	4.2±0.4
Thrombocytes, thousand	456.2±79.7	445.6±77.7	445.6±49.1	442.0±74.5
Leukocytes, thousand/mm^3, including:	11.7±1.9	11.6±0.6	12.4±2.5	12.7±1.6
Basophiles, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Eosinophiles, %	2.4±1.7	2.0±1.4	1.6±1.7	2.2±1.5
Young, %	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Stab, %	2.0±1.4	2.0±1.4	2.0±1.4	2.0±1.4
Segmented, %	22.0±2.0	22.0±2.8	21.2±3.0	21.6±2.6
Lymphocytes, %	67.4±3.3	68.0±2.4	69.2±2.3	68.6±3.6
Monocytes, %	6.2±1.1	6.0±1.4	6.0±1.4	5.6±0.9

Analysis of hematological indices of males and females has demonstrated that Killevir-16 caused a minor reversible increase in the total number of segmented neutrophils and decrease in the total number of lymphocytes only in male rats in 100fold equitherapeutic dose.

2.3. Blood serum biochemistry

After 90 days of dosing in male rats of treated groups the following statistically reliable differences compared to control were noted:

- at dose 0.57 mg/kg/day – increase in glucose and cholesterol levels, decrease in AsAT activity;
- at dose 5.7 mg/kg/day – increase in glucose and cholesterol levels, decrease in AsAT activity;
- at dose 57 mg/kg/day – increase in glucose level (Table 7).

Table 7

Biochemical indices of male rats after exposure period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Total protein, g/l	93.04±8.71	97.68±5.57	96.40±10.14	89.26±7.75
Glucose, mmol/l	9.28±0.66	11.74±1.33*	11.22±0.77*	10.32±0.22*
Urea, μmol/l	5.92±0.67	5.22±0.52	6.38±0.74	5.16±0.42
Cholesterol, mmol/l	3.91±0.11	5.65±1.42*	5.57±1.21*	4.24±0.79
Bilirubin, μmol/l	18.46±4.80	16.04±1.68	16.28±2.54	16.56±5.05
Creatinine, μmol/l	65.02±36.52	58.16±3.15	62.44±8.22	63.32±11.94
AlAT, U/l	23.50±9.60	23.5±7.18	20.90±2.90	16.10±6.24
AsAT, U/l	23.01±3.23	18.39±1.27*	17.33±3.56*	21.09±1.13
Alkaline phosphatase, U/l	103.86±23.90	79.82±21.54	87.84±13.91	101.12±29.63

In female rats in this term of observation the following statistically reliable differences compared to control were found:

- at dose 0.57 mg/kg/day – increase in glucose and bilirubin levels;
- at dose 57 mg/kg/day – decrease in cholesterol and AlAT activity (Table 8).

Table 8

Biochemical indices of female rats after exposure period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Total protein, g/l	98.12±6.12	104.18±6.12	97.56±16.88	91.92±15.83
Glucose, mmol/l	13.22±1.83	15.68±1.83*	15.10±3.43	14.06±2.38
Urea, μmol/l	7.56±1.28	7.32±1.28	7.40±1.14	6.62±0.65
Cholesterol, mmol/l	5.02±0.86	4.50±0.86	4.86±0.88	3.54±0.40*
Bilirubin, μmol/l	8.64±1.51	11.84±1.51*	11.34±2.16	9.68±0.90
Creatinine, μmol/l	74.00±13.98	61.88±13.98	61.76±11.39	62.78±4.90
AlAT, U/l	9.70±1.88	10.32±1.88	8.92±3.04	6.02±1.75*
AsAT, U/l	17.28±2.79	15.69±2.79	16.85±1.49	16.25±2.75
Alkaline phosphatase, U/l	61.88±13.71	62.60±13.71	60.18±6.85	85.52±20.79

After a post-exposure recovery period a statistically reliable decrease in AsAT activity compared to control was noted in male rats in dose group 5.7 mg/kg/day (Table 9).

Table 9

Biochemical indices of male rats after recovery period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Total protein, g/l	95.72±2.24	102.44±6.54	98.20±6.21	98.70±3.20
Glucose, mmol/l	11.4±1.63	13.08±1.5	11.24±1.85	12.12±2.48
Urea, μmol/l	5.50±0.68	5.38±0.52	4.86±0.49	5.34±0.56
Cholesterol, mmol/l	5.97±1.08	5.57±1.13	6.32±0.75	5.23±1.08
Bilirubin, μmol/l	10.16±2.93	9.34±1.65	9.44±1.58	8.86±1.57
Creatinine, μmol/l	73.24±6.96	74.32±9.32	76.4±13.19	69.08±12.21
AlAT, U/l	11.66±5.93	10.96±4.24	8.34±3.97	8.70±3.43
AsAT, U/l	15.36±1.86	15.14±3.81	11.81±2.84*	12.45±2.28
Alkaline phosphatase, U/l	135.32±64.16	148.02±28.53	139.9±15.01	152.32±32.81

In female rats after recovery period the following statistically reliable differences in values compared to control were found:

- at dose 0.57 mg/kg/day – increase in bilirubin and AsAT activity;
- at dose 5.7 mg/kg/day – increase in AsAT activity and alkaline phosphatase;
- at dose 57 mg/kg/day – decrease in urea levels (Table 10).

Table 10

Biochemical indices of male rats after recovery period

Parameter, units	Substance dose, mg/kg/day			
	0 (vehicle)	0.57	5.7	57
n	5	5	5	5
Total protein, g/l	100.94±12.52	102.8±12.21	103.96±14.73	94.52±6.41
Glucose, mmol/l	11.26±1.66	10.02±1.71	10.96±3.00	12.98±3.38
Urea, μmol/l	6.02±0.25	5.50±0.98	5.42±0.76	5.28±0.52*
Cholesterol, mmol/l	5.41±0.34	5.42±1.16	6.42±1.69	5.20±0.96
Bilirubin, μmol/l	7.80±1.72	11.62±1.19*	10.46±2.24	8.58±0.44
Creatinine, μmol/l	70.68±8.12	71.98±10.41	73.12±10.11	83.22±11.71
AlAT, U/l	7.58±2.10	8.08±3.92	10.54±4.32	6.98±3.75
AsAT, U/l	10.54±2.20	14.43±1.41*	14.74±2.72*	12.04±4.1
Alkaline phosphatase, U/l	89.96±32.14	114.08±44.84	175.44±41.15*	103.76±35.08

Results of blood biochemistry analysis after the exposure and recovery periods demonstrate that all the detected statistically reliable changes in indices of treated animals compared to control are within the normal physiological limits for this animal species.

Analysis of the character and intensity of the revealed changes shows that they are sporadic, not dose-dependent, accidental and are not caused by the test substance.

4. Urine analysis

Relative density and pH indices were subjected to statistical analysis. No statistically reliable differences in relative density and pH in treated and control animals following the exposure and recovery periods were found (Table 11-14).

Table 11

Urine indices of male rats after exposure period

Substance dose, mg/kg/day	n	Relative density	pH	Number of animals with abnormal indices							
				Leukocytes	Nitrites	Protein	Glucose	Ketone bodies	Urobilinogen	Bilirubin	Erhythrocytes
0	5	1.01±1.01	8.40±8.80	4	1	0	0	2	1	0	0
0.57	5	1.01±0.00	1.01±0.45	4	1	2	0	1	0	0	3
5.7	5	1.01±0.00	9.00±0.00	4	1	2	0	0	0	0	3
57	5	1.01±0.00	8.40±0.89	4	0	0	0	0	0	0	2

Table 12

Urine indices of female rats after exposure period

Substance dose, mg/kg/day	n	Relative density	pH	Number of animals with abnormal indices							
				Leukocytes	Nitrites	Protein	Glucose	Ketone bodies	Urobilinogen	Bilirubin	Erhythrocytes
0	5	1.02±0.01	7.60±0.89	4	2	3	0	2	3	1	3
0.57	5	1.01±0.00	8.40±0.89	2	0	2	1	0	2	0	1
5.7	5	1.01±0.01	8.60±0.55	5	1	2	0	0	3	1	2
57	5	1.02±0.01	8.80±0.45	3	0	0	0	1	2	1	1

Table 13

Urine indices of male rats after recovery period

Substance dose, mg/kg/day	n	Relative density	pH	Number of animals with abnormal indices							
				Leukocytes	Nitrites	Protein	Glucose	Ketone bodies	Urobilinogen	Bilirubin	Erhythrocytes
0	5	1.01±0.00	7.30±0.67	5	0	3	0	1	1	0	4
0.57	5	1.01±0.00	8.40±0.89	5	1	1	0	1	0	0	3
5.7	5	1.01±0.00	7.80±0.45	5	1	2	0	0	0	0	5
57	5	1.01±0.00	8.40±0.89	5	0	2	0	1	1	0	3

Table 14

Urine indices of female rats after recovery period

Substance dose, mg/kg/day	n	Relative density	pH	Number of animals with abnormal indices							
				Leukocytes	Nitrites	Protein	Glucose	Ketone bodies	Urobilinogen	Bilirubin	Erhythrocytes
0	5	1.01±0.00	8.00±0.71	5	2	3	0	0	1	0	0
0.57	5	1.01±0.01	8.10±1.24	5	1	1	0	0	1	0	0
5.7	5	1.01±0.00	8.00±0.71	3	1	2	0	0	0	0	0
57	5	1.01±0.00	7.60±0.89	4	2	3	0	1	2	2	0

Results of qualitative analysis of urine samples demonstrate that abnormal indices are equally frequent in both terms of observation in control and in groups of treated animals.

Therefore, no qualitative and quantitative changes in the main urine indices were caused by the substance in the tested doses.

2.5. Necropsy

Results of pathomorphological study following 90-day dosing

At visual inspection and examination of sacrificed experimental and control animals conducted after completion of dosing no differences were noted: hair was smooth and shining, skin elastic, movable, hypodermic cellulose moderate, visible mucous membranes pale, clean, without ulcerations and foreign inclusions, no pathological discharges from natural orifices were observed. Pathoanatomical examination has not revealed any differences between experimental and control groups: organs located in thoracic and abdominal cavities had normal anatomical position; on the site of the test and control substances administration - stomach - no macroscopic abnormalities were found.

No statistically reliable differences in mass coefficients between treated and control animals were found (Table 15).

Table 15

Mass coefficients of internal organs after exposure period

Substance dose, mg/kg/day	Mass coefficients, g/kg of body weight						
	Heart	Lungs	Liver	Spleen	Kidneys	Thymus	Testicles
	MALES						
0	3.3±0.2	5.9±0.8	33.4±4.4	2.5±0.6	6.1±0.6	1.2±0.2	7.7±0.7
0.57	3.6±0.6	6.1±0.8	30.2±3.2	2.8±0.5	5.8±0.5	1.0±0.2	7.9±0.9
5.7	3.6±0.5	6.5±0.5	28.6±2.4	2.8±0.5	5.9±0.6	1.2±0.3	8.6±1.2
57	3.7±0.3	6.2±1.4	33.0±5.0	2.4±0.3	6.0±1.2	1.4±0.3	8.1±1.0
	FEMALES						
0	3.3±0.3	6.8±0.5	26.4±2.3	2.9±0.6	5.6±0.5	1.3±0.4	-
0.57	3.7±0.3	6.7±0.6	26.8±3.1	2.5±0.7	5.7±0.7	1.3±0.1	-
5.7	3.1±0.6	7.6±1.7	27.6±2.4	2.8±0.8	5.2±0.3	1.2±0.1	-
57	3.7±0.4	6.8±0.4	27.2±1.7	2.6±0.2	6.2±0.7	1.1±0.4	-

At microscopic examination of organs and tissues pathological changes were found in lungs, liver, and kidneys of rats in maximal dose group and in control (Table 16).

Table 16

Results of microscopic pathomorphological analysis of organs and tissues of rats following 90-day exposure

Organ\ tissue	**Substance**	Vehicle (control)				Killevir-16			
	Dose (mg/kg)	0				57.0			
	Sex	Male		Female		Male		Female	
	Number of animals	5		5		5		5	
	Morphological changes	Animals with abnormal indices							
		Ind#	Q-ty	Ind#	Q-ty	Ind#	Q-ty	Ind#	Q-ty
Lung	Focal thickening of the alveolar septa	1,3	2	13,16	2	75,76	2	85,86	2
	Hyperemia of blood vessels and alveolar capillaries	1,2,3,4, 5	5	13,14,15, 16,17	5	73,74,75, 76,77	5	85,86,87, 88,89	5
	Narrowing of bronchial clearance	1,4	2	13,16	2	74	1	88,89	2
	Focal lymphoid infiltration	1	1	-	-	-	-	-	-
Liver	Dilation of sinusoidal capillaries (around central veins)	2,5	2	15	1	73,74,77	3	-	-
	Venous hyperemia, hyperemia of sinusoidal capillaries	1,2,3,4, 5	5	13,14,15, 16,17	5	73,74,75, 76,77	5	85,86,87, 88,89	5
	Lymphoid infiltration in portal tracts	3	1	17	1	-	-	86	1
	Small-drop fatty degeneration of hepatocytes	-	-	13,17	2	-	-	87,89	2
Kidney	Hyperemia of blood vessels and glomerular capillaries	1,2,3,4, 5	5	13,14,15, 16,17	5	73,74,75, 76,77	5	85,86,87, 88,89	5

The changes detected (hyperemia of blood vessels and cappillaries, narrowing of bronchial clearance, thickening of the alveolar septa) are moderately or weakly pronounced and equally frequent in control and treated groups and therefore considered a reaction to the general anesthesia and CO_2 euthanasia. Separate small foci of lymphoid infiltration found in lungs and liver of 3 animals from all control groups and one treated animal by their character and morphological composition may be reffered to a chronical productive inflammatory reaction. In female rats – 2 from control and 2 from treated groups – a moderate diffuse small-drop fatty degeneration of hepatocytes was found. The level and frequency of the detected lesions compared to control suggest that they are not induced by the test substance and may be considered a background effects for this animal sample.

Results of pathomorphological analysis following the 14-day recovery period

Visual inspection and examination of sacrificed animals has not established any differences between treated and control groups: hair was smooth and shining, skin elastic, movable, hypodermic cellulose moderate, visible mucous membranes pale, clean, without ulcerations and foreign inclusions, pathological discharges from natural orifices were not observed. Organs located in thoracic and abdominal cavities had normal anatomical position; on the site of the test and control substances administration - stomach - no macroscopic abnormalities were found.

Analysis of mass coefficients of internal organs (Table 17) revealed increase in heart index in male rats of dose group 5.7 mg/kg/day and decrease in liver index in male and female groups at the same dose. Deviations from control ≤8-12% were not dose-dependent and may be explained by incorrect euthanasia and different time of necropsy conducted after euthanasia.

Table 17

Mass coefficients of internal organs after recovery period

Substance dose, (mg/kg/day)	Mass coefficients, g/kg of body weight						
	Heart	Lungs	Liver	Spleen	Kidneys	Thymus	Testicles
	MALE						
0	3.4±0.3	4.9±0.5	26.2±0.7	2.2±0.3	6.0±0.4	1.2±0.2	7.4±0.6
0.57	3.4±0.3	5.0±0.2	27.5±3.1	2.6±0.5	5.3±0.6	0.8±0.3	7.8±0.5
5.7	3.8±0.2*	5.4±0.6	24.3±1.2 *	2.1±0.2	5.5±0.7	1.1±0.6	8.3±0.9
57	3.7±0.4	5.3±0.5	28.0±2.4	2.0±0.1	5.6±0.5	1.0±0.2	8.1±0.7
	FEMALE						
3.6±0.3	3.6±0.3	7.0±0.8	28.5±1.9	2.6±0.2	6.1±0.7	1.1±0.2	-
3.9±0.5	3.9±0.5	6.3±0.8	27.1±1.9	2.3±0.6	5.5±0.8	1.0±0.2	-

3.5±0.2	3.5±0.2	6.6±1.0	25.2±1.4 *	2.3±0.4	5.7±0.8	1.1±0.3	-
3.6±0.4	3.6±0.4	6.0±1.1	26.2±3.8	2.5±0.6	5.7±0.7	1.5±0.2	-

Note: "*"- statistically reliable differences compared to control at p≤0,05 (by Wilcoxon-Mann- Whitney U-criteria)

At microscopic examination of organs and tissues of animals sacrificed upon completion of the recovery period the detected changes by their character, level, and frequency were analogous to those found after dosing (Table 18). The changes were not caused by the test substance exposure and were considered the life-time background ones for this animal sample and a reaction to the general anesthesia and CO_2 euthanasia.

Therefore, pathomorphological examination has not revealed damaging effects of pharmaceutical substance Killevir-16 on organs and tissues of rats at i/g administration in doses up to 57 mg/kg/day during 90 days. No pathomorphological changes caused by the test substance were revealed after the post-exposure recovery period.

Table 18

Results of microscopic pathomorphological analysis of organs and tissues of rats after recovery period

Organ\ tissue	**Substance**	Vehicle (control)				Killevir-16			
	Dose (mg/kg)	0				57.0			
	Sex	Male		Female		Male		Female	
	Number of animals	5		5		5		5	
	Morphological changes	Animals with abnormal indices							
		Ind#	Q-ty	Ind#	Q-ty	Ind#	Q-ty	Ind#	Q-ty
Lung	Focal thickening of the alveolar septa	7,11	2	19,20	2	-	-	92	1
	Hyperemia of blood vessels and alveolar capillaries	7,8,9,10, 11	5	19,20,21, 22,23	5	79,80,81, 82,83	5	91,92,93, 95,96	5
	Narrowing of bronchial clearance	8,9,10	3	19	1	79,81	2	92,93	2
	Focal lymphoid infiltration	9,11	2	-	-	82	1	-	-
Liver	Dilation of sinusoidal capillaries (around central veins)	9,11	2	20	1	80,81	2	95	1
	Venous hyperemia, hyperemia of sinusoidal capillaries	7,8,9,10, 11	5	19,20,21, 22,23	5	79,80,81, 82,83	5	91,92,93, 95,96	5
	Lymphoid infiltration in portal tracts	8,9,11	3	19,22	2	79,82	2	95	1
	Small-drop fatty degeneration of hepatocytes	-	-	22	1	-	-	91	1
Kidney	Hyperemia of blood vessels and glomerular capillaries	7,8,9,10, 11	5	19,20,21, 22,23	5	79,80,81, 82,83	5	91,92,93, 95,96	5

CONCLUSION

Chronic toxicity of the test substance was studied on outbred white rats at i/g administration during 90 days in doses 0.57, 5.7, and 57 mg/kg/day (1-, 10- and 100fold therapeutic doses for humans). Control animals received a vehicle - 1% starch gel - in the volumes equal to maximal doses of the test substance.

Clinical observations of health status of animals, hematological analysis of peripheral blood, biochemical analysis of blood serum, urine analysis, and pathomorphological examination of internal organs after 90-day administration in 1-, 10- and 100fold clinical doses and 14 days of the recovery period have not revealed major adverse effects caused by the test substance. The only change detected was a minor reversible increase in the total number of segmented neutrophils and decrease in total number of lymphocytes following 90-day exposure in dose 57 mg/kg/day.

The test substance does not have local irritating effect on mucous membranes of gastrointestinal tract at repeated exposure.

Results of the studies conducted suggest that Killevir-16 has a high therapeutic index and is harmless at a long-term treatment.

ANNEX 1

STUDY PROTOCOL

Chronic toxicity study of pharmaceutical substance Killevir-16 at intragastrical administration in rats during 90 days

Study Code:	**O0211**
Substance code	**0611**
Customer:	ZAO "Intelpharm" Closed Corporation "Intelpharm" (ZAO "Intelpharm"). Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia Tel/fax: 8 (8314)30-20-32
Customer Representative	Lev D. Rasnetsov, Director of ZAO "Intelpharm"
Testing facility	State Federal Enterprise for Science "Research Centre for Toxicology and Hygienic Regulation of Biopreparations" (RCT&HRB), Federal Medico-Biological Agency Bld. 102A Lenin str., Serpukhov, Moscow region, 142253, Russia tel/fax: (4967) 39-97-38 toxic@online.stack.net
Study Director	Nikolay M. Onatsky, PhD(Biol) Head of Division for General Toxicology Bld. 102A Lenin str., Serpukhov, Moscow region, 142253, Russia tel/fax: (4967) 70-54-84, 39-97-38 toxic@online.stack.net

1. Key dates

Planned animal receipt date: 6.04.2011
Planned treatment initiation date: 20.04.2011
Planned 1st necropsy date: 19.07.2011
Planned last necropsy date: 03.08.2011
Planned draft report submission: 03.10.2011

2. Responsible Personnel

Study Director	N.M. Onatsky, PhD (Biol), Head of Division for General Toxicology
Preparation of substance doses	L.A. Eremenko, Researcher, Division for Analytical Chemistry and Radiobiology.
Treatment and clinical observation of animals	V.A. Blokhin, Senior Researcher, Division for General Toxicology
Histology	S.P. Rybalkin, Deputy Director in Scientific Affairs, Head of Division for Pathomorphology and Reproductive Toxicology
Clinical observation	N.N. Sivogrivova, Researcher, Division for General Toxicology
Hematology	N.V. Bodrova, Junior Researcher, Division for General Toxicology
Quality assurance	A.S. Karpova, Head of QA Laboratory

3. Study Objective

The study objective is experimental assessment of the level and character of damaging effect of pharmacetical substance Killevir-16 on laboratory animals and safety assessment at repeated exposure.

4. Study design

The study design, test substance, doses and route of administration are agreed with the Customer. Test substances suspension (vehicle – 1% starch gel) will be administered in animals daily during 90 days in accordance with the regimens presented in Table.
Control animals will receive a vehicle.

Group Assignment and Dose Levels

Group #	Substance, code	Animals		Individual # of animals	Dose level (mg/kg)	Concentration of active substance (mg/ml)	Administered volume (ml/rat with weight 200 g)
		Sex	Q-ty				
1	Vehicle	M*	12	1-12	0	0	1.0
2	Vehicle	F**	12	13-24	0	0	1.0
3	0611	M*	12	25-36	0.57	0.114	1.0
4	0611	F**	12	37-48	0.57	0.114	1.0
5	0611	M*	12	49-60	5.7	1.14	1.0
6	0611	F**	12	61-72	5.7	1.14	1.0
7	0611	M*	12	73-84	57	11.4	1.0
8	0611	F**	12	85-96	57	11.4	1.0

F – female, M — male

Clinical observation and pathohistological examination of experimental animals will be performed on the scheduled dates: day 91 and day 105 from initiation of dosing.

5. Guidelines

The study will be performed in compliance with "Rules for Laboratory Practice in the Russian Federation" (Order of Russian Ministry of Health and Social Development #708n of August 23, 2010).

All procedures within the study will be performed in accordance with the approved written study protocol and Standard operating procedures (SOPs).

The study design was made up based on "Methodical guidance on general toxicity studies of pharmaceuticals" /Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev – 2nd edition, corrected and amended; ad. – M.: OAO "Izdatel'stvo Medicina", 2005. – P.41-54.

6. Quality assurance

Quality Assurance Laboratory conducts audit of the principal phases of the study for consistency of procedures with the approved protocol; reliability of the obtained data and correctness of documentation.

7. Humane treatment and use of test animals

Based on the data obtained in this research a veterinary protocol will be written and submitted to the Bioethics Committee of the RCT&HRB for expert assessment and approval.
In the studies only the personnel who have appropriate qualification and skills will be involved.

During the study all manipulations with animals will comply with the procedures of the approved protocol.

8. Justification

8.1. Justification of the study

Test substance - Killevir-16; pharmacotherapeutical group- antiviral preparation.
Chronic toxicity study of the test substance at repeated administration in animals must meet the requirements of the regulatory documents. The study is included in complex nonclinical studies mandatory for the product registration on the territory of the Russian Federation.
The information obtained in the course of the study will not repeat the earlier obtained study results.

8.2. Justification of test species

The study will be conducted on rats as species commonly used for toxicological studies.

8.3 Number of animals

The number of animals in the study will be sufficient for obtaining statistically significant values of the test parameters.

8.4. Route and doses

Intragastric route will be used as the intended route of administration in humans. The study design, test substance, doses and route of administration are agreed with the Customer. Maximal test doses are limited by the substance ability to form a homogeneous substance in starch gel and maximal allowed volumes for i/g repeated administration in rats.

9 Test material

9.1. Test substance

Name	Killevir-16
Manufacturer	ZAO “Intelpharm”
Series	#54 of 22.12.09
Code	0611
Shelf life	5 years
Description	amorphous powder , brown or dark brown
Solubility	almost insoluble in water, 95% ethyl alcohol, 1,2-dichlorbenzene, freely soluble in dimethyl sulfoxide
Storage conditions	≤30°C, protected from light

9.2. Control substance

Name	1% starch gel
Code	vehicle

9.3. Test substance accountability

Pharmaceutical group maintains records on procurement and accountability of the test substances and is responsible for preparation of test solutions for treatment of experimental animals.

9.4. Safety assurance

Any manipulations with substances will comply with standards and rules for safe work with pharmaceuticals.
The manufacturer of the test substance will supply a Material Safety Data Sheet (MSDS) and/or other pertinent documentation regarding safety.

9.5. Disposal of test substance

Upon completion of the testing unused residues of the test material will be returned to the Customer.

9.6. Preparation of doses

Test substance solutions will be prepared by pharmaceutical group prior to administration in aseptic conditions in volumes equal to the total single daily doses.
Test substance for administering in animals will be prepared as suspension in 1% starch gel.

10. Stability

Stability of the test substance for experimental conditions is determined by the Customer.

11. Animals

Species:	rat
Strain:	outbred
Source:	Animal Nursery at SE RCBMT RAMS, "Stolbovaya" branch
Age before treatment start:	7-9 weeks
Weight before the treatment start:	Females 130-170 g, males 140-180 g
Number of males:	48 + 7 reserve
Number of females:	48 + 7 reserve

11.1. Animal care

Animals will be kept in routine conditions in compliance with the rules approved by the Ministry of Health of the Russian Federation on 06.07.73 "Construction, equipping and maintenance of experimental biological clinics (vivariums)".

11.1.1. Cages

Rats will be kept in polypropylene cages, 6 animals per cage. The cages have steel lattice covers with deepenings, steel dispensers for feed and water, and steel label holders.

11.1.2. Bedding

For bedding wood cuts will be used. Thickness of bedding layer in the cage – 10-15 mm.

11.1.3. Diet
The diet for laboratory animals PK-120-1 (GOST P 50258-92) will be given *ad libitum* through the deepenings in the cage cover.

11.1.4. Water
Animals will be given water compliant with GOST 2874–82 "Drinking water". Water will be given *ad libitum* in conventional sterilized vials with steel covers-spouts.

11.1.5. Environmental conditions
Animals will be kept under controlled environmental conditions (tº 18-26°C, relative humidity of air 30-70%) protected from direct sunrays. A 12-hour light/dark cycle will be maintained.

11.1.6. Acclimation
Before the testing the animals received will be kept for adaptation in groups in cages for 10-14 days. In this period visual clinical observation of the animals will be daily performed.
Animals with deviations detected during the inspection will be judged unacceptable for use in the experiments.

11.1.7. Animal grouping

Animals will be assigned to experimental groups at random based on weight and sex.

11.1.8. Animal identification
Each animal will be individually enumerated according to the Table (item 5 of the Protocol) with indelible ink on skin near the tail root from the dorsal side. The individual numbers of animals are indicated on the cage label.

11.1.9. Spare animals

The animals remained after grouping will be included in stock population and used in the current experiments conducted by the organization.

12. Testing methods
12.1. Lifetime manipulations with animals
12.1.1. Administration of substances
Test and control substances will be once daily i/g administered in fixed doses. The volume of administered dose will be calculated individually for each animal based on body weight and adjusted after each weighing.

12.1.2. Clinical observations
Animals will be observed for viability throughout the study and during 14-day recovery period. Cageside observations for health status deviations and deaths will be made and recorded daily.

If no external signs of animal ill-being are observed, a detailed clinical examination of each animal will be performed weekly. In case any animal condition deviations are observed clinical examination will be performed daily; in case of rapid development of adverse signs - no less than twice a day.

12.1.3. Body weight

Animals will be weighed weekly. Body weights of animals fasted overnight prior to necropsy are used for calculation of organs' mass coefficients.

12.1.4. Food consumption

Deviations in food and water consumption in separate cages will be noted daily .

12.1.5. Food deprivation

Animals will be fasted overnight prior to necropsy. Water will be given *ad libitum*.

12.1.6. Collection of blood samples

Whole blood samples will be collected from animals on day 91 and day 105 following the first dosing with test substance prior to the scheduled necropsy. Animal blood for hematological analysis will be taken by tail tip incision without anesthesia. Blood for biochemical analysis will be taken from femoral vein of pre-anesthetized animals. If necessary, blood can be taken from heart.

12.1.7. Urine samples

Urine samples will be taken from 5 animals 1-3 days before euthanasia.

12.2. Clinical observation

Necropsy will be performed on day 91 and day 105 of the study as follows:
– day 91 – half of survived animals from each group;
– day 105 – rest of animals from each group, maximum 5.

12.2.1. Hematology

Blood for hematological studies on Hema-screen-13 hematological analyzer will be sampled into tubes with Diluid Azide Free isotonic diluent (J.T.BAKER, Netherlands). Calculation of reticulocytes and leukocyte formula blood will be performed under microscope in dyed blood smears. For calculation of the number of reticulocytes blood samples will be placed in test tubes with 1% brilliant cresyl blue and after 30 minutes of supravital dyeing blood smears are prepared.

The whole blood samples will be analyzed for the following parameters:

Erhythrocytes
Hemoglobin
Hematocrit
Reticulocytes
Mean corpuscular volume
Thrombocytes

- Leukocytes, including:
- Basophiles level
- Eosinophiles
- Young

- Stab
- Segmented
- Lymphocytes
- Monocytes

12.2.2. Blood serum biochemistry

Blood samples for biochemical analysis will be taken without coagulants added. After retraction of blood clots in blood samples serum is sucked off with a pipette and centrifuged. In blood serum the following parameters will be evaluated on semi-automatic analyzer “Stat Fax 3300”:

- Total protein
- Glucose
- Urea
- Cholesterol
- Creatinine
- Alanine aminotransferase
- Aspartate aminotransferase
- Alkaline phosphatase
- Bilirubin

12.2.3. Urine analysis

The following urine parameters will be analyzed on Urisys 1100 analyzer using test stripes:

- Protein
- pH
- Glucose
- Relative density
- Ketones
- Leukocytes
- Bilirubin
- Nitrites
- Occult blood
- Urobilinogen

12.3. Pathoanatomy and histology

12.3.1. Moribund animals and animals died during the study

If the animal dies during the study it is critical to most accurately determine the time of death and immediately perform the necropsy. If it is impossible at the moment, the dead body is placed in a fridge at +4°C for no more than 12 hours.
The moribund animal can be euthanized by decision of the researcher who conducts clinical observation after consulting with veterinarian. If it is possible, blood and other samples are taken.
For histological analysis samples of modified internal organs will be taken.

12.3.2. Euthanasia

CO_2 inhalation will be used as a means of euthanasia.

12.3.3. Necropsy

Full-scale necropsy of all dead animals will be performed with detailed examination and description of external state of the body, skin, hair, visible mucous membranes, as well as examination of pectoral, abdominal and pelvic cavities with the internal organs.
All abnormalities must be documented. To the discretion of the pathologist the photos of the sites with macroscopic changes can be taken.

12.3.4. Organ weights

In all animals subjected to the planned or emerged euthanasia weights of the following organs is determined:

- Heart
- Liver
- Spleen
- Kidneys
- Lungs
- Thymus
- Testicles

Twin organs are weighed together. Organs of suddenly died animals are not weighed. Mass coefficients of the organs will be evaluated by calculation of the ratio of the organ weight in g to body weight (in kg) determined before necropsy after a night of starvation.

12.3.5. Tissue collection

During necropsy samples of the following organs and tissues will be taken for histological analysis:

- Cerebrum
- Heart
- Lung
- Liver
- Stomach
- Empty intestine including Peyer's patches
- Kidney
- Spleen
- Thymus
- Testicles

All samples of organs and tissues will be placed in 10% buffer solution of formalin.
Organs and tissues or their residues after histology will be archived and shipped to the Customer upon request.

12.3.6. Histology

The fixed samples of organs and tissues will be dehydrated, saturated with paraffin and embedded in paraffin blocks. From blocks slices of thickness 5 μm will be done and dyed with hematoxilin and eosin. Additional methods of dyeing are used by decision of the pathologist.

12.3.7. Examination of histological preparations

Histological preparations will be studied by method of light microscopy. For histological analysis preparations from the animals that received maximal dose of substance and control animals will be taken. In case of abnormalities considered to be caused by the test substance, tissues of these organs from animals treated with lower doses of substance will be examined.

Preparations from animals obtained after recovery period will be examined in the same manner.

13. Data analysis

For all quantitative data obtained in the studies descriptive statistics will be applied; average randomized values and standard deviations will be calculated and presented in the summarized tables. To evaluate reliability of differences between the groups adequate parametric or non-parametric criteria will be used depending on the manner of quantitative data distribution. Analysis will be performed separately for males and females. The differences are evaluated at significance level 0.05.

14. Protocol amendments

Changes in the approved protocol will be discussed by the Study Director and Customer and documented in the form of Amendment which will be approved and will have an effective date. The protocol changes are signed by Study Director and Customer Representative and attached to all approved copies of the protocol. Customer may authorize the changes presented in PDF file by the facsimile machine or by electronic mail.

15. Deviations

Any deviations will be documented in the data sheets with assessment of their affect on the study.

16. Report

A draft report will be submitted to the Customer as soon as possible after the last necropsy. The final report presents full data on the study according to the protocol. Upon completion the Customer will receive two hard copies and one electronic copy (Word) of the report. The study protocol, amendments and significant deviations will be included in the annex to the report.

17. Documentation and archive

All study-related data and documents will be filed. Study Director is responsible for the completeness of data presented and adequacy of documentation. Upon signing of the final report all data, documentation, samples, glasses and tissues in blocks will be archived and stored at the RCT&HRB for 2 years. After 2 years of storage archive materials upon agreement with the Customer are handed to the Customer or destroyed after notifying the Customer.

ANNEX 2

Individual body weights, g

Male rats

Dose, mg/kg/day	Group #	Ind.#	Day of experiment															
			0	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**	**84**	**91**	**98**	**105**
0	1	**1**	165	236	292	329	350	364	386	411	418	433	440	455	467	477		
		2	169	206	253	286	320	343	375	382	386	397	406	425	441	447		
		3	162	227	281	308	335	349	376	404	414	440	450	465	487	491		
		4	161	222	262	300	330	354	385	404	415	430	438	450	478	492		
		5	182	248	300	339	368	391	430	445	461	478	494	491	507	520		
		7	174	237	300	356	390	421	459	471	475	488	500	513	541	544	571	578
		8	170	224	261	305	332	359	380	395	402	416	435	438	457	463	476	486
		9	170	244	307	341	360	383	398	413	420	432	439	445	459	474	473	481
		10	179	241	303	345	376	395	401	403	410	428	444	474	501	515	523	531
		11	159	214	261	290	310	327	350	368	375	375	385	394	407	414	423	431
0.57	3	**25**	173	229	270	299	316	333	365	381	408	418	430	445	471	479		
		26	173	237	310	366	395	429	453	477	488	507	520	544	577	596		
		27	167	222	278	309	331	334	358	369	380	388	405	422	448	462		
		28	162	223	265	289	312	331	352	366	364	375	387	405	425	430		
		29	186	250	309	347	366	399	421	441	450	464	470	473	491	508		
		31	177	233	281	306	327	345	369	380	392	407	420	443	456	465	491	490
		32	161	212	262	300	320	338	373	384	397	402	414	433	444	461	469	468
		33	161	218	258	304	322	335	351	357	365	380	395	413	426	436	440	451
		34	174	227	271	311	329	346	362	383	396	410	425	434	434	439	448	453
		35	166	228	279	323	362	383	411	434	442	418	445	456	475	484	502	513
5.7	5	**49**	184	245	289	320	344	370	404	425	433	448	467	480	496	500		
		50	171	237	285	322	336	368	390	379	413	426	444	461	475	484		
		51	172	234	293	330	349	383	402	402	416	426	442	455	462	477		
		52	168	203	254	285	305	336	357	354	364	382	402	418	431	436		
		53	185	238	285	310	326	353	373	382	400	414	425	437	454	464		
		55	186	240	290	319	346	372	403	406	412	424	438	449	468	477	480	485
		56	154	208	265	305	329	354	385	399	408	425	427	431	445	452	458	458
		57	149	211	270	306	327	341	365	376	390	405	420	432	448	460	457	478
		58	172	231	277	303	320	330	343	353	358	366	364	369	380	382	390	395
		59	167	210	258	295	314	322	336	349	357	370	389	403	413	415	407	405
57	7	**73**	175	229	276	308	327	334	363	379	380	382	388	395	407	403		
		74	181	255	313	352	371	393	410	424	449	463	483	490	515	522		
		75	179	223	268	304	330	349	363	368	373	388	406	412	427	436		
		76	157	230	287	325	354	385	415	441	452	474	487	489	514	527		
		77	188	260	327	370	394	421	441	458	471	480	490	503	524	536		
		79	180	234	295	320	337	349	367	333	360	416	439	457	477	488	503	512
		80	166	233	282	310	323	334	353	324	352	395	415	430	460	466	474	484
		81	176	247	306	346	372	382	415	377	405	442	444	459	480	501	492	498
		82	148	203	249	273	315	340	362	333	354	394	409	428	441	450	453	451
		83	188	214	307	339	365	383	413	374	400	451	471	484	503	507	517	513

Individual body weights, g

Female rats

Dose, mg/kg/day	Group #	Ind.#	Day of experiment															
			0	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**77**	**84**	**91**	**98**	**105**
0	1	**13**	185	227	251	264	266	290	310	314	317	333	340	337	342	344		
		14	163	201	227	241	256	269	282	279	291	300	300	300	314	322		
		15	170	208	230	246	254	251	277	282	285	294	302	309	314	316		
		16	171	205	220	232	241	244	258	269	274	273	284	285	288	285		
		17	150	200	232	248	255	273	296	301	309	325	336	339	343	345		
		19	168	206	238	252	266	283	292	300	294	312	319	324	326	335	341	336
		20	168	207	223	222	239	248	260	257	267	275	275	279	285	287	288	290
		21	157	194	222	222	244	257	273	288	280	291	302	306	312	317	323	321
		22	146	172	186	212	221	228	241	253	258	253	265	266	273	274	286	295
		23	164	194	217	236	248	250	268	279	290	285	306	313	314	317	330	340
0.57	3	**37**	182	210	220	224	240	250	251	247	251	258	259	260	267	274		
		38	168	191	199	204	219	228	238	234	240	256	254	254	266	269		
		39	153	191	223	238	246	259	265	276	280	285	295	298	299	301		
		40	165	200	219	234	251	258	271	277	292	301	306	308	312	314		
		41	170	195	206	219	233	247	259	263	260	277	285	296	292	305		
		43	166	194	209	217	229	243	244	256	273	258	273	270	280	280	286	290
		44	153	176	208	228	237	245	254	266	268	267	278	283	290	290	302	304
		45	176	208	218	231	244	255	265	273	274	281	283	286	290	304	313	317
		46	174	209	233	245	250	266	279	290	290	291	306	310	312	305	317	315
		47	183	210	227	239	242	255	270	272	281	283	295	295	300	304	311	317
5.7	5	**61**	155	186	201	215	228	227	233	247	249	240	254	265	276	271		
		62	166	187	202	203	218	224	232	229	241	244	249	254	260	266		
		63	171	213	242	255	271	277	292	297	307	315	320	327	342	350		
		64	164	202	226	238	251	256	261	268	271	280	281	286	301	323		
		65	181	220	220	226	228	238	241	255	253	258	267	266	267	270		
		67	160	197	233	245	255	272	288	303	312	317	318	321	330	340	344	350
		68	171	215	243	266	274	286	304	315	323	328	328	343	344	351	354	354
		69	169	208	223	254	269	278	290	296	302	296	297	310	319	330	340	339
		70	157	196	225	243	264	270	280	279	280	282	293	299	301	317	318	318
		71	188	222	242	249	263	274	295	299	296	299	318	320	322	324	336	339
57	7	**85**	153	188	206	217	227	230	236	247	255	247	270	277	283	285		
		86	177	226	232	239	256	266	275	229	287	284	292	291	303	302		
		87	165	195	230	253	260	251	267	297	288	287	296	291	309	302		
		88	168	187	208	225	239	248	247	268	271	266	284	283	290	282		
		89	167	199	220	232	243	251	261	255	264	272	281	290	287	293		
		91	165	198	225	237	255	268	286	303	296	300	310	315	330	340	331	351
		92	174	207	225	237	244	246	259	315	280	275	291	294	304	296	307	314
		93	162	203	226	233	247	257	270	296	276	294	300	310	310	317	318	313
		95	171	207	230	258	274	285	295	299	297	300	304	312	314	313	323	330
		96	170	208	237	244	255	265	284	265	301	305	314	325	332	340	351	358

ANNEX 3

Individual hematological indices

Individual hematological indices after exposure period (male rats)

Dose, mg/kg/day	Group #	Ind #	Hemoglobin, mmol/dm^3	Erythro cytes, mln/mm^3	Hemato crit, %	Mean corpu scular volume, μm^3	Reticulo cytes, %	Thrombo cytes, thousand	Leuko cytes, thousand/mm^3	Leukocytes including						
										B %	E %	Yu %	S %	S %	L %	M %
0	1	1	8.6	7.8	38.3	49.0	4.8	440.0	20.1	0	6	0	2	24	60	8
		2	9.1	8.2	41.1	50.0	4.2	566.0	17.5	0	2	0	4	20	66	8
		3	6.3	4.5	26.5	59.0	4.3	330.0	9.6	0	2	0	0	18	74	6
		4	7.3	7.0	33.3	47.0	4.4	523.0	21.6	0	4	0	2	18	70	6
		5	7.5	7.1	34.3	48.0	4.2	476.0	14.8	0	0	0	0	24	70	6
0.57	3	25	8.6	7.1	36.1	51.0	4.0	368.0	17.9	0	2	0	2	26	64	6
		26	9.9	8.7	42.1	48.0	4.6	451.0	21.5	0	2	0	2	20	70	6
		27	8.1	6.8	33.6	50.0	4.8	417.0	20.0	0	2	0	4	20	68	6
		28	8.1	7.1	33.7	48.0	3.5	449.0	16.1	0	0	0	0	18	74	8
		29	8.0	7.0	31.9	48.0	4.1	438.0	16.2	0	4	0	0	18	72	6
5.7	5	49	7.3	5.1	28.3	55.0	4.0	332.0	9.2	0	2	0	2	22	66	8
		50	7.9	6.6	33.0	50.0	4.5	377.0	10.3	0	2	0	0	20	74	4
		51	6.9	5.8	30.3	52.0	3.6	384.0	8.2	0	2	0	2	20	70	6
		52	7.3	6.6	34.2	52.0	4.7	483.0	9.8	0	0	0	4	24	66	6
		53	7.3	6.0	31.7	53.0	3.8	419.0	20.7	0	2	0	2	20	70	6
57	7	73	7.2	6.2	31.9	52.0	5.0	506.0	14.3	0	4	0	0	50	40	6

		74	8.1	7.3	36.4	50.0	4.4	365.0	12.6	0	4	0	0	32	58	6
		75	7.9	7.2	35.9	50.0	6.0	540.0	22.0	0	4	0	2	30	60	4
		76	8.4	7.1	32.9	46.0	4.6	453.0	19.2	0	0	0	6	36	50	8
		77	8.6	6.9	35.5	52.0	3.5	339.0	16.5	0	4	0	0	32	60	4

Individual hematological indices after exposure period (female rats)

Dose, mg/kg/day	Group #	Ind #	Hemoglobin, mmol/dm^3	Erythro cytes, mln/mm^3	Hemato crit, %	Mean corpu scular volume, μm^3	Reticulo cytes, %	Thrombo cytes, thousand	Leuko cytes, thousand/mm^3	Leukocytes including						
										B %	E %	Yu %	S %	S %	L %	M %
0	2	13	8.5	6.8	34.6	51.0	5.4	567.0	11.8	0	6	0	4	18	68	4
		14	7.4	6.1	31.3	51.0	5.8	402.0	12.2	0	2	0	0	20	72	6
		15	7.1	6.2	31.7	51.0	4.0	458.0	21.3	0	2	0	2	28	64	4
		16	8.1	6.2	34.0	55.0	5.3	280.0	15.3	0	6	0	0	24	64	6
		17	8.1	6.1	34.1	56.0	3.8	362.0	15.2	0	0	0	0	34	62	4
0.57	4	37	6.6	5.2	27.9	53.0	3.9	302.0	20.6	0	2	0	2	28	64	4
		38	7.2	5.6	30.3	54.0	4.2	341.0	14.2	0	2	0	2	22	68	6
		39	8.1	6.2	34.6	56.0	4.6	335.0	14.6	0	4	0	2	18	70	6
		40	8.1	6.4	33.9	53.0	5.0	357.0	13.9	0	2	0	2	16	72	8
		41	7.3	5.7	31.1	54.0	3.6	380.0	20.5	0	2	0	2	28	62	6
5.7	6	61	7.3	5.1	28.3	55.0	4.0	332.0	11.2	0	0	0	0	24	70	6
		62	7.9	6.6	33.0	50.0	4.5	377.0	10.3	0	4	0	2	26	64	4
		63	6.9	5.8	30.3	52.0	3.6	284.0	18.2	0	0	0	4	24	64	8
		64	7.3	6.6	34.2	52.0	4.7	483.0	9.8	0	4	0	2	20	70	4

		65	7.3	6.0	31.7	53.0	3.8	419.0	20.7	0	4	0	4	22	64	6
57	8	85	7.1	5.7	30.0	52.0	4.7	195.0	9.6	0	4	0	2	20	70	4
		86	8.2	6.5	34.0	52.0	4.4	368.0	13.5	0	0	0	0	34	62	4
		87	7.4	6.0	30.8	52.0	4.0	373.0	14.8	0	6	0	2	24	62	6
		88	8.1	6.8	34.7	51.0	5.0	410.0	18.6	0	4	0	2	34	58	2
		89	7.9	6.5	39.4	51.0	4.4	415.0	11.2	0	2	0	2	14	74	8

Individual hematological indices after recovery period (male rats)

Dose, mg/kg/day	Group #	Ind #	Hemoglobin, mmol/dm^3	Erythro cytes, mln/mm^3	Hemato crit, %	Mean corpu scular volume, μm^3	Reticulo cytes, %	Thrombo cytes, thousand	Leuko cytes, thousand/mm^3	Leukocytes including						
										B %	E %	Yu %	S %	S %	L %	M %
0	1	7	8.7	7.5	39.9	54.0	4.6	451.0	18.9	0	2	0	4	20	68	6
		8	8.3	7.4	38.3	52.0	3.4	487.0	16.0	0	4	0	2	22	64	8
		9	6.9	6.1	30.5	50.0	4.5	384.0	20.6	0	0	0	0	24	72	4
		10	8.3	6.8	38.7	54.0	4.8	672.0	10.1	0	2	0	2	18	72	6
		11	7.2	6.2	31.9	52.0	5.0	506.0	14.3	0	4	0	2	22	66	6
0.57	3	31	8.1	7.3	36.4	54.0	4.4	365.0	12.6	0	2	0	3	20	67	8
		32	7.9	7.2	35.9	50.0	6.0	540.0	22.0	0	4	0	0	26	66	4
		33	8.2	7.1	39.9	50.0	4.6	453.0	19.2	0	2	0	2	19	71	6
		34	7.6	6.5	35.8	55.0	3.6	399.0	16.2	0	2	0	2	18	72	6
		35	7.8	6.5	32.5	50.0	4.8	520.0	10.9	0	4	0	0	24	66	6
5.7	5	55	8.5	8.7	36.9	50.0	4.2	421.0	12.8	0	0	0	2	22	70	6

		56	7.9	6.8	34.1	54.0	3.8	671.0	11.6	0	2	0	0	18	74	6
		57	7.1	5.7	32.3	50.0	4.2	448.0	17.3	0	2	0	0	22	72	4
		58	6.9	6.5	34.4	53.0	4.8	361.0	20.9	0	0	0	2	24	70	4
		59	8.2	7.1	37.0	52.0	5.1	549.0	14.4	0	6	0	2	20	66	6
57	7	79	8.2	7.3	39.5	54.0	4.4	520.0	17.3	0	4	0	0	18	72	6
		80	7.3	6.8	36.5	50.0	4.2	349.0	15.8	0	0	0	3	22	70	5
		81	8.4	7.5	38.9	52.0	3.4	422.0	20.6	0	2	0	2	20	70	6
		82	8.1	6.2	34.0	52.0	4.0	507.0	18.4	0	4	0	4	24	62	6
		83	7.4	6.6	34.0	51.0	3.2	562.0	15.8	0	4	0	0	20	70	6

Individual hematological indices after recovery period (female rats)

Dose, mg/kg/day	Group #	Ind #	Hemoglobin, mmol/dm^3	Erythro cytes, mln/mm^3	Hemato crit, %	Mean corpu scular volume, μm^3	Reticulo cytes, %	Thrombo cytes, thousand	Leuko cytes, thousand/mm^3	Leukocytes including						
										B %	E %	Yu %	S %	S %	L %	M %
0	2	19	7.9	6.5	37.4	54.0	4.4	415.0	11.2	0	4	0	2	24	64	6
		20	6.9	6.0	33.6	56.0	5.1	548.0	11.7	0	2	0	2	20	70	6
		21	6.8	6.0	33.8	56.0	3.6	535.0	11.4	0	2	0	0	22	68	8
		22	7.4	6.0	30.8	52.0	4.0	373.0	14.8	0	0	0	4	20	71	5
		23	8.1	6.8	34.7	51.0	5.0	410.0	9.6	0	4	0	2	24	64	6
0.57	4	43	8.1	6.5	36.3	54.0	3.8	490.0	10.7	0	2	0	4	18	72	4
		44	7.9	7.4	32.8	51.0	4.7	361.0	11.6	0	2	0	2	24	66	6
		45	7.1	5.9	33.8	58.0	5.0	448.0	12.2	0	2	0	0	24	66	8

		46	8.6	7.1	37.0	54.0	3.2	549.0	12.1	0	0	0	2	24	68	6
		47	7.3	6.0	31.1	54.0	3.6	380.0	11.5	0	4	0	2	20	68	6
5.7	6	67	6.8	5.8	33.6	57.0	4.0	410.0	12.9	0	2	0	0	22	72	4
		68	7.3	6.2	32.7	56.0	3.8	404.0	12.3	0	2	0	4	20	68	6
		69	7.9	6.7	37.6	57.0	4.5	476.0	10.5	0	0	0	2	26	66	6
		70	8.4	6.9	37.6	55.0	3.7	421.0	16.3	0	4	0	2	18	70	6
		71	8.2	6.5	33.8	52.0	5.4	517.0	9.9	0	0	0	2	20	70	8
57	8	91	6.6	5.9	31.7	52.0	4.4	526.0	13.6	0	4	0	2	24	66	4
		92	7.5	6.5	34.8	54.0	3.8	376.0	10.6	0	2	0	4	20	68	6
		93	8.6	7.2	34.1	52.0	4.6	373.0	14.6	0	0	0	2	18	74	6
		95	7.2	6.6	34.6	56.0	3.8	516.0	12.9	0	2	0	0	22	70	6
		96	8.3	6.9	38.7	55.0	4.3	419.0	11.8	0	3	0	2	24	65	6

ANNEX 4

Individual biochemical indices

Individual biochemical indices after exposure period (male rats)

Dose, mg/kg/day	Group #	Ind #	Total protein g/l	Glucose mmol/l	Urea mmol/l	Cholesterol mmol/l	Bilirubin μmol/l	Creatinine μmol/l	AlAT U/l	AsAT U/l	Alkaline phosphatase U/l
0	1	1	105.0	8.7	7.0	3.89	15.5	130.3	36.7	24.27	88.3
		2	84.7	9.9	5.5	4.05	19.7	48.8	21.7	22.06	78.4
		3	90.7	8.9	5.3	3.75	12.0	47.7	14.0	18.32	121.3

		4	85.9	8.8	5.7	3.92	20.7	51.1	15.5	23.26	135.8
		5	98.9	10.1	6.1	3.94	24.4	47.2	29.6	27.16	95.5
0.57	3	25	95.0	10.5	6.0	4.72	17.1	57.8	16.7	16.21	51.2
		26	102.9	12.9	4.7	6.63	15.8	60.6	14.9	19.22	81.2
		27	104.2	12.7	5.2	7.59	16.9	53.9	28.6	19.12	110.6
		28	94.9	12.5	4.8	5.16	17.2	56.7	30.5	18.31	71.7
		29	91.4	10.1	5.4	4.16	13.2	61.8	26.8	19.08	84.4
5.7	5	49	103.7	12.1	7.2	4.94	19.9	65.1	18.8	20.55	83.1
		50	100.3	10.6	6.6	7.63	14.4	62.9	21.4	15.98	87.1
		51	103.6	12.0	6.6	5.66	14.9	69.1	23.7	17.95	80.3
		52	95.0	10.9	5.2	4.89	18.0	48.3	17.1	11.89	76.9
		53	79.4	10.5	6.3	4.72	14.2	66.8	23.5	20.26	111.8
57	7	73	89.7	10.7	5.5	3.80	15.9	60.6	11.6	21.30	115.3
		74	97.0	10.2	4.9	3.47	12.0	61.2	9.5	19.77	125.8
		75	88.9	10.3	5.0	4.19	14.6	71.9	14.0	22.85	92.4
		76	94.0	10.2	4.7	5.55	15.1	46.0	22.2	20.79	119.0
		77	76.7	10.2	5.7	4.21	25.2	76.9	23.2	20.72	53.1

Individual biochemical indices after exposure period (female rats)

Dose, mg/kg/day	Group #	Ind #	Total protein g/l	Glucose mmol/l	Urea mmol/l	Cholesterol mmol/l	Bilirubin μmol/l	Creatinine μmol/l	AlAT U/l	AsAT U/l	Alkaline phosphatase U/l
0	2	13	99.1	14.4	6.7	5.86	9.8	76.9	12.5	21.46	54.2
		14	93.1	10.1	5.8	4.97	6.9	62.3	10.7	18.72	64.3
		15	104.3	13.8	8.9	5.49	7.1	78.1	7.9	16.15	63.1
		16	103.5	14.6	8.4	3.61	9.4	93.8	8.7	15.19	45.5
		17	90.6	13.2	8.0	5.17	10.0	58.9	8.7	14.86	82.3
0.57	4	37	103.9	14.8	6.3	3.79	13.2	54.5	8.1	12.62	41.4

		38	109.5	16.0	8.5	3.71	13.2	58.4	13.5	17.04	44.8
		39	101.6	14.1	7.7	4.91	8.2	66.8	12.4	13.51	63.1
		40	94.0	16.5	7.1	4.57	11.9	61.2	12.9	15.64	74.2
		41	111.9	17.0	7.0	5.50	12.7	68.5	4.7	19.63	89.5
5.7	6	61	104.3	10.6	7.4	4.97	14.4	62.9	8.5	17.13	64.5
		62	86.1	12.8	6.6	3.68	10.8	66.8	12.3	18.27	54.4
		63	73.9	19.0	8.2	5.05	9.1	77.5	9.4	14.34	65.9
		64	111.8	15.5	8.8	6.09	12.6	49.4	10.3	16.96	64.9
		65	111.7	17.6	6.0	4.51	9.8	52.2	4.1	17.57	51.2
57	8	85	89.5	12.2	6.6	3.82	8.3	66.3	8.7	15.25	63.1
		86	112.9	13.4	7.6	4.03	10.0	56.1	3.8	20.21	113.6
		87	69.2	12.9	5.8	3.05	9.3	59.0	6.0	13.73	94.4
		88	90.0	13.6	6.4	3.56	10.6	65.7	5.8	17.92	67.0
		89	98.0	18.2	6.7	3.24	10.2	66.8	5.8	14.16	89.5

Individual biochemical indices after recovery period (male rats)

Dose, mg/kg/day	Group #	Ind #	Total protein g/l	Glucose mmol/l	Urea mmol/l	Cholesterol mmol/l	Bilirubin μmol/l	Creatinine μmol/l	AlAT U/l	AsAT U/l	Alkaline phosphatase U/l
0	1	7	93.8	10.6	4.7	4.39	7.5	82.0	7.5	16.25	39.2
		8	97.3	11.1	5.1	6.83	9.8	65.7	10.6	14.22	135.2
		9	93.0	9.3	6.5	7.12	15.1	71.9	21.8	16.28	151.5
		10	96.3	13.4	5.5	5.59	8.5	78.7	7.3	17.34	218.7
		11	98.2	12.6	5.7	5.94	9.9	67.9	11.1	12.73	132.0
0.57	3	31	95.9	11.7	6.0	4.92	9.3	69.6	13.5	9.42	173.5

		32	96.2	15.4	4.8	4.18	7.7	80.3	11.2	16.55	133.8
		33	102.9	13.4	5.5	5.37	8.1	71.3	15.6	13.6	126.1
		34	106.0	11.8	4.9	6.98	11.9	63.4	10.1	19.47	183.9
		35	111.2	13.1	5.7	6.42	9.7	87.0	4.4	16.68	122.8
5.7	5	55	100.0	10.8	5.1	6.88	10.3	62.3	9.6	13.87	121.1
		56	91.6	8.8	5.4	5.03	9.6	75.8	9.8	11.14	131.4
		57	95.0	12.3	5.0	6.76	11.3	97.2	2.9	15.39	144.3
		58	107.9	10.6	4.7	6.31	7.1	78.1	13.3	10.43	141.5
		59	96.5	13.7	4.1	6.61	8.9	68.6	6.1	8.23	161.2
57	7	79	95.3	9.0	4.9	4.91	6.9	78.2	5.8	14.54	142.6
		80	95.7	12.1	5.0	5.78	9.1	56.1	12.2	10.67	195.7
		81	99.0	10.8	6.0	3.50	10.8	75.2	7.2	9.63	105.2
		82	100.8	13.1	4.9	5.72	7.7	80.3	12.6	12.68	162.1
		83	102.7	15.6	5.9	6.25	9.8	55.6	5.7	14.73	156.0

Individual biochemical indices after recovery period (female rats)

Dose, mg/kg/day	Group #	Ind #	Total protein g/l	Glucose mmol/l	Urea mmol/l	Cholesterol mmol/l	Bilirubin μmol/l	Creatinine μmol/l	AlAT U/l	AsAT U/l	Alkaline phosphatase U/l
0	2	19	120.6	11.8	6.0	5.56	9.8	58.4	6.7	8.92	142.6
		20	94.6	10.5	5.8	4.86	6.1	76.4	4.9	8.95	62.5
		21	86.8	11.3	6.4	5.37	6.9	66.8	10.0	14.12	94.2
		22	101.6	9.1	6.1	5.53	6.7	73.6	6.9	9.51	84.1
		23	101.1	13.6	5.8	5.75	9.5	78.2	9.4	11.18	66.4

0.57	4	43	112.5	10.7	3.9	6.71	10.1	67.9	10.3	14.83	140.0
		44	93.5	7.1	6.5	3.78	10.9	79.7	7.2	15.58	67.0
		45	92.7	10.4	5.4	5.08	13.0	59.5	4.4	15.86	151.7
		46	119.2	11.6	5.9	6.33	11.5	85.4	13.7	12.98	148.3
		47	96.1	10.3	5.8	5.19	12.6	67.4	4.8	12.89	63.4
5.7	6	67	108.1	15.2	4.8	8.39	9.7	72.4	5.5	12.83	128.6
		68	121.1	12.6	6.2	5.62	11.4	86.5	13.1	18.86	181.9
		69	102.3	7.8	6.3	7.68	6.9	58.4	10.0	16.07	234.6
		70	107.6	8.7	5.0	6.32	11.8	71.9	16.4	12.32	186.8
		71	80.7	10.5	4.8	4.11	12.5	76.4	7.7	13.61	145.3
57	8	91	99.1	10.3	5.8	5.97	9.3	71.3	4.5	8.81	155.7
		92	97.1	15.7	5.2	6.15	8.7	100.0	5.5	1.82	102.7
		93	86.8	8.6	5.0	4.61	8.3	73.0	3.1	10.23	56.7
		94	101.0	16.3	4.6	3.85	8.3	84.2	11.9	11.64	101.4
		95	88.6	14.0	5.8	5.41	8.3	87.6	9.9	11.68	102.3

ANNEX 5

Individual urine indices

Individual urine indices after exposure period (male rats)

Dose, mg/kg/day	Ind #.	Relative density	pH	Leukocytes Cells/µl	Nitrites µmol/l	Protein g/l	Glucose mol/l	Ketone bodies mmol/l	Urobilinogen µmol/l	Bilirubin µmol/l	Erhythrocytes Cells/µl
0	1	1.010	9.0	100	neg	neg	norm	neg	norm	neg	neg
	2	1.010	8.0	neg	neg	neg	norm	neg	norm	neg	neg
	3	1.015	8.0	100	pos	neg	norm	neg	norm	neg	neg
	4	1.015	9.0	100	neg	neg	norm	0.5	norm	neg	neg
	5	1.010	8.0	500	neg	neg	norm	1.5	17	neg	neg
0.57	25	1.010	9.0	100	neg	neg	norm	neg	norm	neg	neg
	26	1.015	9.0	25	neg	neg	norm	neg	norm	neg	50
	27	1.005	8.0	neg	neg	neg	norm	neg	norm	neg	10
	28	1.010	9.0	500	pos	0.75	norm	0.5	norm	neg	neg
	29	1.015	9.0	25	neg	0.25	norm	neg	norm	neg	50
5.7	49	1.015	9.0	100	neg	0.25	norm	neg	norm	neg	25
	50	1.010	9.0	100	neg	neg	norm	neg	norm	neg	neg
	51	1.010	9.0	neg	neg	neg	norm	neg	norm	neg	neg
	52	1.010	9.0	25	neg	neg	norm	neg	norm	neg	250
	53	1.010	9.0	100	pos	0.25	norm	neg	norm	neg	50
57	73	1.010	8.0	100	neg	neg	norm	neg	norm	neg	10
	74	1.015	9.0	100	neg	neg	norm	neg	norm	neg	neg
	75	1.015	9.0	neg	neg	neg	norm	neg	norm	neg	neg
	76	1.005	7.0	100	neg	neg	norm	neg	norm	neg	10
	77	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg

Individual urine indices after exposure period (female rats)

Dose, mg/kg/day	Ind #	Relative density	pH	Leukocytes Cells/µl	Nitrites µmol/l	Protein g/l	Glucose mol/l	Ketone bodies mmol/l	Urobilinogen µmol/l	Bilirubin µmol/l	Erhythrocytes Cells/µl
0	13	1.020	7.0	500	pos	0.75	norm	neg	17	neg	50
	14	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg
	15	1.015	7.0	500	neg	1.5	norm	1.5	70	17	250
	16	1.010	8.0	neg	neg	neg	norm	neg	norm	neg	neg
	17	1.020	7.0	100	pos	1.5	norm	1.5	70	neg	10
0.57	37	1.015	9.0	100	neg	0.25	norm	neg	17	neg	neg
	38	1.010	8.0	25	neg	0.25	norm	neg	70	neg	neg
	39	1.020	9.0	neg	neg	neg	norm	neg	norm	neg	neg
	40	1.015	9.0	neg	neg	neg	3	neg	norm	neg	25
	41	1.010	7.0	neg	neg	neg	norm	neg	norm	neg	neg
5.7	61	1.010	9.0	100	neg	0.25	norm	neg	norm	neg	250
	62	1.010	8.0	25	neg	neg	norm	neg	norm	neg	10
	63	1.020	8.0	100	pos	0.25	norm	neg	17	17	neg
	64	1.020	9.0	100	neg	neg	norm	neg	17	neg	neg
	65	1.010	9.0	100	neg	neg	norm	neg	17	neg	neg
57	85	1.020	8.0	500	neg	neg	norm	neg	norm	neg	neg
	86	1.010	9.0	neg	neg	neg	norm	0.5	70	neg	neg
	87	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg
	88	1.020	9.0	25	neg	neg	norm	neg	17	17	10
	89	1.015	9.0	neg	neg	neg	norm	neg	norm	neg	neg

Individual urine indices after recovery period (male rats)

Dose, mg/kg/day	Ind #	Relative density	pH	Leukocytes Cells/µl	Nitrites µmol/l	Protein g/l	Glucose mol/l	Ketone bodies mmol/l	Urobilinogen µmol/l	Bilirubin µmol/l	Erhythrocytes Cells/µl
0	7	1.010	7.0	25	neg	neg	norm	neg	norm	neg	25
	8	1.020	6.5	500	neg	0.75	norm	1.5	70	neg	50
	9	1.010	7.0	500	neg	0.75	norm	neg	norm	neg	10
	10	1.010	8.0	100	neg	0.75	norm	neg	norm	neg	50
	11	1.010	8.0	100	neg	neg	norm	neg	norm	neg	neg
0.57	31	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg
	32	1.005	7.0	100	neg	neg	norm	neg	norm	neg	10
	33	1.010	9.0	25	neg	neg	norm	neg	norm	neg	25
	34	1.010	9.0	100	neg	0.25	norm	1.5	norm	neg	neg
	35	1.015	8.0	25	pos	neg	norm	neg	norm	neg	50
5.7	55	1.010	8.0	100	neg	neg	norm	neg	norm	neg	25
	56	1.015	8.0	25	neg	neg	norm	neg	norm	neg	25
	57	1.010	7.0	500	pos	0.25	norm	neg	norm	neg	10
	58	1.010	8.0	25	neg	neg	norm	neg	norm	neg	10
	59	1.005	8.0	25	neg	0.25	norm	neg	norm	neg	50
57	79	1.015	9.0	100	neg	neg	norm	neg	norm	neg	neg
	80	1.010	9.0	500	neg	0.25	norm	neg	norm	neg	10
	81	1.010	9.0	100	neg	neg	norm	neg	norm	neg	10
	82	1.010	7.0	500	neg	1.5	norm	1.5	17	neg	250
	83	1.020	8.0	100	neg	neg	norm	neg	norm	neg	neg

Individual urine indices after recovery period (female rats)

Dose, mg/kg/day	Ind #	Relative density	pH	Leukocytes Cells/µl	Nitrites µmol/l	Protein g/l	Glucose mol/l	Ketone bodies mmol/l	Urobilinogen µmol/l	Bilirubin µmol/l	Erhythrocytes Cells/µl
0	19	1.010	8.0	25	pos	0.25	norm	neg	norm	neg	neg
	20	1.005	7.0	100	pos	0.25	norm	neg	17	neg	neg
	21	1.010	8.0	25	neg	neg	norm	neg	norm	neg	neg
	22	1.010	9.0	100	neg	neg	norm	neg	norm	neg	neg
	23	1.010	8.0	500	neg	0.25	norm	neg	norm	neg	neg
0.57	43	1.020	6.5	25	neg	neg	norm	neg	norm	neg	neg
	44	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg
	45	1.010	9.0	25	neg	neg	norm	neg	norm	neg	neg
	46	1.010	9.0	25	pos	neg	norm	neg	norm	neg	neg
	47	1.005	7.0	25	neg	0.25	norm	neg	17	neg	neg
5.7	67	1.015	9.0	100	pos	neg	norm	neg	norm	neg	neg
	68	1.015	8.0	neg	neg	neg	norm	neg	norm	neg	neg
	69	1.010	8.0	neg	neg	0.25	norm	neg	norm	neg	neg
	70	1.010	8.0	25	neg	neg	norm	neg	norm	neg	neg
	71	1.010	7.0	25	neg	0.25	norm	neg	norm	neg	neg
57	91	1.010	7.0	500	pos	0.75	norm	0.5	70	17	neg
	92	1.010	7.0	500	neg	0.25	norm	neg	norm	neg	neg
	93	1.020	7.0	25	pos	0.75	norm	neg	70	17	neg
	95	1.015	8.0	25	neg	neg	norm	neg	norm	neg	neg
	96	1.015	9.0	neg	neg	neg	norm	neg	norm	neg	neg

ANNEX 6

Individual weights of organs

Individual organs weights after exposure period (male rats)

Dose, mg/kg/day	Group #	Ind #	Animal weight, g	Organ weight, g						
				Heart	Lungs	Liver	Spleen	Kidneys	Thymus	Testicles
0 (vehicle)	1	1	434	1.5	2.3	13.4	0.9	2.2	0.5	3.6
		2	410	1.3	3.0	13.1	0.8	2.6	0.5	3.4
		3	448	1.6	2.6	12.9	1.5	2.6	0.6	3.6
		4	456	1.4	2.6	16.2	1.1	3.0	0.7	3.0
		5	482	1.7	2.7	19.3	1.3	3.2	0.5	3.6
0.57	3	25	440	1.5	2.8	13.1	1.3	2.6	0.5	3.8
		26	541	1.5	3.1	14.2	1.3	3.0	0.4	4.2
		27	427	1.5	2.1	12.9	1.0	2.2	0.5	3.0
		28	394	1.5	2.6	13.9	1.4	2.4	0.4	2.8
		29	465	2.0	3.2	13.8	1.2	3.0	0.4	4.2
5.7	5	49	464	1.4	2.8	12.1	1.1	2.6	0.6	3.2
		50	450	1.5	3.2	13.6	1.2	2.4	0.4	4.0
		51	431	1.8	2.8	12.8	1.6	2.4	0.7	4.2
		52	402	1.6	2.8	12.5	1.1	2.6	0.5	3.8
		53	426	1.6	2.6	11.0	1.0	2.8	0.5	3.4
57	7	73	377	1.6	3.2	15.4	1.0	3.0	0.6	3.6
		74	480	1.8	2.6	16.4	1.2	2.8	0.6	3.8
		75	398	1.5	2.4	11.1	1.1	2.2	0.7	3.0
		76	408	1.7	2.2	14.1	1.1	2.2	0.5	4.0
		77	488	1.7	3.1	16.1	1.0	3.0	0.6	3.4

Individual organs weights after exposure period (female rats)

Dose, mg/kg/day	Group #	Ind #	Animal weight, g	Organ weight, g					
				Heart	Lungs	Liver	Spleen	Kidneys	Thymus
0 (vehicle)	2	13	318	1.1	2.0	9.5	0.7	1.8	0.5
		14	294	0.9	2.1	7.4	1.1	1.4	0.4
		15	294	1.1	1.9	7.7	0.9	1.8	0.2
		16	268	0.9	2.0	7.2	0.7	1.6	0.3
		17	316	1.0	2.1	7.5	0.9	1.8	0.5
0.57	4	37	250	0.9	1.8	6.2	0.4	1.6	0.3
		38	248	1.0	1.4	7.4	0.6	1.6	0.3
		39	280	0.9	1.9	7.3	0.6	1.6	0.4
		40	291	1.1	1.9	6.7	0.8	1.4	0.4
		41	278	1.1	2.0	8.4	1.0	1.4	0.4
5.7	6	61	254	0.6	2.2	7.1	0.5	1.4	0.3
		62	241	0.8	2.0	6.3	0.6	1.2	0.3
		63	319	1.1	1.7	8.7	0.9	1.6	0.4
		64	289	1.1	2.7	9.1	1.2	1.6	0.4
		65	250	0.7	1.6	6.3	0.7	1.2	0.3
57	8	85	264	1.0	2.0	7.1	0.6	1.6	0.4
		86	280	1.2	1.8	7.4	0.8	1.6	0.2
		87	279	0.9	1.9	7.3	0.7	1.6	0.3
		88	267	0.9	1.8	7.0	0.7	2.0	0.2
		89	269	1.1	1.8	8.1	0.7	1.6	0.4

Individual organs weights after recovery period (male rats)

Dose, mg/kg/day	Group #	Ind #	Animal weight, g	Organ weight, g						
				Heart	Lungs	Liver	Spleen	Kidneys	Thymus	Testicles
0 (vehicle)	1	7	536	1.8	2.7	13.9	1.1	3.2	0.6	3.8
		8	449	1.6	2.4	11.8	1.1	3.0	0.4	3.8
		9	446	1.3	2.4	11.2	0.9	2.6	0.6	3.0
		10	494	1.8	2.0	13.3	1.0	2.8	0.5	3.6
		11	402	1.4	2.0	10.7	1.1	2.4	0.6	3.0
0.57	3	31	454	1.5	2.3	13.0	1.1	2.6	0.3	3.4
		32	428	1.5	2.0	10.6	0.9	2.0	0.3	3.2
		33	417	1.4	2.2	10.0	1.4	2.0	0.2	3.4
		34	426	1.6	2.2	12.2	1.0	2.6	0.5	3.2
		35	475	1.4	2.3	15.0	1.4	2.4	0.4	3.2
5.7	5	55	442	1.7	2.4	10.3	0.9	2.2	0.7	3.0
		56	427	1.5	2.2	10.7	0.8	2.0	0.8	3.4
		57	439	1.7	2.2	10.6	1.0	2.4	0.2	4.0
		58	362	1.3	2.3	8.3	0.7	2.2	0.2	3.2
		59	385	1.6	1.9	10.0	0.9	2.4	0.5	3.4
57	7	79	480	1.8	2.3	14.5	0.9	3.0	0.4	3.4
		80	450	1.9	2.5	13.3	1.0	2.6	0.5	3.6
		81	465	1.6	2.3	13.6	0.9	2.2	0.5	4.0
		82	419	1.6	2.5	10.3	0.9	2.4	0.6	3.8
		83	473	1.5	2.5	12.6	1.0	2.6	0.4	3.8

Individual organs weights after recovery period (female rats)

Substance daily dose, mg/kg	Group #	Ind. #	Animal weight, g	Organ weight, g					
				Heart	Lungs	Liver	Spleen	Kidneys	Thymus
0 (vehicle)	2	19	323	1.0	2.1	9.5	0.8	2.0	0.4
		20	270	1.1	1.8	7.1	0.7	1.6	0.2
		21	306	1.1	1.9	8.8	0.9	1.8	0.4
		22	263	1.0	2.0	7.1	0.6	1.4	0.3
		23	307	1.1	2.5	9.5	0.8	2.2	0.3
0.57	4	43	262	1.1	1.9	7.9	0.9	1.6	0.2
		44	283	1.2	1.6	7.4	0.5	1.2	0.3
		45	296	1.1	1.6	8.0	0.7	1.6	0.3
		46	292	0.9	2.0	7.9	0.6	1.8	0.3
		47	292	1.2	1.8	7.3	0.6	1.6	0.4
5.7	6	67	325	1.1	2.6	7.9	0.7	1.4	0.2
		68	329	1.2	2.4	8.4	0.7	2.0	0.4
		69	320	1.2	1.8	8.6	0.6	2.0	0.4
		70	297	1.1	1.9	7.7	0.9	1.8	0.3
		71	317	1.0	1.8	7.4	0.7	1.8	0.4
57	8	91	326	1.1	1.8	9.7	0.9	1.8	0.5
		92	281	1.2	2.2	7.4	0.7	1.6	0.4
		93	295	1.0	1.5	6.5	0.5	1.4	0.4
		95	305	1.0	1.8	6.9	0.7	2.0	0.4
		96	332	1.3	1.9	10.0	1.1	2.0	0.6