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(SFES RCT&HRB FMBA RF)

Approved:

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prof. N.R. Dyadishchev, Dr. Sci (Med)
Director of SFES RCT&HRB
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REPORT

Experimental study of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats

Study Director:

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RESPONSIBLE PERSONNEL

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SUMMARY

***Report* – 13 pages, 4 tables, 3 annexes on 11 pages**

***Key words**: pharmaceutical substance Killevir-16, embryotoxic and teratogenic effects, Wistar rats*

The study objective is experimental assessment of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats on day 1-20 of pregnancy.

Killevir-16 was perorally administered in pregnant Wistar rats once daily in 2 doses – 5.7 mg/kg and 57.0 mg/kg of body weight.

Visual examination of fetuses taken out after necropsy from euthanized rats on day 21 of pregnancy did not reveal developmental pathologies or anomalies in all experimental groups. The values of main parameters of embryonic development – body weight and cranio-caudal size – were comparable in both dose groups and in control. The values of such embryotoxicity parameters as the number of yellow bodies in testicles and the number of implants, and, consequently, preimplantation embryo mortality did not reliably differ in treated animals and in control.

Study of skeletal system development in all groups including control revealed rare cases of delay in ossification of hyoid bone and separate segments of sternum. The level and character of delay of ossification in the indicated zones did not significantly differ in dose groups and control and were within the norm for the given term of pregnancy in rats.

Study of internal organs by method of serial sections revealed single cases of hyperemia of vessels and lungs in control group and in dose group 5.7 mg/kg that is not a substance-induced pathology. No other abnormalities or developmental deficiencies were found in any of experimental and control groups.

The study conducted has not revealed the signs of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 at peroral administration in rats in doses up to 57.0 mg/kg of body weight from 1 to 20 days of pregnancy.

INTRODUCTION

The study objective is experimental assessment of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats on day 1-20 of pregnancy.

The intended clinical dose of Killevir-16 for humans - 40 mg divided into 2 doses (1 capsule of 20 mg twice daily).

1. MATERIALS AND METHODS

1.1. General conditions for conducting the studies

The study was conducted in compliance with the following regulatory documents:

- Rules for Laboratory Practice in the Russian Federation (Order of Russian Ministry of Health #708n of August 23, 2010).
- Methodical guidance on assessment of reproductive toxicity 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. 87-100.
- Methodical guidance on hygienic assessment of new pesticides. USSR Ministry of Health. All-Union Research Institute for Hygiene and Toxicology of Pesticides, Polymers, and Plastics, Kiev, 1988.
- Prenatal Developmental Toxicity Study. OECD guideline for the testing of chemicals. Proposal for updating guideline 414. Adopted: 22nd January 2001.

Test substance

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

Molecular mass: 1500

Description: amorphous powder, brown or dark brown

Decomposition temperature without melting: 400-450 °C

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

pH: 5.2

Amino-caproic acid: 3-3.5 %

Chlorides: ≤0.2%

Total ash: ≤0.5%

Hard metals: ≤0.001%

Residual vehicles:

1, 2- dichlorbenzene ≤0.032%

Nitrogen: 5.0-5.4%

Elemental analysis:

%C 69.52

%H 4.82

%N 5.20

%Cl no

Batch #54, produced December 22, 2009

Shelf life – 5 years

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

Control substance (vehicle)

1% starch gel

Prepared by pharmaceutical group, sterilized, stored at 4°C.

Animals

Species: rat (*Rattus sp.*)

Stock: Wistar

Source: Animal Nursery of SE RCBT RAMS, "Stolbovaya" branch

Handling and feeding

Animals were housed in a room separately from other species (temperature 20-22°C, relative humidity of air 55-60%, 12 h light/12 h dark regime, air exchange – 18/h) and acclimated to laboratory conditions for 7 days prior to the start of dosing. Animals were given conventional extruded diet (OOO "Laboratorkorm", Russia). The diet was given *ad libitum* through the deepenings in the steel cage cover.

Obtaining animals with definite terms of gestation

To obtain rats with definite terms of gestation, male rats were placed with females (2 females per 1 male) in the evening, and next morning vaginal smears were taken and examined under

microscope. The fact of mating was confirmed by the presence of sperm in the smears. The day when the fact of fertilization was established is considered the first day of pregnancy.

Animal grouping

Females confirmed to be pregnant were assigned to equal size experimental groups at random based on body weight. Animals were not considered for assignment if out of the ±20% range from their corresponding group mean body weight.

Animal identification

Each animal was individually enumerated with indelible ink on the animal skin near the tail root from the dorsal side. The individual numbers of animals are indicated on the cage label.

1.2. Conditions for conducting key phases of the study

Based on smear analysis, animals were assigned to two experimental and one control groups (20 animals per group).

Killevir-16 and vehicle were daily perorally administered in females from the 1^{st} to the 20^{th} day of pregnancy in 2 doses - 5.7 mg/kg and 57.0 mg/kg of body weight. Control animals received 1% starch gel in the volume equal to the maximal dose of test substance calculated per 1 kg of body weight.

The animals were weighed on day 1, 5, 12, 16, and 20 of pregnancy.

On day 21 after mating female rats were euthanized to detect antenatal developmental deficiencies and abnormalities. From rats confirmed pregnant the uterus with gonads were taken out to determine:

- number of yellow bodies in gonads;
- total number of fetuses;
- total number of implants;
- number of live and dead fetuses;
- number of early and late resorptions;
- mass of each fetus;
- cranio-caudal size of each fetus;
- sex of each fetus;
- visual developmental deviations and abnormalities in fetuses.

After visual inspection about a half of fetuses from each litter were fixed in 96^{o} ethanol and used for examination of skeletal system (staining with alizarin by Dawson method modified by RIEM AMS, USSR). Second half of fetuses were placed in Bouin liquid to examine internal organs by Wilson method modified by RIEM AMS.

Uteruses of animals not having fetuses or distinct implants were taken out and stained with ammonium sulfide by Salewski method for visualization of possible implants (early complete resorptions).

During statistical processing a unit of observation was a litter, i.e., the outcome obtained at examination of one female. Data were given as mean values with standard deviation. The differences were determined using Statistica 8 (StatSoft) program and Mann-Whitney U-test at confidence probability 95%. The rats not confirmed pregnant were not taken for calculations.

The details are given in Study protocol (Annex 1).

2. STUDY RESULTS

During the whole period of pregnancy no signs of pathology were noted in experimental and control rats. Administration of Killevir -16 did not cause changes in general physical state of animals. Consumption of feed and water and behavioral reactions were within the norm.

Dynamics of body weights was comparable in dose groups and control (Table 1).

Calculations of mean body weights were made not considering the weights of rats not confirmed pregnant.

Table 1

Body weights of rats during pregnancy at peroral administration of Killevir-16 (embryotoxicity study)

Dose, mg/kg	Number of pregnant rats	Body weights of rats during the experiment, g				
		day 1	day 5	day 12	day 16	day 20
0	17	216.1±13.7	232.1±13.9	251.2±14.0	273.6±14.2	307.7±14.7
5.7	16	211.9±14.3	228.4±14.2	247.9±13.5	269.7±15.9	302.6±15.6
57.0	17	216.2±13.6	229.7±14.1	248.8±14.9	273.3±17.2	309.2±21.6

Post-mortem examination on day 21 after mating revealed the absence of pregnancy in 3 rats of 20 in control group and in dose group 57.0 mg/kg; in dose group 5.7 mg/kg - in 4 rats of 20. Staining by Salewsky did not reveal any implants. Fertilization index in control group was 85%, in treated groups - 80% (5.7 mg/kg) and 85% (57.0 mg/kg).

Results of Killevir-16 embryotoxicity evaluation are given in Table 2.

Visual inspection of fetuses did not reveal any developmental abnormalities or pathologies in rats of experimental groups. All fetuses had normal anatomic structure. The

number of viable fetuses per female in treated groups did not differ from control, no dead fetuses were found.

Values of body weights and cranio-caudal sizes did not differ between groups. It follows from the data presented that such parameters as the number of yellow bodies in gonads and the number of implants, and, consequently, the level of preimplantation embryo mortality did not statistically reliably differ between experimental groups and control. No statistically reliable differences are noted between experimental and control groups in the number of resorbed fetuses. The values of postimplantation mortality did not reliably differ from control.

Table 2

Embryotoxicity parameters of Killevir-16 at peroral administration in rats

Test parameter	Dose (mg/kg)		
	DMSO	5.7	57.0
Number of pregnant rats	17	16	17
number of embryos	8.7±2.7	9.0±2.4	9.3±1.9
Results of post-mortem examination (for one female)			
Yellow bodies	10.2±2.2	10.5±1.4	10.6*1.3
Implantation sites	9.2±2.6	9.4±2.0	9.9±1.4
Live fetuses	8.7±2.7	9.0±2.4	9.3±1.9
Resorbed fetuses	0.5±0.2	0.4±0.2	0.5±0.2
Pre-implantation mortality, %	11.3±3.4	11Л±3.0	7.1±2.1
Post-implantation mortality, %	5.9±2.7	4.9±2.5	5.7±2.3
Body weight, g	3.8±0.2	3.7±0.2	3.6±0.3
Craniocaudal size, cm	3.9±0.1	4.0±0.2	3.9±0.2
Number of females in a litter	4.3±1.6	4.6±1.6	4.9±1.5
Number of males in a litter	4.7±1.2	4.4±1.4	4.5±1.3
Results of visual inspection			
Number of examined fetuses	148	144	159
Number of fetuses with developmental anomalies: total/%	0/0	0/0	0/0
Examination of skeletal system of fetuses			
Number of examined fetuses	74	72	80
Number of fetuses with developmental anomalies: total/%	5/6.7	6/8.3	6/7.5
Examination of internal organs of fetuses			
Number of examined fetuses	74	72	79
Number of fetuses with developmental anomalies: total/%	1/1.3	2/2.8	0/0

Fetuses of all experimental groups had normal anatomic structure. Study of skeletal system development in all groups including control revealed rare cases of delay in ossification of hyoid bone and separate segments of sternum (Table 3). The level of delay of ossification in the

indicated zones did not significantly differ and were within the norm for the given term of pregnancy in rats.

Table 3

Defects in fetal skeletal system in late antenatal period

Dose, mg/kg	Number of examined fetuses	No ossification (abs / %)		
		hyoid bone	5th sternal segment	6th sternal segment
0	74	3/4.0	2/2.7	-
5.7	72	2/2.8	3/4.2	1/1.4
57.0	80	3/3.7	1/1.3	2/2.5

Examination of internal organs by method of serial sections revealed single not substance-induced deficiencies (Table 4).

Table 4

Developmental anomalies in fetal internal organs in late antenatal period

Dose, mg/kg	Number of examined fetuses	Number of fetuses with developmental anomalies: abs/%
		hyperemia of vessels in lungs
0	74	1/1.3
5.7	72	2/2.8
57.0	79	-

CONCLUSION

The study objective is experimental assessment of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats on day 1-20 of pregnancy.

During the whole period of pregnancy no signs of pathology were noted in experimental and control groups. Administration of Killevir-16 did not affect general physical state of animals. Consumption of feed and water and behavioral reactions were within the norm.

Dynamics of body weights was comparable in dose groups and control.

Visual examination of fetuses taken out from euthanized rats did not reveal developmental pathologies or anomalies in all experimental groups.

The values of such embryotoxicity parameters as the number of yellow bodies in testicles and the number of implants, and, consequently, preimplantation embryo mortality did not reliably differ in treated animals and in control. No statistically reliable differences are noted between experimental and control groups in the number of resorbed fetuses. The values of postimplantation mortality did not differ from control.

The values of main parameters of embryonic development – body weight and cranio-caudal size – were comparable in both dose groups and in control.

The level of delay in ossification of hyoid bone and separate segments of sternum did not significantly differ in dose groups and in control; study of internal organs by method of serial sections revealed single cases of pathologies not induced by the substance.

The study conducted has not revealed any signs of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 at peroral administration in rats in doses up to 57.0 mg/kg of body weight from 1 to 20 days of pregnancy.

ANNEX 1

STUDY PROTOCOL
Experimental study of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats

Study code: KO411

Test substance code: 0611

Sponsor: ZAO Intelpharm
Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia

Sponsor Representative:

Lev D. Rasnetsov,
Director of ZAO "Intelpharm"
Tel/fax: 8 (8314)30-20-32

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:

Elena V. Kovaleva, senior researcher, Division for Pathomorphology and Reproductive Toxicology, RCT&HRB, tel/fax: (4967) 39-97-38

1. Key dates

Planned study initiation date: May, 2011
Planned study completion date: September, 2011
Planned experimental starting date: June, 2011
Planned experimental completion date: August, 2011

2. Responsible personnel:

- Animal grouping - Elena V. Kovaleva, senior researcher,

Division for Pathomorphology and Reproductive Toxicology

- Administration of the preparation and clinical observation - Elena V. Kovaleva
- Necropsy and visual analysis - Elena V. Kovaleva
- Analysis of internal organs – Elena V. Kovaleva
- Skeletal system analysis - Elena V. Kovaleva
- Quality Assurance – Vladimir V. Kapranov, Head of QA Laboratory
- Preparation of doses – Larisa A. Eremenko, senior researcher, Division for Analytical Chemistry and Radiobiology.

3. Study Objective

Experimental study of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats

4. Methodical guidances

- Methodical guidance on assessment of reproductive toxicity 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. 87-100;
- Methodical guidance on hygienic assessment of new pesticides. Kiev, 1988, approved by USSR Ministry of Health 13.03.1987, #4263-87;
- Prenatal Developmental Toxicity Study. OECD guideline for the testing of chemicals. Proposal for updating guideline 414. Adopted: 22nd January 2001;
- Rules for Laboratory Practice in the Russian Federation (Order of Russian Ministry of Health #708n of August 23, 2010).

All procedures in the study will be compliant with the given written protocol and after its approval – with standard operating procedures (SOPs).

4.2. Humane treatment and use of test animals

Based on the data from this research protocol a veterinary protocol will be written and submitted to the Bioethics Committee of the RCT&HRB for expert assessment and approval. Procurement of animals will be performed after approval of the veterinary protocol.
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.

5. Justification of test species and test substance delivery conditions

The study will be conducted on Wistar rats as species commonly used for toxicity evaluations. The number of animals in experimental and control groups – at least 20 females with known term of gestation. For administration of substance peroral route will be used as the intended route of administration in humans.

Group Assignment and Dose Levels

Group #	Number of animals	Ind. #	Dosing conditions				Administration regime
			Substance	Dose (mg/kg)	Concentration (mg/ml)	Volume (ml) per 200 g of body weight	
1	≥20*	1-20	1% starch gel (vehicle)	0	0	0.1	once daily perorally
2	≥20	21-40	0611	5.7	1.14	0.1	
3	≥20	41-60	0611	57.0	11.4	0.1	

*Note: actual number of rats in each group at equal assignment will depend on results of controlled mating

6. Test material

6.1. **Test substance –** pharmaceutical substance Killevir-16

Prepared by pharmaceutical group, sterilized, stored at 4°C.

Manufacturer: ZAO Intelpharm

Molecular formula: $C_{60}(NH(CH2)_5COOH)_n$, where n=4-6
Molecular mass: 1500
Description: amorphous powder, brown or dark brown
Decomposition temperature without melting: 400-450 °C
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

pH: 5.2
Amino-caproic acid: 3-3.5 %
Chlorides: ≤0.2%
Total ash: ≤0.5%
Hard metals: ≤0.001%
Residual vehicles:
1, 2- dichlorbenzene ≤0.032%
Nitrogen: 5.0-5.4%
Elemental analysis:
%C 69.52
%H 4.82
%N 5.20
%Cl no
Batch #54, produced December 22, 2009
Shelf life – 5 years
Storage conditions: dry, light-protected, temperature ≤30°C

6.2. Control substance (vehicle)

1% starch gel, prepared by pharmaceutical group, sterilized, stored at 4°C.

6.3. Characteristics of test substance

The pharmaceutical substance is produced by the Sponsor in accordance with the pharmacopoeia article of the organization. Sponsor is responsible for the composition and stability characteristics of the test substance.

6.4. Test substance accountability

The test substance will be issued upon submission of the request and registration in the log. Daily consumption of the preparation must be equal to the total volume of single daily doses calculated based on body weight. Allowed excess - 20%.

6.5. Substance specimens

Pharmaceutical group maintains specimens of each lot of test and control substances in allocated room under proper storage conditions. Person in charge - Eremenko L.A.

6.6. Safety assurance when handling test substance

No special measures are required. In case of the substance getting on skin and mucous membranes rinse with warm water and soap is recommended.

6.7. Disposal of test substance

Upon completion of the testing unused residues of the test material in crystals can be returned to the Sponsor upon his request. Residues of solutions will be discharged in sewage system.

6.8. Preparation of test substance for administering in animals

Test and control substance solutions are administered in animals in doses indicated in Item 5 of the Study Protocol. Single doses for each group are calculated based on individual body weight and corrected after every weighing (allowed excess - 20%). Test solutions are prepared by pharmaceutical group prior to administration in aseptic conditions in volumes indicated by the researcher in "Requirements to formulations for administering in animals…'. Solutions are poured in labeled vials of requested volumes.

Dosing calculations are raw data subjected to archiving.

7. Animals

Species:	Rat (*Rattus sp.)*
Strain:	Wistar
Source:	Animal Nursery at SE RCBMT RAMS, "Stolbovaya" branch
Weight before mating	males - 220-250 g, females - 180-220 g
Number of males:	30
Number of females:	60

To obtain rats with definite terms of gestation, male rats are placed with females (2 females per 1 male) in the evening, and next morning vaginal smears are taken and examined *ex tempore* under the microscope. The fact of mating is confirmed by the presence of sperm in the smear. The day when the fact of mating is established is considered the first day of pregnancy.

7.1. Feeding and handling conditions

Animals will be housed in a room separately from other species in compliance with the rules and conditions approved by the Ministry of Health of the Russian Federation on 06.07.73 "Construction, equipping and maintenance of experimental biological clinics (vivariums)" in compliance with "Guide for Care and Use of Laboratory Animals" (ILAR publication, 1996. National Academy Press, USA). 3-4 days prior to delivery each of the pregnant rats will be placed in individual cages. All routine procedures on handling animals within the study will be performed and documented in accordance with the vivarium Standard operating procedures (SOPs).

7.1.1. Cages

Rats will be kept in polycarbonate Type-4 cages on stain steel racks (LabProdex, USA), 5-6 animals in each cage. The cages have steel lattice covers with deepenings, steel dispensers for feed and water, and steel label holders.

7.1.2. Bedding

For bedding mixed wood cuts will be used. Thickness of bedding layer in the cage – 15-20 mm. Sanitary-bacteriological laboratory performs periodical examination of the bedding for microbiological contamination. The data obtained are documented and stored at the laboratory.

7.1.3. Diet

For feeding animals conventional extruded diet (OOO “Laboratorkorm”) will be used. The diet will be given *ad libitum* through the deepenings in the cage cover. Sanitary-bacteriological laboratory performs periodical examination of diet for microbiological contamination. The data obtained will be documented and stored at the laboratory.

7.1.4. Water

Animals will be given water compliant with GOST 28.74–73 "Drinking water". Filtered tap water will be given in conventional sterilized vials with steel covers-spouts.
No contaminants are known to be present in the water that would interfere with the results of the study. Sanitary-bacteriological laboratory performs periodical examination of water for microbiological contamination. The data obtained will be documented and stored at the laboratory.

7.1.5. Environmental conditions

Animals will be kept under controlled environmental conditions (tº 18-22°C, relative humidity of air 30-70%). Temperature and humidity will be monitored in each experimental room and documented in data sheets kept in the room. A 12-hour light/dark cycle will be maintained; the airflow in animal rooms will be 18 air changes per hour.

7.1.6. Quarantine and adaptation

All animals will be kept in groups in cages and acclimated to laboratory conditions for a minimum of 7 days prior to the start of dosing. During that period, the health status of the animals will be visually evaluated. Animals with deviations detected during the inspection will be kept separately until a decision is taken by the Study Director and veterinarian on their use.

7.1.7. Animal grouping

Animals confirmed to be pregnant will be assigned to equal size experimental groups at random based on weight (item 5 of the Protocol). No animal will be considered for assignment if out of the ±20% range from their corresponding group mean body weight.

7.1.8. Animal identification

After the 1st day of pregnancy is determined (confirmed mating) each animal will be individually enumerated according to the Table (item 5 of the Protocol) with indelible ink on the animal skin near the tail root from the dorsal side. The individual numbers of animals are indicated on the cage label.

7.1.9. Spare animals

The animals remained after grouping and males placed with females for mating after group assignment will be taken to the other room and used for the other purposes.

8. Testing methods

8.1. Lifetime manipulations with animals

8.1.1. Administration of substances

Route	peroral
Regime	once daily
Duration	during day 1-20 of pregnancy
Dose correction	after every weighing on day 5, 12, and 16 of pregnancy

8.1.2. Assessment of animal state

Animals will be observed for viability once daily prior to administration of the substance in the morning.

8.1.3. Clinical observations

If no external signs of animal ill-being are observed detailed clinical examination of each animal will be performed weekly. In case any animal condition deviations are observed clinical examination will be performed daily; if rapid development of adverse signs is observed clinical examination will be performed not less than two times a day.
Any raw data of clinical observation will be documented.

8.1.4. Body weight

Animals will be weighed on day 1, 5, 12, 16, and 20 of pregnancy.

8.1.5. Food consumption

Deviations in food and water consumption in separate cages will be noted. In case of evident decrease in consumption a daily volume of consumption is determined by weighing the feed/water introduced and left after 24 hours.

8.2. 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 Study director after consulting with veterinarian.

8.3. Euthanasia

Pregnant rats are euthanized on day 21 of pregnancy by CO_2 inhalation followed by cervical dislocation.

8.4. Collection of data for the experiment

8.4.1. Evaluation of parameters of embryotoxic and teratogenic effects

From rats confirmed pregnant the uteruses with gonads will be taken out to determine:

- number of yellow bodies in gonads;
- total number of fetuses;
- total number of implants;
- number of live and dead fetuses;
- number of early and late resorptions;
- number of each sex in a litter;
- mass of each fetus;
- cranio-caudal size of each fetus;
- sex of each fetus;
- possible visual developmental deviations and abnormalities in fetuses.

After visual inspection, about a half of fetuses from each litter will be placed in 96^{o} ethanol, second half of fetuses - in Bouin liquid.

Uteruses of animals not having fetuses or distinct implants will be taken out and stained with ammonium sulfide by Salewski method for visualization of possible implants (early complete resorptions).

8.4.2. Study of skeletal system and internal organs

In fetuses fixed in ethanol examination of skeletal system by Dawson method modified by RIEM AMS, USSR is performed in compliance with SOP.04-067-1. In fetuses fixed in Bouin liquid internal organs are examined by Wilson method modified by RIEM AMS (SOP.04-068-1).

9. Statistical analysis of data

For all data the method of parametric or nonparametric analysis that allows to compare two independent samples of pair observations and determine the level of reliability of the detected differences (confidence probability 95%) will be applied.

10. Protocol amendments

Changes in the approved protocol will be discussed by the Study Director and Sponsor, documented, signed by the Study Director and Sponsor representative and dated. The personnel is informed about the approved amendments to the protocol and its effective date. The changes are attached to the protocol and report.

11. Deviations

Any deviations will be documented in the data sheets with assessment of their affect on the study. The list of deviations that can affect on the study is attached to the report.

12. Report

The final report will be submitted to the Sponsor after completion of the study and analysis of the obtained material. The final report presents full data on the study according to the protocol. The study protocol, amendments and significant deviations will be included in the annex to the report.

13. Documentation and archive

All study-related data and documents will be filed by persons responsible for separate stages of the study. 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:

- Protocol, amendments, and deviations
- Test article and formulation records
- Animal procurement records including existing prior health records
- Group assignment
- Records on cageside observation of animals and physical examination
- Body weight data
- Necropsy data
- Records on assessment of embryotoxic and teratogenic parameters
- Letters or any written communication concerning the study
- Final report

ANNEX 2

INDIVIDUAL BODY WEIGHTS

Individual body weights of rats at peroral administration of Killevir-16

Dose, mg/kg	Animal #	Body weights of rats during the experiment, g				
		day 1	day 5	day 12	day 16	day 20
0	1	213	225	244	258	290
	2	221	239	262	279	311
	4	209	218	234	266	295
	5	218	232	251	275	306
	6	228	245	266	287	331
	7	210	222	240	263	299
	9	221	236	255	279	313
	10	188	210	234	260	296
	11	216	234	252	281	319
	12	233	252	268	289	325
	13	209	221	240	259	294
	14	225	244	263	285	316
	16	238	256	275	299	338
	17	202	217	236	257	296
	18	219	236	255	277	302
	19	192	213	230	249	288
	20	232	246	265	288	312
5.7	22	203	221	242	259	294
	23	228	242	260	282	320
	24	215	238	257	279	303
	25	187	202	222	240	284
	27	208	225	246	261	296
	28	214	233	252	274	303
	29	232	251	270	296	331
	30	201	220	242	262	297
	31	192	208	230	251	289
	32	212	229	251	274	306
	34	195	210	229	249	282
	35	218	237	246	268	291
	36	235	249	267	290	328
	38	207	222	241	264	291
	39	216	230	252	276	302
	40	228	237	260	290	325
57.0	41	211	230	249	274	303
	42	207	218	236	260	296
	43	225	238	259	285	320
	45	219	230	247	280	311
	46	214	223	240	262	289
	47	230	244	262	284	312
	48	203	215	233	254	285
	49	220	238	260	281	316
	51	216	230	247	282	329
	52	199	215	235	256	296
	53	227	242	261	294	341
	54	188	199	216	238	269
	55	202	215	237	259	290
	56	236	250	274	302	346
	57	214	227	245	259	293
	58	229	243	263	286	326
	59	236	248	265	290	334

ANNEX 3

INDIVIDUAL EMBRYOTOXICITY PARAMETERS

Individual embryotoxicity parameters at peroral administration of Killevir-16 in rats

Dose, mg/kg	Ind.#.	Main gestation parameters			
		Yellow bodies	Implants	Live fetuses	Resorptions
0	1	11	8	6	2
	2	10	9	9	0
	4	4	2	2	0
	5	12	11	10	1
	6	12	12	12	0
	7	9	7	7	0
	9	12	10	10	0
	10	11	8	8	0
	11	12	12	11	1
	12	11	11	11	0
	13	8	7	7	0
	14	9	9	9	0
	16	13	13	13	0
	17	11	10	8	2
	18	10	10	10	0
	19	8	8	5	3
	20	11	10	10	0
5.7	22	9	7	7	0
	23	11	10	10	0
	24	10	10	9	1
	25	9	7	5	2
	27	11	7	7	0
	28	10	9	9	0
	29	12	12	12	0
	30	8	8	8	0
	31	12	11	11	0
	32	10	10	10	0
	34	9	7	5	2
	35	10	8	7	1
	36	11	11	11	0
	38	13	13	13	0
	39	11	8	8	0
	40	12	12	12	0
57.0	41	10	10	9	1
	42	9	8	8	0
	43	11	11	11	0
	45	10	10	10	0
	46	9	9	7	2

47	12	10	10	0
48	10	9	6	3
49	11	11	10	1
51	13	11	11	0
52	11	8	8	0
53	12	12	12	0
54	10	10	9	1
55	9	8	7	1
56	13	13	13	0
57	10	8	8	0
58	11	10	10	0
59	10	10	10	0

ANNEX 4

Preclinical Study Quality Assurance Report

Test substance code: 0611
Study code: K0411

Experimental study of embryotoxic and teratogenic effects of pharmaceutical substance Killevir-16 in antenatal period at peroral administration in rats

Audits conducted:

Study phases	Date	Date of report submission to Study Director and to the organization management
Audit of Study protocol	18.05.2011	18.05.2011
Inspection of active phase:		
Administration of substance	28.06.2011	28.06.2011
Weighing of animals	09.07.2011	09.07.2011
Necropsy	19.07.2011	19.07.2011
Audit of final report: 27.09.2011		

(Signature)

V.V. Kapranov
Head of QA Laboratory
RCT&HRB

28.09.2011

Bound and sealed
25 pages

(Signature)

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