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FEDERAL MEDICO-BIOLOGICAL AGENCY OF RUSSIA
(SFES RCT&HRB FMBA RF)

Approved:

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prof. N.R.Dyadishchev, Dr. Sci(Med)
Director of SFES RCT&HRB
"____"_____2011

Report

Study of acute toxicity and local irritating effects of new pharmaceutical substance Killevir-16 at peroral administration in laboratory animals

(Contract # ZT 22/2011)

Study Director: Nikolay M. Onatsky, PhD (Biol)

Serpukhov 2011

RESPONSIBLE PERSONNEL

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SUMMARY

Report: 10 pages, 2 tables, 2 annexes

Key words: *Killevir-16, substance, acute 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 single 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.

Acute toxicity of the test substance was studied on outbred white mice and rats after single intragastrical administration of the test substance in doses 200, 1000, and 5000 mg/kg. Control animals were dosed with a vehicle - 1% starch gel - in the volumes equal to maximal doses of the test substance.

The substance in tested doses did not cause animal intoxication or death. LD_{50} of Killevir-16 for mice and rats is greater than maximal tested dose – 5000 mg/kg; so it is more than 8700 times higher than a single therapeutic dose (0.57 mg/kg) for humans. No species- or sex-dependent differences in sensitivity to the test substance administered in up to 8700fold equitherapeutic doses are revealed. The substance does not have local irritating effect on mucous membranes of gastrointestinal tract at single exposure.

Therefore, Killevir-16 has a high therapeutic index and can not cause acute poisoning in case of accidental overdose.

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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 single 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 studies were conducted in accordance 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.

MATERIALS AND METHODS

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

Test substance:

1. **Molecular formula**: $C_{60}(NH(CH2)_5COOH)_n$, 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 white outbred mice and 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 complience 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 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 they were kept at room temperature (no longer than 3 hours).

Test substance was intragastrically administered in mice and rats in doses 200, 1000, 5000 mg/kg. Maximal test doses are limited by the substance ability to form a homogeneous substance in starch gel and maximal allowed volumes for i/g administration in rats.

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 taking into account individual body weight of animals. Each dose was tested on 5 per sex animals.

During the observation period (14 days following treatment) the health status of the animals was evaluated by motor activity, consumption of feed and water, state of hair and mucous membranes, and body weight.

Upon completion of the observation period animals treated with the test substance in dose 5000 mg/kg and control substance (vehicle) were euthanized by CO_2 inhalation. Post-mortem examination was performed within 1 hour after euthanasia. Morphological examination of internal organs was conducted visually during necropsy.

Statistical treatment of the obtained results was performed by methods of variation statistics using Student’s criterion.

STUDY RESULTS

No clinical symptoms of poisoning were noted at all tested doses. In the period of observation no deaths were recorded; there were no differences in health status of animals in treated and control groups. Animals were eating the diet willingly, uniformly gained body weight; no statistically significant differences in mean group values of body weight were noted between test and control animal (Tables 1, 2).

Table 1

Body weights of mice

Substance dose, mg/kg	Animal body weight, g (M±SD)		
	Day 0	Day 7	Day 14
MALES			
0 (vehicle)	25.2±1.4	29.1±2.5	31.5±2.4
200	25.4±2.6	29.0±1.1	31.3±1.4
1000	25.3±1.3	29.6±0.6	32.5±0.9
5000	25.4±2.9	30.2±1.0	32.3±1.0
FEMALES			
0 (vehicle)	22.4±1.5	24.8±1.1	25.9±1.5
200	22.7±1.1	23.9±1.7	24.2±2.0
1000	22.2±1.1	24.2±2.6	25.8±2.8
5000	22.8±1.4	25.2±1.8	26.5±2.5

Table 2

Body weights of rats

Substance dose, mg/kg	Animal body weight, g (M±SD)		
	Day 0	Day 7	Day 14
MALES			
0 (vehicle)	182±13.5	233±18.5	268±20.7
200	181±12.8	229±24.8	260±30.0
1000	181±5.5	226±14.7	260±14.6
5000	183±9.5	238±7.6	262±16.2
FEMALES			

0 (vehicle)	168±6.7	202±18.3	222±17.9
200	170±13.7	198±13.4	214±9.4
1000	169±10.1	201±14.8	218±18.0
5000	168±14.3	194±11.6	217±11.7

It was established that LD_{50} of the test substance at intragastrical administration for mice and rats regardless the sex is greater than maximal tested dose 5000 mg/kg.

Post-mortem examination of mice and rats was conducted after 14 days following single intragastrical administration of the test substance. Since there were no animal deaths in groups regardless the dose administered, only mice and rats from maximal dose group 500 mg/kg and control animals were assigned to necropsy. In each experimental and control group 5/sex animals were examined.

At visual inspection of mice and rats of treated and control groups the picture was as the same: hair was smooth and shining, skin elastic, movable, hypodermic cellulose moderate, visible mucous membranes pale, without ulcerations and foreign inclusions, pathological discharge from natural body orifices were not observed.

Post-mortem examination has not established any differences between treated mice and rats and control groups. Organs located in thoracic and abdominal cavities had normal anatomical position and macrostructure; no abnormalities were found. In the site of test substance administration– stomach – no signs of damage were detected.

Therefore, post-mortem examination conducted has not revealed any signs of damaging effect of test substance at single intragastrical administration in mice and rats in doses up to 5000 mg/kg.

CONCLUSION

Acute toxicity of pharmaceutical substance Killevir-16 was studied on outbred mice and rats at single intragastrical administration in doses 200, 1000, 5000 mg/kg. Control animals received a vehicle – 1% starch gel in the volumes equal to maximal doses of the test substance.

The substance in tested doses did not cause animal intoxication or death. LD_{50} of Killevir-16 for mice and rats is greater than maximal tested dose – 5000 mg/kg, so it is more than 8700 times higher than a single therapeutic dose (0.57 mg/kg) for humans. No species- or sex-dependent differences in sensitivity to the test substance in up to 8700fold equitherapeutic doses are revealed. The substance does not have local irritating effect on mucous membranes of gastrointestinal tract at single exposure.

Therefore, KILLEVIR-16 has a high therapeutic index and can not cause acute poisoning in case of accidental overdose.

ANNEX 1

STUDY PROTOCOL

Study of acute toxicity of pharmaceutical substance **Killevir-16** at intragastrical administration in mice and rats

Study code: J0211

Substance code: 0611

Sponsor: ZAO "Intelpharm"
Closed Corporation "Intelpharm" (ZAO "Intelpharm").
Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia
Tel/fax: 8 (8314)30-20-32

Sponsor 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
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Study Director:
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1. Key dates

Planned animal receipt date: 05.04.2011
Planned treatment initiation date: 19.04.2011
Planned necropsy date: 03.05.2011
Planned draft report submission: 03.06.2011

2. Key Personnel:

- Preparation of substance doses – Eremenko L.A., Senior Researcher, Division for Analytical Chemistry and Radiobiology
- Treatment and clinical observation of animals – Blokhin V.A., Senior Researcher, Division for General Toxicology
- Histopathological examination – Rybalkin S.P., Deputy Director in Scientific Affairs, Head of Division for Pathomorphology and Reproductive Toxicology
- Quality Assurance – Kapranov V.V., Head of QA Laboratory

3. Study Objective

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

4. 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.

4.1. Quality assurance

QA Laboratory will check compliance of the key phases with procedures of the approved study protocol, determine reliability of the data obtained and appropriateness of the documentation submitted.

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.
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 mice and rats as species commonly used for nonclinical toxicity evaluations.

The number of animals in the study will be sufficient for registration of the observed effects.
The study design, test substance, doses and route of administration are agreed with the Sponsor.
Maximal test doses are limited by the substance ability to form a homogeneous substance in starch gel and maximal allowed volumes for i/g administration in mice and rats.
Test and control substances in fixed doses will be administered in animals once intragastrically in accordance with the regimens presented in Tables 1 and 2.

Table 1

Study design (mice)

Group #	sex	Number of animals	Animal #	Substance	Dose of active substance (mg/kg)	Concentration of active substance (mg/ml)	Administered volume (ml per animal, mass 20 g)
1	m	5	1-5	Vehicle	0	0	1.0
2	f	5	6-10	Vehicle	0	0	1.0
3	m	5	11-15	0611	200	4	1.0
4	f	5	16-20	0611	200	4	1.0
5	m	5	21-25	0611	1000	20	1.0
6	f	5	26-30	0611	1000	20	1.0
7	m	5	31-35	0611	5000	100	1.0
8	f	5	36-40	0611	5000	100	1.0

m – male, f – female

Table 2

Study design (rats)

Group #	sex	Number of animals	Animal #	Substance	Dose of active substance (mg/kg)	Concentration of active substance (mg/ml)	Administered volume (ml per animal, mass 200 g)
1	m	5	1-5	Vehicle	0	0	5
2	f	5	6-10	Vehicle	0	0	5
3	m	5	11-15	1509	200	8	5
4	f	5	16-20	1509	200	8	5
5	m	5	21-25	1509	1000	40	5
6	f	5	26-30	1509	1000	40	5
7	m	5	31-35	1509	5000	200	5
8	f	5	36-40	1509	5000	200	5

m – male, f – female

6. Test material

6.1. Test substance

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

6.2. Control substance

Name	1% starch gel
Code	vehicle

6.3. 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.

6.4. Substance specimens

Pharmaceutical group maintains specimens of each lot of test and control substances in allocated room under proper storage conditions.

6.5. Safety assurance when handling test substance

Any manipulations with substances will comply with standards and rules for safe work with pharmaceuticals.

6.6. Disposal of test substance

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

6.7. Preparation of test substance for administering in animals

Test substance suspensions are prepared by pharmaceutical group in aceptic conditions, poured in labeled vials and stored at room temperature no longer than 3 hours before treatment.
Test substance for administring in animals will be prepared as a suspension in 1% starch gel.

6.8. Stability

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

7. Test animals

Species	mouse
Strain	outbred
Source	Animal Nursery at SE RCBMT RAMS, “Andreevka” branch
Mass before treatment	17-25 g
Number of males	20 + 2 reserve
Number of females	20 + 2 reserve

Species	rat
Strain	outbred
Source	Animal Nursery at SE RCBMT RAMS, “Stolbovaya” branch
Mass before the treatment	170-250 g
Number of males	20 + 2 reserve
Number of females	20 + 2 reserve

7.1. Feeding and handling conditions

Animals will be kept in conventional conditions in compliance with the rules approved by Ministry of Health of the Russian Federation on 06.07.73 on construction, equipping and maitenance of experimental biological clinics (vivariums).
Animals will be fed *ad libitum* by extruded feed PK-120-1 prepared in accordance with GOST P 50258-92 and drink water in accordance with GOST "Drinking water" 2874–82.

7.2. Quarantine and adaptation

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.

7.3. Animal grouping

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

7.4. Animal identification

Each animal will be individually enumerated according to the Table 1 (item 5 of the Protocol). The number of testing, number of experimental group and individual numbers of animals are indicated on the cage label.

7.5. Spare animals

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

8. Testing methods

8.1. Lifetime manipulations with animals

8.1.1. Administration of substances

Test and control substances will be injected in fixed doses once intragastrically using a disposal syringe and a steel probe. The volume of administered dose will be calculated individually for each animal based on body weight.

8.1.2. Assessment of animal state, mortality

Registration of physical state and deaths of animals will be performed once a day before midday.

8.1.3. Clinical observations

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.

8.1.4. Body weight

Animals will be weighed weekly.

8.1.5. Food consumption

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

8.1.6. Food deprivation

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

8.2. Pathoanatomy and histology

8.2.1. 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.

8.2.2. Euthanasia

CO_2 inhalation will be used as a means of euthanasia.

8.2.3. Necropsy

Necropsy will be conducted:

- In all animals found dead within 1-2 days following treatment (macroscopic study of internal organs);

- In case of delayed death on day 3-14 after exposure (macro- and microscopic examination of internal organs);
- Scheduled necropsy on day 15 following treatment in all animals treated by vehicle or maximal dose and survived (macroscopic examination of internal organs and microscopic examination of modified organs' tissues).

8.2.4. Collection of specimens for histological assessment

Specimens of organs and tissues will be taken for histological assessment only in case macroscpic changes are detected during necropsy. Specimens of modified organs and tissues will be placed in 10% buffered formalin solution.

8.2.5. Histological preparations for microscopy

The manner of specimens' preparation for histological assessment and method of staining is chosen to the discretion of the pathologist depending on affected tissue and the character of changes.

8.2.6. Examination of histological preparations

Histological preparations of modified tissues will be studied by method of light microscopy.

9. Statistical analysis of data

For all quantitative data obtained in the studies descriptive statistics will be applied. The differences are evaluated at confidence probability 95%.

10. Protocol amendments

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

11. Deviations

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

12. Report

A draft report will be submitted to the Sponsor as soon as possible after the last necropsy. The final report presents full data on the study according to the protocol. Upon completion the Sponsor 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.

13. Documentation and archive

All study-related data and documents are filed by the Study Director. Upon signing the final report all relevant documentation, samples, glasses and specimens of tissues in blocks are handed to the RCT&HRB archive. All the study data and materials are stored in the RCT&HRB archive for 2 years.

After 2 years of storage archive materials upon agreement with the Sponsor are handed to the Sponsor or destroyed after notifying the Sponsor.

Annex 2

Individual body weights, g

Male mice

Substance dose (mg/kg)	Group #	Animal #	Day 0	Day 7	Day 14
0	1	1	24.0	27.4	29.8
		2	26.0	31.3	34.3
		3	25.2	28.1	31.7
		4	23.9	26.4	28.5
		5	27.1	32.2	33.4
200	3	11	28.3	30.4	31.7
		12	27.7	27.4	29.2
		13	23.1	29.3	32.4
		14	25.1	29.2	32.5
		15	22.6	28.8	30.7
1000	5	21	26.0	29.3	32.4
		22	26.8	30.4	33.3
		23	23.4	29.0	32.1
		24	25.5	29.2	31.3
		25	24.6	30.1	33.4
5000	7	31	28.3	31.2	33.3
		32	22.8	29.3	31.1
		33	26.4	30.4	31.6
		34	22.0	29.1	32.2
		35	27.7	31.2	33.4

Female mice

Substance dose (mg/kg)	Group #	Animal #	Day 0	Day 7	Day 14
0	2	6	22.1	25.2	27.1
		7	24.8	26.0	26.6
		8	22.4	25.5	27.2
		9	22.1	24.3	24.8
		10	20.6	23.1	23.9
200	4	16	23.3	24.4	25.3
		17	21.0	20.9	20.9
		18	22.9	25.1	23.7
		19	23.9	24.8	25.4
		20	22.6	24.2	25.8
1000	6	26	22.4	23.3	25.1
		27	21.5	28.0	30.2
		28	24.0	24.6	26.1
		29	21.7	20.8	22.4
		30	21.4	24.4	25.3
5000	8	36	21.4	23.4	24.0
		37	23.1	24.3	24.2
		38	21.3	24.0	26.1
		39	24.4	27.1	29.2
		40	23.7	27.0	29.1

Male rats

Substance dose (mg/kg)	Group #	Animal #	Day 0	Day 7	Day 14
0	1	1	173	221	252
		2	162	206	240
		3	190	243	288
		4	193	246	275
		5	190	248	283
200	3	11	162	188	210
		12	193	254	290
		13	174	229	262
		14	183	237	269
		15	191	238	271
1000	5	21	179	201	238
		22	187	231	254
		23	184	234	266
		24	173	228	276
		25	184	238	266
5000	7	31	170	234	277
		32	187	238	269
		33	180	228	252
		34	196	248	239
		35	182	242	274

Female rats

Substance dose (mg/kg)	Group #	Animal #	Day 0	Day 7	Day 14
0	2	6	166	207	221
		7	172	228	248
		8	162	182	202
		9	178	205	230
		10	163	187	210
200	4	16	168	206	225
		17	158	182	203
		18	167	193	206
		19	193	217	221
		20	162	194	213
1000	6	26	186	224	243
		27	160	183	192
		28	163	196	218
		29	167	201	217
		30	168	201	218
5000	8	36	160	178	209
		37	167	195	216
		38	190	204	222
		39	171	205	234
		40	152	186	204