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

Approved:

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Director of SFES RCT&HRB
"____"_____2011

REPORT

Assessment of immunotoxic effects of pharmaceutical substance Killevir-16

at peroral administration

(Contract ZT 22/2011)

Study Director:

Larisa V. Mikhina, PhD (Biol)

Serpukhov – 2011

RESPONSIBLE PERSONNEL

L.V. Mikhina, PhD	Principal Scientist
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A.V. Tretiakova	Senior Researcher
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ABBREVIATIONS

AP	–	activity of phagocytosis
HDT	–	hypersensitivity of delayed type
IR	–	index of reaction
IP	–	intensity of phagocytosis
PM	–	peritoneal macrophages
TD	–	therapeutic dose
SE	–	sheep erythrocytes

SUMMARY

Report: 20 pages, 6 tables, 2 references, 3 annexes

Keywords: *preclinical trials, pharmacological preparations, immunotoxic effect, Killevir-16 substance*

Immunotoxic potential of Killevir-16 substance was assessed on laboratory animals. The study was conducted in compliance with "Methodical guidance on immune 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.: Federal State Enterprise "Research Center for Pharmaceutical Expertise", 2005. P.70 – 86.

Test substance was administered in first-generation hybrid F1 (CBA×C_{57}Bl/6) female mice in doses equivalent to a daily therapeutic dose for humans (1 TD = 0.57 mg/kg) calculated for mice based on body weight.

The substance effects on humoral immunity at a single intraperitoneal dose, as well as on humoral, cellular immunity and phagocytic activity of peritoneal macrophages at peroral administration during 30 days were assessed. Killevir-16 was administered in the form of suspension in 1% starch gel; animals from groups of negative control were dosed with 1% starch gel. Number of animals in group – 10.

During the study of Killevir-16 substance effects on humoral immunity titers of antibodies to sheep erythrocytes (log_2 titer) in blood serum of mice obtained after 14 days following immunization were compared. After a single intraperitoneal administration of the test substance in high doses – 28.5 mg/kg, 57.0 mg/kg and 142.5 mg/kg (50 TD, 100 TD, and 250 TD) titers of antibodies to SE in blood serum of mice did not reliably differ from negative control. After peroral administration during 30 days the titers were higher than in control (8.30±0.48):

– at dose 5.7 mg/kg (10 TD) – 9.20±0.88, however, the difference was not statistically reliable,

– at dose 57. 0 mg/kg (100 TD) – 9.11±0.60, the difference is statistically reliable ($p<0.05$).

At immunization of mice with SE after the recovery period (14 days after the last dose) the levels of antibodies to SE in blood serum did not differ from negative control.

Study of the substance effects on cellular immunity demonstrated that values of IR calculated based on results of paw edema test were higher in treated groups than in groups of negative control (9.34±1.71):

– at dose 5.7 mg/kg (10 TD) – 6.31±1.99, the difference is statistically reliable ($p<0.05$),
– at dose 57.0 mg/kg (100 TD) – 6.53±0.85, the difference is statistically reliable ($p<0.05$).

In mice immunized with SE after the recovery period (14 days after the last dose), cellular immune response to SE did not reliably differ from negative control.

In the study of Killevir-16 effects on phagocytic activity of macrophages the activity and intensity of phagocytosis with peritoneal macrophages of heat inactivated *S.aureus* cells was assessed. In mice treated with the test substance in doses up to 57.0 mg/kg (100 TD) both parameters did not reliably differ from negative control.

The studies conducted have revealed the effects of Killevir-16 substance on cellular and humoral immunity at a long-term – 30-day – peroral exposure. Stimulating effect of the substance on humoral immunity and suppression of cellular immunity were noted. The revaled effects had a reversible character: in 14 days after dosing the test parameters were found to be comparable to negative control.

CONTENTS

INTRODUCTION

Immunotoxic effects of pharmaceuticals are uncompensated disorders in the structure and functions of immune system that can lead to reduced resistance of organism to infections, increased cancer risk factors, and development of autoimmune pathologies. Assessment of a new drug influence on functioning of immune system of laboratory animals is a mandatory link in preclinical trials of new therapeuticals.

The study objective is assessment of immunotoxic effects of pharmaceutical substance Killevir-16 at peroral administration in first-generation hybrid female mice F1 (CBA×C_{57}Bl/6) during 30 days.

1. MATERIALS AND METHODS

Experiments were conducted in accordance with the study protocol (Annex 1, study code N 0511, substance code 0611) designed in compliance with the guidance document [1].

Test substance:

Substance code: 0611

Name: Killevir-16

Manufacturer: ZAO Intelpharm

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

Molecular mass: 1500

pH: 5.2

Description: amorphous powder, brown or dark brown

Decomposition temperature without melting: 400-450 °C

Admixtures:

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

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

Batch #54, produced December 22, 2009

Shelf life – 5 years

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

Negative control

Vehicle of the test substance at peroral administration

Code	C^{-}
Name	1% starch gel

Preparation of substances for administering in animals

For administering in animals a test substance suspension in 1% starch gel was used. To make the suspension the test substance was ground in a mortar to a homogeneous powder.

Pharmaceutical group prepared working solutions of test substance in aseptic conditions prior to dosing.

Animals

Strain : hybrid mice F_1 (CBA×C_{57}Bl/6)

Source: Animal Nursery of SCBMT RAMS, "Andreevka" branch, Solnechnogorsky district, Moscow region

Weight before the experiment: 18-22g

Number, sex: 260 female mice (including reserve)

Identification: ear tags

Animal groups and doses

Animals were assigned to experimental groups at random based on body weight (deviation- ±10%). Number of animals in group – 10.

Routes of administration, repeatability, duration:

1) single intraperitoneal administration;
2) peroral administration (as intended route for clinical practice) once daily during 30 days. Test and control substances were applied on a tongue root with a variable volume pipettes with removable tips.

Substance doses:

therapeutic dose of Killevir-16: 40 mg/day = 0.57 mg/kg. Test substance was administered in a single 50 TD, 100 TD, and 250 TD (maximal possible). At a long-term exposure 2 doses were tested: 10 TD and 100 TD.

Weighing of animals:

at assignment to groups, then once a week for adjustment of the test substance dose.

SE immunization:

A single dose of SE suspension in physiological saline was administered intraperitoneally in volume 0.5ml/animal; in Experiments #1 and #2 - $1x10^8$ cells/animal, in Experiment #3 - $2x10^6$ cells/animal.

Experimental groups of hybrid F_1 (CBA×C_{57}Bl/6) mice

Experiment, #	Number of animals				
	50 TD	100 TD	250 TD	C^-	Total
Experiment # 1 Assessment of humoral immune response to SE at single i/p administration of the test substance in high doses	10	10	10	10	40

Experiment, #		Number of animals			
		10 TD	100 TD	C^-	Total
Experiment #2 Assessment of humoral immune response to SE at peroral administration of the test substance during 30 days and during 14 days of recovery period		20	20	20	60
	SE immunization after dosing	10	10	10	
	SE immunization after 14 days of recovery period	10	10	10	
Experiment # 3 Assessment of cellular immune response to SE at peroral administration of the test substance during 30 days and during 14 days of recovery period		20	20	40	80
	SE immunization after dosing	10	10	20	
	SE immunization after 14 days of recovery period	10	10	20	
Experiment # 4 Assessment of functional activity of peritoneal macrophages at peroral administration of the test substance during 30 days <u>Assessment of phagocytic activity of PMP:</u> on the following day after the last dose and after 14-day recovery period		20	20	20	60
				Total mice in experiments #1-#4:	**240**

Killevir-16 dose/volume correlation (body weight of mouse 20 g)

Species	Substance dose			Suspensions	Route	Volume
	(TD)	(mg/kg)	(mg/animal)			
Mice	10 TD	5.7	0.11	C=5.70 mg/ml	per os	0.02 ml
Mice	100 TD	57.0	1.14	C=57.00 mg/ml	per os	0.02 ml
Mice	50 TD	28.5	0.57	C=1.14 mg/ml	i/p*	0.5 ml
Mice	100 TD	57.0	1.14	C= 2.28 mg/ml	i/p	0.5 ml
Mice	250 TD	142.5	2.85	C=5.70 mg/ml	i/p	0.5 ml

Note*: i/p –intraperitoneal administration

Clinical observations:

- daily visual inspection of physical state (motor activity, eye mucosa and skin, defecations);
- at parenteral administration – the site of administration in 24 hours after administration; in case any animal condition deviations are observed clinical examination is performed daily up to normalization.

Assessment of immunological indices

Assessment of Killevir-16 substance effects on humoral immunity

In order to assess the effects of Killevir-16 on humoral immune response, mice were immunized with SE suspension (a single i/p dose of 1×10^8 cells/animal):

- in 1 h after a single i/p dose (Experiment # 1),
- in 1 h after the last dose (Experiment #2),
- in 14 days after the last dose (Experiment #2).

In 14 days after immunization the animals were euthanized and blood samples were taken to obtain blood serum. Blood samples were placed in test tubes without anticoagulant, kept for an hour at room temperature, then a blood clot was separated from the tube wall and the samples were stored in a fridge overnight. Blood serum was taken in microtubes and kept at -20^0C.

The level of hemagglutinins in blood serum of laboratory animals was determined in reaction of heamagglutination. The reaction was performed in microplates in series of 2fold serum dilutions using 2 % SE suspension in physiological saline. Blood serum was analyzed after a single unfreezing. Hemagglutinine titers in treated and control animals were compared.

Test substance effects on cellular immunity

For assessment of test substance effects on cellular immune response mice were immunized intraperitoneally with a single dose of suspension of SE diluted in physiological saline (2×10^6 cells/animal):

- in 1 h after the last dose (Experiment #3);
- in 14 days after the last dose (Experiment #3).

In 5 days after immunization cellular immunity was assessed in paw edema test in response to administration of resolving dose of antigen (0.5×10^8 SE in 0.05 ml of physiological saline). In control animals physiological saline was injected. Reaction was assessed after 24 hours following RD administration. Index of reaction (IR) was calculated by the difference between weights of experimental (D_e) and control (D_c) legs using the formula:

$$IR = \frac{D_e - D_c}{D_c} \times 100 \quad (1)$$

IR values in experimental and control groups were compared.

Test substance effects on activity of peritoneal macrophages

For assessment of phagocytic activity of cells of mononuclear phagocytes peritoneal macrophages (PMP) of mice were used.

PMP monolayer was formed on the cover glass after cultivating a peritoneal exudate (1×10^6 cells/ml) in medium 199 with 10% embryonic blood serum (37^0C, 60 min).

The phagocytosis object were heat inactivated cells of daily *Staphylococcus aureus* culture (30 min, 96^0C).

After adding PMP of *S. aureus* suspension (50 m. cells/macrophage) in culture medium phagocytosis was running for 40 min. Then PMP monolayer was 5 times washed up to remove bacteria from the medium, fixed in 96^0 ethanol, dyed with azure-eosin and analyzed under immersion system of light microscope. At least 100 macrophages in each cytological preparation were analyzed.

The index of phagocytosis activity (AP - percent of macrophages that absorbed bacteria) and the index of phagocytosis intensity (IP – average number of bacteria in one phagocyte) were calculated.

Statistical data processing

Statistical processing of experimental data was performed using Student's criterion, the differences were determined at 95% significance level [2].

2. STUDY RESULTS

The effects of Killevir-16 substance on humoral and cellular immunity and functional activity of peritoneal macrophages of first-generation hybrid F_1(CBA×C_{57}Bl/6) female mice were studied.

The test substance was administered in animals in 1% starch gel in doses multiple to a daily therapeutic dose (1 TD) for humans: 40 mg or 0.57 mg/kg. The doses for animals were calculated based on correlation of human and animal body weights. Animals from negative control received 1% starch gel.

2.1. Study of Killevir-16 effects on humoral immunity

Killevir-16 substance effect on humoral immunity was assessed on a model immune response to SE. The levels of hemagglutinins in blood serum were evaluated using 2 routes of the test substance administration:

- 50TD, 100TD and 250 TD (maximal possible) – single intraperitoneal doses,
- 10 and 100 TD - peroral doses during 30 days.

After a single intraperitoneal dose of the substance the levels of hemagglutinins in blood of treated mice did not reliably differ from negative control (Table 1).

Table 1

Humoral immunity in mice following a single intraperitoneal dose of Killevir-16

Dose		Number of animals in group	Immunizing dose of SE (cells/animal)	Levels of hemagglutinins in blood serum (log_2 titer), M±m*
(TD)	(mg/kg)			
50 TD	28.5	10	2×10^8	8.20±0.45
100 TD	57.0	10	2×10^8	8.00±0.58
250 TD	142.5	10	2×10^8	7.90±0.63
Negative control		10	2×10^8	8,10±0,23

*: here and further: random mean value ± error of mean

After a 30-day peroral administration a stimulating effect of the substance on humoral immunity was noted: the level of hemagglutinins in blood serum of animals was higher than in negative control (Table 2); in maximal dose group (100 TD) the difference was reliable ($p<0.05$). After the recovery period (14 days) the index of humoral immunity in treated mice did not differ from negative control (Table 3).

Table 2

Humoral immunity in mice after completion of peroral administration of Killevir-16

Dose		Number of animals in group	Immunizing dose of SE (cells/animal)	Levels of hemagglutinins in blood serum (log_2 titer), M±m*
(TD)	(mg/kg)			
10 TD	5.7	10	2×10^8	9.20±0.88
100 TD	57.0	10	2×10^8	9.11±0.60*
Negative control		10	2×10^8	8.30±0.48

*p<0.05 in relation to negative control

Table 3

Humoral immunity in mice in 14 days after completion of peroral administration of Killevir-16

Dose		Number of animals in group	Immunizing dose of SE (cells/animal)	Levels of hemagglutinins in blood serum (log_2 titer), M±m*
(TD)	(mg/kg)			
10 TD	5.7	10	2×10^8	8.90±0.53
100 TD	57.0	10	2×10^8	8.60±0.54
Negative control		10	2×10^8	8.50±0.38

Therefore, at peroral administration during 30 days Killevir-16 substance stimulated humoral immunity in mice; this effect was reversible and after 14 days of recovery period the indices of humoral immunity did not differ in experimental and control groups.

2.2. Study of test substance effects on cellular immune response

Killevir-16 substance effect on cellular immunity was assessed on a model immune response to SE. The substance was administered perorally during 30 days in doses 10 TD and 100 TD. Cellular immunity was assessed in paw edema test by index of reaction (IR) calculated by formula (1).

It was found that cellular immune response to SE in treated mice was lower than in negative control (Table 4). The difference is reliable ($p<0.05$) for each experimental group. After the recovery period (14 days) cellular immunity index in treated animals did not reliably differ from negative control (Table 5).

Table 4

Cellular immunity in mice after completion of peroral administration of Killevir-16

Dose		Number of animals in group	Immunizing dose of SE (cells/animal)	IR, %, M±m
(TD)	(mg/kg)			
10 TD	5.7	10	2×10^6	6.31±1.99*
100 TD	57.0	10	2×10^6	6.53±0.85*
Negative control		10	2×10^6	9.34±1.71

*p<0.05 in relation to negative control

Table 5

Cellular immunity in mice in 14 days after completion of peroral administration of Killevir-16

Dose		Number of animals in group	Immunizing dose of SE (cells/animal)	IR, %, M±m
(TD)	(mg/kg)			
10 TD	5.7	10	2×10^6	8.71±1.01
100 TD	57.0	10	2×10^6	9.15±0.76
Negative control		10	2×10^6	9.07±1.03

Therefore, Killevir-16 substance had a suppressing effect on cellular immunity of mice at peroral administration during 30 days; this effect was reversible and after 14 days of recovery period the indices of cellular immunity did not differ in experimental and control groups.

2.3. Study of test substance effects on functional activity of peritoneal macrophages

The effects of Killevir-16 on phagocytic activity of peritoneal macrophages were assessed at peroral administration during 30 days in doses 10 TD and 100 TD. PMP were isolated on the following day after last dose. The index of activity of phagocytosis (AP) (percent of the number of macrophages that adsorbed bacteria) and the index of phagocytosis intensity (IP) (the mean of bacteria number in one phagocyte) were evaluated (Table 6).

Table 6

Phagocytic activity of peritoneal macrophages after completion of peroral administration of Killevir-16

Dose		Number of animals in group	AP (%), M±m	IP, M±m
(TD)	(mg/kg)			
10 TD	5.7	10	62.40±1.55	7.67±0.31
100 TD	57.0	10	62.00±1.72	7.41±0.22
Negative control		10	61.40±1.27	61.40±1.27

No statistically significant differences in AP and IP indices were revealed between experimental and control groups. Killevir-16 substance does not have the ability to change functional activity of peritoneal macrophages at repeated peroral administration in doses up to 100 TD.

As no changes in the test parameters were noted, there was no need in the following assessment of phagocytosis indices after the recovery period.

CONCLUSION

Immunotoxic effects of pharmaceutical substance Killevir-16 were assessed on first-generation F_1(CBA×C_{57}Bl/6) hybrid female mice. The test substance was administered in doses multiple to a daily therapeutic dose for humans 0.57 mg/kg. The doses for animals were calculated based on correlation of human and animal body weights.

The test substance effects on humoral immunity at a single intraperitoneal exposure as well as on cellular immunity and phagocytic activity of peritoneal macrophages at peroral exposure during 30 days were assessed. Experimental animals received the test substance suspension in 1% starch gel, animals from negative control - 1% starch gel. Number of animals in group – 10.

During the study of Killevir-16 humoral immunotoxicity titers of antibodies to sheep SE (log_2 titer) in blood serum of mice obtained after 14 days following immunization were compared. After a single intraperitoneal administration of the test substance in high doses – 28.5 mg/kg, 57.0 mg/kg and 142.5 mg/kg (50 TD, 100 TD, and 250 TD) titers of antibodies to SE in blood serum of mice did not reliably differ from negative control. After peroral administration during 30 days the titers were higher than in control (8.30±0.48):

– at dose 5.7 mg/kg (10 TD) – 9.20±0.88, however, the difference was not statistically reliable,

– at dose 57. 0 mg/kg (100 TD) – 9.11±0.60, the difference is statistically reliable ($p<0.05$).

At immunization of mice with SE after the recovery period (14 days following completion of peroral administration of the test substance) the levels of antibodies to SE in blood serum did not differ from negative control.

Study of the substance effects on cellular immunity demonstrated that IR index calculated based on results of paw edema test was higher in treated groups than in groups of negative control (9.34±1.71):

– at dose 5.7 mg/kg (10 TD) – 6.31±1.99, the difference is statistically reliable ($p<0.05$),

– at dose 57.0 mg/kg (100 TD) – 6.53±0.85, the difference is statistically reliable ($p<0.05$).

In mice immunized with SE after recovery period (14 days after completion of peroral administration of the test substance) cellular immune response to SE did not reliably differ from negative control.

In the study of phagocytic activity of macrophages the activity and intensity of phagocytosis with peritoneal macrophages of heat inactivated *S.aureus* cells was assessed. In mice exposed to the test substance in doses up to 57.0 mg/kg (100 TD) both parameters did not reliably differ from negative control.

Results of the studies conducted revealed the effects of Killevir-16 substance on cellular and humoral immunity at a long-term – 30-day – peroral exposure. Stimulating effect of the substance on humoral immunity and suppression of cellular immunity were noted. The revealed effects had a reversible character: in 14 days after dosing the test parameters were found to be comparable to negative control.

REFERENCES

1. “Methodical guidance on immune 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.: Federal State Enterprise “Research Center for Pharmaceutical Expertise”, 2005. P.70 – 86.

2. “Methodical guidance on statistical treatment of preclinical trials data” /Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev – 2nd edition, corrected and amended; ad. – M.: Federal State Enterprise “Research Center for Pharmaceutical Expertise”, 2005. P.774 – 826.

ANNEX 1

STUDY PROTOCOL

Immune toxicity assessment of Killevir-16 at peroral administration

Study Code: N0511

Test substance code: 0611

Customer: **ZAO Intelpharm**

Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia

Fax: +7 8-831-433-00-03

Tel.: +7 8-831-430-02-06, 8-831-430-38-57

e-mail: info@intelpharm.ru

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:
Larisa V. Mikhina, PhD
Head of Immunological Laboratory
tel/fax: (4967) 70-54-84, 39-97-38
toxic@online.stack.net

1. Key dates

Planned study initiation date: April 18, 2011

Planned first dose date: April 25, 2011

Planned dosing completion date: July 22, 2011

Planned study completion date: August 12, 2011

2. Responsible personnel:

Administration of test substances, clinical observation –
S.V. Melnikova, Junior Researcher, Immunological Laboratory,
E.N. Sokolova, Junior Researcher, Immunological Laboratory
Immunological evaluations –
A.V. Tretiakova, Senior Researcher, Immunological Laboratory,
Yu.S. Korobovtseva, researcher, Immunological Laboratory,
S.V. Melnikova
Necropsy –
S.V. Melnikova
Animal care and veterinary control –
I.E. Selivanova, Head of Vivarium
Procurement and accountability of the test substances -
L.A. Eremenko, researcher, Division for Analytical Chemistry and Radiobiology, Head of Pharmaceutical Group
Quality Assurance –
V.V. Kapranov, Head of QA Laboratory

3. Study Objective

Immune toxicity assessment of Killevir-16 substance on laboratory animals at peroral administration during 30 days (1^{st} stage)

4. Guidelines

The study will be guided by the following documents:

1. "Rules for Laboratory Practice" (Order of Russian Ministry of Health and Social Development #708n of August 23, 2010);

2. GOST P 53434-2009. GLP principles. M.: Standartinform.- 2010. – 12p.

The study design was made up based on "Methodical guidance on immunotoxicity assessment of pharmaceuticals" /Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev. – M.: OAO "Medicina Publishing House", 2005. p. 70-86.

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

4.1. Humane treatment and use of test animals

Animals will be handled in compliance with:

- RCT&HRB SOPs on animal care and maintenance;

- Statutory documentation of RCT&HRN Bioethics Committee;
- Sanitary rules approved by the Ministry of Health of the Russian Federation on 06.07.73 on construction, equipping and maintenance of experimental biological clinics (vivariums).

A veterinary protocol will be written and submitted to the Bioethics Committee of the RCT&HRB for expert assessment and approval. Laboratory animals for the studies will be purchased 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.

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.

4.2. 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.

5. Test and control substances

Substance code : 0611

Name: Killevir-16

Manufacturer: ZAO Intelpharm

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

Molecular mass: 1500

pH: 5.2

Description: amorphous powder, brown or dark brown

Decomposition temperature without melting: 400-450 °C

Admixtures:

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

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

Batch #54, produced December 22, 2009

Shelf life – 5 years

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

5.2. Control substances (negative control)

Negative control: test substance vehicle for peroral administration

Name	1% starch gel
Code	C^-

Positive control: a substance with mutagenic activity

Name	cyclophosphamide
Code	C^+

5.3. Composition and stability

The composition and stability of the test substance are the responsibility of the customer. Results of a component analysis are submitted to the Study Director and stored with raw data.

5.4. Substance specimens

Pharmaceutical group maintains records on procurement and accountability of the test substances and maintains specimens of each lot of test and control substances in allocated room under proper storage conditions.

Reserve specimens of test substance are stored with the other study materials.

5.5. Preparation of substances for administering in animals

The test substance suspension in 1% starch gel will be administered in animals. To prepare a suspension the substance will be ground in a mortar to homogenous powder.

Working solutions will be prepared in aseptic conditions by pharmaceutical group prior to dosing.

6. Animals

Species: hybrid F_1 (CBA×C_{57}Bl/6) mice
Source: Research Center for Biomedical Technologies, RAMS, "Andreevka" branch, Solnechnogorsk district, Moscow region

Weight before study: 18 - 22 g

Number, sex: 270 one-sex animals including reserve

Identification: ear tags

Justification of animal species: in accordance with guidance [1].

6.1. Animal care

Animals will have free access to feed and water. Animal care and maintenance will comply with guidance documents. All routine procedures will be performed in accordance with the SOPs of the RCT&HRB.

Housing

Animals will be kept in polycarbonate cages 26×17×12 cm with bedding, 5-6 animals per cage. The cages have steel lattice covers with deepenings, steel dispensers for feed and water, and steel label holders.

Diet

Animals will be fed by conventional granulated feed "Combikorm PK-120 for laboratory rats, mice, and hamsters" (OOO "Laboratorkorm", Russia, 115478, Moscow, Kashirskoye shosse, 24).

Water

Animals will be given water in accordance with GOST "Drinking water" 2874–73.

Environmental conditions

Laboratory animals will be kept in controlled conditions, temperature 18-20^{0}C, relative humidity 50-60%. Temperature and humidity are monitored in each experimental room and data are recorded by special computer program and once daily in manual datasheets. Light- artificial, 12 h light/12 h dark regime, air exchange – 15/h.

Quarantine: 14 days.

6.2. Animal grouping and doses

Animals will be assigned to experimental groups at random based on body weight (deviation $\pm$ 10%). In the experiments only young, healthy, sexually mature animals will be taken. Animal species is chosen in compliance with the Methodical guidance (see item 4). Number of animals in group – 10 of one sex.

Routes of administration, repeatability, duration

1) a single intraperitoneal administration;

2) peroral administration (as intended route in clinical practice) once daily during 30 days.

Test and control substances will be applied on a tongue root with a variable volume pipettes with removable tips.

Doses

Test substance will be administered in doses multiple to the equivalent to therapeutic dose (1 TD) for humans (70 kg). Therapeutic dose of 0611 substance: 40 mg/day = 0.57 mg/kg. Test substance will be administered in a single 50 TD, 100 TD, and 250 TD, and 1250[1] TD. At a long-term exposure 2 doses were tested: 10 TD and 100 TD.

Table 1. 0611 substance dose/volume correlation (body weight of mouse 20 g)

Species	0611 substance dose			Suspensions	Route	Volume
	(TD)	(mg/kg)	(mg/animal)			
Mice	10 TD	5.71	0.11	C=5.71 mg/ml	per os	0.02 ml
Mice	100 TD	57.14	1.14	C=57.14 mg/ml	per os	0.02 ml
Mice	50 TD	28.57	0.57	C=1.14 mg/ml	i/p*	0.5 ml
Mice	100 TD	57.14	1.14	C= 2.29 mg/ml	i/p	0.5 ml
Mice	250 TD	142.86	2.86	C=5.71 mg/ml	i/p	0.5 ml
Mice	1250 TD	714.29	14.29	C=28.57 mg/ml	i/p	0.5 ml

Note*: i/p –intraperitoneal administration

Immunization with sheep erythrocytes (SE)

A single intraperitoneal dose of SE in the form of suspension in physiological saline will be administered in volume 0.5 ml. In experiments ##1, 2 mice will receive 1×10^8 SE/animal, in experiment #3 – 2×10^6 SE.

Table 2. Experimental groups of animals

Experiment, task	Number of animals					
	Doses (TD), control (C^-)					Total
	10 TD	50 TD	250 TD	1250 TD	C^-	
Experiment # 1 Study of humoral immune response to SE at single intraperitoneal administration of 0611 substance in high doses	10	10	10	10	10	50

Experiment, task	Number of animals			
	Doses (TD), control (C^-)			
	10 TD	100 TD	C^-	
Experiment # 2 Study of humoral immune response to SE at peroral administration of 0611 substance during 30 days followed by a 14-day recovery period	20	20	20	60
SE immunization after completion of dosing	10	10	10	
SE immunization after 14 days of recovery period	10	10	10	

Experiment # 3 Study of cellular immune response to SE at peroral administration of 0611 substance during 30 days followed by a 14-day recovery period	20	20	40	80
SE immunization after completion of dosing	10	10	20	
SE immunization after 14 days of recovery period	10	10	20	
Experiment # 4 Study of functional activity of peritoneal macrophages at peroral administration of 0611 substance during 30 days Assessment of phagocytic activity of PMP on the following day after the last dose and after recovery period (14 days)	20	20	20	60
Total mice, experiments ##1-4:				250
[1]In accordance with Amendment #1 1250TD dose was not tested				

6.3. Clinical observation

- daily observation of physical state of animals (motor activity, eye mucosa, skin, defecation);

- at parentheral administration - inspection of test substance administration site – in 24 hours after dosing; in case of deviations – daily up to normalization.

6.4. Completion of the experiments: Euthanasia, necropsy

Upon completion of dosing animals will be euthanized by CO_2 inhalation. Material for *in vitro* studies will be taken immediately after euthanasia.

Cellular immune response to SE will be assessed in 5 days following immunization. Index of HDT reaction to SE in paw edema test will be determined.

Humoral immune response to SE will be assessed in 7-14 days following immunization, the level of antibodies to SE will be determined in blood serum in reaction of hemagglutination.

Functional activity of peritoneal macrophages will be assessed *in vitro*; the object of phagocytosis will be *S. aureus* cell cultures heat inactivated for 30 min at temperature 96-100^{0}C.

7. 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 personnel will be informed on the approved amendments and effective date. The protocol changes will be attached to all approved copies of the protocol and to the report.

8. Deviations

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

9. Report

Report will be submitted to the Customer upon completion of the study in accordance with all items of the Study Plan and analysis of the obtained material. The study plan, all amendments and deviations will be attached to the report.

10. Statistical analysis of data

Statistical treatment of the obtained results will be performed using Student's criterion. A comparative mean-group analysis will be performed, the differences will be determined at confidence probability 95%.

11. Documentation and archive

Study protocol, amendments and deviations, initial data and reporting materials will be stored in the RCT&HRB archive for 2 years.

12. Protocol approval

Customer representative:

_____	_____	_____
Name	Signature	Date

Study Director:

_____ _____	_____	_____
Name	Signature	Date

Director of SFES RCT&HRB:

_____	_____	_____
Name	Signature	Date

ANNEX 2

Amendment #1 to

STUDY PROTOCOL

Immunotoxicity assessment of Killevir-16 at peroral administration

(1st stage)

Study Code: NO511

Test substance code: 0611

The part of the protocol subject to correction

6.2. Animal groups and doses

Doses

0611 substance will be administered in a single 10TD, 50 TD, 250 TD, and 1250 TD.

Table 1. Experimental groups of animals

Experiment, task	Number of animals					
	Doses (TD), control (C^-)					Total
	10 TD	50 TD	250 TD	1250 TD	C^-	
Experiment # 1 Study of the test preparation single intraperitoneal dose on humoral immunity	10	10	10	10	10	50

Description of corrected information

6.2. Animal groups and doses

Doses

0611 substance will be administered in a single 10TD, 50 TD, and 250 TD (maximal possible dose for intraperitoneal administration).

Table 1. Experimental groups of animals

Experiment, task	Number of animals				
	Doses (TD), control (C^-)				Total
	10 TD	50 TD	250 TD	C^-	
Experiment # 1 Study of the test preparation single intraperitoneal dose on humoral immunity	10	10	10	10	40

Reason/justification of amendment: adjustment of 0611 substance doses for intraperitoneal administration connected with physico-chemical properties of the substance.
Administration of 1250 TD is impossible as dense suspensions of the test substances can hardly pass through the needle (22Gx$1_{1/2}$").

Effective date:

Study Director:

Signature

Date

Informed personnel:

Name	**Подпись**	**Дата**
Melnikova S.V.		
Dmitrieva Z.M.		

ANNEX 3

Quality Control Report

ANNEX 3

Table 1

Individual IR values obtained in reaction of hemagglutination (acute experiment, a single intraperitoneal administration of Killevir-16)

Substance, dose	**Ind#**	**Serum titer (titer log_2)**
Negative control + SE	1	9
	2	8
	3	8
	4	8
	5	8
	6	8
	7	8
	8	8
	9	8
	10	8
Killevir-16 substance, 50 TD + SE	11	7
	12	9
	13	8
	14	8
	15	9
	16	8
	17	8
	18	8
	19	8
	20	9
Killevir-16 substance, 100 TD + SE	21	7
	22	9
	23	8
	24	7
	25	8
	26	9
	27	8
	28	8
	29	7
	30	9
Killevir-16 substance**,** 250 TD + SE	31	8
	32	9
	33	6
	34	8
	35	9
	36	8
	37	8
	38	8
	39	8
	40	7

Table 2

Individual hemagglutination reaction indices (30-repeated peroral administration of Killevir-16 substance, SE immunization on the day of last dose)

Substance, dose	**Ind #**	**Serum titer (titer $\log_2$)**
Negative control + SE	71	9
	72	9
	73	8
	74	7
	75	8
	76	8
	77	9
	78	8
	79	8
	80	9
Killevir-16 substance, 10 TD + SE	81	10
	82	8
	83	9
	84	7
	85	11
	86	11
	87	9
	88	9
	89	9
	90	9
Killevir-16 substance, 100 TD + SE	91	9
	92	9
	93	9
	94	8
	95	8
	96	10
	97	10
	98	10
	99	9
	100	no

Table 3

Individual hemagglutination reaction indices (30-repeated peroral administration of Killevir-16 substance, SE immunization in 14 days after dosing)

Substance, dose	**Ind #**	**Serum titer (titer $\log_2$)**
Negative control + SE	161	9
	162	8
	163	8
	164	8
	165	8
	166	9
	167	9
	168	9
	169	9
	170	8
Killevir-16 substance, 10 TD + SE	171	8
	172	9
	173	9
	174	9
	175	9
	176	10
	177	8
	178	10
	179	8
	180	9
Killevir-16 substance, 100 TD + SE	181	8
	182	8
	183	10
	184	9
	185	8
	186	9
	187	9
	188	9
	189	8
	190	8

Table 4

Individual IR obtained in paw edema test (30-repeated peroral administration of Killevir-16 substance, SE immunization on the day of last dose)

Substance, dose	**Ind #**	**IR (%)**
Negative control + SE	41	4.55
	42	8.93
	43	10.00
	44	7.58
	45	12.70
	46	9.23
	47	12.07
	48	11.29
	49	7.81
	50	9.23
Killevir-16 substance**,** 10 TD + SE	51	10.94
	52	2.22
	53	6.15
	54	9.68
	55	2.99
	56	7.76
	57	6.15
	58	4.55
	59	5.00
	60	7.69
Killevir-16 substance**,** 100 TD + SE	61	5.60
	62	5.97
	63	5.60
	64	8.20
	65	4.84
	66	7.27
	67	8.47
	68	6.25
	69	7.14
	70	5.97

Table 5

Table 5. Individual IR indices in paw edema test
(30-repeated peroral administration of Killevir-16 substance, SE immunization in 14 days after dosing)

Administered substance, dose	**Ind #**	**IR (%)**
Negative control + SE	131	9.09
	132	10.17
	133	11.38
	134	8.33
	135	7.09
	136	10.45
	137	9.91
	138	8.47
	139	8.87
	140	6.92
Killevir-16 substance, 10 TD + SE	141	9.17
	142	9.92
	143	9.32
	144	7.38
	145	8.40
	146	10.24
	147	10.53
	148	6.40
	149	8.80
	150	6.98
Killevir-16 substance, 100 TD + SE	151	10.62
	152	8.40
	153	7.87
	154	10.32
	155	9.09
	156	9.60
	157	9.17
	158	9.68
	159	7.21
	160	9.52

Table 6. Individual phagocytosis indices after peroral administration of Killevir-16 substance

Administered substance, dose	**Ind #**	**AP (%)**	**IP (number of bacteria/cell)**
Negative control	101	62	7.60
	102	65	7.40
	103	60	7.90
	104	60	7.80
	105	61	7.40
	106	62	7.30
	107	62	7.20
	108	63	8.00
	109	60	7.40
	110	59	7.70
Killevir-16 substance**,** 10 TD	111	60	7.70
	112	65	7.60
	113	66	7.10
	114	64	7.20
	115	62	7.50
	116	61	7.90
	117	60	7.40
	118	61	8.20
	119	64	8.50
	120	61	7.60
Killevir-16 substance**,** 100 TD	121	59	7.90
	122	64	7.50
	123	64	7.00
	124	65	7.00
	125	62	7.30
	126	60	7.40
	127	63	7.50
	128	58	7.40
	129	61	7.90
	130	64	7.20