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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 Killevir-16 substance allergizing effect at peroral administration

(Contract ZT 22/2011)

Study Director:

Larisa V. Mikhina, PhD (Biol)

Serpukhov – 2011

RESPONSIBLE PERSONNEL

L.V. Mikhina, PhD	Head of Immunological Laboratory
A.V. Tretiakova	Senior Researcher
L.A. Eremenko	Researcher
S.V. Melnikova	Junior Researcher
E.N. Sokolova	Junior Researcher
Z.M. Dmitrieva	Laboratory assistant

ABBREVIATIONS

ACA	–	active cutaneous anaphylaxis
HDT	–	hypersensitivity of delayed type
HIT	–	hypersensitivity of immediate type
IR	–	index of reaction
DMSO	–	dimethyl sulfoxide
RD	–	resolving dose
TD	–	therapeutic dose

SUMMARY

***Report* – 17 pages, 2 tables, 2 references, 3 annexes.**

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

Allergizing properties of Killevir-16 substance were assessed at peroral administration in Balb/c mice and albino guinea pigs. The substance was administered in doses multiple to equivalent to a daily therapeutic dose for humans (40 mg) calculated for animals based on body weight: 0.57 mg/kg (1 TD) and 5.7 mg/kg (10 TD). The test substance was administered in the form of suspension in 1% starch gel; control animals received 1% starch gel.

In the testing of mice for HDT it was found that after completion of administration in dose 1 TD the mean values of IR of HDT in male and female groups did not reliably differ from negative control. In 3 of 10 male mice individual HDT IR values were higher than maximal values in control group; in female mice they were not higher. After exposure in dose 10TD HDT IR in male groups were statistically reliably higher than in group of negative control ($p<0.05$), in female mice they did not differ from negative control. In 5 male mice of 10 individual IR values were higher than maximal values in control; this allows to consider the test substance as a potential allergen.

HIT in mice after a 30-day exposure in doses up to 5.7 mg/kg was not detected in reaction of active cutaneous anaphylaxis.

Testing of guinea pigs using conjunctiva and intracutaneous tests, as well as reaction of general anaphylaxis did not reveal hypersensitivity to the test substance in all tested groups.

Therefore, in experiments on mice it was demonstrated that at repeated peroral administration Killevir-16 substance can cause allergizing reaction expressed in formation of hypersensitivity of delayed type. It is also established that male mice are more sensitive to allergizing effect of the substance.

In experiments on guinea pigs no allergizing effect of the substance at peroral administration during 30 days in doses up to 5.70 mg/kg (10 TD) is established. Based on the results obtained Killevir-16 substance can be referred to potential allergens.

CONTENTS

INTRODUCTION

Hypersensitivity is a possible side effect emerging at medical treatments. Detection of potential allergic reactions is a mandatory stage in preclinical trials of newly developed therapeuticals.

The study objective is assessment of allergizing properties of Killevir-16 substance on laboratory animals at peroral administration during 30 days.

The experiments were conducted on two animal species: mice and guinea pigs.

1. MATERIALS AND METHODS

The experiments were conducted in accordance with the study protocol (Annex 1, study code M 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

The composition and stability of the test substance are the responsibility of the customer.

Negative control

1. Vehicle of the test substance at peroral administration

Code	$C1^{-}$
Name	1% starch gel

2. Vehicle of the test substance at administering of resolving doses

Code	C2
Name	5% DMSO solution in physiological saline

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.

To detect hypersensitivity in animals resolving doses of the test substance in 5% DMSO diluted with physiological saline were administered.

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

Experimental animals

1. Species: Balb/c mice

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

Weight before the experiment: 18-22g

Number, sex: 130 male and female mice (including reserve)

Identification: ear tags

2. Albino guinea pigs

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

Weight before the experiment: 300-350 g

Number, sex: 70 male and female guinea pigs (including reserve)

Identification: ear tags

Animal groups and doses

Group assignment

Animals were assigned to experimental groups at random based on body weight (deviation- ±10%), 10 animals in group, 5 per sex. Exception: for HDT detection in mice 10 male and 10 female mice were taken for each dose and control.

Route of administration

For administering the test substance a peroral route was used as an intended route for clinical practice. Test substance was applied on a tongue root with a variable volume pipettes with removable tips.

Repeatability, duration

Test and control substances were administered once daily during 30 days.

Doses

Daily therapeutic dose of Killevir-16 for humans (70 kg) – 40 mg, equitherapeutic dose for laboratory animals (1 TD) - 0.57 mg/kg. Two doses were tested: 1 TD and 10 TD.

Weighing of animals:

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

Experimental groups of animals (male/female- 1:1)

Animal species	Experiment #, task	Number of animals in group		
		1 TD	**10 TD**	**C1⁻**
Inbred mice Balb/c	**Experiment # 1** Study of the test substance ability to cause HDT at peroral administration during 30 days. HDT detection: in 5 days after completion of dosing in paw edema test (with evaluation of HDT IR in 24 h after administration of resolving dose)	20	20	20
	Experiment #2 Study of the test substance ability to cause HIT at peroral administration during 30 days. HDT detection: in 14-20 days after completion of dosing in ACA reaction	10	10	10
	Selection of RD for HDT and HIT detection	20		
	Total mice:	**110**		
Albino guinea pigs	**Experiment # 3** Study of the test substance ability to cause HIT at peroral administration during 30 days. HDT detection: in 14-20 days after completion of dosing in reaction of general anaphylaxis (anaphylactic shock)	10	10	10

	Experiment # 4 Peroral administration of the test substance during 30 days, detection of hypersensitivity in conjunctiva test and at repeated testing in 10 days in intracutaneous test	10	10	10
	Selection of RD	10		
	Total guinea pigs:	**70**		

Killevir-16 dose/volume correlation (body weight of mouse 20 g, guinea pig - 330 g)

Species	**Substance dose**			**Suspensions**	**Route**
	(TD)	**(mg/kg)**	**(mg/animal)**		
Mice	10 TD	5.70	0.11	C=5.70 mg/ml	0.02 ml
Mice	1 TD	0.57	0.01	C=0.57 mg/ml	0.02 ml
Guinea pigs	10 TD	5.70	1.90	C=38.1 mg/ml	0.05 ml
Guinea pigs	1 TD	0.57	0.19	C=3.8 mg/ml	0.05 ml

Clinical observations:

- daily visual inspection of physical state (motor activity, eye mucosa and skin, defecations);
- daily examination for detection of signs of allergic response to the administered substance (anxiety, state of mucous membranes and skin).

Detection of hypersensitivity in animals

Detection of HDT in mouse paw edema test

HDT detection was conducted in 5 days after completion of dosing. Resolving dose of substance (100 μg in 0.05 ml of 5% DMSO) was administered in paw of rear leg, in the opposite leg (control) – 0.05 ml of 5% DMSO was administered.

After 24 hours, index of reaction of HDT was determined by the difference between weights in experimental (P_e) and control (P_c) paws using the formula:

$$IR = \frac{P_e - P_c}{P_c} \times 100 \quad (1)$$

RD of the test substance not causing strong nonspecific inflammation (IR up to 10%) was adjusted beforehand on intact mice.

The obtained mean random IR values in experimental and control groups were compared.

Detection of HIT in mice in reaction of active cutaneous anaphylaxis

HIT was detected in ACA reaction in 15 days after completion of dosing.

Each animal was intracutaneously injected in the body side with 2 resolving doses of the test substance (100 μg and 200 μg in 0.05 ml of 5% DMSO), in control point – 0.05 ml of 5% DMSO were injected. Resolving doses of substance not causing non-specific increase of blood capillaries permeability were adjusted beforehand on intact mice.

In 20 min after RD administering in tail vein of mice 0.2 ml of 1% Evans blue were injected. 30 min later the animals were euthanized by CO_2 inhalation and the reaction was evaluated by the diameter of excudate spot dyed with Evans blue on the inner skin surface on the site of RD administration.

The difference between the sizes of excudates' spots formed in response to administration of RD and 5% DMSO in experimental and control animals was evaluated.

Hypersensitivity detection in conjunctiva test

In 16 days after completion of dosing under the upper eyelids of experimental guinea pigs RD of the test substance (200 μg in 0.02 ml of 5% DMSO) was administered, in the other (control) eye - the same volume of 5% DMSO. RD not causing non-specific inflammation within 24 hours was adjusted beforehand on intact guinea pigs.

The reaction was evaluated within the first 30 min (HIT assessment), then in 6, 24, and 48 hours (HDT assessment) after RD administering according to the following scale (in points):

1 – slight redness of tear duct;

2 – redness of tear duct and sclera towards cornea;

3 – redness of the whole conjunctiva and sclera.

Hypersensitivity detection in intracutaneous test

Guinea pigs were intracutaneously injected in the body side with a resolving dose of test substance (200 μg in 0.05 ml of 5% DMSO), in control point – 0.05 ml of 5% DMSO were injected. Resolving doses of substance not causing non-specific inflammation within 24 hours was adjusted beforehand on intact guinea pigs.

The reaction on the skin surface was evaluated within the first 60 min, then in 6, 24, 48 and 72 hours after RD administering according to the following scale (in points):

0 – no visible reaction;

1 – pale pink erythema on the site of administration or in the periphery;

2 – bright rose erythema on the site of administration or in the periphery;

3 – red erythema on the site of administration;

4 – infiltration and skin edema (thickening of skin fold) at the presence or absence of erythema;

5 – erythema, pronounced infiltration, focal ulcerations (necrosis).

Hypersensitivity detection in reaction of general anaphylaxis

In 21 days after dosing RD of the test substance (300 μg in 0.6 ml of 5% DMSO) was administered in guinea pigs by intracardiac injection. Non-toxic dose was adjusted beforehand.

The intensity of anaphylactic shock was evaluated using Weigle table:

+ + + +	Lethal shock
+ + +	Severe shock (convulsions, asphyxia, shaky, falling on bodyside, no death)
+ +	Moderate shock (small convulsions, pronounced bronchospasm)
+	Weak shock (anxiety, frequent breathing, scratching face, involuntary urination and defecation, rumpled hair)
0	no shock (absence of signs)

Statistical data processing

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

2. STUDY RESULTS

Allergizing effect of Killevir-16 substance was assessed on 2 animal species – mice and guinea pigs. Peroral route of administration was used as an intended route for clinical practice. Duration of dosing – 30 days.

The test substance was administered in doses multiple to the equivalent to a daily therapeutic dose for humans (40 mg) calculated for experimental animals based on body weight: 0.57 mg/kg (1 TD) and 5.7 mg/kg (10 TD).

Upon completion of dosing the animals were tested to detect hypersensitivity of delayed and immediate types (HDT, HIT).

2.1. Study of allergizing effect of Killevir-16 substance on mice

To detect HDT to the test substance in mice paw edema test was used. Number of animals in each dose group and control– 10 per sex. IR of HDT was calculated by formula (1); results are given in Table 1.

Table 1

HDT IR in mice after 30-day peroral exposure to Killevir-16

Dose		Number of animals, male/female	HDT IR, % (M±m)*	
(TD)	(mg/kg)		Male	Female
1 TD	0.57	10/10	3.89±2.0	2.10±1.12
10 TD	5.70	10/10	4.74±1.85**	2.13±1.14
0 (negative control)		10/10	2.45±0.96	2.69±1.48

*: random mean value ± error of mean
**: $p<0.05$ in relation to negative control

Individual values of HDT IR in mice after equitherapeutic dose did not reliably differ from negative control. In 3 of 10 male mice individual HDT IR values were higher than maximal values in control group, in female mice they were not higher.

After 10fold equitherapeutic dose the HDT IR in male group was statistically higher than in negative control, in female group it did not differ from control. In 50% of male mice exposed to Killevir-16 in dose 10 TD individual values of IR were higher than maximal values of IR in control; this allows to consider the test substance as a potential allergen.

To detect HIT in mice the reaction of active cutaneous anaphylaxis (ACA) was used. Number of animals in each dose group and control – 5 per sex.

It was found that in mice dosed with the test substance and in negative control diameters of dyed with Evans blue exudate spots appeared in points of RD administering did not differ and were not greater than 3 mm. Negative ACA reaction indicates the absence of hypersensitivity of immediate type to the test substance in animals after 30-day exposure in doses up to 5.70 mg/kg (10TD).

Therefore, in experiments on mice it was demonstrated that repeated peroral administration Killevir-16 substance can cause allergizing reactions expressed in formation of hypersensitivity of delayed type. It is also demonstrated that male mice are more sensitive to the allergizing effect of the test substance.

2.2. Assessment of Killevir-16 substance allergizing effect on guinea pigs

Hypersensitivity to the test substance in guinea pigs was assessed in conjunctiva test, intracutaneous test and in reaction of general anaphylaxis. Number of animals in group – 5 per sex.

Conjunctiva test

Within the first 30 min and in 6, 24, and 48 hours after the test substance RD administration no signs of animals' anxiety and changes in tear duct and conjunctiva were observed.

Therefore, no allergic reaction was detected by conjunctiva test in guinea pigs after 30-day peroral exposure to Killevir-16 in doses up to 5.70 mg/kg (10TD).

Intracutaneous test

Within the first 30 min and in 6, 24, and 48 hours after the test substance RD administration in animals treated with test substance no signs of animals' anxiety and changes in tear duct and conjunctiva were observed.

Therefore, no allergic reaction was detected by intracutaneous test in guinea pigs after 30-day peroral exposure to Killevir-16 in doses up to 5.70 mg/kg (10TD).

Reaction of general anaphylaxis

Within 60 min following intracardiac injection of RD no signs of anaphylactic shock in guinea pigs were noted (0 points by Weigle table); hypersensitivity to the test substance in animals was not detected.

The study conducted on guinea pigs has not revealed allergizing effects of Killevir-16 at peroral administration in doses up to 5.70 mg/kg (10 TD) during 30 days.

Table 2

Hypersensitivity in guinea pigs at peroral administration of Killevir-16 substance during 30 days

Method	Dose	Number of animals, male/female	Number of animals with hypersensitivity	
			Male	Female
Conjunctiva test	1 TD	5/5	0	0
	10 TD	5/5	0	0
	0 (negative control)	5/5	–	–
Intracutaneous test	1 TD	5/5	0	0
	10 TD	5/5	0	0
	0 (negative control)	5/5	–	–
Reaction of general anaphylaxis	1 TD	5/5	0	0
	10 TD	5/5	0	0
	0 (negative control)	5/5	–	–

CONCLUSION

Allergizing properties of Killevir-16 substance were assessed at peroral administration in Balb/c mice and albino guinea pigs. The substance was administered in doses multiple to equivalent to a daily therapeutic dose for humans (40 mg) calculated for animals based on body weight: 0.57 mg/kg (1 TD) and 5.7 mg/kg (10 TD). The test substance was administered in the form of suspension in 1% starch gel; control animals received 1% starch gel.

In the testing of mice for HDT it was found that after completion of administration in dose 1 TD the mean values of IR of HDT in male and female groups did not reliably differ from negative control. In 3 of 10 male mice individual HDT IR values were higher than maximal values in control group; in female mice they were not higher. After exposure in dose 10TD HDT IR in male groups were statistically reliably higher than in group of negative control ($p<0.05$), in female mice they did not differ from negative control. In 5 male mice of 10 individual IR values were higher than maximal values in control; this allows to consider the test substance as a potential allergen.

HIT in mice after a 30-day exposure in doses up to 5.7 mg/kg was not detected in reaction of active cutaneous anaphylaxis.

Testing of guinea pigs using conjunctiva and intracutaneous tests, as well as reaction of general anaphylaxis did not reveal hypersensitivity to the test substance in all tested groups.

Therefore, in experiments on mice it was demonstrated that at repeated peroral administration Killevir-16 substance can cause allergizing reaction expressed in formation of hypersensitivity of delayed type. It is also established that male mice are more sensitive to allergizing effect of the substance.

In experiments on guinea pigs no allergizing effect of the substance at peroral administration during 30 days in doses up to 5.70 mg/kg (10 TD) is established. Based on the results obtained Killevir-16 substance can be referred to potential allergens.

REFERENCES

1. Methodical guidance on assessment of allergizing properties of pharmaceuticals / Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev – M.: Federal State Enterprise “Research Center for Pharmaceutical Expertise”. – 2005. – p. 54 – 69.

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

Study of allergizing properties of Killevir-16 at peroral administration

Study code: M0511

Substance code: 0611

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 12, 2011
Planned first dose date: April 18, 2011
Planned dosing completion date: August 15, 2011
Planned study completion date: September 15, 2011

2. Responsible personnel:

Administration of test substances, clinical observation –
S.V. Melnikova, Junior Researcher, Immunological Laboratory,
E.N. Sokolova, Junior Researcher, Immunological Laboratory
Hypersensitivity detection –
Yu.S. Korobovtseva, researcher, Immunological Laboratory,
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. Abbreviations:

ACA	–	active cutaneous anaphylaxis
HDT	–	hypersensitivity of delayed type
HIT	–	hypersensitivity of immediate type
DMSO	–	dimethyl sulfoxide
RD	–	resolving dose
TD	–	therapeutic dose

4. Study Objective

Assessment of Killevir-16 allergizing effects on laboratory animals at peroral administration during 30 days

5. 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 allergenicity assessment of pharmaceuticals” /Guidance on experimental (preclinical) study of new pharmaceuticals. Ed. by prof. R.U. Khabriev. – M.: OAO “Medicina Publishing House”, 2005. p.100 – 104.

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

5.1. Humane treatment and use of test animals

Animals will be housed and 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.

5.2. 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.

6. 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.

6.2. Control substances (negative control)

1) Vehicle of the test substance at peroral administration

Code	$C1^{-}$
Name	1% starch gel

2) Vehicle of the test substance at administering RD

Code	$C2^{-}$
Name	3-5% DMSO in physiological saline

6.3. 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.

6.4. 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.

6.5. 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 up to homogenous powder.

Solutions of test substance in 5% DMSO will be prepared for intracardiac administration of resolving doses in reaction of general anaphylaxis and for intracutaneous administration of RD in ACA reaction.

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

7. Animals

1. **Species**: Balb/c mice
   Source: Animals Nursery of SCBMT RAMS, “Andreevka” branch, Solnechnogorsky district, Moscow region
   Weight before study: 18-22 g
   Number, sex: 130 animals including reserve, equal numbers of each sex
   Identification: ear tags
2. **Species**: guinea pigs, albino or varicolored with non-pigmentation spots 40-50%
   Source: Animals Nursery of SCBMT RAMS, “Andreevka” branch, Solnechnogorsky district, Moscow region
   Weight before study: 300-350 g
   Number, sex: 70 animals including reserve, equal number of each sex
   Identification: ear tags

7.1. Animal care and maintenance

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 with bedding: mice in 26×17×12 cm cages, 5-6 animals per cage, guinea pigs in 45×24×20 $см^3$ cages, 2-3 animals per cage. The cages have steel lattice covers with deepenings, steel dispensers for feed and water, and steel label holders.

Diet

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: mice – for a minimum of 14 days, guinea pigs –21 days prior to the start of dosing.

8. Study design

Animal grouping and doses

Animals will be assigned to experimental groups at random based on body weight (deviation ±20%). In the experiments only young, healthy, sexually mature animals of 2 species – mice and guinea pigs – will be taken. Animal species is chosen in compliance with the Methodical guidance (see item 5). Male and female animals may have different sensitivity to the same preparation, so in the experiments equal numbers of animals of both sexes will be taken. Number of animals in group – 10 of one sex.

Doses

Test substance 0611 will be administered in 2 doses: a dose equivalent to a daily therapeutic dose for humans (1 TD) and in dose equivalent to 10 TD.
The intended daily therapeutic dose for humans (70 kg) – 2 capsules of 20 mg~40 mg~0.57 mg/kg. The doses will be calculated for animals based on body weight.

Route of administration - peroral as an intended route for use in clinical practice.

Dosing frequency

Duration of dosing – 30 days, interval 24 h

Weighing: at assignment to groups and once weekly for adjustment of doses.

Detection of sensitization reactions

HDT in mice will be assessed in paw edema test conducted in 5 days after the last dose.

HIT in mice will be assessed in ACA reaction in 14-20 days after the last dose.

Hypersensitivity in guinea pigs will be assessed in 14-20 days after the last dose in reaction of general anaphylaxis (anaphylactic shock), conjunctiva test and intracutaneous test.

Table 1. Experimental groups of animals (male/female 1:1)

Animal species	Experiment #, task	Number of animals in group		
		0611 substance 1 TD	**0611 substance 10 TD**	**C1⁻**
Inbred mice Balb/c	**Experiment # 1** Study of 0611 substance ability to cause HDT at peroral administration during 30 days. HDT detection: in 5 days after completion of dosing in paw edema test (with evaluation of HDT IR in 24 h after administration of resolving dose)	20	20	20
	Experiment #2 Study of 0611 substance ability to cause HIT at peroral administration during 30 days. HDT detection: in 14-20 days after completion of dosing in ASA reaction	10	10	10
	Selection of RD for HDT and HIT detection	20		
	Total mice:	**110**		
Albino guinea pigs	**Experiment # 3** Study of 0611 substance ability to cause HIT at peroral administration during 30 days. HDT detection: in 14-20 days after completion of dosing in reaction of general anaphylaxis (anaphylactic shock)	10	10	10
	Experiment # 4 Peroral administration of 0611 substance during 30 days, detection of hypersensitivity in conjunctiva test and at repeated testing in 10 days in intracutaneous test	10	10	10
	Selection of RD	10		
	Total guinea pigs:	**70**		

Table 2. Dose/volume correlation (body weight of mouse 20 g, guinea pig - 330 g)

Species	Substance dose			Suspensions	Route
	(TD)	**(mg/kg)**	**(mg/animal)**		
Mice	10 TD	5.70	0.11	C=5.70 mg/ml	0.02 ml
Mice	1 TD	0.57	0.01	C=0.57 mg/ml	0.02 ml
Guinea pigs	10 TD	5.70	1.90	C=38.1 mg/ml	0.05 ml
Guinea pigs	1 TD	0.57	0.19	C=3.8 mg/ml	0.05 ml

8.1. Clinical observations:

- daily visual inspection of physical state (motor activity, eye mucosa and skin, defecations);
- daily examination for detection of signs of allergic response to the administered substance (anxiety, state of mucous membranes and skin);
- observation of animals while testing to detect sensitization reactions (on the site of RD administration, signs of anaphylactic shock).

8.2. Completion of the experiments, Euthanasia

Upon completion of dosing animals will be euthanized by CO_2 inhalation.

9. 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. Personnel will be informed on the approved amendments to the protocol and their effective date. The protocol changes are signed by Study Director and Customer Representative and attached to all approved copies of the protocol.

10. Deviations

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

11. 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.

12. 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%.

13. Documentation and archive

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

14. Protocol approval

Customer representative:

_____	_____	_____
Name	Signature	Date

Study Director:

_____ _____	_____	_____
Name	Signature	Date

Director of SFES RCT&HRB:

_____	_____	_____
Name	Signature	Date

ANNEX 2

QA report

(will be attached to the hard copy)

ANNEX 3

Table 1. Individual values of HDT IR (paw edema test following peroral administration of Killevir-16 in mice during 30 days)

Substance, dose	FEMALE		MALE	
	Ind#	IR (%)	Ind#	IR (%)
	21	4.67	51	5.08
	22	4.24	52	2.68
	23	2.22	53	2.40
Negative control	24	0.00	54	1.75
	25	3.64	55	1.72
	26	4.35	56	3.33
	27	0.00	57	1.59
	28	2.46	58	3.33
	29	0.00	59	2.61
	30	5.30	60	0.00
	1	2.40	31	2.40
	2	0.00	32	5.65
	3	0.80	33	0.00
Killevir-16 substance	4	1.82	34	4.00
	5	3.39	35	9.38
1 TD	6	2.40	36	2.40
	7	1.69	37	6.92
	8	4.35	38	1.60
	9	0.00	39	2.21
	10	4.17	40	4.39
	11	1.69	41	3.05
	12	0.00	42	7.91
	13	2.65	43	5.60
Killevir-16 substance	14	1.63	44	1.69
	15	1.92	45	7.19
10 TD	16	3.45	46	3.23
	17	1.85	47	6.15
	18	2.61	48	8.33
	19	5.45	49	1.69
	20	0.00	50	2.56