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REPORT
Study of the Killevir preparation substance and medicinal form immunotoxic effect
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LIST OF PROVISIONAL ABBREVIATIONS

APh – activity of a phagocytosis

HDT – hypersensitivity of a delayed type

DMSO – dimethylsulfoxide

IR – index of reaction

Iph – intensity of a phagocytosis

PMPh – peritoneal macrophages

TD – therapeutic dose

ER – erythrocytes of a ram

PAPER

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Key words: preclinical investigations, immunotoxic effect, fullerenepolyaminocaproic acid, medicinal preparation Killevir

The aim of the present investigation is a detection of the killevir preparation substance and two medicinal form immunotoxic effect.

The killevir preparation substance is a fullerenepolyaminocaproic acid. An injectional form of the killevir preparation is a substance solution in a DMSO 3%, the killevir preparation, suppositoria rectal, contains a vitepsol beside a DMSO.

An ability of the first generation mouse hybrid immune system F_1(CBA×C_{57}B1/6) to respond to a non-infectious antigen infusion was assessed at a study of the investigating medicinal preparation immunotoxic effect. The ram erythrocytes, bring the experimental T-dependent test-antigen, most fully modeling the different variants of a foreign agent, were used as an antigen. The levels of a cellular and humoral immune response to the ram erythrocytes were revealed. A functional activity of the mononuclear phagocyte system and a complement level in the blood sera (on the guinea-pigs) were also assessed.

The immune system reactivity was studied after the killevir preparation substance and injectional form infusion – 14-fold intraperitonealy, suppositoria – 14-fold rectally. The investigated level of doses: the presumed in a clinic therapeutic ones (according to a substance content – 0,7 mg/kg for the preparation injectional form and 0,14 mg/kg – for the suppositoria rectal) and 10 times them exceeding.

As a result of the conducted investigation it was established, that the killevir preparation substance (a fullerenepolyaminocaproic acid), injectional form and the suppositoria rectal do not possess of an immunotoxic effect on the mammals. No cases of the studied parameter deviation from the control level were revealed in the experimental animals.

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INTRODUCTIN

The side, dangerous for a heath non-compensated disturbances in a structure and function of the immune system, which can lead to a decrease of the organism resistance to infection, an increase of the oncologic disease risk, a development of the autoimmune pathology, are understood under the pharmacologic preparation immunotoxicity. A detection of such lesions of the immune system in the elaborating pharmacologic preparations is an obligatory stage of their preclinical assessment.

The aim of the present investigation is a detection of the killevir preparation substance and two medicinal form immunotoxic effect. The preparation is presumed to be used as an antiviral, specifically, antiherpetic medicinal preparation.

The killevir substance is a fullerenepolyaminocaproic acid.

The killevir preparation composition, injectional form:

- a fullerenepolyaminocaproic acid – 50 mg,
- a DMSO – 1 ml,
- a water for injections – 2 ml.

The killevir preparation composition, suppositoria rectal:

- a fullerenepolyaminocaproic acid – 10 mg,
- a DMSO – 200 mg,
- a vitepsol – up to 2 g.

The work is accomplished according to the “Methodical instructions on the pharmacologic substance immunotoxic effect assessment” of the RF MPH Pharmacologic committee [1].

1. MATERIALS AND METHODS

The work is made according to the Methodical instructions[1].

Laboratory animals

The experiments are performed on the first generation mice hybrids F_1(CBA×C_{57}B1/6) with a body mass of 25±2 g and on the guinea-pigs with a body mass of 380±40 g. The animals are acquired in the nurseries "Stolbovaya" (mice) and "Kryukovo" (guinea-pigs). The animal maintenance in the TBP SRC vivarium corresponded to the sanitary rules, approved by the USSR MPH on 73.07.06, on arrangement, equipping and maintenance of the experimentally-biological clinics (vivaria) [4].

The rodents were fed with a full-rational mixed feed, a plumbing water was used for drinking. The animals have passed a quarantine and acclimatization in the vivarium conditions during not less than 14 days.

The experimental groups of animals were formed by a method of the random selections, considering a body mass as a leading value.

Preparation of solutions of the investigating pharmacologic preparations

Presented for investigation:

1) the killevir preparation substance - a fullerenepolyaminocaproic acid;
2) the killevir medicinal preparation concentrate, injectional form:

- a fullerenepolyaminocaproic acid – 50 mg,
- a DMSO – 1 ml,
- a water for injections – 2 ml;

3) the killevir medicinal preparation, suppositoria rectal:

- a fullerenepolyaminocaproic acid – 10 mg,
- a DMSO – 200 mg,
- a vitepsol – up to 2 g.

The substance and medicinal form solutions for infusion were prepared and packaged according to the rules of antiseptics and aseptics, in a laminar box at a vertical flow of a sterile air.

Substance

A concentrate of a substance solution was thus prepared in a DMSO, that a rated dose can be infused into a DMSO 3% at a further dilution in a physiologic salt solution.

Medicinal preparation for the tests in a clinic therapeutic dose of the killevir preparation is 0,7 mg/kg, according to a substance content (1 TD); and a dose, 10-times its exceeding, is 7 mg/kg (10 TD).

The concentrate (3 ml) was dissolved in 30 ml of a water for injections before infusion, the received solution is a solution A.

10 TD:

- At a mouse body mass of 20 g, a dose is 0,14 mg/specimen, according to a substance content. 0,14 mg of a substance is contained in 0,08 ml of the Solution A. The Solution A was infused by 0,08 ml into each specimen at a body mass of 20 g.
- At a guinea-pig body mass of 330 g, a dose is 2,3 mg/specimen (according to a substance content). 2,3 mg of a substance are contained in 1,4 ml of the Solution A. The Solution A 1,4 ml was infused into each specimen at a body mass of 330 g.

1 TD:

- At a mouse body mass of 20 g, a dose is 0,014 mg/specimen, according to a substance content. The Solution B was received by a 10-fold dilution of the Solution A with a DMSO 3%. The solution B contains 0,014 mg of a substance in 0,08 ml. The Solution B was infused by 0,08 ml into each specimen at a body mass of 20 g.
- At a guinea-pig body mass of 330 g, a dose is 0,23 mg/specimen, according to a substance content. 0,23 mg of a substance is contained in 0,14 ml of the Solution A. The Solution A was infused by 0,14 ml into each specimen at a body mass of 330 g.

Medicinal preparation Killevir, suppositoria rectal

The presumed for the tests in a clinic therapeutic dose of the Killevir preparation in the suppositoria rectal, according to a substance content: 1 TD - 0,14 mg/kg and a dose 10-times exceeding it, 10 TD - 1,4 mg/kg.

10 TD:

- At a mouse body mass of 20 g, a dose is 0,028 mg/specimen, according to a substance content, the pointed dose is contained in 5,6 mkl of a medicinal form. The killevir preparation was infused by 6,0 mkl into each specimen at a body mass of 20 g.
- At a guinea-pig body mass of 330 g, a dose is 0,467 mg/specimen, according to a substance content, the pointed dose is contained in 93 mkl of a medicinal form. The killevir preparation was infused by 95 mkl into each specimen at a body mass of 330 g.

1 TD:

- At a mouse body mass of 20 g, a dose is 0,0028 mg/specimen, according to a substance content, the pointed dose is contained in 0,56 mkl of a medicinal form. A medicinal form with a use of placebo for a rectal form was 10-times diluted (Dilution C), the Dilution C was infused by 6,0 mkl into each specimen at a body mass of 20 g.
- At a guinea-pig body mass of 330 g, a dose is 0,047 mg/specimen, according to a substance content, the pointed dose is contained in 9,3 mkl of a medicinal form. A medicinal form with a use of placebo for a rectal form was 10-times diluted (Dilution C), the Dilution C was infused by 95 mkl into each specimen at a body mass of 330 g.

Placebo

1) A placebo for injectional form is a DMSO 3% solution, prepared on water for injections. It was infused by 0,08 ml/specimen at a body mass of 20 g (mice) or by 1,4 ml at a body mass of 330 g (guinea-pigs).
2) A placebo for suppositoria:
   - a DMSO – 200 mg,
   - a vitepsol – up to 2 g.

It was infused by 6,0 mkl/specimen at a body mass of 20 g (mice) or by 95 mkl/specimen at a body mass of 330 g (guinea-pigs).

Assessment of the medicinal preparation effect on the cellular immune response

The first generation mice hybrids F_1(CBA×C_{57}B1/6) were once intraperitonealy immunized with the ER suspension in a dose of 2×10^6 of cells in a physiologic salt solution after the investigating medicinal preparation last infusion.

The cellular immunity was assessed in 5 days after immunization in a test of a mouse paw edema, revealing a HDT in response to the antigen resolving dose infusion – 0,5-1×10^8 of erythrocytes in 0,05 ml of a physiologic salt solution. A HDT reaction was assessed in 24 hours, calculating the index of reaction (IR) according to the difference in weight of the experimental (R_{ex}) and control (R_c) paws according to the formula:

$$IR = \frac{R_{ex} - R_c}{R_c} \times 100$$

The meanings of the IR values in the experimental and control groups were compared.

Assessment of the medicinal preparation effect on the humoral immune response

The first generation mice hybrids F_1(CBA×C_{57}B1/6) were once intraperitonealy immunized with the ER suspension in a dose of 1×10^8 of cells in a physiologic salt solution after the investigating medicinal preparation last infusion. A humoral immune response was revealed in 7 days according to a level of the hematogglutinins in the experimental animal blood sera.

A hemagglutination reaction was conducted in the microplanes, in the rows of the serum two-fold dilutions, using a 2% suspension of the ER in a physiologic salt solution. A difference of the hemagglutinin titers in the groups of mice, receiving the investigating medicinal preparations, and in the control groups was assessed.

Assessment of the mononuclear phagocyte system cell phagocytic activity values

The guinea-pig PMPh, excreted after the testing medicinal preparation effect, were used for assessment of the mononuclear phagocyte system cell phagocytic activity.

The cells of a daily culture Staphylococcus aureus, inactivated by heating, were used as an object of phagocytosis. The macrophage monolayer was received on a covering glass, cultivating the cells of a peritoneal exudate, resuspended in a medium 199 (1×10^6 kl/ml), at 37°C during 60 min. A phagocytosis has been passing during 40 min. after an addition of the S. aureus suspension to a cultivation medium in a ratio of microbic cell: macrophage = 50:1. Then the PMPh monolayer was 5-times washed off the remaining bacteria in a cultural medium, fixed with the ethanol 96°, stained with the azur-eosin and observed under an immersional system of a luminous microscope. Not less than 100 macrophages were observed in each cytologic preparation.

An activity of a phagocytosis (APh, percent of the macrophages, absorbing the bacteria) and intensity of a phagocytosis (IPh, average number of bacteria in a phagocyte) were calculated.

Assessment of a complement level in the blood sera

A complement level in the guinea-pig blood sera was assessed in a test-system with a hemolytic serum, was added to the microplane small cavities with the investigating blood serum dilutions. The erythrocyte hemolysis has been assessed visually during an hour after the microplane exposure at 37°C. The last (in the two-fold row) serum dilution, causing a full hemolysis of the erythrocytes, was taken for a complement titer in the investigating serum.

Statistical treatment of the data

A statistical treatment of the received experimental data was made with a use of the Student's criterium [3].

2. RESULTS OF INVESTIGATIONS

An immunotoxic effect of the killevir preparation substance and two medicinal forms was studied on two types of the laboratory animals (mice and guinea-pigs) at a 14-fold rectal and intraperitonealy infusion.

The investigated doses are selected according to the presumed therapeutic doses of the killevir preparation: for an injectional form – 0,7 mg/kg (according to a substance content), for the suppositoria rectal – 0,14 mg/kg (according to a substance content).

An assessment of the immune system state was made in the recommended tests [2] on a termination of the testing medicinal preparation infusion in a therapeutic dose and in a dose, 10-times exceeding it.

2.1. STUDY OF THE KILLEVIR PREPARATION SUBSTANCE AND TWO MEDICINAL FORM EFFECT ON THE IMMUNE RESPONSE AGAINST THE RAM ERYTHROCYTES

The investigation is conducted on the females of the first generation mice hybrids F_1(CBA×C_{57}B1/6).

A substance and injectional form of the killevir preparation were 14-fold intraperitonealy infused into mice in doses of 0,70 mg/kg and 7,00 mg/kg, according to a substance content. The killevir preparation, suppositoria rectal, was 14-fold rectally infused into mice in doses of 0,14 mg/kg and 1,40 mg/kg (according to a substance content). The control groups №1 are presented by the animals, receiving placebo: a DMSO 3% - for a substance and injectional medicinal form, a vitepsol in a DMSO – for the suppositoria, the control groups №2 are presented by the intact animals. By 20 animals were taken to each experimental and control groups. The mice of the experimental and control groups were once intraperitonealy immunized with the ram erythrocytes after the medicinal preparation last infusion.

The cellular immune response was assessed in a HDT reaction according to a size of a mouse paw edema in response to the antigen resolving dose infusion.

The ER immunizing dose for the groups of mice, designed for detection of the cellular immune response, containing 2×10^6 of cells in 0,5 ml of the physiologic salt solution, the ER resolving dose – $0,5\times10^8$ of cells in 0,05 ml of the physiologic salt solution were infused in 5 days after immunization, the index of reaction (IR) was detected in 24 hours.

As a result of the conducted investigation it is established, that in a group of mice, undergoing an effect of each of the testing medicinal preparations in a dose, 10-times exceeding the therapeutic one, the HDT IR meanings in the control groups №2 (Table 1). The same data was received for a group of mice, receiving the killevir preparation injectional form in a therapeutic dose. At a comparison of the HDT IR pointed meanings with the control groups №1 (Table 1), a difference was unreliable. In such cases they say of the alterations, stipulated by a stressor effect of an infusion durative course and not of an effect of the medicinal preparation itself.

Thus, the investigating medicinal preparations in the presumed for a use in a clinic therapeutic doses and in doses, 10-times exceeding them, did not have an effect on the cellular immune response of the mice against the ram erythrocytes.

Table 1.

Assessment of the cellular immune response to the ram erythrocytes

Medicinal preparation		ER, dose/specimen		HDT IR, %, M±m
Name	Dose, mg/kg	immunizing	resolving	
Substance	0,70	2×10^6	$0,5\times10^8$	32,64±2,26
	7,00	2×10^6	$0,5\times10^8$	31,93±1,37*
	Control №1 (placebo)	2×10^6	$0,5\times10^8$	27,39±1,61
	Control №2 (intact)	2×10^6	$0,5\times10^8$	25,90±1,36
Killevir (injectional form)	0,70	2×10^6	$0,5\times10^8$	34,61±2,97*
	7,00	2×10^6	$0,5\times10^8$	34,81±1,71*
	Control №1 (placebo)	2×10^6	$0,5\times10^8$	28,57±2,62
	Control №2 (intact)	2×10^6	$0,5\times10^8$	25,90±1,36
Killevir (suppositoria rectal)	0,14	2×10^6	$0,5\times10^8$	25,94±2,26
	1,40	2×10^6	$0,5\times10^8$	32,64±3,01*
	Control №1 (placebo)	2×10^6	$0,5\times10^8$	25,67±2,62
	Control №2 (intact)	2×10^6	$0,5\times10^8$	20,34±1,43

*Note: trustworthy higher than the control level №2.

The humoral immune response was assessed according to a level of the hemegglutinins in the blood sera, received in 7 days after immunization. The

immunizing dose for the mouse groups, designed for the humoral immune respons detection, was 1×10^8 of the ER in a volume of 0,5 ml.

The received results are presented in the hemagglutinin titer $\log_2$ (Table 2). The antibody level to the ER in the mouse groups, undergoing the investigating medicinal preparation effect, did not differ from the meanings in the control groups.

Table 2

Assessment of the humoral immune response to the ram erythrocytes

Medicinal preparation		Immunizing	$\log_2$ of a serum titer, M±m
Name	Dose, mg/kg		
Substance	0,70	1×10^8	8,82±0,17
	7,00	1×10^8	8,52±0,25
	Control №1 (placebo)	1×10^8	8,72±0,22
	Control №2 (intact)	1×10^8	8,70±0,23
Killevir (injectional form)	0,70	1×10^8	7,82±0,43
	7,00	1×10^8	7,42±0,35
	Control №1 (placebo)	1×10^8	7,82±0,34
	Control №2 (intact)	1×10^8	7,58±0,42
Killevir (suppositoria rectal)	0,14	1×10^8	9,12±0,25
	1,40	1×10^8	9,62±0,15
	Control №1 (placebo)	1×10^8	9,52±0,13
	Control №2 (intact)	1×10^8	9,28±0,21

The killevir preparation substance and both medicinal forms in the therapeutic doses, presumed for a use in a clinic, and in doses, 10-times exceeding them, did not influence on a level of the mouse humoral immune response to the ram erythrocytes.

It is not necessary to assess the immune status in 21 days after the medicinal preparation infusion cessation, because no alterations in the levels of the cellular and humoral immune response to the ram erythrocytes, compared to the control, were noted at the investigating medicinal preparation infusion.

2.2. STUDY OF THE KILLEVIR PREPARATION SUBSTANCE AND TWO MEDICINAL FORM EFFECT ON A PHARMACOLOGIC ACTIVITY OF THE MONONUCLEAR PHAGOCYTE SYSTEM CELLS

An investigation is made on the guinea-pigs. The killevir preparation substance and injectional form were 14-fold intraperitonealy infused into the

guinea-pigs in doses of 0,70 mg/kg and 7,00 mg/kg, according to a substance content. The killevir preparation in the suppositoria was 14-fold rectally infused into the guinea-pigs in doses of 0,14 mg/kg and 1,40 mg/kg, according to a substance content. The control groups are presented by the animals, receiving a placebo: a DMSO 3% - for a substance and injectional medicinal form, a vitepsol in a DMSO – for the suppositoria. By 10 animals were taken to each experimental and control groups.

The peritoneal macrophages were excreted after the last infusion of a medicinal preparation, a phagocytic activity was assessed in vitro relating to the killed with a heating cells Staphylococcus aureus. An activity and intensity of a phagocytosis were calculated.

As a result of the conducted investigation, no trustworthy differences in meanings of the macrophage phagocytic activity values in animals from the experiment and control were revealed (Table 3). An absorbtive ability of the mononuclear phagocyte system cells after a 14-fold infusion of the investigating medicinal preparations was not altered.

The killevir preparation substance and both medicinal forms in the therapeutic doses, presumed for a use in a clinic and in doses, 10-times them exceeding, did not influence a functional activity of the macrophages.

Table 3

Assessment of the guinea-pig peritoneal macrophage phagocytic activity

Medicinal preparation		APh (%), M±m	IPh (bacterial cells), M±m
Name	Dose, mg/kg		
Substance	0,70	86,0±2,5	7,2±0,8
	7,00	85,3±1,8	7,7±1,1
	Control (placebo)	87,5±2,1	7,3±1,4
Killevir (injectional form)	0,70	83,2±2,4	6,9±0,6
	7,00	85,8±2,4	7,2±0,9
	Control (placebo)	84,3±2,7	7,2±1,2
Killevir (suppositoria rectal)	0,14	91,3±2,2	7,7±1,1
	1,40	87,4±2,0	8,0±1,1
	Control (placebo)	88,5±2,8	7,8±1,3

2.3. STUDY OF THE KILLEVIR SUBSTANCE AND TWO MEDICINAL FORM EFFECY ON A COMPLEMENT LEVEL IN THE GUINEA-PIG BLOOD SERA

An investigation is made on the guinea-pigs. The killevir preparation substance and injection form were 14-fold intraperitonealy infused into the guinea-pigs in doses of 0,70 mg/kg and 7,00 mg/kg (according to a substance content). The killevir preparation in suppositoria was 14-fold rectally infused into the guinea-pigs in doses of 0,14 mg/kg and 1,40 mg/kg, according to a substance content. The control groups are presented by the animals, receiving a placebo: a DMSO 3% - for a substance and injectional medicinal form, a vitepsol in a DMSO

– for the suppositoria. By 10 animals were taken to each experimental and control groups.

A blood was taken from an otic vein of the guinea-pigs at the following day after the medicinal preparation last infusion, a complement level was revealed in a test system with a hemolytic serum. A reaction was assessed visually, the last dilution of a serum (in the two-fold row), causing a full hemolysis of the erythrocytes, was taken for a complement titer.

The present in a Table 4 data testifies to a lack of alterations in a complement level in the guinea-pigs after a 14-fold infusion of the investigating medicinal preparations in a therapeutic dose and a dose, 10-times its exceeding. The killevir preparation substance and both medicinal forms in the therapeutic doses, proposed for a use in a clinic, and in doses, 10-times them exceeding, did not influence a functional activity of the macrophages.

Table 4

Assessment of a complement level in the guinea-pig blood sera

Medicinal preparation		Complement level (log_2 of a titer), M±m
Name	Dose, mg/kg	
Substance	0,70	5,60±0,37
	7,00	5,60±0,37
	Control (placebo)	5,50±0,51
Killevir (injectional form)	0,70	5,80±0,56
	7,00	6,20±0,45
	Control (placebo)	5,70±0,48
Killevir (suppositoria rectal)	0,14	5,70±0,35
	1,40	5,90±0,41
	Control (placebo)	5,60±0,37

CONCLUSION

The side, dangerous for a health non-compensated disturbances in a structure and function of the immune system, which can lead to a decrease of the organism resistance to infection, an increase of the oncologic disease risk, a development of the autoimmune pathology, are understood under the pharmacologic preparation immunotoxicity. The main task of the potential medicinal preparation immunotoxicity preclinical control consists in that, that in experiments on animals to prove or exclude a possibility of the immune system reaction undesirable consequences, caused by the medicinal preparations or their metabolites.

The aim of the present investigation is a detection of the killevir preparation substance and two medicinal form immunotoxic effect.

The killevir preparation substance is a fullerenepolyaminocaproic acid.

A composition of the killevir preparation, injectional form:

- a fullerenepolyaminocaproic acid – 50 mg,
- a DMSO – 1 ml,

- a water for injections –2 ml.

A composition of the killevir preparation, suppositoria rectal:

- a fullerenepolyaminocaproic acid – 10 mg,
- a DMSO – 200 mg,
- a vitepsol – up to 2 g.

An ability of the first generation mouse hybrid immune system F_1(CBA×C_{57}B1/6) to respond to the non-infectious antigen infusion was assessed at a study of the investigating medicinal preparation immunotoxic effect. That process, in which all the main cells of the immune system are participating in a cooperative interaction, includes the immune reaction main stages (phagocytosis, recognition, proliferation, synthesis of the non-specific antibodies and etc.) The ram erythrocytes, being the experimental T-dependent test-antigen, most fully modeling the different variants of a foreign agent, were used as an antigen. The levels of the cellular and humoral immune response to the ram erythrocytes were revealed. A functional activity of the mononuclear phagocyte system and a complement level in the blood sera on the guinea-pigs were also assessed.

A reactivity of the immune system was studied after the killevir preparation substance and injectional form 14-fold, intraperitonealy infusion, the killevir preparation in suppositoria – the 14-fold rectal infusion. The investigated level of doses: the presumed in a clinic therapeutic ones, according to a substance content, are 0,7 mg/kg for the preparation injectional form and 0,14 mg/kg – for the suppositoria rectal and 10-times them exceeding.

As a result of the conducted investigation, it was established, that the killevir preparation substance and both medicinal forms: injectional one and suppositoria rectal do not possess of an immunotoxic effect on the mammals. No cases of the studied parameter deviation from the control level are revealed in the experimental animals.

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