RUSSIAN MINISTRY OF HEALTH

RESEARCH INSTITUTE FOR INFLUENZA

(FSFI RII)

APPROVED

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«___» _____2015

REPORT

Study of fullerene polyaminocaproic acid substance effects on reproductive function of rats at peroral administration

Non-clinical study- LBL/RT-026/15

Sponsor: ZAO "Intelpharm"

Legal address: Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, Russia

Postal address: Kostin str. 4, Nizhny Novgorod, Russia

Testing facility:

Federal State-Financed Institution "Research Institute for Influenza"

Ministry of Health of the Russian Federation

Legal address: Professor Popov str. 15/17, St. Petersburg, 197376, Russia

Study Director:

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SUMMARY

Report: 19 pages, 5 tables, 8 references, 1 annex.

SUBSTANCE, REPRODUCTIVE TOXICITY, PERORAL ADMINISTRATION, RATS.

The study objective was assessment of reproductive toxicity (character and intensity of damaging effect at the stage of progenesis) of fullerene polyaminocaproic acid substance (ZAO «Intelpharm», Russia) at peroral administration in rats.

It was demonstrated that daily repeated peroral administration of the substance during 15 (females) and 48 (males) days in dose 67 mg/kg did not cause the development of noticeable toxic effects on reproductive function of animals. During the dosing period no decrease in animal body mass gain, changes in behavior, or animal deaths in experimental groups (males or females) were noted. Pregnancy index was not reliably different in all experimental groups. No malfunctions in reproductive system of male и female rats were found.

CONTENTS

GUIDANCE DOCUMENTS

– Federal Law of April 12 2010 #61 “On circulation of therapeutics”;
– Rules for Laboratory Practice in the Russian Federation” (Order of Russian Ministry of Health #708n of August 23, 2010);
– The Russian Federation Standard (GOST 53434-2009 of 02.12.2009) “GLP Principles of Laboratory Practice”, Moscow, Standartinform, 2010;
– “Laboratory animals” (RAMS Guidance, Moscow, 2003);
– Guidance on conducting non-clinical trials of therapeutics (Immunobiological drugs), Part II: - Grif &Co, Moscow, 2012. P. 536;
– Guide for the care and use of laboratory animals, FELASA, 2010;
– Sanitary rules on designing, equipment and maintenance of experimental-biological clinics (vivaria) SP 2.2.1.3218-14 RF, approved August 29, 2014, № 51.

ABBREVIATIONS

RT — reproductive toxicity
FPACA — Fullerene polyaminocaproic acid

INTRODUCTION

Study title

Study of reproductive toxicity of fullerene polyaminocaproic acid substance produced by ZAO «Intelpharm» at peroral administration in rats.

Study objective

Assessment of fullerene polyaminocaproic acid substance effects on reproductive function at intragastrical administration in female white rats during 15 days before mating and in male rats during 48 days before mating in doses 59 times exceeding maximal daily dose for humans.

Study tasks

- determine the number of pregnant females, number of yellow bodies, number of implants; number of live fetuses; number of resorptions;
- determine the number of offspring; deaths of infant rats in the period of feeding; number of male and female infant rats; body mass of infant rats at birth; the days of ear auricle detachment, first hair, tooth eruption, eye opening, drop of testicles, vagina opening, and body mass of infant rats during 30 days of observation.

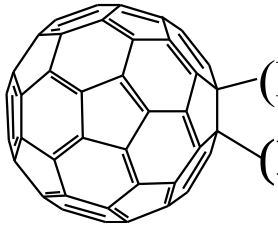
Study subject

Fullerene polyaminocaproic acid substance, batch 16SB (for non-clinical trials).

Chemical name:

N-fullerene-poly-6-aminocaproic acid

Structural formula



(H)

$(NH(CH_2)_5COOH)_3 \cdot (NH_2(CH_2)_5COOH)_3$

N- fullerene poly-amino-caproic acid is a mixture of position isomers of covalently bound amino acid groups, in which polar groups of amino acid fragments are coordinated on fullerene-amino acid derivatives with formation of either ion bounds between carboxyl group of fullerene amino acid and ammonium group, (NH_3^+-), amino acids, or hydrogen bounds of L-COO ---H----NH2-L' type. Composition of the product corresponds to fullerene: coordinated amino acid ratio 1:6.

Empirical formula

$C_{96}H_{72}O_{12}N_6$

Molecular mass 1500 +(nH_2O)

Description

The substance is amorphous brown or dark brown powder.

Physical properties

Decomposition temperature without melting - 200^{o} C

Solubility:

- freely soluble in dimethyl sulfoxide (DMSO)
- soluble in dimethyl formamide (10 mg of substance in 10 ml of DMF)
- almost insoluble in water, 95% ethyl alcohol, and 1,2-dichlorbenzene
- the best mixture for dissolving is DMSO – H2O (1:10), DMFA-H2O (1:10), ethanolamine, polyethylene glycol. The substance is dissolved in polar solvents due to the carboxyl group reduction in the association. That is why alkali salts, ammonium salts, and salts of organic amines (for example, Tris-amine) of fullerene-amino acids (FAA) have considerable higher water solubility (Na-FAA solubility is about 50 mg/ml). The solutions are bright red-brown. For the analysis buffer solution with pH = 8.9 ("boronic" buffer) can be used.

pH: 5.2

The substance characteristics

The data characterizing Fullerene polyaminocaproic acid substance (production, purification, composition, stability) can be provided by the Sponsor.

Planned dosing regimen of the substance as a component of the preparation in the form of tablets for clinical use

Killevir preparation, sublingual tablets 20 mg (by the substance content).

In HIV infection therapy - 2 tablets twice daily.

Pharmaco-therapeutic group

Antiviral preparations with systemic action.

Pharmacological effect

The studies of FPACA mechanism of antiviral action on HIV-1, HIV-2 demonstrated inhibition of joining, confluence and entrance at the same time.

In in vitro studies Fullerene polyaminocaproic acid demonstrated activity against all wild HIV strains. Average EC_{50} was 0.6 μg/ml, average EC_{95} was 1.33 μg/ml. No cytotoxicity was revealed when used in concentrations up to 100 μg/ml.

Test substance producer:

ZAO «Intelpharm»

Legal address: Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia

Sponsor:

ZAO «Intelpharm»

Legal address: Pushkin str. 36, Nizhegorodskaya region, Chkalovsk, 606540, Russia

Testing facility

FSFI "Research Institute for Influenza"

Ministry of Health of the Russian Federation

Legal address: Professor Popov str. 15/17, St. Petersburg, 197376, Russia

Study design, dose substantiation, routes of administration

are described in Study Plan #LBL/RT-026/15 (Annex)**.**

Study procedures

During the study all procedures complied with the approved written protocol and SOPs developed by the laboratory for therapeutic safety. Test substance was administered using the route of administration planned to be used in clinical practice. Upon receive of the test substance Pharmaceutical group kept records on its consumption. All manipulations with test substance were compliant with the safety rules.

Data storage

All raw data and a properly certified copy of the Non-clinical Study Protocol, study reports, conclusion of Bioethics Committee and Quality Assurance group are stored in a specially allocated room. Storage life is specified by the local act of the organization conducting the study.

Terms of the Study

Study initiation date – December 23, 2014

First dose date – February 26, 2015

Clinical observation completion date - June 11, 2015

1 MATERIALS AND METHODS

1.1 Animal test system and its handling

Outbred white rats (male and female).

1.1.1 Source, date of purchase and complementary documents

Veterinary certificate 247 № 0138906 of 18.02.2015 Animal Nursery "Rappolovo" RAS. Before shipping animals were under quarantine during 30 days. In this period animal material was tested at Federal State-Financed Institution "Leningrad Interregional Veterinary Laboratory".

1.1.2 Humane treatment and use of test animals

Laboratory animals were handled in compliance with "Sanitary rules on construction, equipping and maintenance of experimental biological clinics (vivariums)" SP 2.2.1.3218-14 RF, approved August 29, 2014, № 51", "Guide for the care and use of laboratory animals, FELASA, 2010; "Laboratory animals" (RAMS Guidance, Moscow, 2003).

All animal procedures were considered and approved by Bioethics Committee of FSFI "Research Institute for Influenza" of Russian Ministry of Health (Protocol #28 of January 6, 2015).

1.1.3 Quarantine and adaptation at FSFI "Research Institute for Influenza"

Duration of quarantine (acclimatization period) for all animals was 7 days. During that period, the health status of animals was daily evaluated (behavior, general health state, sickness and death).

1.1.4 Number of animals

Experimental and control groups had 20 female and 10 male rats.

For mating, to experimental and control females or males intact animals were added (1 male for 2 female rats) for no longer than 2 weeks; in total 80 female and 40 male rats. This number of animals was sufficient for complete description of the test effects and statistical processing of the obtained data.

1.1.5 Weight and age of animals

Rats: 180-200 g, age 12-18 weeks

1.1.6 Handling

Animals were housed in vivarium rooms at FSFI "Research Institute for Influenza" under controlled environmental conditions - temperature and humidity - at 12-hour light/dark cycle throughout the whole period of the study. Animals were handled and cared in accordance with SOP.

1.1.7 Cages

Animals were kept in cages, 3 in a cage (mating), cage type IV (floor square – 1815 cm^2). Females got pregnant were kept in individual cages. The cages had steel lattice covers with deepening, steel dispensers for feed and water.

1.1.8 Bedding

For bedding a natural granulated material made of one-year vegetable cultures,

Rehofix®, (J.Rettenmaier & SÖHNE GmbH+CO, Germany) was used.

1.1.9 Diet

Animals were given *ad libitum* a full-ration diet, formulation #PK-120-2_173 (OOO "Laboratorkorm", Moscow). No contaminants were known to be present in the diet that could interfere with the results of the study.

1.1.10 Water. Animals were given *ad libitum* pure (filtered) drinking water. No contaminants were known to be present in the water that could interfere with the results of the study.

1.1.11 Acclimatization

All animals were temporarily kept in special rooms and acclimated to laboratory conditions for 7 days prior to the start of dosing. During that period, the health status of the animals was daily visually evaluated. Animals with deviations detected during the inspection were not used in the experiment.

1.1.12 Assigning to groups

Animals were assigned to groups at random based on body weight. No animal was considered for assignment if out of the ±10% range from mean body weight for males and females.

1.1.13 Identification

All animals in group were identified by individual numbers indicated on the cage label. The animals were marked using biological paints.

1.1.14 Moribund animals and animals died during the study

If the animal died during the study it was critical to most accurately determine the time of death. Dead body was weighed and necropsy was performed immediately to specify the cause of the death. If it was impossible at the moment, the dead body was placed in a fridge at +2- +8°C for no more than 12 hours.

Animals found in agony were weighed and after euthanasia were necropsied. The moribund animal was euthanized by decision of the Study director after consulting with veterinarian.

Final report contains the conclusions on causes of deaths of the animals died during the study or subjected to unplanned euthanasia.

1.1.15 Euthanasia

On day 20 of pregnancy half of female rats from each group were sacrificed by one-step cervical dislocation. After necropsy the number of yellow bodies, live and dead fetuses and pre- and post-implantation mortality were evaluated in accordance with the "Guidance …, 2012".

All procedures with animals were considered and approved by Bioethics Committee of FSFI “Research Institute for Influenza” of Russian Ministry of Health for compliance with the guidance documents.

1.2 Test parameters

Criteria of reproductive toxicity assessment were as follows:

- number of pregnant females;
- body mass of pregnant females;
- number of yellow bodies;
- number of live fetuses;
- number of offspring;
- number of live and dead newborns;
- pre-implantation mortality;
- post-implantation mortality;
- number of infant males и femals;
- deaths of infant rats during the feeding period;
- body mass of infant rats;
- days of ear auricle detachment, first hair, tooth eruption, eye opening, drop of testicles, and vagina opening.

1.2.1 Lifetime observation

1.2.1.1 Appearance, behavioral reactions, death of animals

Health status of animals was examined twice daily. The number of dead animals and time of death were recorded. General state of animals, their behavior, character and motive activity were examined.

1.2.1.2 Body mass

Females were weighed on day 1, 7, 14 and 20 of pregnancy. Infant rats were weighed after birth, on day 4, 7, 14 and 21 of life. The accuracy of balance was verified before the experiment. Each animal was weighed prior to the study and once weekly during the experiment.

1.2.2 Autopsy

Half of females from each group were necropsied on day 20 of pregnancy. Numbers of yellow bodies in testicles, number of implants, dead and live fetuses were counted. All data obtained were recorded in logs and protocols.

1.3 Raw data and statistical processing

Raw data were processed using MSExcel 2010 for their further accumulation and preparation for the analysis. Statistical processing of results was performed using IBM SPSS Statistics software, version 21. Statistic characteristics of groups were mean values, standard errors of mean values, medians and interquartile range, frequencies and sampling sizes. For comparison of quantitative parameters of FPACA-treated and control groups non-parametrical Mann-Whitney U criterion and parametrical Student’s t-criterion were used depending on the distribution type. Normality of distribution was checked using Shapiro–

Wilk test. Equality of variances was evaluated by Levene's test. To compare parameters Fisher's exact test was used. Accepted critical significance level was 0.05. The data in tables in the report were given as median (M) and median error (±m).

1.4 Archive

All study-related data and documents (Non-clinical Study Plan, amendments and deviations, report, materials of Quality Assurance Group, conclusion of Bioethics Committee, veterinary certificate, diet certificate, all raw data, a copy of the contract for conducting non-clinical study) are collected in a dossier. All documents and a specimen of the test substance were passed to the FSFI “Research Institute for Influenza” archive and stored for 5 years. After 5 years of storage the documents will be destroyed.

1.5 Quality Assurance

The study was conducted under internal quality control. Observance of SOPs during the studies was a responsibility of the Study Director.

Quality Assurance Group inspected the Study plan, key phases of the study, raw data, and the study report. Quality Assurance Group report is available at the archive of FSFI “Research Institute for Influenza” of the Russian Ministry of Health.

2 STUDY RESULTS

2.1 The effect of FPACA substance on reproductive function

Results of FPACA substance reproductive toxicity study are presented in Tables 1-3.

1 The effect of FPACA substance on fertility of rats

Parameters	**Experimental groups**			
	Administered in female rats		**Administered in male rats**	
	placebo	**FPACA**	**placebo**	**FPACA**
Number of females placed with males	20	20	20	20
Number of impregnated females	20	20	20	20
Number of pregnant females	17	16	17	17
Pregnancy index, %	85.0	80.0	90.0	85.0
No reliable differences between experimental and control values are found ($p \geq 0.05$)				

In experimental groups a sufficient number of impregnated females were noted. Pregnancy index was within physiological limits for the given animal species and sex as well as for the season of the experiment.

The data presented demonstrate the absence of toxic effects of FPACA substance on fertility of male and female rats at the stage of progenesis.

Table 2 The effects of FPACA substance on dynamics of pregnant rats mass (M±m)

Date	**Experimental groups**			
	Administered in female rats		**Administered in male rats**	
	placebo	**FPACA**	**placebo**	**FPACA**
Background	208.41±2.80	209.94±2.30	206.00±8.57	233.06±4.27
Day 7	222.06±3.81	215.44±3.08	273.35±6.52	250.59±5.58
Day 14	249.77±4.40	251.69±3.10	264.59±7.59	280.94±6.35
Day 19	292.94±6.83	271.19±4.79*	293.06±5.42	301.06±8.28
* - Differences between mean values in the experiment and control are reliable at $p<0.05$				

Analysis of data from Table 2 shows that pregnant female rats in all experimental groups had positive dynamics in body mass gain. Slight but reliable difference in body mass gain on day 19 in group of experimental animals dosed with FPACA compared to control ($p=0.023$ by t-test for equal dispersions) was noted.

In group of female rats fertilized by males dosed with test substance no differences in dynamics of body mass were reveled ($p \geq 0.05$).

Table 3 The effects of FPACA substance on reproductive function of rats (M±m)

Parameters	**Experimental groups**			
	Administered in female rats		**Administered in male rats**	
	placebo	**FPACA**	**placebo**	**FPACA**
Number of yellow bodies	9.92±0.61	9.92±0.61	9.50±0.91	8.57±0.92
Number of live fetuses	9.00±0.71	9.25±0.61	9.00±0.87	8.00±0.93
Number of dead fetuses	0	0	0	0
Number of resorptions	0.50±0.20	0.08±0.08	0.13±0.13	0.43±0.20
Pre-implantation deaths	4.86±1.84	5.64±2.14	3.75±2.63	3.57±3.57
Post-implantation deaths	6.99±2.71	1.39±1.39	1.14±1.14	4.60±2.17
No reliable differences between experimental and control values are found ($p \geq 0.05$)				

From Table 3 it follows that FPACA substance did not have negative effects on reproductive function of both female and male rats. The number of dead fetuses and resorptions as well as pre- and post-implantation mortality did not considerably differ in groups after dosing with placebo and test substance in male and female rats ($p \geq 0.05$).

The absence of reliable differences in test parameters between experimental groups of animals shows that FPACA substance does not have effect on reproductive ability of male and female rats at administration in maximal dose 67 mg/kg.

2.2 Study of the substance effects on the offspring development

Results of the study of the substance effects on development of offspring from FPACA-treated parents are given in Tables 4-5.

Table 4 Effects of FPACA substance on development of offspring (females, M±m)

Parameters	**Experimental groups**	
	Administered in female rats	
	placebo	**FPACA**
Number of litters	7	9
Average number of infant rats	7.57±0.89	9.11±0.92
Including live	7.29±0.78	8.89±1.05
Deaths of infant rats in the period of feeding, abs. %	 2 3.77%	 2 2.44%
Average number of females	2.71±0.52	3.22±0.49
Average number of males	4.57±0.37	5.78±0.68
Sex ratio (F/M)	0.59	0.56
Day of ear auricle detachment	2±0	2±0
First hair	4±0	4±0
Day of tooth eruption	9.57±0.14	9.56±0.11
Day of eye opening	14.80±0.09	14.59±0.09
Day of drop of testicles	24.09±0.20	23.55±0.16
Day of vagina opening	34.63±0.29	33.86±0.26
Body mass of infant rats (g)		
on day 1	6.22±0.08	5.93±0.07
on day 4	12.92±0.31	11.31±0.26
on day 7	19.07±0.29	18.11±0.27
on day 14	33.98±0.52	30.94±0.47
on day 21	56.93±0.83	55.38±0.92
Death of females in the period of feeding	0	0
No reliable differences between experimental and control values are found ($p \geq 0.05$)		

Analysis of the data obtained demonstrated the absence of reliable differences in the values of test parameters in experimental and control animals. Mortality of infant rats during the period of feeding, sex ratio, time of sense organs maturation and dynamics of body weight gain did not considerably differ. Statistical differences between infant rats born from females dosed with placebo and the test substance were not revealed ($p \geq 0.05$).

Table 5 FPACA substance effects on development of offspring (male rats, M±m)

Parameters	**Experimental groups**	
	Administered in male rats	
	placebo	**FPACA**
Number of litters	9	10
Average number of infant rats	10.11±0.66	10.10±0.72
including live	10.11±0.65	9.30±0.63
Deaths of infant rats in the period of feeding, abs. %	 1 1.08%	 2 1.98%
Average number of females	3.89±0.68	4.40±0.72
Average number of males	6.33±0.75	5.70±0.33
Sex ratio (F/M)	0.61	0.77
Day of ear auricle detachment	1.77±0.04	1.9±0.06
First hair	4.6±0.09	5.1±0.11
Day of tooth eruption	8.81±0.12	9.93±0.33
Day of eye opening	14.96±0.10	15.61±0.09
Day of drop of testicles	24.35±0.18	24.23±0.18
Day of vagina opening	33.86±0.28	33.35±0.26
Body mass of infant rats (g)		
on day 1	6.07±0.07	6.13±0.07
on day 4	10.58±0.25	10.51±0.25
on day 7	15.80±0.23	16.27±0.20
on day 14	29.50±0.49	29.12±0.31
on day 21	43.04±0.8	43.01±0.49
Death of females in the period of feeding	0	0
No reliable differences between experimental and control values are found ($p \geq 0.05$)		

Analysis of the data obtained demonstrated the absence of reliable differences in the values of the test parameters in experimental and control animals. Mortality of infant rats during the period of feeding, sex ratio, time of sense organs maturation and dynamics of body weight gain did not considerably differ. Statistical differences between infant rats born from males dosed with placebo and the test substance were not revealed ($p \geq 0.05$).

CONCLUSION

Daily repeated peroral administration of FPACA substance during 15 (females) and 48 (males) days in dose 67 mg/kg did not cause development of noticeable toxic effects on reproductive function of animals. During the period of the substance dosing no decrease in animal body mass gain, changes in behavior, or animal deaths in experimental groups (males or females) were noted. Pregnancy index was not reliably different in all experimental groups. No malfunction of reproductive system of male и female rats was found.

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