Research Article

Search and evaluation of pharmacodynamic and pharmacokinetic parameters of selective blocker of TRPA$_1$ ion channels from the group of substituted pyrazinopyrimidinones

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Abstract

Introduction. Doctors of almost all specialties have to deal with the problem of pain and its relief. According to the literature, almost 30 million people daily take analgesics from the group of non-opioid analgesics, but in more than half of them 4-6 hours after taking the medication, the severity of pain is unchanged.

Objective. to search for the most active molecules potential selective inhibitors of the TRPA1 ion channel with further investigation of their pharmacodynamic effects, toxicological safety, pharmacokinetic parameters and organ distribution, as well as to assess their impact on the psychoemotional state, general locomotor activity levels and anxiety in laboratory animals.

Materials and methods. According to the results of in vitro tests, the most active molecule under code ZC02-0012 was selected from the pool of candidates. Further its analgesic activity was evaluated using an acetic acid-induced writhing test and a hot plate test; its anti-inflammatory activity was studied in the acute exudative paw edema model; in the open field and elevated plus-maze tests the influence of ZC02-0012 on the general locomotor activity levels and the anxiety of the laboratory animals was studied. The pharmacokinetic parameters and organ distribution of the substance ZC02-0012 were studied using a liquid chromatograph with an operating pressure range of 0-60 mPa (Thermo Scientific Dionex UltiMate 3000).

Results and discussion. According to the results of in vitro tests, it was found that IC50 of the TRPA1 selective inhibitor under laboratory code ZC02-0012 was 91.3 nmol. The preclinical studies showed that ZC02-0012 possessed pronounced analgesic and anti-inflammatory activities and absence of the influence on the behavior and anxiety of the laboratory animals. Absolute bioavailability of ZC02-0012 in rabbits was 47%, while ZC02-0012 was intensely distributed into organs and tissues with a high level of blood circulation. The highest content of ZC02-0012 is typical of liver, kidneys and lungs, the lowest – for muscle tissue. Most of the substance is undergone rapid biotransformation and excreted as metabolites.

Introduction

Doctors of almost all specialties have to deal with the problem of pain and its relief. Pain is the cause of health encounter in 90% of cases worldwide (Mamchur and Kovalenko 2016). According to the results of the European epidemiological study of the prevalence of mental disorders, which was attended by 1659 respondents younger than 75 years, it was found that for the study group the problem of pain was relevant (55.2%), exceeding the level of depression and anxiety (11,6%) (Briggs et al. 2016, König et al. 2010). Surveys of patients in German hospitals showed that more than 80% (n=438) of them suffered from pain during the 3 months prior to hospitalization, and in more than 60% the pain syndrome was the cause of hospitalization (Kotova 2013). According to some reports, 19% of the European population suffer from the chronic pain (Leadley et al. 2014). In the USA, by the number of patients, chronic pain syndrome outnumbers even such common pathological conditions as diabetes mellitus, cardiovascular diseases and cancer (Gatchel 2014). At the same time, pain is not only a negative feeling, but also reduces the quality of life of the patient, negatively affecting family and interpersonal relationships, leading to a decrease in and even loss of ability to work and is associated with huge economic problems for health care.

However, despite all modern research in the field of pharmacology and pharmacotherapy of the pain syndrome, guidelines and practical recommendations designed for doctors on the principles of adequate pain relief, patients are often left without proper therapy (Cascorbi 2015). According to the literature, almost 30 million people daily take analgesics from the group of non-opioid analgesics (Garland 2014, Mamchur and Kovalenko 2016), but in more than half of them, 4-6 hours after taking the medication, the severity of pain remains unchanged (Moore et al. 2015).

Despite the current recommendations for the treatment of the pain syndrome, indicating the need for pathogenetic therapy, most practitioners continue to prescribe non-steroidal anti-inflammatory drugs (NSAIDs) even when there is no tissue damage or inflammation, although the optimal use of NSAIDs, opioid analgesics or a combination of drugs in patients with chronic pain is still the subject of research (Dhalla et al. 2012, Wehling 2014).

The mechanism of analgesic action of opioid (narcotic) analgesics is mainly due to the interaction with μ_1-receptors. However, not only analgesic effect of this group of drugs, but also a number of side effects, such as respiratory depression, nausea and vomiting, the development of euphoria, drug dependence, increased smooth muscle tone are realized through interaction with opioid receptors (Arbuh 2017).

NSAIDs are widely used for pain relief at all stages of medical care. This group of drugs is especially popular in the treatment of diseases of the musculoskeletal system and joints, which are based on inflammation. Realization of drugs of this group is carried out mainly through pharmacies (about 90% of sales), and over-the-counter sale makes them easily accessible and increases the risk of complications and side effects of pharmacotherapy (Zhurakhovskaya et al. 2014).

Therefore, today it is actually to search for new molecules that can selectively block the "targets", directly detecting pain stimuli and inflammatory mediators. One of these targets is a $TRPA_1$ ion channel.

Objective

To search for the most active molecules candidates for selective inhibitors of the $TRPA_1$ ion channel with further investigation of their pharmacodynamic effects, toxicological safety, pharmacokinetic parameters and organ distribution, as well as to assess their impact on the psychoemotional state, general locomotor activity levels and anxiety in laboratory animals.

To achieve this objective, it is necessary to solve the following research tasks:

1. To select molecules candidates for selective inhibitors of the $TRPA_1$ ion channel according to the selectivity criteria in *in vitro* tests.
2. To carry out the dosage selection of the most active and safe compounds from the group of substituted pyrazinopyrimidinones based on the results of the study of LD_{50}, and calculation of therapeutic doses.
3. To investigate the analgesic activity of the $TRPA_1$ selective inhibitor under laboratory code ZC02-0012 in intragastric and intramuscular administration using the hot plate test.
4. To investigate the analgesic activity of the $TRPA_1$ selective inhibitor under laboratory code ZC02-0012 in intragastric and intramuscular administration using the acetic acid-induced writhing test.
5. To investigate the anti-inflammatory activity of the $TRPA_1$ selective inhibitor under laboratory code ZC02-0012 in intragastric and intramuscular administration using the acute exudative paw edema model.
6. To study the influence of the $TRPA_1$ selective inhibitor under laboratory code ZC02-0012 on emotional and behavioral activity, the general locomotor activity levels and the anxiety in intragastric and intramuscular

administration using the open field and elevated plus maze tests.

7. To conduct preclinical studies of pharmacokinetics, organ distribution and metabolism of the TRPA$_1$ selective inhibitor under laboratory code ZC02-0012.

Materials and methods

The experiments were performed at the Research Institute of the Pharmacology of the Living Systems of Belgorod State National Research University and ChemRar High-Tech Center. All the experiments were approved by the Ethical Committee of Belgorod State National Research University. Vivisection was carried out in accordance with the ethical principles of handling laboratory animals "The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. CETS No. 170".

Initially, the most promising candidates for selective ion channel antagonists, which showed the percentage of inhibition of the cellular calcium response above 50% - 3*SDmaximum and own agonistic activity less than 10% + 3*SDminimum, were selected from the 1.5 million library of small organic molecules at the disposal of ChemRar High-Tech Center using the high throughput molecular screening. Then their mechanism of action and specific activity were confirmed in cells with superexpression of TRPA$_1$ ion channel. For hits, the IC$_{50}$ value was determined in GraphPad Prism (GraphPad Software, Inc., San Diego, CA). To construct the concentration dependence, the equation was chosen (Formula 1):

$$Y = Bottom\ curve + \frac{Top\ curve - Bottom\ curve}{1 + 10^{logIC50-X}} \times Curve\ slope \quad (1)$$

The test substances were synthesized by ChemRar High-Tech Center, under the leadership of General Director Dmitriy V. Kravchenko. The structures of the synthesized potential molecules are given in Table 1.

According to the results of *in vitro* tests, a molecule under laboratory code ZC02-0012 was selected as the most active for further studies in laboratory animals.

The study of a general toxical action was performed in white mice of both sexes weighing 20±2 g. Animals were administered the prepared solution of the active pharmaceutical substance ZC02-0012 using a metal stomach tube with a smooth olive on the end at the maximum for mice and their body weight volume of 0.5 ml, four-time administration during the day with 1 hour intervals. The maximum possible dose for the administration of the test substance was 5000 mg/kg.

The toxic effect of the substance was evaluated by the general condition of the animals and their survival, LD$_{50}$. Survivors and dead animals were calculated within 3 days after the priming of the substance, followed by keeping watch over the surviving animals for two weeks after the priming.

The study of the analgesic activity of the selective inhibitor of the TRPA$_1$ ion channel, active pharmaceutical substance under code ZC02-0012 was performed in two tests: hot plate and acetic acid-induced writhing test.

A. Hot plate Test

The experiments were performed in white laboratory mice of either sex weighing 22 to 28 g. To simulate the pain syndrome, the animals were placed on a preheated 55°C hot plate (Hot-Plate LE7406, Panlab Harvard Apparatus, Spain), and the time in seconds was recorded until the pain behavior (licking and pulling up the legs, bouncing, squeaking) started. Then all the animals were divided into groups, depending on the administered substance and administration way:

Group I – control, the intragastric administration of PEG-400 (n=15);

Group II – the intragastric administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 1 mg/kg (n=15);

Group III – the intragastric administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 3 mg/kg (n=15);

Group IV – the intragastric administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 9 mg/kg (n=15);

Group V – the intragastric administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 3.48 mg/kg (n=15);

Group VI – control, the intramuscular administration of PEG-400 (n=15);

Group VII – the intramuscular administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 1 mg/kg (n=15);

Group VIII – the intramuscular administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 3 mg/kg (n=15);

Group IX – the intramuscular administration of the selective inhibitor of TRPA$_1$ ion channel at a dose of 9 mg/kg (n=15);
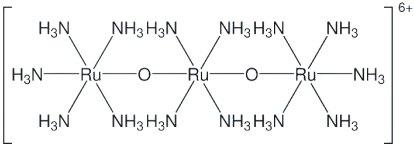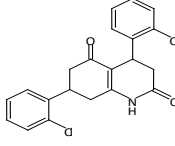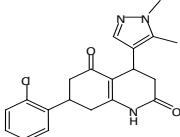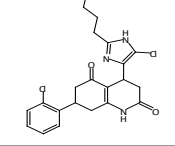
Group X – the intramuscular administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 3.48 mg/kg (n=15).

At intervals of 30, 60, 90 and 120 minutes after administration of the test substances, the experimental animals were re-placed on a plate heated to 55ºC, and the time in seconds until the pain behavior was recorded. The analgesic action was evaluated by increasing the exposure time of the animals on the hot plate.

B. Acetic acid-induced writhing test

The experiments were performed in white inbred rats of both sexes weighing 200-220 g. The animals were administered by the test substances intramuscularly or intragastrrically.

Table 1. Chemical Structures of the Test Substances.

Substances	Structures
Ruthenium red	
C276-1460	
ZC02-0007	
ZC02-0011	
ZC02-0012	
ZC02-0020	

Group I – control, the intragastric administration of poly-ethylene glycol 400 (PEG-400);

Group II – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group III – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IV – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);

Group V – the intragastric administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15);

Group VI – control, the intramuscular administration of PEG-400;

Group VII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group VIII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IX – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);
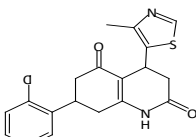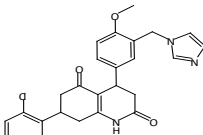
Group X – the intramuscular administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15).

Thirty minutes after intramuscular administration and 1 hour after intragastric administration of the test substances to simulate the pain syndrome, the experimental animals was injected intraperitoneally with 0.75% solution of acetic acid at the rate of 1 ml per 100 g weight of the animal. A count of the number of "writhes" started 15 min later and was carried out for 30 min. Writhes are the characteristic movements of animals, including contractions of abdominal muscles, alternating with their relaxation, the stretching of hind paws and bowing of the back (Mironov et al. 2012). Analgesic effect was estimated by a reduced number of writhes compared to those in the control group and calculated by Formula 2:

$$E_{analg} = \frac{\tilde{N}_{\ell} - \tilde{N}_{f}}{\tilde{N}_{\ell}} \times 100\% \qquad (2)$$

where: C_o is the number of "writhes" after test substances administration and C_κ is the number of "writhes" before test substances administration.

The experiments to study the anti-inflammatory activity were performed in white laboratory mice of both sexes weighing 22-28 g. The anti-inflammatory effect was evaluated in a model of acute aseptic inflammation of the mice paw by a degree of inhibiting an increase in paw edema on the background of drugs administration compared with the control group of the untreated animals. The experimental animals were administered intragastrically or intramuscularly with the studied substances.

Group I – control, the intragastric administration of PEG-400 (n=15);

Group II – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1 mg/kg (n=15);

Group III – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 3 mg/kg (n=15);

Group IV – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 9 mg/kg (n=15);

Group V – the intragastric administration of the reference drug Diclofenac sodium (Chemopharm, Serbia) at a dose of 13.91 mg/kg (n=15);

Group VI – control, the intramuscular administration of PEG-400 (n=15);

Group VII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1 mg/kg (n=15);

Group VIII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 3 mg/kg (n=15);

Group IX – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 9 mg/kg (n=15);

Group X – the intramuscular administration of the reference drug Diclofenac sodium (Chemopharm, Serbia) at a dose of 13.91 mg/kg (n=15).

Exudative edema was caused 45 min after administration of the test substances by subplant injection into the right hind paw of mice with 0.02 ml of 2% formaldehyde solution. The mass of the paw was measured 4 hours (peak edema) after injection of a phlogistic, using electronic scales with an accuracy of 1 mg; the left paw of the same animal, which was injected with an equal volume of isotonic NaCl along with the phlogistic injection, was used as a control. The inhibitory effect was calculated by Formula 3:

$$E_{ing} = \frac{(\Delta M_{\hat{e}} - \Delta M_0) \times 100\%}{\Delta M_{\hat{e}}} \qquad (3)$$

where: E_{ing} is the inhibitory effect, ΔM_0 is the average weight gain of the edematous paw in the experimental group and $\Delta M_{\hat{e}}$ is the average weight gain of the edematous paw in the control group.

The study of the behavioral reactions in the open field test was performed in white male inbred rats weighing 200-220 g. Twenty-four hours before the study, the animals were randomized and organized into experimental groups; with each rat individually labled. The test substances were administered intragastrically or intramuscularly 1 hour before testing, and then the animals were placed into a quiet, poorly lit room. During this period, the regrouping of the animals, feeding, picking them up and other active manipulations were excluded.

All animals were divided into groups:

Group I – control, the intragastric administration of PEG-400;

Group II – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group III – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IV – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);

Group V – the intragastric administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15);

Group VI – control, the intramuscular administration of PEG-400;

Group VII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group VIII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IX – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);

Group X – the intramuscular administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15).

Registration of the general locomotor activity of the animals was performed in the open field box (Open Field LE800S, PanLab Harvard Apparatus, Spain) for 10 minutes. The lighting was done by a 100 W lamp, suspended at a height of 1.5 m from the bottom of the arena. The animals were placed in the open field near the wall of the arena. A zero-group animal was placed in the arena and allowed to freely examine the box for 5 minutes immediately before testing the rats of the experimental and control groups to provide equal footing in terms of olfactory signal between the first and subsequent animals. After the "zero" and each subsequent animal, the arena and the walls of the box were wiped with a moist rag. For the data analysis, we used the program Smart v.3.0.03. (Panlab Harvard Apparatus, Spain).

For the convenience of processing and simplifying the presentation of the results, the values of the registered parameters were generalized (Shevkunova et al. 2013). The following behaviors were analyzed: horizontal locomotion by the distance traveled in the center and outer zone of the field, vertical locomotion by the number of rearing behaviors, the percentage of time spent in each zone, reflecting the general locomotor activity of the animals, as well as the number of defecations and the total duration of grooming, characterizing the emotional state of the animal.

The study of behavioral reactions in the elevated-plus maze test was performed in white male inbred rats weighing 200-220 g. Twenty-four hours before the study, the animals were randomized and organized into experimental groups, with each rat individually labled, and then coops with the animals were transferred from the vivarium to the room where the experiment was conducted. The test substances were administered intragastrically or intramuscularly 1 hour before the test. All animals were divided into groups:

Group I – control, the intragastric administration of PEG-400;

Group II – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group III – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IV – the intragastric administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);

Group V – the intragastric administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15);

Group VI – control, the intramuscular administration of PEG-400;

Group VII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 0.46 mg/kg (n=15);

Group VIII – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 1.38 mg/kg (n=15);

Group IX – the intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel at a dose of 4.15 mg/kg (n=15);

Group X – the intramuscular administration of the reference drug Ketorolac (Dr. Reddy's laboratories Ltd, India) at a dose of 1.6 mg/kg (n=15).

Registration of the animals' activity was carried out in the elevated-plus maze test LE840 (PanLab Harvard Apparatus, Spain) for 5 minutes. The animals were placed in the center of the maze, with the nose to open arm. It is assumed that the animal will seek to explore a new environment, struggling with the fear of open space and height. A zero-group animal was placed in the arena and allowed to freely examine the box for 5 minutes immediately before testing the rats of the experimental and control groups to provide equal footing in terms of olfactory signal between the first and subsequent animals. After the "zero" and each subsequent animal, the arena and walls of the box were wiped with a moist rag. For the data analysis, we used the program Smart v.3.0.03 (Panlab Harvard Apparatus, Spain).

The following behaviors were analyzed: duration in open arms and closed arms, the number of open and closed arm entries, total duration of grooming, as well as the number of hanging from the open arms and the number of defecations.

To study the pharmacokinetics of the $TRPA_1$ selective inhibitor under code ZC02-0012, 12 rabbits were pre-catheterized into the right ear vein so that blood samples at all time points were taken from the same animals throughout the experiment. Twelve hours before the start of the experiment, the animals were deprived of feed, having free access to water. On the third day after catheterization, the test substance was administered.

Intravenously, the test substance was administered bolus to 6 rabbits in the ear vein at a dose of 10 mg/kg in the form of a solution 10 mg/ml in propylene glycol. Blood was sampled through a catheter in a volume of 0.3 ml into polypropylene tubes containing 20 µl of 5% EDTA prior to the administration, and then 10, 30, 60, 90, 120, 180, 240 and 360 minutes after the injection. Intragastrically, the substance was administered at a dose of 10 mg/kg through stomach tube in the form of a solution of 10 mg/ml in propylene glycol. Blood was sampled through a catheter in a volume of 0.2 ml into polypropylene tubes containing 20 µl of 5% EDTA prior to injection, and then 10, 30, 60, 90, 120, 180, 240 and 360 minutes after the injection. Blood plasma was separated by centrifugation at 5600 g for 10 min and stored at a temperature of 70°C.

The organ distribution of the test substance was studied in 60 male rabbits after a single intravenous administration of ZC02-0012 to the animals at a dose of 10 mg/kg. Whole blood, plasma, heart, spleen, muscles, liver, kidneys, brain and lungs were recovered before administration and 5, 10, 30, 45, 60, 90, 120, 180, 240 minutes after administration of ZC02-0012.

The concentration of ZC02-0012 was determined using the previously developed HPLC method. In the specialized program Chromeleon 7, there were calculated the areas of the peaks of the analyzed substance and the internal standard, then the data was transferred to the Microsoft office Excel 2010, in which we calculated the equation of the calibration curve, statistically estimated deviations, graphically displayed the results. Concentrations of substance ZC02-0012 in the studied objects were calculated in the program Microsoft office Excel 2010 by calibration curves.

The outliers in each time point were identified using Grubb's test. If for any sample, the value of Z was greater than the critical value for the given number of measure-

ments N, this sample was excluded from further calculations of pharmacokinetic parameters. Thus, for N=6, the critical value of Z was 1.89, so samples with Z>1.89 were considered as outliers (Bland 2000).

The concentration of ZC02-0012 in the homogenates of organs and tissues was also evaluated using the calibration curves, then the concentration was recalculated in μg/g of tissue taking into account dilution (the final concentration was multiplied by two in accordance with the method of analysis).

The concentration of ZC02-0012 in excrement was detected by the same way, and the total amount of the substance in excrement was calculated in the observation intervals.

The main pharmacokinetic parameters were calculated in accordance with the guidelines for the preclinical studies of drugs under the editorship of A.N. Mironov, using Microsoft office Excel 2010. On the basis of experimentally obtained data, there were calculated pharmacokinetic parameters and organ distribution.

The outliers in each time point were identified using Grubb's test (Grubb's Test for Detecting Outliers. URL: http://graphpad.com/quickcalcs/GrubbsExplain.cfm). There were calculated mean values and CV of the pharmacokinetic parameters for 6 animals.

The results of the experiments were subjected to statistical processing in accordance with modern concepts of mathematical processing of medical research data (Glantz 1998, Kochetov et al. 2012), using IBM SPSS Statistics 21 (USA).

The main objective of the statistical study was to compare the indicators of ZC02-0012 groups with those of the control and reference drugs groups in order to solve the question of accepting a statistical hypothesis (H0/H1) in relation to a specific parameter. The hypothesis of significant difference of values (HI) was accepted if its probability was equal to or greater than 95%. In other cases, the null hypothesis (H0) was accepted.

The analysis of the quantitative parameters assumed, first of all, determination of their mean values (M), median (Me), first and third quartiles (Q1 and Q3) and standard errors. A normality of distribution of quantitative parameters was assessed using Shapiro-Wilk W test. In order to accept a statistical hypothesis, Student's t-test was calculated and compared with the critical value. In cases when the law of probability significantly differed from the normal one, the statistical hypothesis was accepted on the basis of the results of the nonparametric Kruskal-Wallis test. The Holm-Bonferroni correction for multiple testing was used to correct the effect of multiple comparison.

Results and discussion

The mechanism of action and specific activity of the new synthesized molecules were tested in cells with superexpression of the $TRPA_1$ ion channel (Fig. 1). The obtained results confirm that the mechanism of action of all mo-
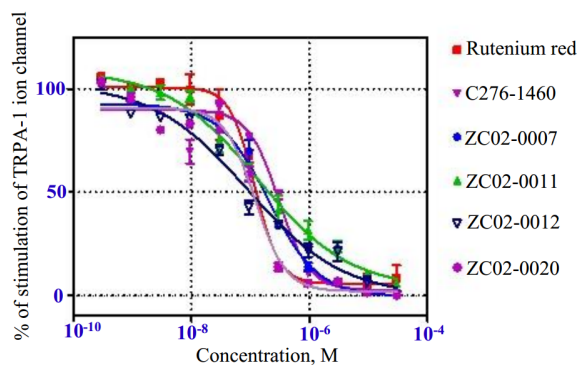

Figure 1. Concentration dependence of antagonistic activity on the concentration of the test substances. Each concentration point is an average of two repeats.

Table 2. Activity of the Test Substances to Suppress the Activity of the $TRPA_1$ ion Channel.

Substance	IC_{50}, nmol
Ruthenium red	118.2
C276-1460	303.3
ZC02-0007	204.0
ZC02-0011	135.2
ZC02-0012	91.3
ZC02-0020	120.2

lecules is the antagonism of the $TRPA_1$ ion channel. The activity of the substances is summarized in Table 2.

Thus, using *in vitro* screening, the specific activity of several synthesized substances was investigated. It is shown that all of them are antagonists of the $TRPA_1$ ion channel. IC_{50} for all the substances was within the range of ~100-300 nmol. Since the substance under laboratory code ZC02-0012 showed the greatest activity (IC_{50} 91.3 nmol), this compound was chosen as the main candidate for preclinical studies.

The performed studies of acute toxicity showed that after four-time with an interval of 1 hour intragastric administration to mice of active pharmaceutical substance ZC02-0012 in the largest for this species of animals and administration way dose of 5000 mg/kg, the deaths of animals were not observed within two weeks after acute priming.

Consequently, the results of keeping watch over the experimental animals in the intoxication period in the study of acute toxicity allow grading the investigated selective inhibitor of the $TRPA_1$ ion channel ZC02-0012 to the V class of drugs (Hodge 1975).

In accordance with the experiment design, to study the analgesic effects in the hot plate test, the intact white laboratory mice were placed on a plate heated to 55° in order to determine the pain threshold. Then divided into groups animals were administered the test substances, and the hot plate test was repeated 30, 60, 90 and 120 minutes afterwards. The results are presented in Tables 3 and 4.

It was found that the test substance at a dose of 1 mg/kg in both intragastric and intramuscular administration does

Table 3. Dynamics of Values of the Hot Plate Test in Intragastric Administration of the Test Substances (M±m).

Substance	Initial value	30 min	60 min	90 min	120 min
PEG-400	7.7±0.4	8.0±0.6	8.2±0.4	8.9±0.5	8.8±0.4
ZC02-0012 1 mg/kg	7.6±0.4	9.2±0.4	10.9±0.6	10.5±0.5	9.6±0.5
ZC02-0012 3 mg/kg	7.2±0.5	15.1±0.9**	22.2±1.0**	16.4±0.8**	14.3±0.7**
ZC02-0012 9 mg/kg	7.5±0.5	15.4±0.7**	22.9±1.0**	17.7±1.3**	13.5±1.2**
Ketorol3,48 mg/kg	7.1±0.4	10.8±0.4*	16.6±0.6*	13.8±0.5*	10.8±0.4

Note: * - p<0.05 in comparison with PEG-400; ** - p<0.05 in comparison with Ketorol

Table 4. Dynamics of Values of the Hot Plate Test in Intramuscular Administration of the Test Substances (M±m).

Substance	Initial value	30 min	60 min	90 min	120 min
PEG-400	8.4±0.3	9.1±0.5	9.6±0.5	9.9±0.3	9.8±0.4
ZC02-0012 1 mg/kg	7.3±0.4	10.8±0.9	9.6±0.5	8.5±0.3	8.4±0.5
ZC02-0012 3 mg/kg	7.3±0.5	22.4±1.0**	17.3±0.8**	14.6±0.8**	12.4±0.8**
ZC02-0012 9 mg/kg	7.0±0.5	22.7±1.4**	17.7±1.1**	14.1±0.8**	11.5±0.8**
Ketorol3,48 mg/kg	7.2±0.5	17.6±0.9*	12.7±0.7*	9.8±0.6	8.3±0.6

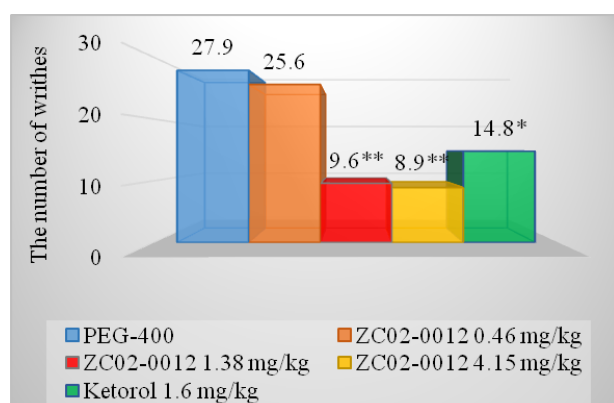*Note:* * - p<0.05 in comparison with PEG-400; ** - p<0.05 in comparison with Ketorol.

not have an analgesic effect. At the same time, at the doses of 3 mg/kg and 9 mg/kg, the selective inhibitor of the TRPA1 ion channel has an analgesic effect that exceeds that of the reference drug Ketorolac. In this case, both intragastric and intramuscular administration of selective antagonist ZC02-0012 retains its analgesic effect throughout the study period, up to 120 minutes, while for Ketorolac this effect is offset by the 90th minute with intramuscular administration and by the 120th minutes with intragastric administration. However, we did not find a significant difference in the intensity of the analgesic effect between the doses of 3 mg/kg and 9 mg/kg of the selective inhibitor of TRPA1, a substance under code ZC02-0012.

The acetic acid-induced writhing test is one of the most common in the evaluation of analgesic activity of non-narcotic analgesics. The pain reaction in this test is due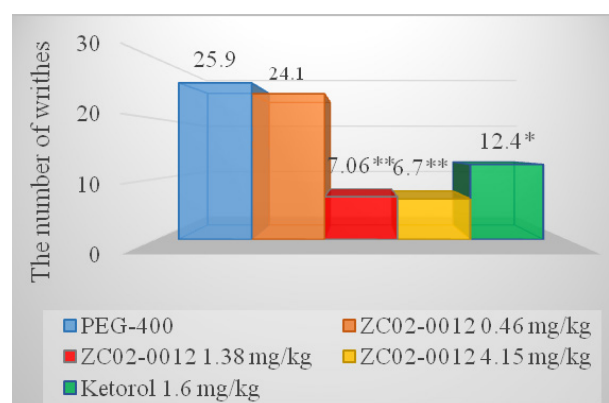 to the activation of prostaglandin biosynthesis as a result of moderate short-term irritation of the peritoneum with a dilute solution of acetic acid.

Intraperitoneal injection of acetic acid solution to the control untreated rats causes a pain reaction in the form of writhes, expressed in the contraction of abdominal muscles and stretching the hind paws. The number of writhes in such group within a 30-minute observation period is usually from 20 to 30.

In accordance with the experiment design, to study the analgesic effects in the acetic acid-induced writhing test, the males of inbred laboratory rats were administered the test substances in the studied doses, and then, 30 minutes after intramuscular administration and 60 minutes after intragastric administration there was performed intraperitoneal administration of 0.75% solution of acetic acid at the rate of 1 ml per 100 g of animals' weight. The count of the number of writhes started 15 minutes after the injecti-

Figure 2. Analgesic activity of the selective inhibitor of TRPA₁ ion channel, substance under code ZC02-0012, and the reference drug Ketorolac using the acetic acid-induced writhing test with intragastric administration.
Note: * – p<0.05 in comparison with PEG-400; ** – p<0.05 in comparison with Ketorol.

Figure 3. Analgesic activity of the selective inhibitor of TRPA₁ ion channel, substance under code ZC02-0012, and the reference drug Ketorolac using the acetic acid-induced writhing test with intramuscular administration.
Note: * – p<0.05 in comparison with PEG-400; ** – p<0.05 in comparison with Ketorolac.

on of acetic acid and lasted for 30 minutes. The results are presented in Figures 2 and 3.

As the data presented in Figures 2 and 3 evidence, pretreatment with a selective inhibitor of $TRPA_1$ ion channel, substances under code ZC02-0012, at a dose of 0.46 mg/kg has no analgesic effect either in the intragastric or in intramuscular administration. At the same time, the test substance ZC02-0012 at doses of 1.38 mg/kg and 4.15 mg/kg and the reference drug Ketorolac, both in the intragastric and intramuscular administration, cause a analgesia, manifested in a decreased number of writhes compared to the control group, but in the selective antagonist of $TRPA_1$ ion channel, substance under code ZC02-0012, this effect is more pronounced than in Ketorolac. However, differences in analgesic effect between the doses of 1.38 mg/kg and 4.15 mg/kg of substance ZC02-0012 in both administration ways were not statistically significant.

The results of the study of anti-inflammatory activity of ZC02-0012 showed that after a phlogistic was injected under the aponeurosis of the hind paw of the mice, all the experimental animals developed severe edema. The data obtained are presented in Tables 5 and 6.

From the tables above it can be seen that a single intragastric and intramuscular administration of the selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012 at a dose of 1 mg/kg does not have a statistically significant effect on the exudative phase of inflammation, that is practically does not reduce the severity of edema of the hind paw in mice after the injection of 2% formaldehyde solution. At the same time, a single intragastric and intramuscular administration of the substance under code ZC02-0012 at a dose of 3 and 9 mg/kg, as well as Diclofenac sodium at a dose of 13.91 mg/kg statistically significantly suppressed the severity of edema of the hind paw of the animal. However, there was no significant difference in the intensity of the inhibitory effect between the test substance under code ZC02-0012 and Diclofenac sodium and between the doses of 3 mg/kg and 9 mg/kg of the substance under code ZC02-0012. Thus, the selective inhibitor of the $TRPA_1$ ion channel, substance under code ZC02-0012, is comparable to the most active anti-inflammatory drug in the group of NSAIDs, Diclofenac sodium.

In the study of the general locomotor activity levels of animals in the open field test, the arena was divided into two zones – central and outer. It is known that placing an animal into a new environment contributes to the research behavior and emotions of fear, so in the open field test, during the first minutes of the study, the motor activity of animals is limited mainly to the outer zone and movement along the walls of the arena. The level of fear gradually decreases, and the animal begins to explore the central zone. In this regard, to study the effect of the test substances on the adaptive capabilities of the experimental animals, the distance traveled in each zone, as well as the time spent in each zone, was measured. The results of the study showed no significant differences in the horizontal activity of the

Table 5. Anti-inflammatory Activity of the Selective Inhibitor of $TRPA_1$ ion Channel, Substance Under Code ZC02-0012 and the Reference Drug Diclofenac sodium in Intragastric Administration in the model of Formalin Edema of the Mice Paw.

Substances and doses	Weight of intact paw, M±m	Weight of edematous paw, M±m	Weight gain, g M±m	Weight gain, % M±m	Inhibitory effect, %
PEG-400	118.8+3.1	179.3+6	60.5+3.8	50.8+2.8	–
ZC02-0012 1 mg/kg	114.1+3.6	169.5+4	55.3+2.6	49.3+2	2.73%
ZC02-0012 3 mg/kg	120.3+1.6	142.3+2*	22+1.6*	18.4+1.4*	63.78%
ZC02-0012 9 mg/kg	116.7+2.2	135+2.3*	18.3+1.0*	15.8+1.0*	68.78%
Diclofenac sodium 13.91 mg/kg	122.3+1.9	141.5+2.5*	19.1+1.8*	15.7+1.5*	69.05%

Note: * – p<0.05 in comparison with PEG-400.

Table 6. Anti-inflammatory Activity of the Selective Inhibitor of $TRPA_1$ ion Channel, Substance Under Code ZC02-0012 and the Reference Drug Diclofenac in Intramuscular Administration in the Formalin Edema Model in Mice Paw.

Substances and doses	Weight of intact paw, M±m	Weight of edematous paw, M±m	Weight gain, g M±m	Weight gain, % M±m	Inhibitory effect, %
PEG-400	115.2+3.8	173.9+6.8	58.7+4.1	50.8+3.2	-
ZC02-0012 1 mg/kg	114.3+3.7	169.6+4.8	57.7+4.0	49.1+3.3	3.5%
ZC02-0012 3 mg/kg	123.1+1.9	144.4+2.6*	21.3+1.4*	17.3+1.1*	66%
ZC02-0012 9 mg/kg	116.5+1.3	134+2*	17.5+1.7*	15+1.5*	70.4%
Diclofenac sodium 13.91 mg/kg	123.2+2.4	141.9+2.6*	18.7+1.3*	15.3+1.2*	69.85%

Note: * – p<0.05 in comparison with PEG-400.

animals in the experimental groups, compared with that in the control, both in intragastric and intramuscular administration of the test substances (Tables 7, 8).

In addition to horizontal locomotor activity, the vertical motor activity of the experimental animals was also studied by the number of rearing behaviors, the analysis of which showed no statistically significant differences between the experimental and control groups, both in intragastric and intramuscular administration of the substances (Tables 7, 8). To assess the emotional status of the animals in the open field test, the number of defecation acts and the total duration of grooming acts were evaluated. No significant differences of these criteria in comparison with the control were detected in any experimental group, neither in intragastric nor in intramuscular administration of the test substances (Tables 7, 8).

When testing animals in the elevated plus maze test, a high level of locomotor activity was registered, the criteria of which did not differ significantly among different groups.

All studied test parameters had no significant differences in the experimental and control groups (Tables 9, 10).

Thus, the results of the open field and elevated plus-maze tests showed that intragastric and intramuscular administration of the selective inhibitor $TRPA_1$, substance under laboratory code ZC02-0012, at the doses of 0.46, 1.38 and 4.15 mg/kg, as well as reference drug Ketorol (Dr. Reddy's Laboratories Ltd., India) at a dose of 1.6 mg/kg does not have a negative effect on the behavioral reactions of animals, their level of anxiety and the general locomotor activity levels.

The pharmacokinetic of ZC02-0012 was studied in blood plasma of rabbits in a single intravenous administration at a dose of 10 mg/kg and intragastric administration at a dose of 10 mg/kg. The concentration of ZC02-0012 in plasma is presented in Tables 11 and 12. The averaged pharmacokinetic curves (Figure 4) were constructed on the basis of the obtained data, and the main pharmacokinetic parameters were calculated.

Table 7. Results of the Open Field Test in Intragastric Administration of the Test Substances, Me [Q1; Q3].

Test criteria	Administered substances and doses					P value
	PEG-400 n=15	ZC02-0012 0.46 mg/kg n=15	ZC02-0012 1.38 mg/kg n=15	ZC02-0012 4.15 mg/kg n=15	Ketorol 1.6 mg/kg n=15	
Horizontal locomotion in central zone (m)	1.67 [0.54; 2.2]	1.14 [0.03; 1.34]	0.86 [0.08; 1.3]	1.23 [0.18; 2.12]	1.24 [0.0; 1.96]	0.603
Horizontal locomotion in outer zone (m)	64.21 [50.19; 85.07]	64.1 [47.34; 85.82]	59.79 [44.87; 67.6]	55.41 [39.35; 69.93]	74.8 [49.99; 83.39]	0.297
Time spent in central zone (%)	0.33 [0.19; 0.93]	0.15 [0.02; 0.53]	1.11 [0.12; 1.88]	0.92 [0.5; 1.68]	1.1 [0.02; 1.79]	0.088
Time spent in outer zone (%)	99.67 [99.07; 99.81]	99.85 [99.47; 99.98]	98.89 [98.12; 99.88]	99.09 [98.32; 99.5]	98.9 [98.21; 99.98]	0.089
Rearing behavior	8 [7; 10]	9 [8; 13]	9 [7; 13]	9 [6; 12]	10 [8; 11]	0.657
Total duration of grooming	17 [14; 29]	26 [19; 29]	27 [16; 32]	24 [18; 31]	21 [16; 25]	0.417
Number of defecations	2 [0; 3]	1 [0; 2]	1 [0; 2]	1 [0; 3]	2 [1; 3]	0.729

Table 8. Results of the Open Field Test in Intramuscular Administration of the Test Substances, Me [Q1; Q3].

Test criteria	Administered substances and doses					P value
	PEG-400 n=15	ZC02-0012 0.46 mg/kg n=15	ZC02-0012 1.38 mg/kg n=15	ZC02-0012 4.15 mg/kg n=15	Ketorol 1.6 mg/kg n=15	
Horizontal locomotion in central zone (m)	1.03 [0.44; 2.61]	1.3 [0.74; 2.71]	1.81 [0.0; 2.53]	1.58 [0.74; 2.66]	1.51 [1.08; 2.19]	0.955
Horizontal locomotion in outer zone (m)	60.93 [58; 75.45]	55.18 [48.06; 70.96]	81.12 [63.56; 84.3]	59.87 [49.04; 75.86]	67.85 [52.17; 77.27]	0.165
Time spent in central zone (%)	1.11 [0.1; 1.93]	0.84 [0.15; 1.44]	0.64 [0.0; 1.39]	0.84 [0.13; 1.27]	1.29 [0.32; 1.58]	0.619
Time spent in outer zone (%)	98.89 [98.07; 99.9]	99.16 [98.56; 99.89]	99.37 [98.61; 100]	99.17 [98.73; 99.87]	98.71 [98.42; 99.69]	0.615
Rearing behavior	9 [7; 10]	9 [7; 10]	10 [7; 13]	9 [9; 11]	10 [8; 12]	0.401
Total duration of grooming	25 [18; 35]	25 [20; 35]	27 [20; 28]	22 [12; 36]	28 [21; 30]	0.946
Number of defecations	1 [0; 3]	0 [0; 2]	3 [0; 3]	1 [1; 2]	2 [1; 2]	0.101

Table 9. Results of the Elevated Plus-maze Test in Intragastric Administration of the Test Substances, Me [Q1; Q3].

Test criteria	Administered substances and doses					P value
	PEG-400 n=15	ZC02-0012 0.46 mg/kg n=15	ZC02-0012 1.38 mg/kg n=15	ZC02-0012 4.15 mg/kg n=15	Ketorol 1.6 mg/kg n=15	
Time spent in closed arms (sec)	248.95 [234.57; 270.91]	262.27 [248.98; 279.31]	253.86 [241.49; 276.38]	253.31 [246.58; 268.86]	259.99 [250.43; 263.56]	0.546
Time spent in open arms(sec)	42.84 [22.29; 58.02]	30.06 [18.75; 46.91]	38.43 [16.59; 52.05]	39.3 [25.44; 47.05]	36.79 [31.64; 42.99]	0.610
Number of open arm entries	2 [1; 3]	3 [2; 3]	2 [1; 2]	2 [1; 2]	2 [2; 3]	0.082
Number of closed arm entries	4 [3; 5]	3 [3; 4]	5 [3; 5]	4 [3; 4]	4 [3; 4]	0.194
Total duration of grooming	16.25 [11.22; 20.1]	16.12 [13.98; 19.13]	14.49 [11.34; 19.9]	17.5 [11.84; 22.21]	12.23 [10.2; 18.77]	0.487
Number of hangings from the open arms	5 [4; 7]	5 [3; 7]	6 [5; 6]	5 [4; 7]	5 [4; 7]	0.5
Number of defecations	1 [0; 2]	1 [0; 3]	2 [0; 3]	2 [0; 3]	2 [0; 3]	0.823

Table 10. Results of the Elevated Plus-maze Test in Intramuscular Administration of the Test Substances, Me [Q1; Q3].

Test criteria	Administered substances and doses					P value
	PEG-400 n=15	ZC02-0012 0,46 mg/kg n=15	ZC02-0012 1,38 mg/kg n=15	ZC02-0012 4,15 mg/kg n=15	Ketorol 1,6 mg/kg n=15	
Time spent in closed arms (sec)	255.64 [238.05; 270.43]	261.96 [237.22; 279.34]	251.71 [231.77; 277.31]	244.69 [236.69; 276.54]	259.33 [242.28; 277.69]	0.873
Time spent in open arms(sec)	36.84 [26.35; 53.93]	32.0 [13.34; 54.2]	40.36 [15.82; 60.66]	49.21 [15.99; 59.17]	35.14 [19.19; 51.22]	0.872
Number of open arm entries	2 [2; 3]	2 [1; 3]	2 [1; 3]	2 [1; 3]	2 [1; 3]	0.521
Number of closed arm entries	4 [2; 4]	3 [3; 4]	4 [3; 5]	3 [3; 5]	3 [2; 4]	0.503
Total duration of grooming	16.43 [13.31; 21.89]	19.1 [14.51; 21.39]	16.46 [11.79; 20.26]	13.7 [11.98; 21.89]	18.03 [13.27; 20.23]	0.804
Number of hangings from the open arms	6 [5; 7]	5 [4; 7]	5 [3; 6]	5 [4; 7]	5 [4; 6]	0.486
Number of defecations	1 [1; 2]	2 [1; 3]	2 [0; 3]	1 [0; 2]	2 [0; 3]	0.692

The results obtained show that the maximum concentration of ZC02-0012 in blood plasma of rabbits was reached on average within 10 minutes after intragastric administration. The half-life was short (20.6 minutes). The mean absorption time (MAT) of ZC02-0012 was 4.2 minutes. Absolute bioavailability f_a (%) of ZC02-0012 in intragastric administration in rabbits was 47%.

The organ distribution of ZC02-0012 was studied after a single extravascular (intragastric) administration of the substance to rabbits at a dose of 10 mg/kg. The substance was well distributed to the organs. The maximum concentration in the studied organs was reached within 30 minutes after administration of ZC02-0012. The highest levels of ZC02-0012 were observed in liver (f_t=216.9), kidney (f_t=399.4) and lung (f_t=332.0) tissues. The profiles of the distribution curves of ZC02-0012 in the heart, brain and spleen are close. These organs are characterized by a close maximum concentration of ZC02-0012 and f_t within 50-90. The lowest distribution of the substance ZC02-0012 was recorded for muscles, for which f_t was 32. The average residence time of substance ZC02-0012 in tissues ranged from 90 to 140 minutes. The highest values are typical of kidneys, spleen and lungs.
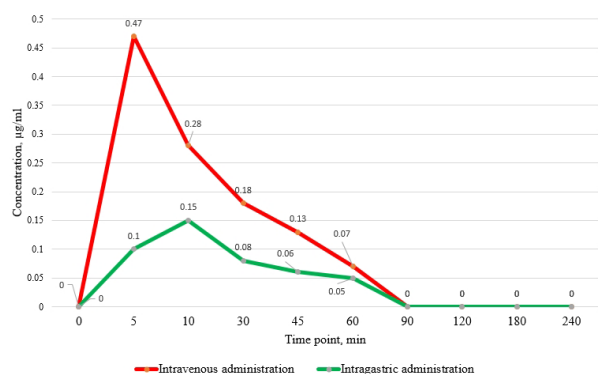
Excretion of ZC02-0012 was studied after a single extravascular (intragastric) administration of the substance to rabbits at a dose of 10 mg/kg. The substance is found unchanged in urine in trace amounts (0.7% of the dose). Thus, nonrenal clearance is typical of ZC02-0012. With feces, ZC02-0012 is excreted mainly in the period of 8-24 hours. The total amount of ZC02-0012 in the faeces collected over 24 h is about 3.5 % of the administered dose. Taking into account the results obtained, it is obvious that most of the drug undergoes biotransformation in the liver and is excreted as metabolites.

Table 11. Concentration of ZC02-0012 in Blood Plasma of Rabbits after a Single Intravenous Injection at a Dose of 10 mg/kg.

Statistical values	Concentration of ZC02-0012, µg/ml									
	Time point, min									
	0	5	10	30	45	60	90	120	180	240
Mean	0.00	0.47	0.28	0.18	0.13	0.07	0.00	0.00	0.00	0.00
SD	0.00	0.10	0.03	0.04	0.08	0.01	0.00	0.00	0.00	0.00
Median	0.00	0.48	0.27	0.19	0.11	0.07	0.00	0.00	0.00	0.00
CV, %	-	21.97	11.29	21.48	58.68	14.38	-	-	-	-

Table 12. Concentration of ZC02-0012 in Blood Plasma of Rabbits after a Single Extravascular Administration at a Dose of 10 mg/kg.

Statistical values	Concentration of ZC02-0012, µg/ml									
	Time point, min									
	0	5	10	30	45	60	90	120	180	240
Mean	0.00	0.10	0.15	0.08	0.06	0.05	0.00	0.00	0.00	0.00
SD	0.00	0.04	0.03	0.02	0.01	0.00	0.00	0.00	0.00	0.00
Median	0.00	0.08	0.14	0.07	0.06	0.05	0.00	0.00	0.00	0.00
CV, %	-	38.76	19.47	24.96	12.68	9.77	-	-	-	-

Figure 4. Averaged pharmacokinetic curves of ZC02-0012 in blood plasma of rabbits after a single administration.

Conclusion

1. The substance under laboratory code ZC02-0012 from the group of substituted pyrazinopyrimidinones *in vitro* had the most pronounced inhibitory activity against the $TRPA_1$ ion channel at a concentration of 91.3 nmol, as its full inhibitor.

2. The results of the study of acute toxicity of the selective inhibitor of $TRPA_1$ ion channel, substances under code ZC02-0012, showed that the maximum possible dose for intragastric administration to mice of 5000 mg/kg no animal deaths were recorded within two weeks after acute priming. The results of keeping watch over the experimental animals in the intoxication period, when studying acute toxicity, make it possible to attribute the test substance of the selective inhibitor of $TRPA_1$ ion channel, ZC02-0012, to the V drug class

3. Selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012, has an analgesic effect, superior to that of the reference drug Ketorolac, having the greatest analgesic activity in the group of NSAIDs, comparable with opioid analgesics, according to some

sources. Analgesic effect was manifested in an increased latent period of white laboratory mice staying on the plate heated to 55º in the hot plate test and a decreased number of writhes in response to intraperitoneal administration of a dilute solution of acetic acid to the inbred laboratory rats.

4. Selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012, has an anti-inflammatory activity comparable in intensity to Diclofenac sodium, a drug with the greatest anti-inflammatory activity in the NSAID group. The anti-inflammatory effect was manifested in the suppression of the hind paw edema of the mouse in response to the subaponeurotic injection of 2% formaldehyde solution after the administration of the test substances.

5. There was neither analgesic nor anti-inflammatory activity of the selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012, at the dose of 1 mg/kg, and it was maximally expressed at the dose of 3 mg/kg. At the same time, there was no significant difference in the severity of the effects between the doses of 3 mg/kg and 9 mg/kg, which evidences the absence of a pronounced dose-dependent action of the selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012.

6. Selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012, according to the results of behavioral tests – open field test and elevated plus-maze test – has no effect on the general locomotor activity levels and the anxiety of the laboratory animals.

7. The bioavailability of the selective inhibitor of $TRPA_1$ ion channel, substance under code ZC02-0012, in inintragastric administration is relatively low, whereas ZC02-0012 is intensely distributed into organs and tissues with a high level of blood circulation. The highest concentration of ZC02-0012 is typical of liver, kidney and lungs, the lowest is of muscle tissue. Most of the substance undergoes rapid biotransformation and is excreted as metabolites.

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