

UDC: 615.224

DOI: 10.18413/2500-235X -2016-2-3-28-45

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Pokrovskii M.V.²**CARDIOVASCULAR EFFECTS OF AN ARGINASE II SELECTIVE
INHIBITOR**

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ABSTRACT

For the first time in vitro experiments there were studied the inhibitory activity and safety of potential molecules arginase II selective inhibitors from the group of norleucine derivatives. Also first the substance under the code ZB49-0010C from the group of norleucine derivatives showed the greatest selectivity and inhibitory activity against arginase II in experiments in vitro. However, this substance in vivo exerts dose-dependent hypotensive action and cardioprotective and endothelial protective effects on the L-NAME induced and homocysteine-induced endothelial dysfunction (ED), which are most pronounced at a dose of 10 mg/kg in intragastric administration. Endothelial protective effect consists of in preventing the increase of coefficient of endothelial dysfunction (CED) and the decrease in the concentration of stable metabolites of nitric oxide in the blood plasma. Cardioprotective effect consists of in preventing the increase of the level of left ventricular pressure during the test for adrenoactivity and reducing of the cardiac reserve during the overload resistance test, and also in preventing the development of the left ventricular hypertrophy. As part of the study there was investigated the dose-dependent anti-ischemic effect of the arginase II selective inhibitor, substance ZB49-0010C on the chronic limb ischemia in rats, which most pronounced at a dose of 10 mg/kg and consists of in preventing the fall in microcirculatory level on the 29th day of the experiment in the ischemic limb and a protective effect based on the morphological examination of the muscle tissue.

Key words: arginase II selective inhibitors, ZB49-0010C, L-NAME, endothelial dysfunction, nitric oxide, methionine, homocysteine, ischemia, cardioprotective effect.

To date, the morbidity and, most importantly, the disablement and mortality from cardiovascular diseases have no tendency to decrease, despite the presence of a huge number of tools and methods like medication and non-drug therapy, which in itself reflects the limited therapeutic possibilities of modern medicine, or about the faulty and deadlock the paradigm of pharmacotherapy of cardiovascular diseases. As long as modern medicine will not find adequate and effective treatment modalities of cardiovascular diseases, the search for new therapeutic targets and molecules will not lose its relevance and priority for innovation cardiopharmacology.

According to the most progressive of existing paradigms, a key element in the pathogenesis of the majority of such cardiovascular diseases as atherosclerosis, arterial hypertension, atherosclerotic cardiovascular disease (CAD), acute cerebrovascular event, dyscirculatory encephalopathy (DEP), chronic arterial insufficiency of the lower extremities is an ED [1, 2, 3, 4]. ED is the imbalance between humoral

factors having vasodilatory and antiplatelet action (NO, endothelium-derived hyperpolarizing factor, prostaglandins), and vasospastic and haemostatic system activation factors (endothelin-1, thromboxane A₂, superoxide anion). Thus, a key element of the pathogenesis of ED is the deficit of endogenous NO. Normally functioning endothelium continuously produces NO via endothelial NO synthase (eNOS) from L-arginine [5, 6, 7, 8, 9].

However, along with deficit of NO production, a decrease in its bioaccessibility has a significant importance in the pathogenesis of ED, which may be due to increase level in plasma asymmetric dimethylarginine (ADMA), an endogenous competitive eNOS inhibitor. Another mechanism of NO reduction is associated with increased production of oxygen free radicals [4, 9, 10].

As you know, L-arginine as the only substrate for NO synthesis, is actively biotransformed under the influence of arginase. The concentration of L-arginine in the blood plasma varies depending on a

great many corrected and non-corrected factors [5, 6, 7, 8, 11, 12, 13].

It is known that the metabolism of L-arginine in the cells occurs in two ways (the first way L-arginine is hydrolyzed by arginase to ornithine and urea. The second L-arginine is metabolized to nitric oxide and citrulline that is catalyzed by NO synthase. Enzymes arginase and NO-synthase compete for the common substrate L-arginine [4, 9, 14, 15].

Arginase has a high activity exceeding the activity of NO-synthase. It is represented as two isoforms: arginase I is liver and arginase II is the extrahepatic form, localized more frequently in the kidney, prostate, small intestine. According to several contemporary researchers, the increased activity of arginase II has been observed in much pathology such as diabetes, asthma, glomerulonephritis, psoriasis. As you know, arginase II inhibits eNOS, preventing the production of nitric oxide. A number of studies have established that inhibition of this enzyme increases the nitric oxide production and the prevention of dysfunctional disorders in the endothelium [12, 13, 14, 16, 17, 18, 19, 23].

Natural arginase inhibitors include amino acids such as ornithine, leucine, valine, lysine, isoleucine and norvaline. All inhibitors of arginase are divided into selective and non-selective, and specific acting directly on the enzyme and block its active site, and non-specific, acting on the enzyme indirectly. With the aim of inhibiting arginase there was used N ω -hydroxy-L-arginine (NOHA), which is an intermediate in the synthesis of NO. However, due to the NOHA is a coenzyme of cytochrome P₄₅₀, its administration was very difficult, and in return, he was synthesized N ω -hydroxy-nor-L-arginine (nor-NOHA) [10, 24]. Also as an arginase inhibitor there was investigated ϵ -difluoromethylornithine (DFMO) in vitro and in vivo. DFMO inhibits ornithine decarboxylase (ODC) and increases the amount of L-arginine through the urea cycle. However, to reduce the activity of arginase large concentrations of DFMO are required, and it contributes to the independent vascular responses and accumulation of ornithine. Such substances as S-(2-boronoethyl)-L-cysteine (BEC) and 2(S)-amino-6-borono-hexanoic acid (ABH) showed a high activity in reducing the activity of arginase. Another inhibitor of arginase is L-norvaline [1, 14, 15, 16, 24, 25].

However, the described inhibitors of arginase are low-selective or non-selective and inhibit the activity of arginase II and arginase I. A decrease in the activity of arginase I associated with a number of side effects. In experiments with gene knockout of

arginase I in mice there were observed symptoms of hyperammonemia. All animals died within 10 to 14 days of postnatal development [26].

That is why it is important to search for new highly selective inhibitors of arginase II, the study of their cardioprotective and endothelial protective effects. To date, the global pharmaceutical market has no drugs from the group of arginase II selective inhibitors. That is why the aim of our study is increase the efficiency of pharmacological correction of ED using arginase II selective inhibitors, norleucine derivatives.

RESEARCH OBJECTIVES

1. To carry out the selection of potential molecules arginase II selective inhibitors according to the criteria of selectivity and safety tests in vitro.

2. In acute pharmacological experiments to select antihypertensive doses of the most active and safe agent from the group of norleucine derivatives.

3. To study endothelial protective and cardioprotective effects of arginase II selective inhibitor under the code ZB49-0010C in dose flexibility on the L-NAME induced deficit of endogenous NO.

4. To study endothelial protective and cardioprotective effects of arginase II selective inhibitor under the code ZB49-0010C in dose flexibility on homocysteine-induced ED.

5. To evaluate the antiischemic activity of the arginase II selective inhibitor under the code ZB49-0010C on the chronic limb ischemia on the background of L-NAME induced deficit of NO.

MATERIALS AND METHODS

The experiments were performed in the laboratory of preclinical studies of the Center for preclinical and clinical studies of Belgorod State University in 520 male Wistar rats (weight 200 \pm 10 g, age 4-5 months) and 40 white mice of both sexes (weight 20 \pm 2 g; age 5-6 weeks). All experiments were approved by the Ethics Committee of Federal Autonomous Educational Institution of Higher Education Belgorod State University. Vivisection was carried out in accordance with the ethical principles for the treatment of laboratory animals of "The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. CETS No. 123". The investigated substances were synthesized by High-Technology Center "CHEMRAR", under the leadership of the director general Dmitry Vladimirovich Kravchenko. Structures of the synthesized potential molecules are given in table 1.

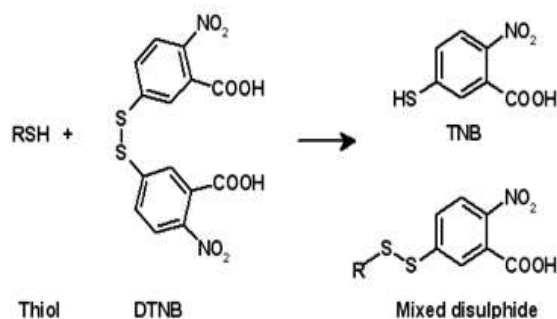
Table 1

Chemical structure of the investigated substances

Laboratory code	Full chemical name of agent	Structure	Molecular weight
Z991-0104	1,2,4,7-tetraze- 5-tertbutylamino -6-[4H-2,6- dimethoxyphenyl] pyrazine		319
ZB49-0009	2-{1-[3-(thio isoxazole -5-yl) propyl] piperidine -4-yl}-6-(dihydroxyboryl) norleucine		378
ZB49-0010C	2-{1-[3-(3-chloro isoxazole -5-yl) propyl] piperidine -4-yl }- 6-(dihydroxyboryl) norleucine dihydrochloride		471.5

Research methods in vitro. Study the specific activity of ZB49-0010C

was carried out using the following biochemical reactions:



Arginase II hydrolyses L-arginine to mercaptovalerate, which in the presence of a 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) is metabolized to the 5-thio-2-nitrobenzoic acid the characteristic yellow color. The activity of the arginase II is in direct ratio to the amount of 5-thio-2-nitrobenzoic acid and measures spectrophotometrically by the intensity of yellow color at a wavelength of 405 nm. A known arginase II inhibitor ABH was used as a positive control and to determine a value of minimum signal of the experiment. IC_{50} (concentration of substance in μm which causes a 50% maximal effect) for ZB49-0010C was determined from the graph of the concentration dependence of the inhibiting effect of the substance. The experimental data were analyzed in the GraphPad Prizm program (GraphPad Software,

Inc., San Diego, CA). To graph concentration dependence there was chosen the equation:

$Y = \text{Bottom curve} + (\text{Top curve} - \text{Bottom curve}) / (1 + 10^{((\text{Log}IC_{50} - X) * \text{Curve slope}))}$.

To verify the most promising molecules according to the criterion of safety it is necessary primary to study of the binding potential molecules arginase II selective inhibitors hERG channel of cardiomyocytes, as blocking of this channel leads to fatal consequences such as the disturbance of repolarization, QT interval increase and the subsequent development of asystole.

Determination of the effect of the studied substances on the hERG channel was performed using in vitro test systems Invitrogen® Predictor™ hERG. There was investigated the binding substance the hERG channel in the composition of the

membrane fraction when the concentration of the drug 10 and 40 μmol . The method is based on changing the detection polarization of the fluorescence, which decreases as the displacement high-affinity fluorescent ligand tracer by the test substance. Experimental procedures were performed in accordance with the manufacturer's instructions to test the system PredictorTM hERG.

Research methods in vivo. Experiments to study general toxic action in the acute toxicity of the arginase II selective inhibitor under the code ZB49-0010C were performed on white mice of both sexes weighing 22 ± 2 g in accordance with the applicable guidelines on preclinical study of new pharmacological substances (Mironov A. N., 2012, R. U. Khabriev, 2005).

Therapeutic dose calculation of ZB49-0010C. According to the study in vitro activity, ZB49-0010C at a concentration of 30 μmol has a 100% high selective inhibitory activity against the arginase II. On the basis of molecular weight 471.5, when the concentration of the solution equal to 30 μmol , dose of the agent in the volume of distribution of entire body is 14 mg/kg. As the circulating blood volume in rats is approximately 8-10% of body weight, the actual dose may be reduced to 10 times; therefore, a minimal therapeutic dose is 1 mg/kg.

To study the hypotensive effect and the selection of effective doses there was performed acute pharmacological experiment. Thus arginase II selective inhibitor ZB49-0010C was administrated intragastric in doses of 1 mg/kg, 5 mg/kg and 10 mg/kg. Measurement and record of the systolic blood pressure, diastolic blood pressure and heart rate were made for 30 min before the intragastric administration of ZB49-0010C, as well as after 15 and 30 min, 1 and 2 hours after on the tail by a sensor and hardware-software complex MP 150 "Biopac-systems" (USA), in animals without anesthesia in a holder at a temperature of 28° C, which was maintained with the help of a special heating chamber ("Biopac-systems", USA).

1. To simulate ED male rats were administrated intraperitoneal eNOS inhibitor L-NAME at a dose of 25 mg/kg of body weight daily, 1 time per day for 7 days. To simulate homocysteine-induced ED rats were administrated intragastric methionine (Polisintez, LLC, Belgorod) daily in a dose of 3 g/kg body weight, 1 time per day. On the 8th day from the start of the experiment under noninhalation anesthesia (chloral hydrate 150 mg/kg + zoletil 60 mg/kg) the left carotid artery was catheterized for

registration of hemodynamic parameters. Systolic blood pressure, diastolic blood pressure and heart rate were measured continuously by a sensor, the computer program AcqKnowledge 4.1 and hardware-software complex MP 150 "Biopac-systems" (USA). There were performed functional tests for endothelium-dependent and endothelium-independent vasodilatation with the following calculation of the CED [8, 27].

To study the contractile activity of the myocardium and hemodynamic parameters on the back of ED, in rats with anesthesia and automatic breathing there was performed catheterization of the left ventricle and there were measured continuously by a sensor, the computer program AcqKnowledge 4.1 and hardware-software complex MP 150 "Biopac-systems" (USA) hemodynamic parameters, such as left ventricular pressure, maximum contraction rate (+dp/dt max), maximum relaxation rate (-dp/dt max) and heart rate. There were performed functional stress tests in the following sequence:

2. The test for adrenoactivity
 3. The overload resistance test (the clamping of the ascending aorta for 30 sec) [8, 27].
 4. Quantification of stable metabolites of NO.
- As part of the study there was used a modification of the method for the quantification of stable metabolites of NO (Metelskaia V. A., 2005).

Simulation the chronic limb ischemia of the hind limb in rats was performed with anesthesia (chloral hydrate 150 mg/kg and zoletil 60 mg/kg) by ligation with the intersection of the femoral artery beneath the inguinal ligament and removal of the portion of the great vessel, including the femoral, popliteal artery and primary departments of the crural arteries (Kolesnik, I. M., 2010). Assessment of the microcirculation level in the tibiotarsus muscles of the rats was performed with noninhalation anesthesia (chloral hydrate 150 mg/kg and zoletil 60 mg/kg) using a laser Doppler flowmeter "Biopac-systems" MP 150, needle sensor TSD-144 and program AcqKnowledge 4.1 [28, 29].

To confirm the development of simulated pathological processes and integrated assessment of the effectiveness of their correction in all series of experiment there was performed histomorphometric studies of the heart, kidneys and muscles of the hind limb.

The results of the studies were statistically analysed. They calculated arithmetic means and standard errors. The significance of differences was

evaluated in Student t-test, Wilcoxon and Mann-Whitney tests. IC_{50} was calculated by linear regression analysis. For the calculations there was used program for statistical analysis Microsoft Excel 2007.

THE RESEARCH DESIGN

To study dose-dependent pharmacological activity, ZB49-0010C was administered intragastrically at doses of 1 mg/kg, 5 mg/kg and 10 mg/kg in acute pharmacological experiment and in the study of pharmacodynamic action in the long-term experiment on the L-NAME induced and homocysteine-induced ED, as well as on the chronic limb ischemia.

The study protocol of cardioprotective and endothelial protective effects of the arginase II selective inhibitor ZB49-0010C included the following sections:

1. Simulation L-NAME induced and homocysteine-induced ED and its correction by the arginase II selective inhibitor ZB49-0010C for 7 days.

2. Assessment of endothelium-dependent and endothelium-independent reactions of arterial pressure on simultaneous intravenous introduction of acetylcholine and sodium nitroprusside with chloral hydrate anesthesia.

3. Automatic breathing of animals, catheterization of the left ventricle, registration of the left ventricular pressure, $+dp/dt$, $-dp/dt$, heart rate. There were performed functional stress tests to assess functional reserves of the contractile activity of the myocardium.

4. Blood sampling from the right ventricle for biochemical analysis and euthanasia of animal.

5. Sample collection of the heart and kidneys for morphological studies.

In the study of the antiischemic activity of the arginase II selective inhibitor ZB49-0010C there was developed the following research design:

1. The group of intact animals
2. The sham-operated group of animals
3. The group with simulated limb ischemia
4. The group with simulated limb ischemia + intraperitoneal administration of L-NAME at a dose of 12.5 mg/kg daily for 28 days.
5. The group with simulated limb ischemia + intraperitoneal administration of L-NAME at a dose

of 12.5 mg/kg daily for 28 days + ZB49-0010C at a dose of 1 mg/kg

6. The group with simulated limb ischemia + intraperitoneal administration of L-NAME at a dose of 12.5 mg/kg daily for 28 days + ZB49-0010C at a dose of 5 mg/kg

7. The group with simulated limb ischemia + intraperitoneal administration of L-NAME at a dose of 12.5 mg/kg daily for 28 days + ZB49-0010C at a dose of 10 mg/kg

8. The group with simulated limb ischemia + intraperitoneal administration of L-NAME at a dose of 12.5 mg/kg daily for 28 days + L-norvaline at a dose of 1 mg/kg

The study protocol of the antiischemic activity of the arginase II selective inhibitor under the code ZB49-0010C included the following sections:

1. Mixed noninhalation anesthesia (chloral hydrate intraperitoneally 150 mg/kg + zoletil 60 mg/kg)

2. Dissection of skin and fascia

3. Measurement of microcirculation by LDF

4. Blood sampling from the right ventricle for test concentration of stable metabolites of nitric oxide

5. Sample collection of the muscles for morphological study.

RESEARCH RESULTS

Study of specific activity and mechanism of action of bioactive molecule ZB49-0010C. The activity of the arginase II is directly proportional to the amount of 5-thio-2-nitrobenzoic acid and it was measured spectrophotometrically by the intensity of yellow color at a wavelength of 405 nm. A known inhibitor of the arginase II ABH was used as a positive control to determine value of minimum signal of the experiment. IC_{50} for ZB49-0010C was determined from the graph of the concentration dependence of the inhibitory effect of the substance (figure 1 and table 2). Concentration dependence of the inhibitory effect of the substance ZB49-0010C and control substance ABH in relation to the arginase II is presented in figure 1. Each concentration point represents the average value for the two repetitions. Specific activity of ZB49-0010C in respect of the arginase II obtained in 2 independent experiments is shown in table 3.

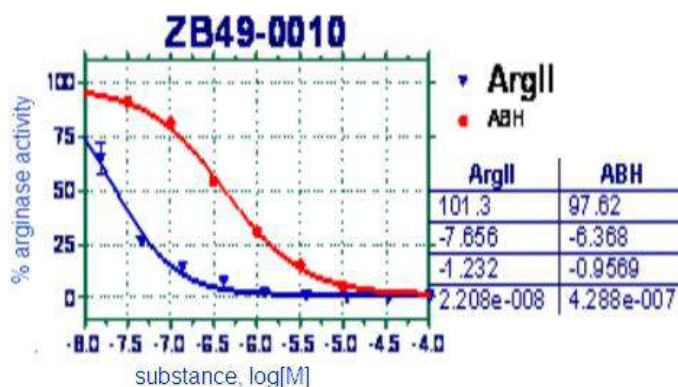


Figure 1. Concentration dependence of the inhibitory effect of the substance ZB49-0010C and control substance ABH in relation to the arginase II

Table 2

Specific activity of ZB49-0010C in relation to the arginase II, obtained in two independent experiments

Substance	Max testing concentration (μmol)	Substance activity in max concentration (%)	IC ₅₀ , (nmol)	Numerical order of the experiment
ZB49-0010C	30	99	22.0	1
ZB49-0010C	30	100	19.7	2

As can be seen from figure 1 and table 2, ZB49-0010C has a nanomolar inhibitory activity against arginase II, as a complete inhibitor of the enzyme.

The results of the binding potential molecules arginase II selective inhibitors hERG channel at

concentrations of 10 and 40 μm in the composition of the membrane fractions using a test system Invitrogen® Predictor™ hERG are presented in table 3.

Table 3

The results of the binding potential molecules arginase II selective inhibitors hERG channel in the composition of the membrane fractions using a test system Invitrogen® Predictor™ hERG

Substance	hERG- dependence fluorescence polarization				
	Concentration (μmol)	Polarization, mP. Mean value (n=20)	SD	CV, %	Non-binding hERG, %
Z991-0104	40	275	5.1	1.9	86
ZB49-0009	40	245	5.0	2.1	87
ZB49-0010	40	265	6.9	2.6	100
Z991-0104	10	287	10.1	3.5	96
ZB49-0009	10	271	5.4	2.0	97
ZB49-0010C	10	268	3,0	1,0	100

The data indicate the absence of the binding ZB49-0010C hERG channel at concentrations up to 40 μmol (table 3).

Conducted study of general toxicological action in the acute toxicity showed that after a single intragastric administration to mice of the active pharmacological substance ZB49-0010C at the dose of 1438 mg/kg animal deaths were not observed within two weeks after administration of ZB49-0010C. According to the classification Sidorov, K. K., active pharmacological

substance ZB49-0010C can be attributed to low-toxic substances.

The results of the dynamic of the systolic blood pressure, diastolic blood pressure and heart rate in acute pharmacological experiment in animals without anesthesia treated ZB49-0010C are presented in figure 2.

As shown by the results of acute pharmacological experiment (figure 2), ZB49-0010C has a dose-dependent hypotensive effect, most pronounced at a

dose of 10 mg/kg, reaching its maximum after 30 min after a single intragastric administration, and not

changing significantly up to 2 hours measurements of blood pressure.

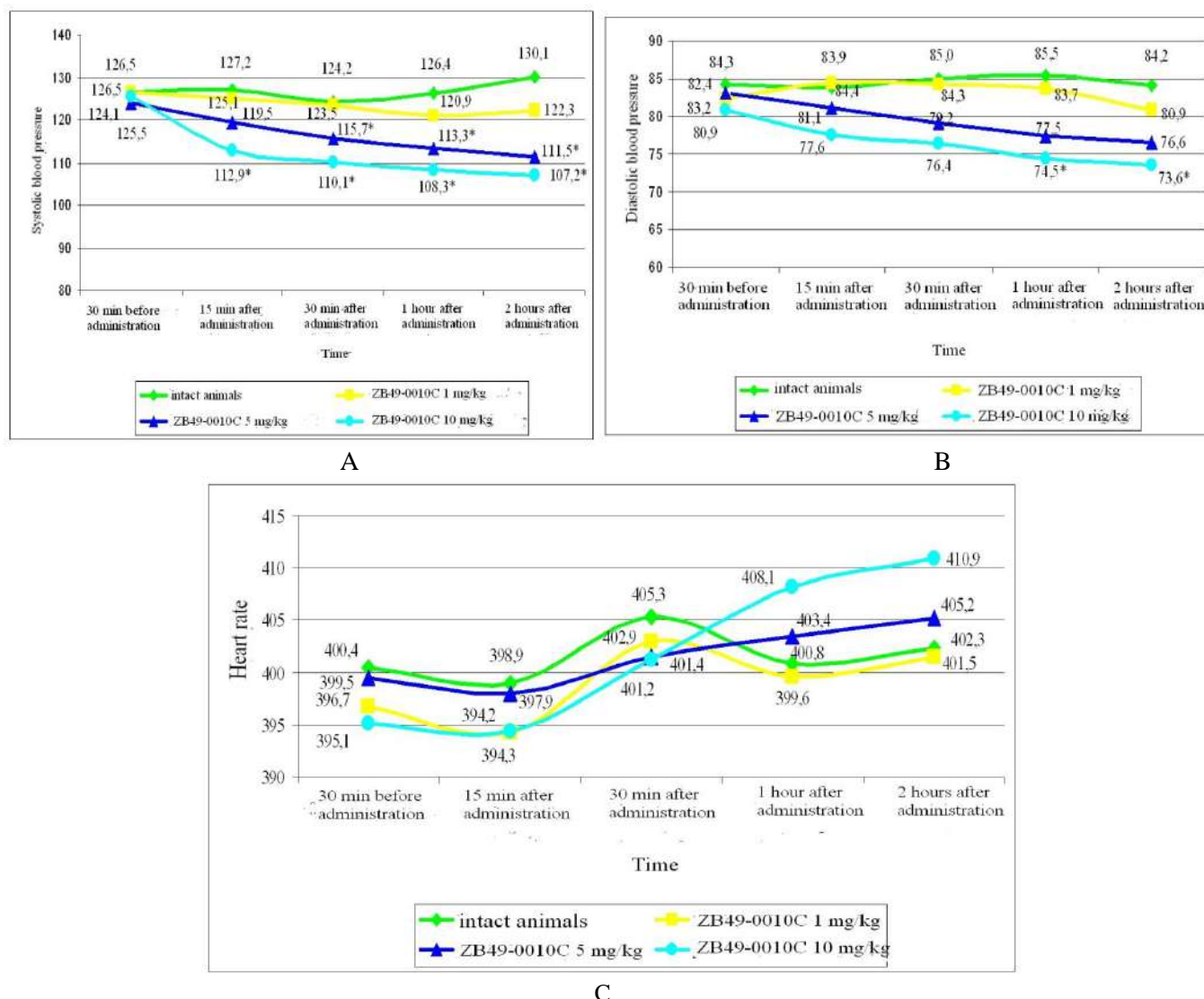


Figure 2. The dynamic of dose-dependent effect of the ZB49-0010C on the systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) in rats without anesthesia in the acute pharmacological experiment.

Comment: * – p<0.05 in comparison with group of intact animals

Study of dose-dependent cardioprotective and endothelial protective effects of the arginase II selective inhibitor substances under the code ZB49-0010C on the L-NAME induced deficit of endogenous NO.

Dose-dependent effects of the ZB49-0010C at baseline blood pressure, CED, Total NO, adrenoreactivity, cardiac reserve and the diameter of cardiomyocytes in comparison with L-Norvaline in rats with anesthesia on the back of L-NAME-induced ED are presented in figure 3.

Found that arginase II selective inhibitor substance under the code ZB49-0010C has a dose-

dependent endothelial protective effect, most pronounced at a dose of 10 mg/kg, which manifested in prevention of the increase in CED and approached of its values to the group of intact animals. Also there was found a dose-dependent antihypertensive action of ZB49-0010C, which is maximal at a dose of 10 mg/kg on the back of L-NAME-induced deficit of endogenous NO. There was established a dose-dependent cardioprotective effect of this pharmacological agent, which is most pronounced at a dose of 10 mg/kg and manifested in preventing the increase of the left ventricular pressure during the test for adrenoreactivity and the reduction of cardiac

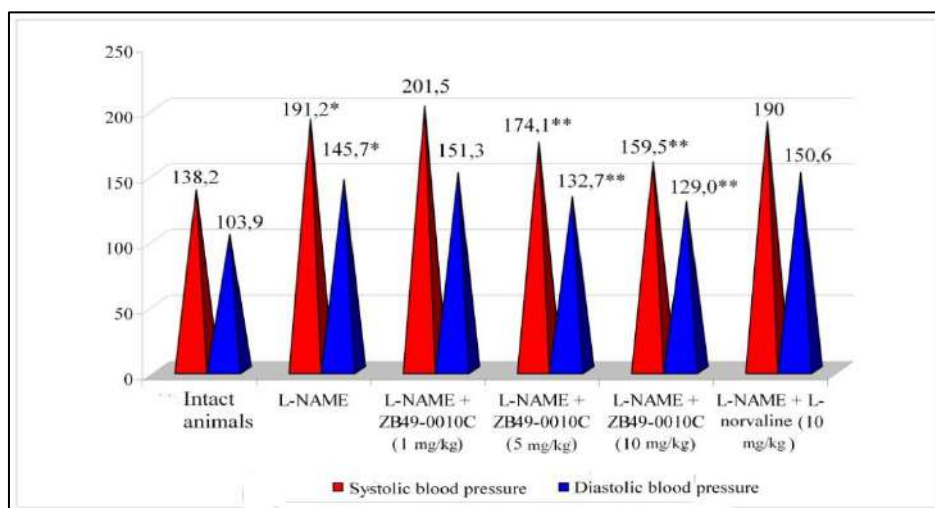
reserve during the overload resistance test, and preventing the development of the left ventricular hypertrophy, identified by histological examination.

The study of the dose-dependent cardioprotective and endothelial protective effects of the arginase II selective inhibitor substances under the code ZB49-0010C on the homocysteine-induced endothelial dysfunction.

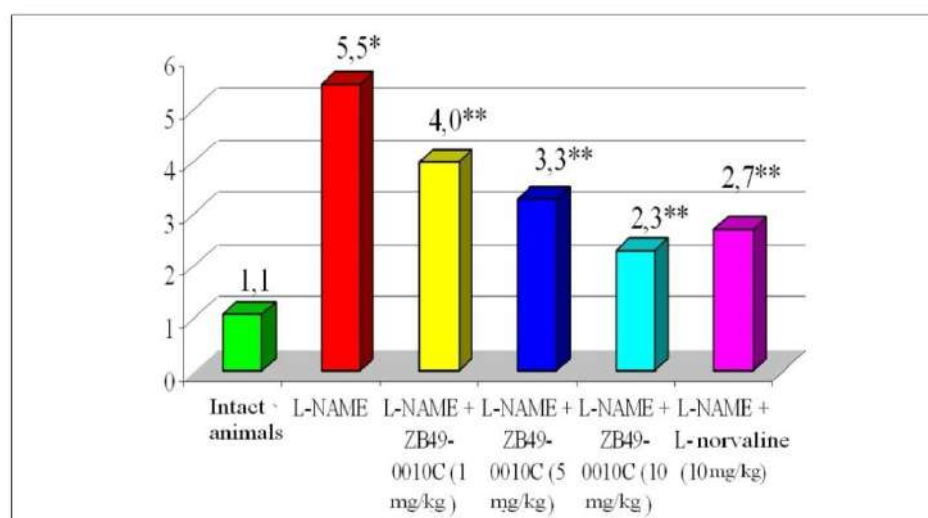
Dose-dependent effect of ZB49-0010C at baseline CED, Total NO, level of homocysteinemia, adrenoreactivity, cardiac reserve and the diameter of cardiomyocytes in comparison with L-Norvaline in rats with anesthesia on the back of homocysteine-induced ED is presented in figure 4.

Found that arginase II selective inhibitor substances under the code ZB49-0010C has a dose-

dependent endothelial protective effect, most pronounced at a dose of 10 mg/kg, which manifested in prevention of the increase in CED and approached of its values to the group of intact animals, and prevented the increase of the level of homocysteine in the blood plasma of rats and reduced the concentration of stable metabolites of nitric oxide. At the same time there was discovered a dose-dependent cardioprotective effect of this pharmacological agent, which is most pronounced at a dose of 10 mg/kg and manifested in preventing the increase of the left ventricular pressure during the test for adrenoreactivity and the reduction of cardiac reserve during the overload resistance test, and preventing the development of the left ventricular hypertrophy, identified by histological examination.

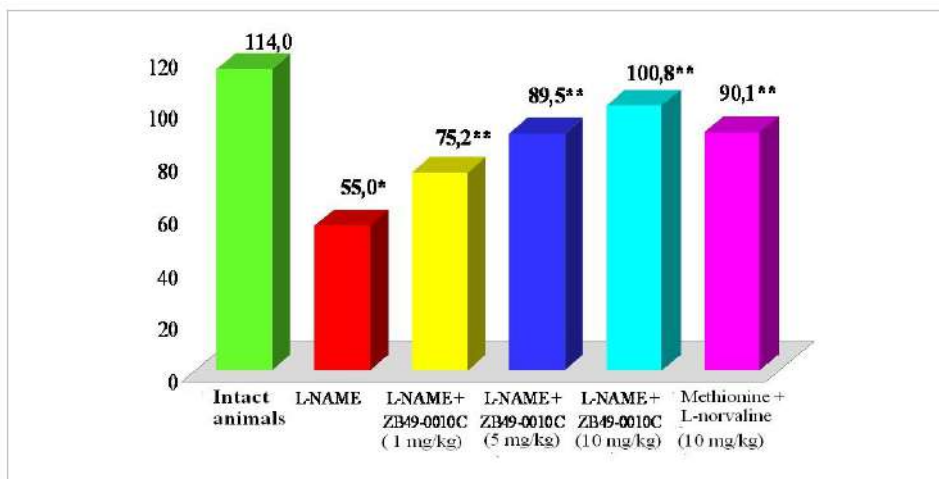


A) Arterial pressure

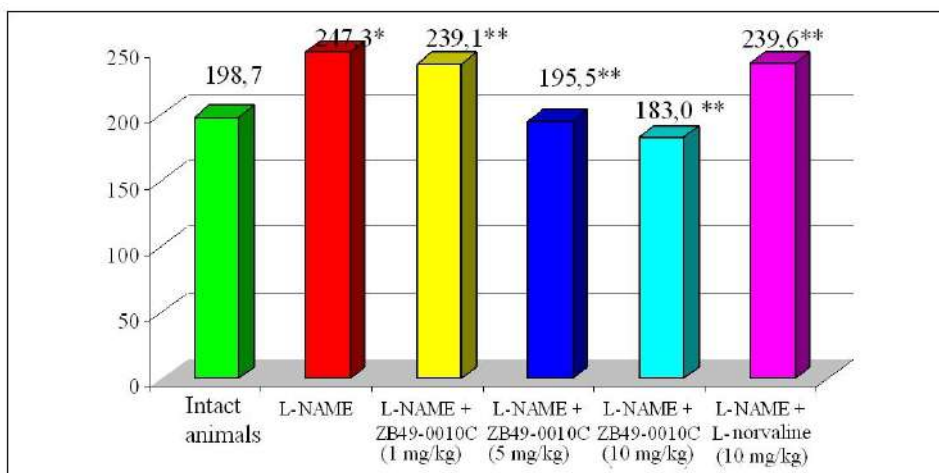


B) Coefficient of endothelial dysfunction

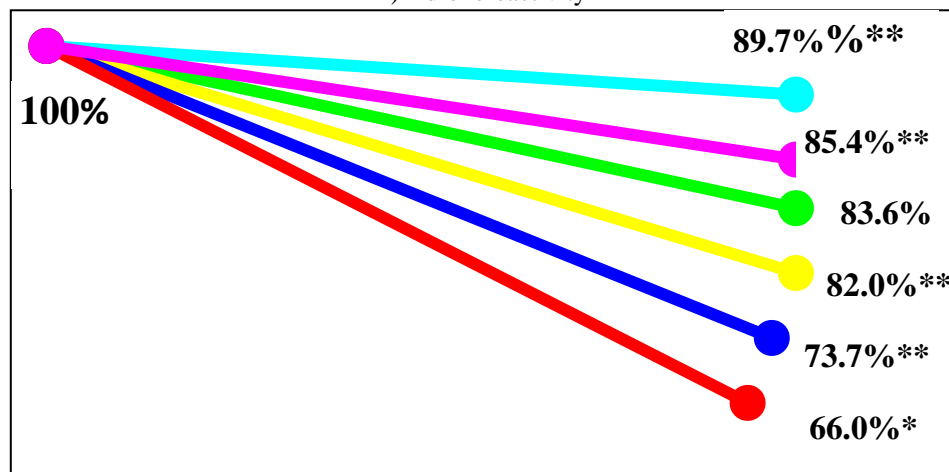
Figure 3. Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline arterial pressure (A), CED (B), Total NO (C), adrenoreactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of L-NAME-induced ED



B) Total NO

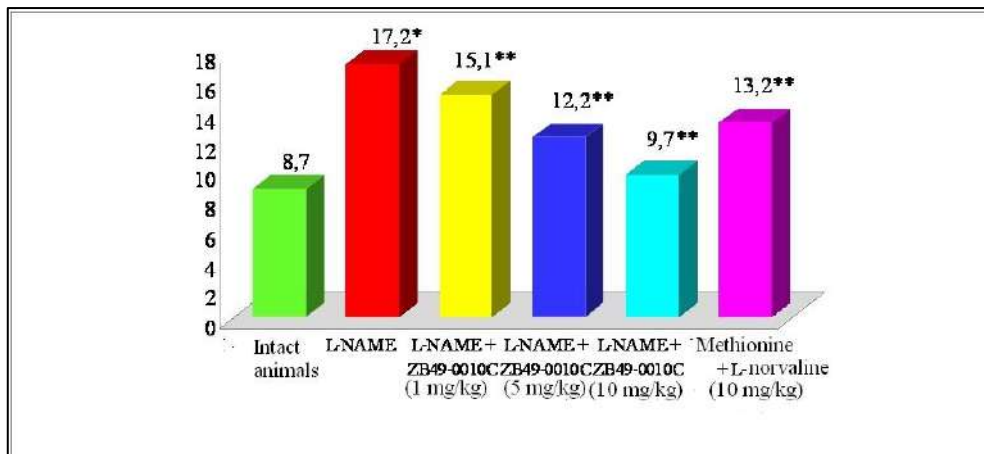


D) Adrenoreactivity



E) Cardiac reserve

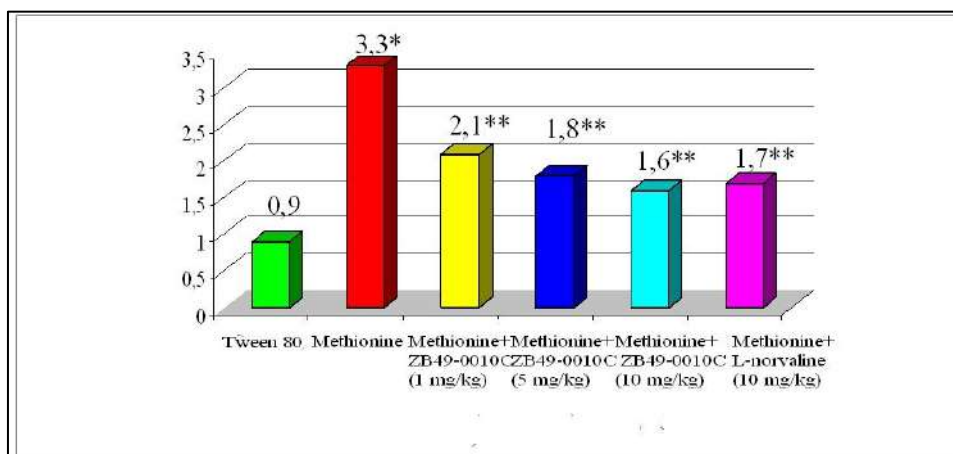
Figure 3 (continued). Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline arterial pressure (A), CED (B), Total NO (C), adrenoreactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of L-NAME-induced ED



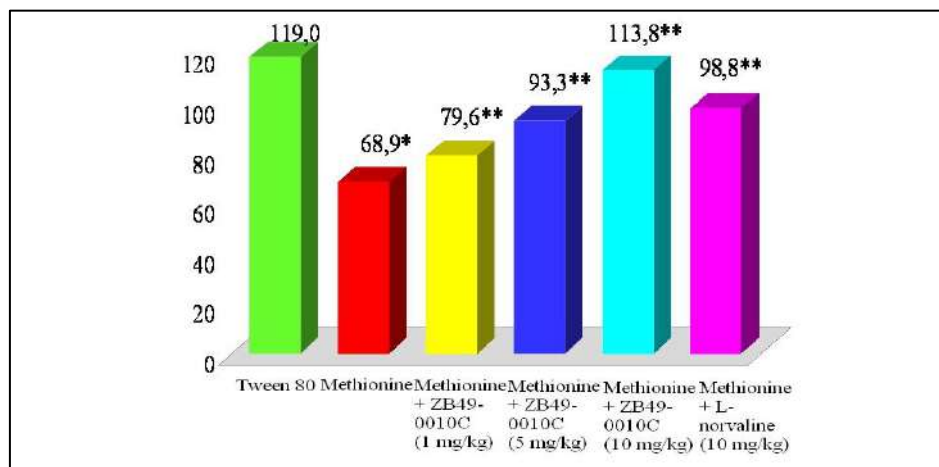
F) Diameter of cardiomyocytes

Figure 3 (continued). Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline arterial pressure (A), CED (B), Total NO (C), adrenoactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of L-NAME-induced ED

Comment: * – $p < 0.05$ in comparison with group of intact animals; ** – $p < 0.05$ in comparison with the group of animals with L-NAME-induced ED.

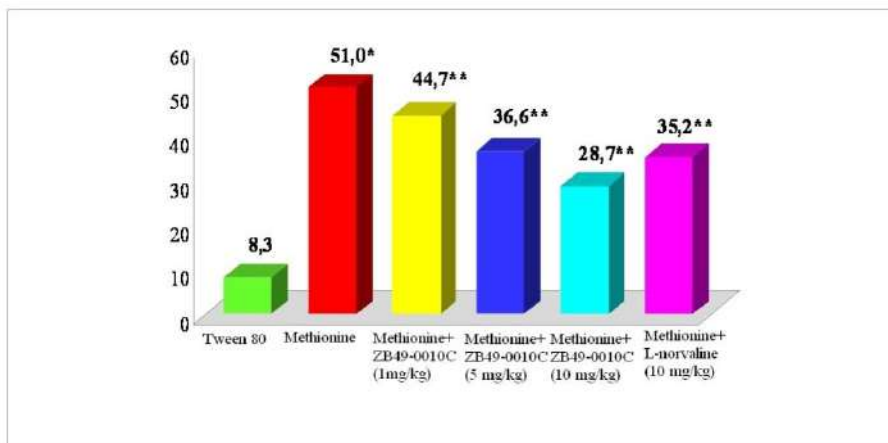


A) Coefficient of endothelial dysfunction

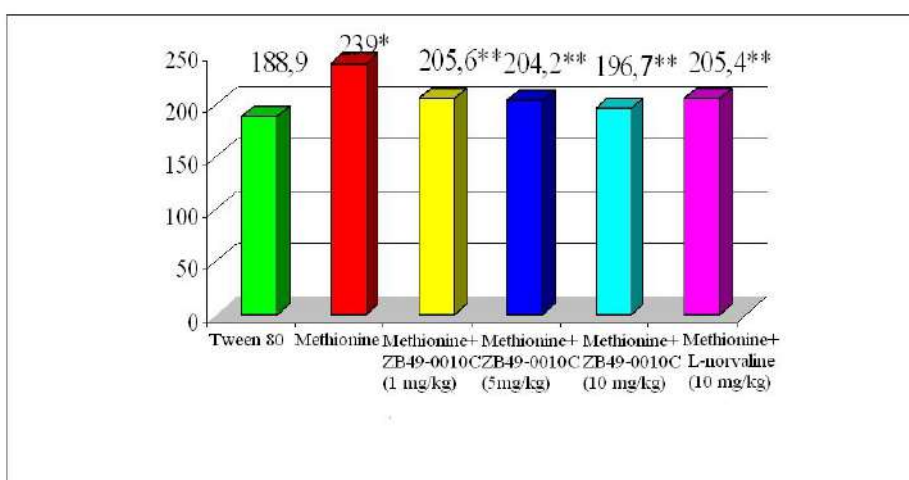


B) Total NO

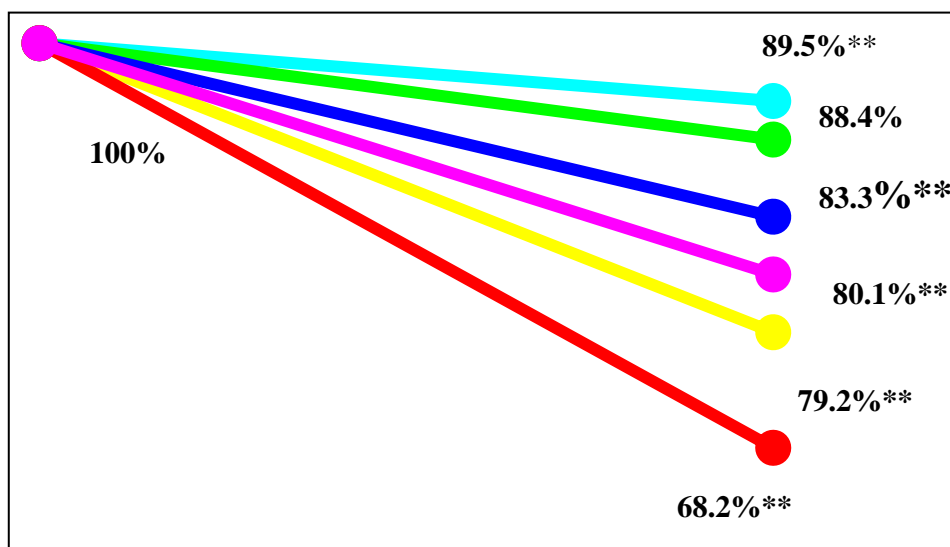
Figure 4. Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline CED (A), Total NO (B), level of homocysteine in the blood plasma (C), adrenoactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of homocysteine-induced ED.



C) Level of homocysteine in the blood plasma

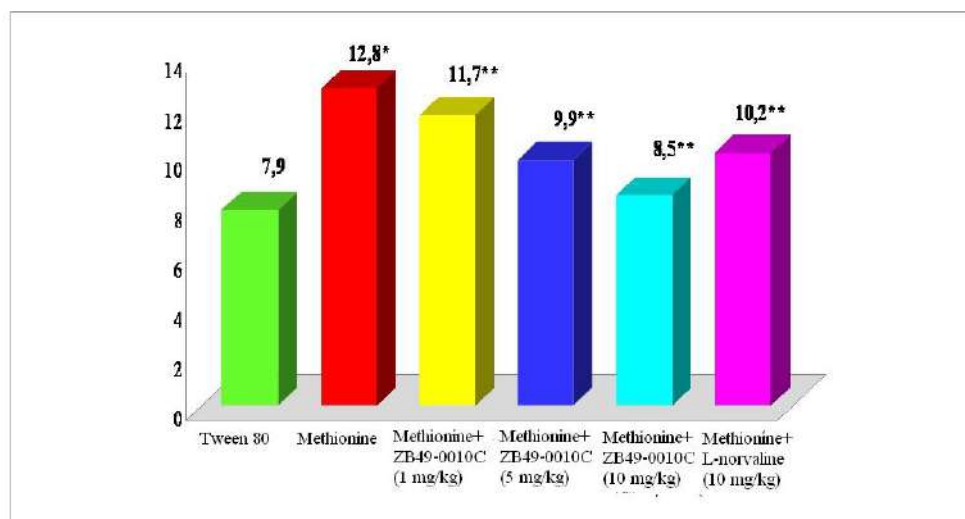


D) Adrenoreactivity



E) Cardiac reserve

Figure 4 (continued). Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline CED (A), Total NO (B), level of homocysteine in the blood plasma (C), adrenoreactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of homocysteine-induced ED



F) Diameter of cardiomyocytes

Figure 4 (continued). Dose-dependent effects of the ZB49-0010C compared with L-Norvaline on baseline CED (A), Total NO (B), level of homocysteine in the blood plasma (C), adrenoactivity (D), cardiac reserve (E), the diameter of cardiomyocytes (F) in rats with anesthesia on the back of homocysteine-induced ED

Comment: * – $p < 0.05$ in comparison with group of Tween; ** – $p < 0.05$ in comparison with the group of animals with homocysteine-induced ED.

The effects of the arginase II selective inhibitor, substances ZB49-0010C at the microcirculation level in the ischemic limbs on the back of L-NAME-induced deficit of endogenous NO in experiment.

The results of the evaluation of the microcirculation level in animals on day 29 in the

ischemic limb on the back of L-NAME-induced deficit of endogenous NO and its correction by arginase II selective inhibitor, substances ZB49-0010C at doses of 1, 5 and 10 mg/kg are presented in table 4.

Table 4

The results of the evaluation of the microcirculation level in the tibiotarsus muscles and the concentration of stable metabolites of NO (Total NO) in the blood plasma of rats on day 29. (M±m), PU (perfusion units)

Group	Perfusion units	Total NO, umol/l
Intact animals (n=20)	542.1 ±18.5	117.7 ±3.1
Sham-operated animals (n=20)	540±3.7	120.3±4.0
Animals with simulated limb ischemia (n=20)	360.8 ±8.7*	119.2 ±3.8*
Simulated limb ischemia + L-NAME 12.5 mg/kg (n=20)	281.4±25.6**	61.3±4.2***
Simulated limb ischemia +L-NAME 12.5 mg/kg + ZB49-0010C 1 mg/kg (n=20)	386.9 ±30.7***	73.8 ±4.2***
Simulated limb ischemia +L-NAME 12.5 mg/kg + ZB49-0010C 5 mg/kg (n=20)	583.6±36.7***	91.6±6.4***
Simulated limb ischemia +L-NAME 12.5 mg/kg + ZB49-0010C 10 mg/kg (n=20)	616.9±24.0***	118.7±4.3***
Simulated limb ischemia +L-NAME 12.5 mg/kg + L-norvaline 10 mg/kg (n=20)	496.8±29.6***	96.6±8.4***

Comment: * – $p < 0.05$ in comparison with group of intact animals; ** – $p < 0.05$ in comparison with the group of animals with simulated limb ischemia; *** – $p < 0.05$ in comparison with the group of animals with simulated limb ischemia+L-NAME.

As can be seen from table 4, the minimum microcirculation level in the tibiotarsus muscles was noted in the group of animals with simulated limb ischemia+ L-NAME. The influence of the arginase II selective inhibitor, substances ZB49-0010C at the

microcirculation level was dose-dependent, maximum effect was observed at a dose of 10 mg/kg.

Found that the arginase II selective inhibitor, substance ZB49-0010C has dose-dependent antiischaemic effect, which most pronounced at a dose of 10 mg/kg and consists of in preventing the

fall in microcirculatory level, and also a protective effect on histoarchitecture of the striated muscle tissue in the ischemic limb on the results of morphological studies (figure 5). It was found that

one of the mechanisms of the antiischemic effect of ZB49-0010C is the activation of synthesis of endogenous NO due to increasing bioaccessibility of L-arginine.

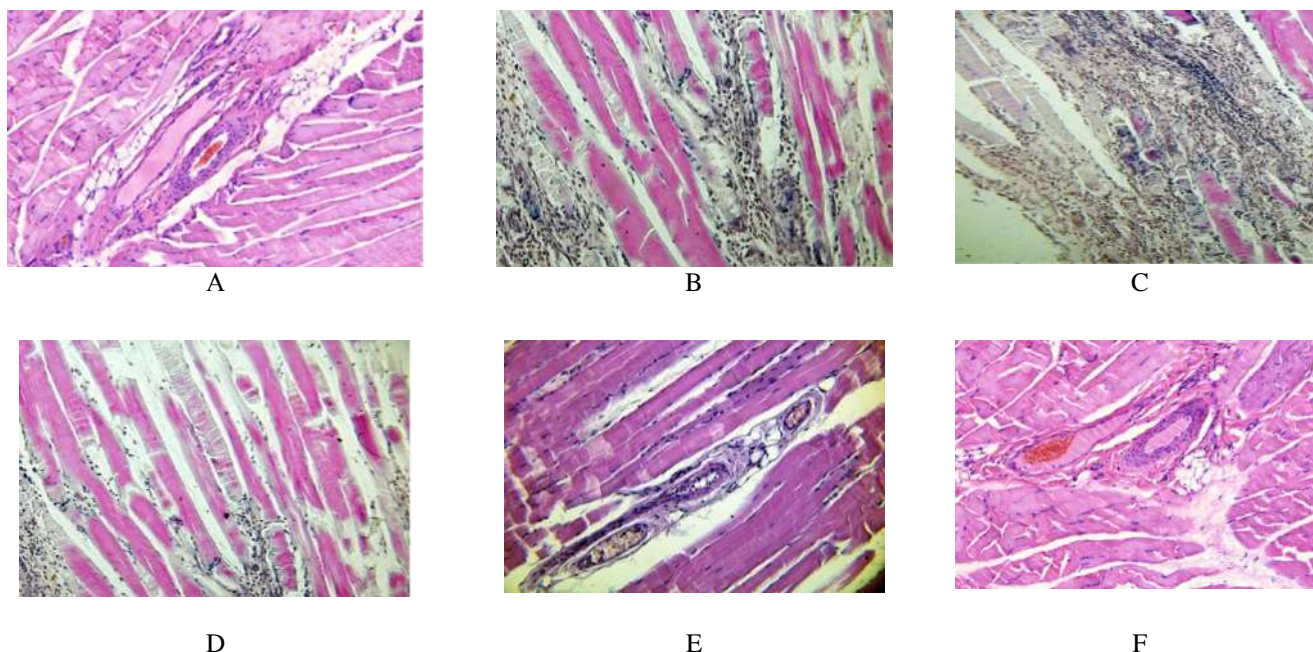


Figure 5. Photomicrography 200 x: Morphological examination of the tibiotarsus muscles of rats, 29 days of the experiment.

A – group of intact animals; B – group with simulated limb ischemia; C – group with simulated limb ischemia + L-NAME; D – group with simulated limb ischemia + L-NAME + ZB49-0010C 1 mg/kg; E – group with simulated limb ischemia + L-NAME + ZB49-0010C 5 mg/kg; F – group with simulated limb ischemia + L-NAME + ZB49-0010C 10 mg/kg. Hematoxylin and eosin stain.

Thus, in the study of the 3 most promising potential molecules, according to results of testing in vitro, a substance under laboratory code ZB49-0010C from the group of norleucine derivatives shows most pronounced inhibitory activity (at a concentration of 30 μ mol) against arginase II as its full inhibitor. This agent in vitro doesn't bind hERG- channel and not inhibit it at concentrations up to 40 μ mol, thus not affecting the membrane potential and the cardiac repolarization that indicates its potential safety.

During a comprehensive in vivo studies, simulation L-NAME induced and homocysteine-induced ED, as well as chronic limb ischemia on the back of deficit of endogenous NO, there was found that arginase II selective inhibitor substance under laboratory code ZB49-0010C from the group of norleucine derivatives has a dose-dependent antihypertensive action, endothelial protective, cardioprotective, antiischemic effects, most pronounced at a dose of 10 mg/kg.

EVALUATION

According to current data, ED is the main risk prediction of the overwhelming majority of

cardiovascular diseases, a key role in the pathogenesis of which, as already mentioned, is played the deficit of endogenous NO. As you know, L-arginine as the only substrate for NO synthesis is actively metabolized by arginase II. Increased activity of arginase II leads to a decrease in NO and, consequently, to the development of ED. According to several contemporary authors, the increased activity of arginase is observed in bronchial asthma, arthritis, glomerulonephritis, psoriasis, with the development of diabetic erectile dysfunction. Also there was confirmed the link to the high activity of arginase with the development of ED in rats. Inhibition of this enzyme increases the NO synthesis and prevents the dysfunctional disorders in the endothelium (figure 6) [4, 13, 14, 15, 16, 17, 18, 19, 20, 21]. Therefore, the most promising and expedient pathogenetically in the prevention and complex therapy of cardiovascular diseases is the use of highly selective arginase II inhibitors. As you know, today the world pharmaceutical market has no one drug from the group of arginase II selective inhibitors. There is a variety of different reasons, the main of which, in

our opinion, is the failure of the search of molecules possessing on the one hand, the high selectivity in respect of arginase II and pharmacological activity and, on the other hand, a wide therapeutic range and toxicological safety. That is why the search for active highly selective and safe potential molecules arginase II selective inhibitors requires not only a huge set of mathematical, physicochemical methodology, aimed at the synthesis and selection of the most innovative and promising agents, but its long term test both in vitro and in vivo in various models of disease. Therefore, we have consistently used complex methodological approaches as methodologies of selection of the most selective and safe substances in tests in vitro and with the use of validated models of pathology in vivo, which are directed to the study of the pharmacological activity of the most effective and safe molecule.

In vitro tests established that the substance ZB49-0010C from the group of norleucine derivatives showed most pronounced inhibitory activity at a concentration of 30 mmol against arginase II, as its full inhibitor. This agent doesn't bind hERG channel of cardiomyocytes and not inhibit it at concentrations up to 40 μmol that indicates its potential safety in respect of cardiac electrical stability.

In our study, endothelial protective and cardioprotective effects of arginase II selective inhibitor ZB49-0010C were studied on L-NAME induced and homocysteine-induced ED. To simulate homocysteine-induced ED experimental animals were administered intragastrical the methionine at dose of 3 g/kg once a day for 7 days. A metabolite of methionine homocysteine, due to its high peroxide activity has a damaging effect on the endothelium. Free radicals react with synthesized in the endothelium NO and reduce its biological activity. Also homocysteine can intervene in synthesis of disulfides that, in addition to functional disorders of endothelial cells, stimulates proliferation of vascular smooth muscle cells, causing its remodeling. Endothelium-independent vasodilatation in homocysteine-induced ED is not as pronounced as in L-NAME-induced deficit of NO. Perhaps this is due to reduced availability of exogenous NO and the smaller values of the initial arterial pressure. Apparently, this can explain the higher values of CED in L-NAME-induced ED. In the body, homocysteine promotes the synthesis and

accumulation in the cell membranes and intercellular space of LDL and VLDL α and their oxidation and reduction the synthesis of sulfur containing glucosaminoglycans that leads to vessel wall laxity. Simulation of homocysteine-induced deficit of endogenous NO leads to increase of CED to 3.4 ± 0.2 (0.9 ± 0.1 in the group of intact animals), and at the same time to increase the concentration of homocysteine in the blood plasma up to 51.0 ± 2.0 $\mu\text{mol/l}$ (8.3 ± 4.3 $\mu\text{mol/l}$ in the control group animals). Also simulation of homocysteine-induced ED leads to a sharp decrease in the concentration of stable metabolites of NO (Total NO) in plasma of rats to the level of 68.9 ± 4.3 (19.0 ± 4.3 in the group of intact animals). With the administration of methionine there was observed a sharp decline in the functional parameters of the myocardium after stress tests that shows the development of latent cardiac insufficiency.

Simulation of ED has also been performed intraperitoneal injection of the eNOS inhibitor L-NAME to male rats at a dose of 25 mg/kg for 7 days. L-NAME in the body is converted to the active inhibitor L-NNA, which inhibits NO synthesis by inhibiting the eNOS. Deficit of NO causes a reduction of endothelium-dependent vasodilators effects and increases the vasoconstrictor influences, disturbances of systemic and regional hemodynamic, increases blood pressure, leads to heart dysfunction, increases the expression of endothelial cell adhesion molecules and others.

As a result of experiments, a seven-day blockade of NO-synthase with L-NAME led to the development of arterial hypertension (systolic blood pressure 191.2 ± 6.4 ; diastolic blood pressure 145.7 ± 3.8 mm Hg, compared to the group of intact animals, systolic blood pressure 138.2 ± 3.6 , diastolic blood pressure 103.9 ± 2.7 mm Hg). In addition, develop of NO deficit manifested by the increase in CED fivefold in comparison with its values in the group of intact animals, the negative dynamics in the stress tests, the decrease in the concentration of stable metabolites of NO in the blood, myocardial hypertrophy and hypertrophy of VSMC. However, CED in animals with homocysteine-induced deficit of endogenous NO was lower than in animals with L-NAME – induced ED that makes you think about more severe violation of the ratio of endothelium-dependent and endothelium-independent vasodilatation associated with administration of

L-NAME at a dose of 25 mg/kg than with the administration of methionine in dose 3 g/kg.

The main objectives of our research were to study the specific activity of the substance most selective and safe molecule arginase II inhibitor ZB49-0010C from the group of norleucine derivatives on the ED and limb ischemia in experiment. Mechanism of action of arginase II selective inhibitor is due to its structural similarity with ornithine, which is one of the products of metabolism of the urea cycle. Indirect effects of the arginase II selective inhibitor to arginase activity are associated with inhibition of ornithine carbamyl transferase, which catalyzes the metabolism of ornithine citrulline in the urea cycle. The result is an excessive accumulation of ornithine, which inhibits arginase. Moreover, arginase selective inhibitor increases the endogenous synthesis of L-arginine from citrulline by inhibiting argininosuccinate synthetase. The suppression of the activity of argininosuccinate synthetase by the arginase II selective inhibitor leads to increase endogenous synthesis of L-arginine and restoration of NO synthesis [4, 10, 14, 15, 16, 17, 18, 19, 24, 25]. In our study arginase II selective inhibitor ZB49-0010C were studied to identify its hypotensive action, endothelial protective and cardioprotective effects and anti-ischemic effect. Hypotensive effect of arginase II selective inhibitor ZB49-0010C was dose-dependent and most pronounced at a dose of 10 mg/kg and was shown to prevent the increase in arterial pressure due to the blockade of eNOS. Endothelial protective effect of arginase II selective inhibitor ZB49-0010C was also dose-dependent and most pronounced at a dose of 10 mg/kg, which was manifested by decrease of CED to 2.3 ± 0.3 in L-NAME – induced ED and to 1.6 ± 0.2 in homocysteine-induced ED. Endothelial protective effect of ZB49-0010C is due to the suppression of the activity of arginase II, thereby there is an increase the synthesis of the main vasodilator of endothelium NO.

The results of the research have been identified cardioprotective effect of the arginase II selective inhibitor ZB49-0010C in both models of ED, which manifested positive dynamic in the stress tests (test for adrenoreactivity, overload resistance test). It was possible to identify the prevention of increase of adrenoreactivity and reduce cardiac reserve. Cardioprotective effect of the arginase II selective inhibitor ZB49-0010C, in

our opinion, may be associated with increase endogenous synthesis of L-arginine by inhibiting activity of argininosuccinate synthetase and accumulation of L-arginine due to inhibition of arginase. In turn, L-arginine has several properties that are beneficial for the activity of the cardiovascular system, it facilitates membrane depolarization of endothelial cells and regulates the pH in these cells, as well as the pH of the blood, reduces blood viscosity, reduces the production of free radicals and removes them from the endothelial cells [3, 4, 5, 6, 7, 9, 14, 30].

A morphological study demonstrates prevention under the influence of arginase II selective inhibitor ZB49-0010C increase in transverse diameter of the cardiomyocytes in animals with L-NAME-induced and homocysteine-induced ED. Also under the action of arginase II selective inhibitor ZB49-0010C has been observed a prevention of development of negative pathological processes in the kidneys which was most pronounced at a dose of 10 mg/kg.

In the study of biochemical markers of ED in the blood plasma there was observed a prevention of the reduce of the concentration of stable metabolites of NO in L-NAME – induced pathology. In simulation of homocysteine-induced pathology there was also observed a prevention of the reduce of the concentration of stable metabolites of NO and an increase the level of homocysteine in the blood plasma which was most pronounced at a dose of 10 mg/kg.

In the study of the anti-ischemic effect of arginase II selective inhibitor ZB49-0010C there was established prevention of falling of the microcirculation on the 29th day in the chronic limb ischemia which was the most significant at a dose of 10 mg/kg. A morphological study also revealed the most pronounced at a dose of 10 mg/kg prevention the development of necrotic and atrophic processes in skeletal muscle of the ischemic limb and the protective effect on histoarchitecture of striated muscle.

These effects are primarily associated with the increase of bioaccessibility of L-arginine as the main substrate for NO synthesis, due to the block of its biotransformation by arginase II. It leads to an increase of NO synthesis, which is an important factor that activates neoangiogenesis by increasing production of endothelial growth factor that stimulates the proliferation, migration of endothelial cells and activates local blood flow [5, 28, 29, 30].

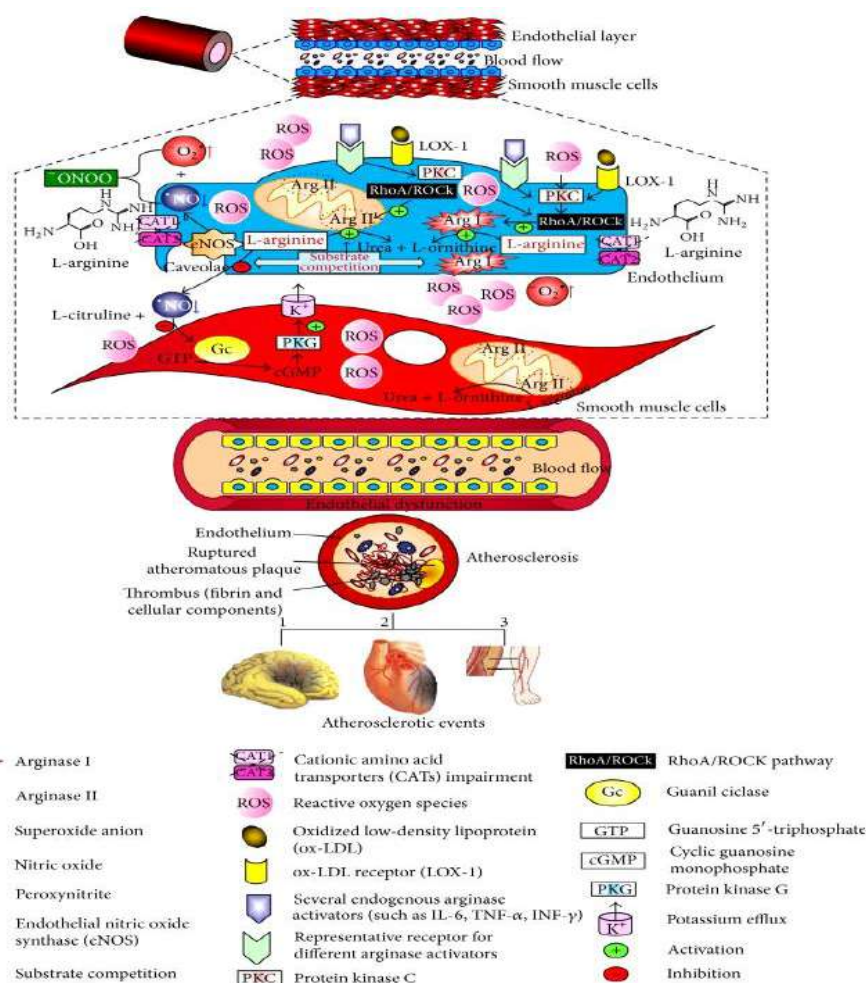


Figure 6. The pathogenesis of endothelial dysfunction and the application points of the action potential endothelial protective agents (L. Santhanam, D. W. Christianson, et al., 2008)

The obtained data fully coincide with the results of other authors [13, 15, 17, 18, 21, 25, 31, 32, 33, 34, 35, 36] and indicate the prospects for drugs suppressing the activity of arginase II. The selectivity of the studied drug makes it more preferable because there is no impact on the urea exchange [4, 14, 16, 26].

ABBREVIATION LIST

- +dp/dt max – maximum contraction rate
- dp/dt max – maximum relaxation rate
- ABH – 2(S)-amino-6-borono-hexanoic acid
- ADMA – asymmetric dimethylarginine
- BEC – S-(2-boronoethyl)-L-cysteine
- CAD – atherosclerotic cardiovascular disease
- CED – coefficient of endothelial dysfunction
- DEP – dyscirculatory encephalopathy
- DFMO – ϵ -difluoromethylornithine
- DTNB – 5, 5'-dithio-bis-(2-nitrobenzoic acid)
- ED – endothelial dysfunction
- eNOS – endothelial NO synthase
- L-NAME – L-Nitro-Arginine Methyl Ester
- L-NNA – N ω -nitro-L-arginine

- LDF – laser doppler flowmetry
- LDL – low-density lipoprotein
- NO – nitric oxide
- nor-NOHA – N ω -hydroxy-nor-L-arginine
- NOHA – N ω -hydroxy-L-arginine
- ODC – ornithine decarboxylase
- PU – perfusion units
- VLDL α – very low-density lipoproteins
- VSMC – vascular smooth muscle cells

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