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Study of acute toxicity, endothelial- and cardioprotective properties of phenolic and thiophenolic derivatives of 2*H*-imidazoles

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The aim. To study the acute toxicity, endothelial- and cardioprotective properties of phenolic and thiophenolic derivatives of 2*H*-imidazoles.

Materials and methods. The study was performed on white laboratory female BALB/c mice (*n*=57) and male C57Bl/6 mice (*n*=66). Acute toxicity was assessed according to the interstate standard GOST 32644-2014 with histological evaluation of internal organs. Endothelial dysfunction was modeled by 7-day intraperitoneal administration of *N*-nitro-*L*-arginine methyl ester (L-NAME). The studied small molecules were administered intragastrically using a probe. To assess the endothelial protective effect, the levels of systolic and diastolic blood pressure were evaluated, as well as the coefficient of endothelial dysfunction; for the cardioprotective effect, the results of stress tests on the myocardium were evaluated.

Results. The study of acute toxicity of the studied small molecules allowed us to classify them as class 4 and 5. The administration of compounds **1(a–d)** and **2(a–c)** to mice at a dose equal to 1/10 of LD50 led to changes in blood pressure and restoration of the dynamics of pharmacological tests in response to the administration of acetylcholine and sodium nitroprusside. Molecules **1b** and **2c** showed statistically significant endothelial protective activity in 3 doses (1/10, 1/50 and 1/100 of LD50). Also, these hit compounds demonstrated cardioprotective effects, recorded by the restoration of the functional capabilities of the myocardium in response to load and in the adrenoreactivity test, and to a lesser extent, during resistance exercise.

Conclusion. The studied compounds have low toxicity and have endothelial- and cardioprotective effects. This study may contribute to the formation of an idea about further directions in the study of the pharmacological activity of these molecules from the group of phenolic and thiophenolic derivatives of 2*H*-imidazoles.

Keywords: azaheterocyclic compounds; small molecules; endothelial dysfunction; imidazoles; phenols; thiophenols

Abbreviations: ED — endothelial dysfunction; CVD — cardiovascular diseases; L-NAME — *N*-nitro-*L*-arginine-methyl ester; LD_{50} — median lethal dose; NO — nitric oxide; SBP — systolic blood pressure; DBP — diastolic blood pressure; CED — coefficient of endothelial dysfunction; ACh — acetylcholine; SNP — sodium nitroprusside.

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Исследование острой токсичности, эндотелио- и кардиопротективных свойств фенольных и тиофенольных производных 2*H*-имидазолов

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Цель. Изучить острую токсичность, эндотелио- и кардиопротективные свойства фенольных и тиофенольных производных 2*H*-имидазолов.

Материалы и методы. Исследование выполнено на белых лабораторных мышах-самках линии BALB/c (*n*=57) и на мышах-самцах линии C57Bl/6 (*n*=66). Исследование острой токсичности проводилась по межгосударственному стандарту ГОСТ 32644-2014 с гистологической оценкой внутренних органов. Эндотелиальную дисфункцию моделировали при помощи 7-дневного внутрибрюшинного введения N-нитро-L-аргинин-метиловый эфира (L-NAME). Исследуемые малые молекулы вводились внутрижелудочно с помощью зонда. Для оценки эндотелиопротективного действия оценивали уровни систолического и диастолического артериального давления, а также коэффициент эндотелиальной дисфункции, для кардиопротективного эффекта — результаты нагрузочных проб на миокард.

Результаты. Изучение острой токсичности исследуемых малых молекул позволило отнести их к 4 и 5 классу. Введение мышам соединений **1(a–d)** и **2(a–c)** в дозе, равной 1/10 от LD_{50}, привело к изменению уровня артериального давления и восстановлению динамики фармакологических проб в ответ на введение ацетилхолина и нитропруссида натрия. Статистически значимую эндотелиопротективную активность в 3 дозах (1/10, 1/50 и 1/100 от LD_{50}) показали молекулы **1b** и **2c**. Также данные соединения-хиты продемонстрировали кардиопротективные эффекты, регистрируемые восстановлением функциональных возможностей миокарда в ответ на нагрузку в объёме и в пробе на адренореактивность, и в меньшей степени — при проведении нагрузки сопротивлением.

Заключение. Исследуемые соединения имеют низкую токсичность и обладают эндотелио- и кардиопротективным действием. Данное исследование может поспособствовать формированию представления о дальнейших направлениях в изучении фармакологической активности данных молекул из группы фенольных и тиофенольных производных 2*H*-имидазолов.

Ключевые слова: азагетероциклические соединения; малые молекулы; эндотелиальная дисфункция; имидазолы; фенолы; тиофенолы

Список сокращений: ЭД — эндотелиальная дисфункция; ССЗ — сердечно-сосудистые заболевания; L-NAME — N-нитро-L-аргинин-метиловый эфир; LD_{50} — полулетальная доза; NO — оксид азота; САД — систолическое артериальное давление; ДАД — диастолическое артериальное давление; КЭД — коэффициент эндотелиальной дисфункции; АХ — ацетилхолин; НП — нитропруссид натрия.

INTRODUCTION

Endothelial dysfunction (ED) makes a significant contribution to the development of socially significant cardiovascular diseases (CVD). The search for effective methods of pharmacological therapy and prevention of this pathology is extremely relevant at present. Various risk factors, such as hypercholesterolemia, hyperhomocysteinemia, hyperglycemia, hypertension, smoking, inflammation, and aging, contribute to the development of ED. The impaired structure and function of the endothelium play the main role in the pathogenesis of diseases such as arterial

hypertension, atherosclerosis, diabetes mellitus, and many others. Any disturbance affecting the equilibrium of the endothelium as a physical barrier, as well as metabolism, synthesis, and release of biologically active substances, can contribute to the development of ED, which leads to the progression of CVD [1, 2]. It is obvious that multiple mechanisms are involved in ED, namely inflammation, increased reactive oxygen species, cell apoptosis, increased production of vasoconstrictors, decreased production of vasodilators, and vascular remodeling. However, a decrease in the bioavailability of nitric oxide (NO) appears to play the central role in the development of this pathology [3, 4].

Researchers around the world are constantly searching for new biologically active molecules to develop medicines based on them for the treatment of a wide range of socially significant diseases, including cardiovascular pathologies. Chemicals used in pharmaceuticals based on small organic molecules are of particular importance.

Small molecules are low molecular weight chemical compounds that have the ability to regulate or affect certain biological processes. Azaheterocyclic six- and five-membered systems rightfully occupy a special place among numerous known substances, on the basis of which a large number of known and effective Chemicals used in pharmaceuticals have been created [5]. Among five-membered molecules, bifunctional derivatives based on imidazoles, modified with various biogenic fragments (amines, carboxyl groups, phenols, indoles, and their other analogs), which are used in the therapy of inflammatory, viral, bacterial, neurodegenerative, and other pathological processes, have recently deserved special attention [6, 7]. At the same time, researchers pay special attention to ensuring that the methods for obtaining these molecules are effective, economical, environmentally friendly, and also comply with the principles of "green chemistry" [8, 9].

It is worth mentioning that at the moment there are no targeted medicines aimed to correct ED. Pleiotropic effects of known medicines are used, which, favorably affect the endothelium together with the main function. Small molecules that simultaneously affect several targets involved in the pathogenesis of ED (multitarget compounds) are the most promising. Taking in account the dominant role of reducing NO bioavailability in the development of ED, small molecules for the treatment of this pathology should be able to restore NO levels in the endothelium [10]. Thus, the synthesis of small molecules that affect various pathways in the pathogenesis of ED development is promissing in modern CVD therapy.

THE AIM. To study the acute toxicity, endothelial- and cardioprotective activity in vivo of synthesized small molecules from the group of phenolic and thiophenolic derivatives of 2*H*-imidazoles.

MATERIALS AND METHODS

Phenolic and thiophenolic derivatives of 2H-imidazoles **1(a–d) and 2(a–c)** were synthesized in accordance with the previously published method [11, 12]. The previously undescribed hydrochloride salt **1b** was isolated and characterized by NMR and IR spectroscopy, mass spectrometry, and elemental analysis data.

Animals

The study was conducted from November 2023 to April 2024. The acute toxicity was performed on white laboratory BALB/c mice (virgin females) (*n*=57; 3 mice per cage). Pharmacological activity was studied on laboratory male C57Bl/6 mice (*n*=66; 6 mice per cage). Animals were taken into the experiment at the age of 8–10 weeks, weighing 18–22 g, without external signs of disease. All laboratory animals were obtained from the nursery of the Belgorod State National Research University (Belgorod, Russia). After passing a 14-day quarantine regime, the mice were stratified by weight and placed in separate cages. The animals were kept in a standard biologically clean experimental room. Feeding was carried out in accordance with GOST 33216-2014 "Guidelines for the maintenance and care of laboratory animals". The daily diet throughout the study included granulated feed (GOST 50258-92) and filtered water disinfected with UV irradiation. 4 hours before the introduction of the test substances, all experimental animals were subjected to complete food deprivation with free access to water, and feeding was resumed 2 h after the test substance was introduced. On the eve of the day of necropsy at 16:00, the animals were deprived of food with free access to water. The experiments were carried out in compliance with the requirements of Federal Law No. 498-FZ of December 27, 2018 "On responsible treatment of animals and on amendments to certain legislative acts of the Russian Federation", the Rules of Good Laboratory Practice in conducting preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), the Directive of the European Community (86/609 EC), the Rules of International Recommendations of the European Convention for the Protection of Vertebrate Animals Used in Experimental Studies (1997) and the Rules of Good Laboratory Practice (approved by Order of the Ministry of Health of the Russian Federation No. 199n of April 1, 2016).

ОРИГИНАЛЬНАЯ СТАТЬЯ
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All *in vivo* experiments were conducted at the Belgorod State National Research University (Belgorod, Russia). The study was approved by the Commission for the Control of the Maintenance and Use of Laboratory Animals of the Belgorod State National Research University (Belgorod, Russia), expert opinion No. 01-07i/23 dated July 10, 2023.

Study of acute toxicity of synthesized small molecules

In the study of acute toxicity of seven compounds **1(a–d) and 2(a–c),** the interstate standard GOST 32644-2014 "Methods of testing the effects of chemical products on the human body. Acute oral toxicity — method for determining the class of acute toxicity" (OECD, Test No. 423) was observed. Based on the results of these works, the toxicity class was determined in accordance with the globally harmonized system of hazard classification and labeling of chemical products that cause acute toxicity and the range of doses for subsequent determination of LD_{50}. The structural formulas of the studied samples are shown in Figure 1.

At the Federal University named after the First President of Russia B.N. Yeltsin, a preliminary stage of studying the synthesized phenolic and thiophenolic derivatives of 2*H*-imidazoles **1(a–d) and 2(a–c)** was carried out. The results of studies of the acute toxic effect in vitro of these pharmaceutical substances were obtained. Based on the data obtained, a suggestion was made about the low danger of acute toxicity of the substances, which formed the basis for choosing the initial dose — 2000 mg/kg for this study. According to the presented data on the solubility of the studied compounds, obtained during preliminary studies, an aqueous solution of dimethyl sulfoxide (in a ratio of 1:1) was used as a solvent for intragastric administration of the medicines. The studied substances were administered in a volume of 0.05 ml/10 g of weight in one dose using an atraumatic gastric probe. The body weight of the animals was measured and the dose was prepared immediately before the administration of the medicine.

The study of acute toxicity was carried out on BALB/c mice, which were grouped into 3 individuals per cage for each stage of the experiment (*n*=57). Observation of the animals was carried out for 14 days. Within 2 h after the administration of the dose of the medicine, each individual was under individual continuous observation. Then, up to 12 h from the moment of administration of the studied dose, the frequency of individual examination was every 60 min, and then daily in the morning and evening for all 14 days. The observation included an assessment of the general condition with fixation of the clinic of poisoning (changes in the skin and coat, eyes and mucous membranes, assessment of the respiratory, circulatory, autonomic and central nervous systems, as well as somatomotor activity and the nature of behavior) and counting the number of animals that died during the experiment.

Animals that died during the experiment were subjected to autopsy, and survived animals were humanely euthanized at the end of the experiment (overdose of ethyl ether) and subjected to autopsy. The resulting organs and tissues were fixed in 10% buffered formalin. After macroscopic evaluation, histological dissection of the material was carried out, followed by wiring according to the standard scheme using an AGT-11 FMP apparatus (Russia). After wiring, paraffin embedding was performed with the formation of blocks using an MPS/P2 SLEE medical GmbH filling station (Germany). The finished paraffin blocks were subjected to microtomes using an RMD-3000 rotary microtome (Russia). Microtome sections were placed on slides with an adhesive coating, after which standard histological staining was performed on an automated multistainer Histoprocessor TLP-144 (Russia) — hematoxylin + eosin. The stained sections were evaluated using a Nikon Eclipse E200 microscope (Japan).

Study of the endothelial protective activity of the obtained small synthesized molecules on the model of L-NAME-induced endothelial dysfunction

The animal groups included an intact group (*n*=6, without the introduction of medicines and modeling of pathology), a control group (*n*=6, with modeling of L-NAME-induced ED without pharmacological correction) and groups with L-NAME-induced ED with pharmacological correction with the studied substances (*n* =66; 6 individuals per group, 7 groups in the first stage, 4 additional groups in the second stage).

To model ED, an inducer of endothelial dysfunction was used — a non-selective NO-synthase blocker *N*-nitro-*L*-arginine methyl ester (L-NAME, Sigma Aldrich, USA), which was administered daily 1 time per day intraperitoneally at a dose of 60 mg/kg for a week.

In order to correct ED, the synthesized small molecules **1(a–d)** and **2(a–c)** were administered 1 time per day intragastrically using a probe for small laboratory animals for 7 days. The control group was administered intragastrically with water for injection in a volume of 0.05 mL/10 g of weight. The development of ED in experimental animals, as well as the degree of its correction by the studied medicines, was assessed by dynamic non-invasive measurement of blood

pressure on the tail of rodents, as well as using the calculated coefficient of endothelial dysfunction (CED).

The pressure was measured three times, on the day the animals were removed from the experiment on the "Systola" apparatus from Neurobotics (Moscow, Zelenograd). The hardware and software complex has a built-in pump that automatically pumps pressure into the tail cuff until the blood flow pulsations stop, and then, slowly reducing the pressure, measures the systolic and diastolic values based on the readings of the infrared pulse sensor worn on the animals' tail after the cuff. Previously, the animal was fixed using the Teremok restrainer from Neurobotics (Moscow, Russia), and then heated to a temperature of 32–37°C for 10–15 min, being on the Flogiston heating platform from Neurobotics (Moscow, Russia). This made it possible to ensure blood circulation in the tail vessels in the required volume and stabilize blood flow. The obtained hemodynamic parameters using the Systola software (Version 1.3.1) were reflected in the form of graphs of the recording curve of blood pressure and pulse and were used in the future to evaluate the results of the experiment.

On the 8th day of the experiment, after preliminary anesthesia with zolazepam (Virbac, Russia) 2.5 mg/100 g+xylazine (Biogel (Belarus) 2 mg/100 g intraperitoneally, the blood flow velocity in the carotid artery was measured in mice, followed by intravenous functional tests with acetylcholine (ACh) and sodium nitroprusside (SNP), which are used to assess endothelium-dependent and endothelium-independent vasodilation, respectively. Hemodynamic parameters were measured continuously using a perivascular flowmeter sensor Transonic Systems from BIOPAC Systems (USA). The data obtained were evaluated in the AcqKnowledge program. CED is the ratio of the area of the triangle above the blood flow velocity recovery curve in response to the administration of SNP (S(SNP)) to the area of the triangle above the blood flow velocity recovery curve in response to the administration of ACh, i.e. S(ACh))/CED=(S(SNP))/(S(ACh)) (Fig. 2).

Study of the cardioprotective activity of the obtained small synthesized molecules

The myocardial contractility after modeling the pathology was carried out in anesthetized mice on controlled respiration. The cavity of the left ventricle was probed with a needle through the apex of the heart and, using the RX104A sensor "Biopac Systems, Inc." (USA) and using the computer program Biopac Systems, Inc. (USA), cardiogeodynamic parameters were recorded (pressure in the left ventricle, heart rate — HR). To assess the functional capabilities of the myocardium in animals, stress tests were performed: with a volume load, a test for adrenoreactivity (intravenous single administration of a solution of adrenaline hydrochloride 1.0–5 mol/L), with a resistance load (clamping the ascending aorta for 30 seconds).

Statistical processing of results

All the data obtained were subjected to statistical processing using the Microsoft Excel 2010. Using descriptive statistics methods, the data were checked for compliance with the law of normal distribution using the Shapiro–Wilk test. Relative and average values (arithmetic mean [M], median [Me], standard deviation [SD], interquartile range) were also calculated. With a normal distribution, the data were presented as M±SD. Given the normal type of data distribution, Student's test was used to compare two samples. Differences were considered significant at p <0.05.

RESULTS AND DISCUSSION

Acute toxicity

In the group receiving 2000 mg/kg of 1a, 30 minutes after the bait with the medicine, non-lethal signs of toxicity were observed with suppression of the general activity of the animals, bilateral ptosis. Pathological symptoms completely regressed by the end of 2 h after acute baiting. During the next 6 days of the observation period, the animals remained mobile, adequately reacted to external stimuli and manipulations with them. However, on the 7th day after baiting, the activity of the animals decreased, the animals refused to eat, there was a progressive decrease in the reaction to external stimuli, a hunched posture, ruffled coat, and foci of alopecia. At the 2nd stage, one lethal outcome was recorded (on the 12th day) after baiting with the drug. Surviving animals remained lethargic and inactive throughout the remaining observation period. Pathoanatomical examination revealed changes in the renal parenchyma in the form of severe granular and hyaline-droplet dystrophy of the epithelium of the convoluted tubules (Fig. 3), also in the liver there was moderate granularity of hepatocytes with blurred hepatic beams and plethoric sinusoids. The histological picture allows us to conclude that the medicine **1a** in high doses has moderate pneumo- and hepatotoxicity, and allows us to classify it as class 5 — LD_{50}≈2500 mg/kg.

Compound **1b** at a dose of 2000 mg/kg immediately after administration caused an increase in the general activity of the animals, tachypnea, and phenomena of shortness of breath. After 30 min, motor anxiety was replaced by lethargy, the visible mucous membranes and skin acquired a cyanotic shade, and the coat lost its luster and became ruffled. During the next 2 days, the development of tonic-clonic seizures was observed,

ОРИГИНАЛЬНАЯ СТАТЬЯ
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which caused the death of the animals. Survived animals were lethargic, there was a weak reaction to light, tactile and sound stimuli, there was an increased consumption of water and a reduced consumption of feed. The clinic of acute poisoning was observed for up to 48 hours. After that, the animal returned to active life or died. In the interval of 10–16 h after the administration of the medicines, a peak in the mortality of experimental animals was recorded. With further observation for 14 days, no deaths were recorded.

Pathomorphological examination revealed multiple hemorrhages on the mucous and serous membranes of internal organs, enlargement of the liver, the liver was of flabby consistency, grayish in color on the cut, and a decrease in the airiness of the lungs. Microscopically, in the liver, subtotal disruption of histoarchitectonics was noticeable, hepatic beams were difficult to determine, hepatocytes had signs of total severe balloon dystrophy (Fig. 4). At the same time, focal interstitial fibrosis with plethora and mononuclear infiltration was noted in the lungs. The administration of 300 mg/kg of compound **1b** did not cause pathological symptoms during the entire observation period. According to the results of the autopsy, no pathological changes in the internal organs of the animals in this group were found, the general histological picture corresponded to the results of the control group. Thus, compound **1b** can be classified as toxicity class 4 — LD_{50}≈2000 mg/kg, in doses exceeding LD50 1b, it has a pronounced hepatotoxic and moderate pneumotoxic effect.

Upon administration of the maximum dose of substance 1c, initial signs of poisoning appeared after 30 min. The signs of poisoning included a 2–3-minute period of pronounced anxiety, tachypnea, impaired coordination, alternating with a state of general lethargy, decreased motor activity, and suppression of behavioral and exploratory reactions. However, activity recovered within the next 2 days. During dynamic observation over the subsequent 14 days, lethal outcomes were recorded in some animals (on days 7 and 8). A single administration of 300 mg/kg of compound 1c did not cause pathological symptoms during the entire observation period. According to the autopsy results, no pathological changes in the internal organs of animals in this group were found; the overall histological picture corresponded to the results of the control group. Thus, compound **1c** can be classified as toxicity class 4 — LD_{50}≈1000 mg/kg.

During the study of compound **1d** at a dose of 2000 mg/kg, immediately after administration, pronounced anxiety of the experimental animals was observed. Within the next 15–20 min, the respiratory rate increased, the mice stood on their hind legs, sometimes supported by the walls of the cage, and retraction of the intercostal spaces during breathing was observed. After 40 min, the excitement was replaced by a state of general lethargy, decreased motor activity, suppression of behavioral and exploratory reactions, and acrocyanosis was observed. Lethal outcomes were recorded in the interval of 3–4 hours after drug administration. Autopsy revealed signs of general acute venous hyperemia, characterized by plasmorrhages and edema, multiple diapedetic hemorrhages, and necrotic changes in parenchymal organs (Fig. 5). Administration of 300 mg/kg of compound 1d did not cause pathological symptoms during the entire observation period. According to the autopsy results, no pathological changes in the internal organs of animals of this group were found; the overall histological picture corresponded to the results of the control group. Thus, compound **1d** can be classified as toxicity class 4 — LD_{50}≈500 mg/kg; in doses exceeding LD_{50}, **1d** has a toxic effect on the heart muscle, leading to acute cardiovascular failure.

Immediately after administration of compound **2a** at a dose of 2000 mg/kg, an increase in the number of grooming acts was observed in the animals. After 10–15 min, symptoms of conjunctivitis (swelling and hyperemia of the eyelids) were observed. 40 min after medicine administration, the activity of the animals decreased, indicators of orienting-exploratory activity decreased (decrease in the number of stands), and signs of a central depressant effect were observed (decrease in the number of defecation acts). The clinical picture of acute poisoning was observed for up to 24 hours. One lethal outcome was recorded 10 hours after medicine administration. Subsequently, the animal returned to active life. Autopsy revealed no pathological changes in the internal organs of animals in this group; the overall histological picture corresponded to the results of the control group. Thus, compound **2a** belongs to toxicity class 5 — LD_{50}≈2500 mg/kg. Administration of high doses of **2a** close to LD50 has a toxic effect on the central nervous system, with suppression of locomotor and orienting-exploratory activity.

In the group receiving 2000 mg/kg of **2b**, 30 min after medicine administration, non-lethal signs of toxicity were observed with suppression of locomotor and orienting-exploratory activity, as well as the development of postural-kinetic tremor. Pathological symptoms completely regressed by the end of the first day. Throughout the entire subsequent observation period, the animals remained mobile, adequately reacted to external stimuli and manipulations with them, and no lethal outcomes were recorded. Pathological examination of the stomach preparations revealed chronic active inflammation in the base of

the lamina propria and in the submucosal layer (Fig. 6); otherwise, the autopsy results did not differ from the control group, which indicates a local irritant effect of compound **2b** and low overall toxicity and allows it to be classified as class 5 — LD_{50}≈5000 mg/kg.

A single intragastric administration of 2000 mg/kg of 2c after 40–45 min led to a decrease in the locomotor activity of the animals, lethargy, the coat became ruffled, and an increase in the frequency of respiratory acts was noted. By the end of the first day, the visible skin and mucous membranes acquired a yellowish tint. In the interval of 20–24 hours after medicine administration, the peak of the mortality of experimental animals was recorded. Survived animals remained lethargic and inactive for 2–3 days after acute administration; some animals remained lethargic throughout the entire observation period. Autopsy revealed jaundice of the serous and mucous membranes of internal organs, an increase in the size and density of the liver, and the liver had a grayish color on the cut. Histological examination revealed a subtotal disruption of the liver histoarchitecture; the hepatic trabeculae were sharply smoothed, and the boundaries between hepatocytes were practically not defined. Hepatocytes were characterized by extreme polymorphism with pronounced balloon dystrophy; nuclei with dense chromatin were found, as well as pyknotic nuclei. In addition, there was heterogeneous interstitial mononuclear infiltration, as well as moderate hyperemia. After administration of 300 mg/kg of 2c, signs of toxicity with suppression of general activity were observed after 40 minutes. 1 lethal outcome was recorded at the first stage of the study after 36 hours of administration.

The activity of survived animals completely recovered on days 5–6. Pathomorphological examination of the dead mouse revealed signs of toxic liver damage, while surviving animals showed a pronounced disruption of the histoarchitecture of the liver tissue with centrilobular hyperemia, without signs of necrosis (Fig. 7). Based on the results of the study, it can be concluded that the drug has pronounced hepatotoxicity and can be classified as class 4 — LD_{50}≈1000 mg/kg.

The results of assessing the toxicity class of the studied compounds are presented in Table 1.

Evaluation of the Endothelioprotective Activity of Small Synthesized Molecules on a Model of L-NAME-Induced Endothelial Dysfunction

Modeling of ED induced by L-NAME administration contributed to the formation of arterial hypertension and an increase in systolic blood pressure (SBP) by more than 1.35 times (Table 2), which led to a change in pharmacological tests in response to the administration of ACh and NP. The development of these changes was evidenced by a statistically significant (p <0.05) increase in CED by more than 4 times compared to intact animals.

Administration of compounds **1(a–d) and 2(a–c)** to sexually mature male mice at doses equal to 1/10 of LD50 led to a change in blood pressure and restoration of the dynamics of pharmacological tests in response to the administration of ACh and NP. This dose was chosen because it corresponds to the generally accepted practice in screening studies of the pharmacological activity of new compounds[1]. A statistically significant difference in the reduction of blood pressure levels was observed for compounds with laboratory codes **1b and 2c** (p <0.05). A comparable dynamic was also noted for the CED indicator.

At the next stage, a preliminary assessment of the effectiveness of 2 hit compounds (the most active compounds) was carried out in 3 doses: the first dose corresponded to 1/10 of LD50, the second dose — 1/50 of LD50, and the third — 1/100 of LD50. The choice of these ranges is due to the chemical structure of the substance, the peculiarities of studying the pharmacological activity of compounds, as well as the need, when introducing them into clinical practice, for long-term administration of drugs based on the studied compounds[2]. The studied doses for each compound are indicated in Table 3.

The results of this series of experiments demonstrated high endothelioprotective activity of compounds **1b and 2c** at a dose of 1/10 and 1/50 of LD50 according to the dynamics of SBP and DBP levels (p <0.05), as well as according to the CED indicator at a dose of 1/10 and 1/100 of LD50 for compound 1b and at a dose of 1/10 of LD50 for compound **2c** (p <0.05; Table 4).

Based on the results of these data on the CED indicator, LD50 was calculated for each of the hit compounds during experimental modeling of L-NAME-induced NO deficiency (according to the method of B.M. Shtabsky): for EAH-165 — 86.67 mg/kg, for EAH-280 — 28.33 mg/kg [13].

[1] Karkishchenko NN, Karkishchenko VN, Shustov EB, Kapanadze GD, Revyakin AO, Semenov HH, Bolotova VT., Dulya MS. Methodological recommendations Biomedical (preclinical) study of antihypoxic activity of drugs. Moscow: Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency (Svetly Gory); 2017. 97 p.
[2] Ibid.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2024-12-6-394-409

Investigated azaheterocyclic compounds of the 2H-imidazole series

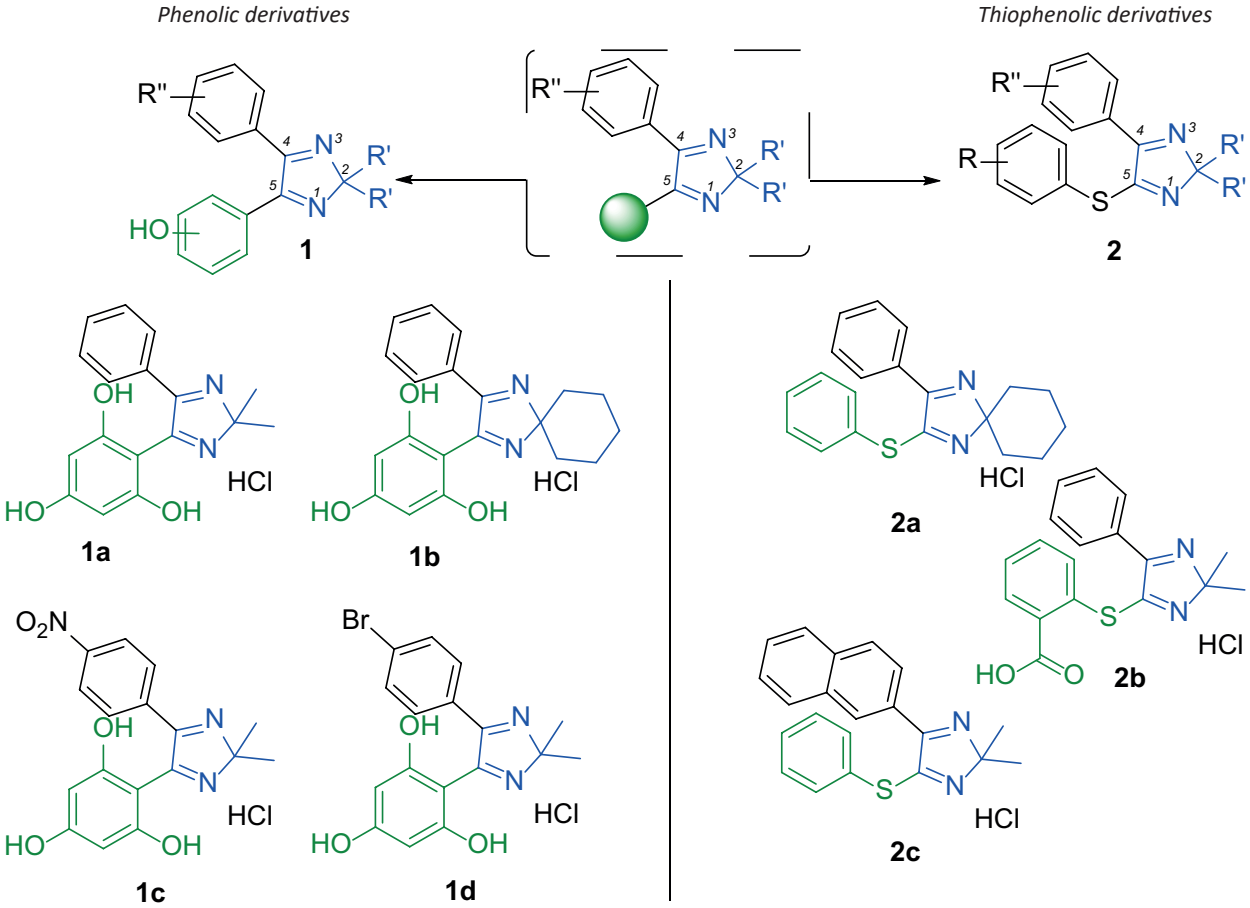

Figure 1 – Structures of the studied phenolic and thiophenolic derivatives of 2H-imidazole.

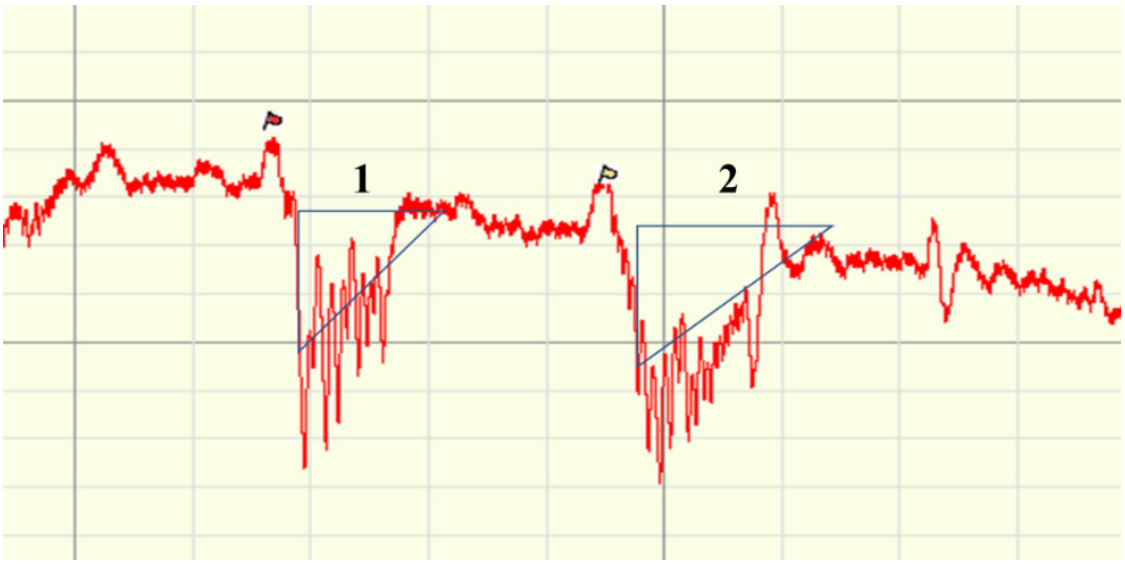

Figure 2 – Dynamics of blood flow velocity in determining the coefficient of endothelial dysfunction in intact animals.

Note: 1 — with the introduction of acetylcholine, 2 — with the introduction of sodium nitroprusside.

ISSN 2307-9266 e-ISSN 2413-2241

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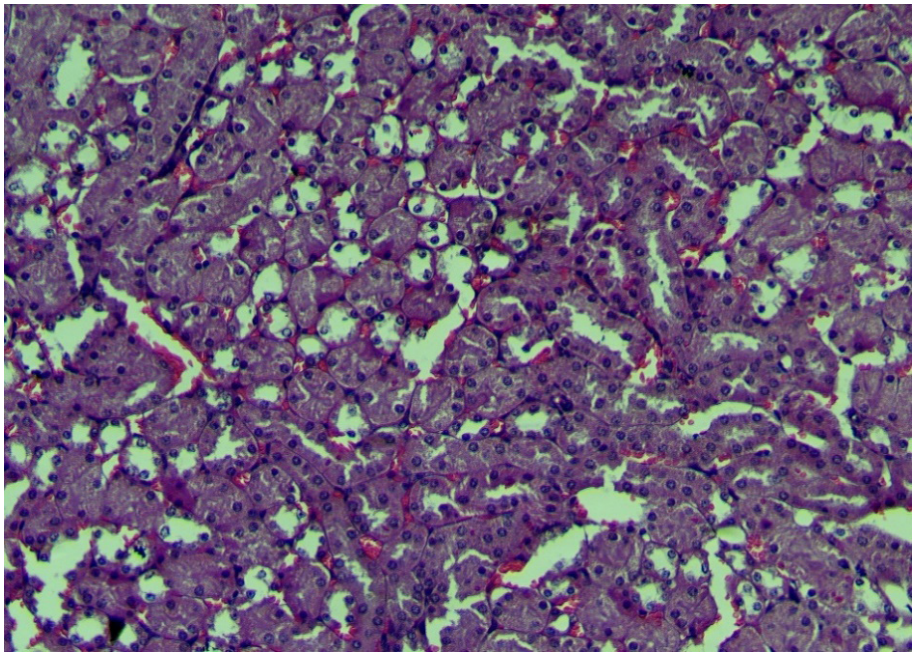

Figure 3 – Histological structure of the renal parenchyma of a mouse after oral administration of 1a at a dosage of 2000 μg/kg. Staining with hematoxylin+eosin, ×200.

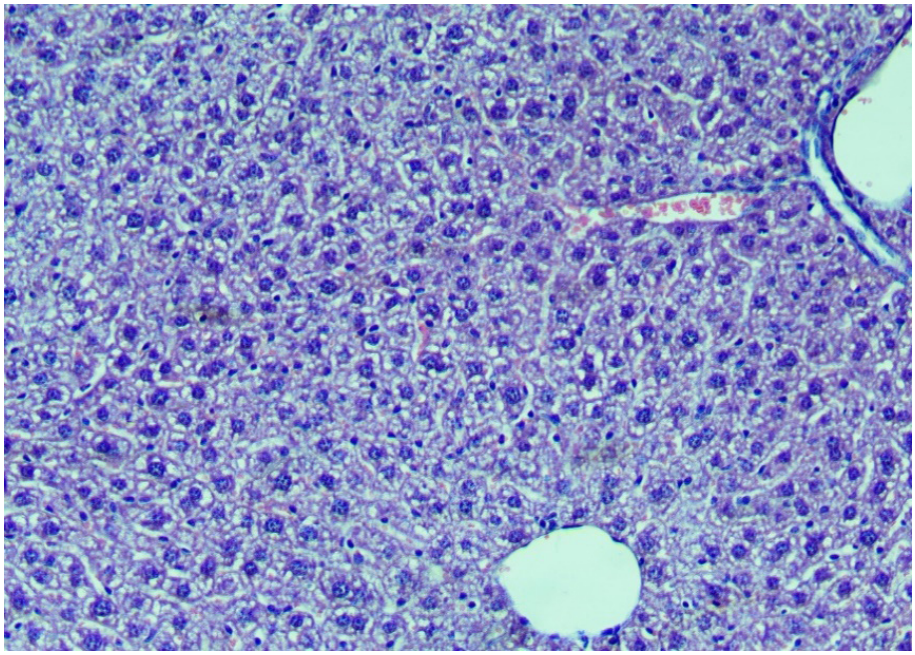

Figure 4 – Histological structure of the liver of a mouse after oral administration of 1b at a dosage of 2000 μg/kg. Staining with hematoxylin+eosin, ×200.

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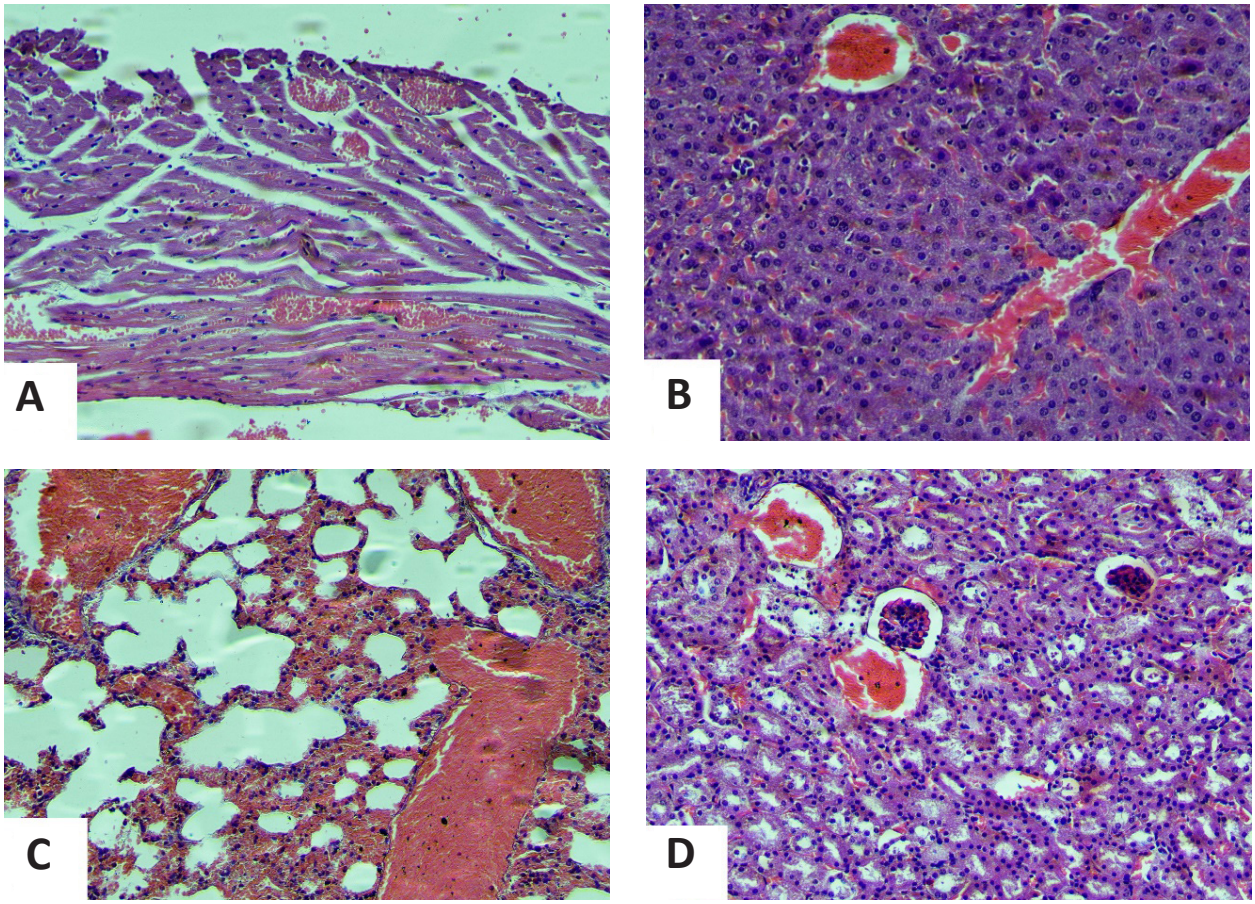

Figure 5 – Venous hyperemia in the parenchymal organs of a mouse after oral administration of 1d at a dosage of 2000 mcg/kg. Hematoxylin+eosin staining, ×200.
Note: A — myocardium; B — liver; C — lung; D — kidney.

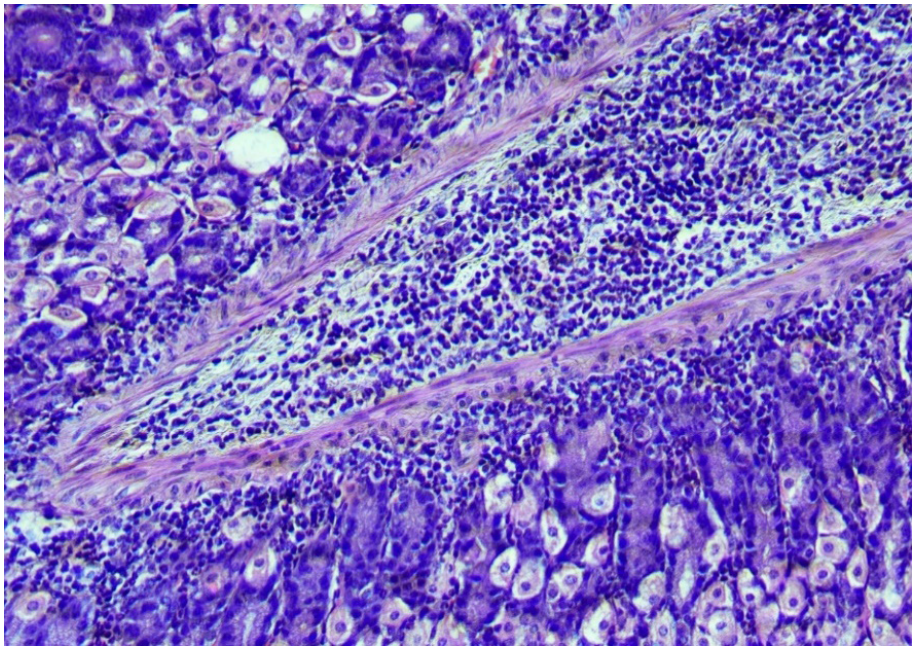

Figure 6 – Histological structure of the stomach wall of a mouse after oral administration of 2b at a dosage of 2000 mcg/kg. Hematoxylin+eosin staining, ×200.

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PHARMACY & PHARMACOLOGY
(ФАРМАЦИЯ И ФАРМАКОЛОГИЯ)

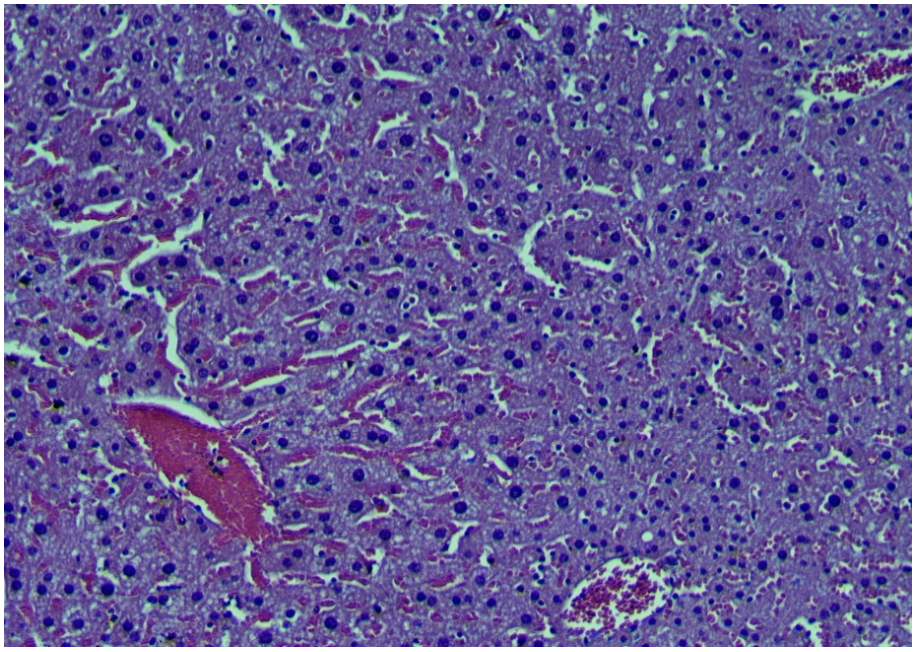

Figure 7 – Histological structure of the liver of a mouse after oral administration of 2c at a dosage of 2000 mcg/kg. Hematoxylin+eosin staining, ×200.

Table 1 – Results of assessing the toxicity class of the studied compounds

Compound code	Number of dead animals			Toxicity class	LD_{50} value
	2000 mg/kg	300 mg/kg	50 mg/kg		
1a	0/1	–	–	class 5: 2000–5000 mg/kg	2500 mg/kg
1b	1/2	0/0	–	class 4: 300–2000 mg/kg	2000 mg/kg
1c	2	0/0	–	class 4: 300–2000 mg/kg	1000 mg/kg
1d	3	0/0	–	class 4: 300–2000 mg/kg	500 mg/kg
2a	1/0	–	–	class 5: 2000–5000 mg/kg	2500 mg/kg
2b	0/0	–	–	class 5 or not classified >2000 mg/kg 5000 mg/kg	5000 mg/kg
2c	2	1/0	–	class 4: 300–2000 mg/kg 1000 mg/kg	1000 mg/kg

Table 2 – Systolic and diastolic blood pressure and endothelial dysfunction coefficient in experimental groups (M±SD)

Group (n=6)	SBP, mm Hg	DBP, mm Hg	CED
Intact	106.5±3.5	64.3±3.3	1.01±0.1
L-NAME	147.5±4.2	89.5±3.8*	4.15±0.5*
L-NAME+1a	137.3±9.7*	84.5±6.0*	3.38±1.1*
L-NAME+1b	118.7±7.7**	72.8±5.1**	1.47±0.4**
L-NAME+1c	132.8±9.39*	83.8±6.3*	2.90±0.8*
L-NAME+1d	136.3±8.4*	83.2±11.5*	3.57±0.7*
L-NAME+2a	129.2±8.6*	83.2±7.9*	2.87±0.9*
L-NAME+2b	130.5±8.4*	85.5±6.3*	3.31±0.9*
L-NAME+2c	119.8±8.2**	72.2±6.1**	1.85±0.6**

Notes: SBP — systolic blood pressure; DBP — diastolic blood pressure. * p <0.05 when compared with the group of intact animals; ** p <0.05 when compared with the L-NAME group.

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2024-12-6-394-409

Table 3 – Range of doses of the studied compounds for conducting studies of pharmacological activity on animal models of endothelium-associated pathology

Compound code	Preliminary LD_{50} value	1/10 of LD_{50}	1/50 of LD_{50}	1/100 of LD_{50}
1a	2500 mg/kg	250 mg/kg	–	–
1b	2000 mg/kg	200 mg/kg	40 mg/kg	20 mg/kg
1c	1000 mg/kg	100 mg/kg	–	–
1d	500 mg/kg	50 mg/kg	–	–
2a	2500 mg/kg	250 mg/kg	–	–
2b	5000 mg/kg	500 mg/kg	–	–
2c	1000 mg/kg	100 mg/kg	20 mg/kg	10 mg/kg

Notes: * p <0.05 when compared with the group of intact animals; ** p <0.05 when compared with the L-NAME group.

Table 4 – Systolic and diastolic blood pressure and CED in experimental groups of hit compounds (M±m)

Group (n=6)	SBP, mm Hg	DBP, mm Hg	CED
Intact	106.5±3.5	64.3±3.3	1.01±0.1
L-NAME	147.5±4.2*	89.5±3.8*	4.15±0.5*
L-NAME + 1b, 200 mg/kg	118.7±7.7**	72.8±5.1**	1.47±0.4**
L-NAME + 1b, 40 mg/kg	119.2±6.4**	72.8±4.7**	1.6±0.4*
L-NAME + 1b, 20 mg/kg	124.2±6.1*	79.6±8.3*	2.3±0.6*. **
L-NAME + 2c, 100 mg/kg	119.8±8.2**	72.2±6.1**	1.85±0.6**
L-NAME + 2c, 20 mg/kg	121.2±7**	77±7.9**	1.6±0.5*
L-NAME + 2c, 10 mg/kg	130.2±8.3*	79.2±3.7*	2.8±0.7*

Notes: SBP — systolic blood pressure; DBP — diastolic blood pressure. * p <0.05 when compared with the group of intact animals; ** p <0.05 when compared with the L-NAME group.

Table 5 – Cardioprotective effects of the studied small molecules during stress tests in experimental groups

Group (n=6)	Volume load, mm Hg	Adrenoreactivity, mm Hg	Resistance load, %
Intact	140.3±6.3	199.70±4.7	87.3±4.5
L-NAME	203.9±7.2*	254.65±8.5*	65.0±5.5*
L-NAME + 1b, 200 mg/kg	165.9±8.6***	216.63±10.7**	83.1±6.7
L-NAME + 1b, 40 mg/kg	170.3±7.2***	214.82±10.5**	79.1±6.3
L-NAME + 1b, 20 mg/kg	192.5±11.5*	225.38±8.9***	73.1±8
L-NAME + 2c, mg/kg	160.6±6.4***	207.65±9.2**	81.4±6.1
L-NAME + 2c, 20 mg/kg	157.9±5.7**	210.58±10.7**	84.3±4.4**
L-NAME + 2c, 10 mg/kg	186.2±2.6***	231.27±10.7*	76.8±4.8

Notes: * p <0.05 when compared with the group of intact animals; ** p <0.05 when compared with the L-NAME group.

Evaluation of the Cardioprotective Activity of Small Synthesized Molecules

Cardioprotective effects were evaluated for hit compounds **1b and 2c** in mice with L-NAME-induced ED. Stress tests were performed and SBP in the left ventricle and heart rate were evaluated. With L-NAME-induced ED, the functional capabilities of the myocardium decreased in animals, as evidenced by a more pronounced increase in SBP in the left ventricle during volume overload — 203.9±7.2 mm Hg in the control group with L-NAME administration; 140.3±6.3 mm Hg in the intact group (p <0.05). During the adrenoreactivity test, a statistically significant increase in left ventricular pressure was also recorded during ED modeling — 254.65±8.5 mm Hg in the control group with L-NAME administration; 199.70±4.7 mm Hg in the intact group (p <0.05). The resistance load test demonstrated a statistically significant decrease in myocardial contractility from 5 to 25 seconds of aortic clamping in the group with L-NAME-induced ED modeling — 65.0±5.5%, compared with the intact group — 87.3±4.5% (p <0.05).

Compounds **1b and 2c** demonstrated cardioprotective effects, recorded primarily by increasing myocardial reserve, restoring the functional capabilities of the myocardium in response to volume load and in the adrenoreactivity test, and to a lesser extent — during the resistance load test. Compound **1b** showed statistically significant results in 2 doses — 1/10 of and 1/50 of LD_{50} in the volume load test,

and in all 3 studied doses in the adrenoreactivity test compared with the L-NAME group, significantly reducing pressure in the left ventricle (p <0.05). Substance **2c** demonstrated a statistically significant cardioprotective effect in the volume load test in all 3 studied doses and in 2 doses — 1/10 and 1/50 of LD_{50} in the adrenoreactivity test, reducing left ventricular pressure (p <0.05). In the resistance load test, only compound **2c** at a dose of 1/50 of LD_{50} significantly increased the frequency of myocardial contractility from 5 to 25 seconds of aortic clamping (Table 5).

Over the past decades, pharmacological interventions for these pathologies have advanced significantly. However, clinical treatment of CVD is still quite complex, as there is no recognized method for improving the condition of the entire vascular bed. The development of medicines is inextricably linked with the constant search, improvement of existing, as well as the development of new methods and approaches for the directed design of molecules [14, 15].

In this study, we demonstrated the endothelioprotective and cardioprotective activity of new synthesized small molecules from the group of azageterocyclic compounds, the presumed mechanism of which is the effect on the synthesis or bioavailability of NO in the vascular bed. Examples of targets that participate in NO metabolism include NOS (NO synthase) [16–18], arginase [19–21], DDAH (dimethylarginine dimethylaminohydrolase) [22, 23], VEGFR2 (vascular endothelial growth factor type 2), PDE5 (phosphodiesterase type 5) [24, 25], B2AR (beta2-adrenergic receptor), ECE1 (endothelin-converting enzyme) [26], AT1 and AT2 (angiotensin 1 and 2 receptors) [27, 28], BH4 [29]. Thus, there is an increasing interest in the search for new and the study of known biomarkers and therapeutic strategies for the prevention and correction of ED. The synthesis of small molecules that affect various pathways in the pathogenesis of ED development is promising in modern pharmacology of CVD.

Limitations of the study

This study was performed on one model of L-NAME-induced ED. To determine the exact mechanism of action of the studied substances, further study of their pharmacological activity on other ED models is required.

CONCLUSION

Synthesized small molecules from the group of phenolic and thiophenolic derivatives of 2H-imidazoles under codes **1(b–d) and 2c** belong to toxicity class 4, and **1a and 2(a, b)** — to class 5. The results of this series of experiments demonstrated that the presented molecules have endothelioprotective properties at a dose of 1/10 of LD50. The most active on the L-NAME-induced ED model were substances **1b and 2c**, which in 3 doses (1/10, 1/50 and 1/100 of LD50) showed not only statistically significant endothelioprotective, but also cardioprotective activity. The conducted study will allow us to form ideas about further directions in order to chemically synthesize and subsequently use small molecules from the group of azageterocyclic compounds. The possibilities and prospects of using synthesized small molecules from the group of azageterocycles in cardiovascular pathologies caused by ED is a promising area of pharmacy and medicine.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

AUTHORS CONTRIBUTION

Olesya A. Puchenkova — participation in the study of acute toxicity, organ harvesting for micro- and macroscopic study, modeling of endothelial dysfunction and carrying out loading myocardial tests, writing the article; Olesya V. Shheblykina — participation in the study of acute toxicity, organ harvesting for micro- and macroscopic study, modeling of endothelial dysfunction and carrying out loading myocardial tests, statistical analysis of results; Daria A. Kostina — modeling of endothelial dysfunction and conducting stress myocardial tests; Anton A. Bolgov — participation in the study of acute toxicity, histological study; Petr R. Lebedev — modeling of endothelial dysfunction and carrying out stress myocardial tests, formalizing the list of references; Vladimir V. Molchanov — animal care, preparation of experimental groups, administration of drugs; Tatyana G. Pokrovskaya — consultation on individual stages of experimental work, ensuring the quality of research; Mikhail V. Korokin — idea, research planning, consultation on individual stages of experimental work, ensuring the quality of research; Egor A. Nikiforov, Nailya F. Vaskina, Tair A. Idrisov — synthesis of experimental substances; Timofey D. Moseev, Vsevolod V. Melekhin — synthesis of experimental substances, writing an article; Mikhail V. Varaksin, Valery N. Charushin, Oleg N. Chupakhin — synthesis of experimental substances, consultation on individual stages of experimental work. All authors confirm their authorship meets the international ICMJE criteria (all authors made a significant contribution to the development of the concept, conduct of the research, and preparation of the article, read and approved the final version before publication).

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