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Research Article

Pharmacological screening of substances with cardioprotective effect in the group of 3-oxypyridine derivatives

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Abstract

Introduction: The search for new compounds with antihypoxic and cardioprotective effects among 3-oxypyridine derivatives is promising.

Research objectives: To study the anti-hypoxic and cardioprotective effects of 3-oxypyridine derivatives.

Materials and methods: The search for compounds with an antihypoxic effect was carried out on blood leukocytes of rats in in vitro. To simulate hypoxia, Oil for Tissue Culture (SAGE) was used, 500 µl of which was applied into wells over a growth medium in order to block gas exchange. The cardioprotective effect of 3-oxypyridine derivatives was studied in the model of coronary-occlusive myocardial infarction (30 minutes of ischemia, 90 minutes of reperfusion). The level of troponin I (Tn I) was determined as a biochemical marker of myocardial damage.

Results and discussion: In the in vitro experiments, when culting white blood cells, the lead compound in the group of 3-oxypyridine derivatives was identified under code LKhT 21–16, which increases the number of viable cells in the presence of hypoxia, surpassing the reference drugs. When confirming the chemical structure of the lead compound, LHT 21–16, a high sensitivity of the NMR spectroscopy method was revealed.

In studying the cardioprotective activity in the model of coronary-occlusive myocardial infarction compound LHT 21–16 exerted a marked cardioprotective effect when reducing the size of the necrotic zone and the level of biochemical marker Tn I.

Conclusions: 3-oxypiridine derivatives have antihypoxic and cardioprotective effects, which shows in a high number of surviving cells in the presence of hypoxia in the in vitro model, a reduced size of the necrotic zone and a reduced level of Tn I in the coronary-occlusive myocardial infarction.

Keywords

3-oxypiridine derivatives, hypoxia, LHT 21–16, myocardial infarction, troponin I.

Introduction

In recent years, the role of free radical oxidation in the normal and pathological conditions has been given considerable attention to (Pajak et al. 2017). Free radicals are formed in the body as a result of the metabolism of oxygen dissolved in tissues, and reactive oxygen species (ROSs) formed thereby cause oxidation of membrane lipids, proteins, polysaccharides, and nucleic acids (Yakusheva et al. 2018). The damaging effect of free radicals is opposed by a multilevel endogenous antioxidant system, which balances the free radical oxidation and antioxidant systems (Chanchayeva et al. 2013). Intensive formation of ROS and insufficient activity of the antioxidant system lead to a condition, accompanied by intensified destructive processes in tissues which go along with oxidative stress (Baiburina et al. 2017). This condition is involved in the pathogenesis of a significant number of diseases (Tyurenkov et al. 2013). In case of oxidative stress, the use of exogenous antioxidants as agents for pharmacological correction of free radical oxidation is pathogenetically substantiated (Godunova et al. 2018).

Drugs of different chemical nature are widely used for pharmacological correction of oxidative stress (Karneev 2007). The chemical structure of a substance determines its targets of action in correcting oxidative stress (Smirnova 2014). Accordingly, taking this relationship into account may be useful when searching for new groups of drugs for correcting oxidative stress. From the chemical point of view, the prerequisites for high efficiency of the compound as an antioxidant are the presence of an aromatic or heteroaromatic cycle of low molecular weight; the presence of one hydroxyl group in the aromatic cycle or a side chain to provide lipophilicity and antiradical activity; good solubility in the medium where free radicals are generated (hydrophilicity); the presence of saturated or unsaturated alkyl chain in the aromatic cycle as a prerequisite for the integration of the compound into the cell membrane (Štěrba et al. 2013, Blinov et al. 2012).

3-oxypiridine derivatives which in the experimental and clinical studies have shown the antioxidant efficacy have the above properties (Yasnetsov and Smirnov 2005). A wide range of pharmacological activity and low toxicity determine the relevance of the search for and development of new original drugs in the group of synthetic pyridine derivatives (Smirnov et al. 1985, Yasnetsov et al. 2017).

Materials and methods

In the first stage, an in vitro analysis of antihypoxic effect among 3-hydroxypyridine derivatives (17 compounds) was performed. The experiment was carried out on the fraction of leukocytes obtained by the conventional method (Frimmel 1987).

The leukocyte suspension was washed using centrifugation at 1000 rpm for 10 min; the supernatant was removed, and the residue was resuspended in 12 ml of growth medium RPMI-1640 with glutamine (PanEco, Russia). Cultivation was carried out in 24-well plastic plates (SPL Life Sciences Co., Ltd, Korea).

The wells with cells in an intact growth medium RPMI-1640 with glutamine (PanEco, Russia) were a group of positive control, a 5% DMSO solution (PanEco, Russia) in a growth medium RPMI-1640 with glutamine (PanEco, Russia) was used as a negative control.

To simulate hypoxia in the in vitro experiments, oil was added over the growth medium to stop oxygen supply. Into each well of the plate, 500 µl of growth medium containing leukocytes was added and 500 µl of Oil for Tissue Culture (SAGE, USA) was applied over the growth medium.

Trimetazidine (Sigma-Aldrich, USA) and Ethylmethylhydroxypyridine succinate (Mexidol, PHARMASOFT, Russia) at a dose of 1 mmol/L as a reference drug, recommended by the Ministry of health of the Russian Federation and the Federal Medical and Biological Agency for biomedical (preclinical) study of antihypoxic activity of drugs (Karkishchenko et al. 2017), were selected.

Cultivation of white blood cells was carried out in a CO_2 incubator filled with a gas mixture (95% air + 5% CO_2) at a temperature of 37°C and a relative humidity of 100% during 6 hours. To assess the viability of the cells, a two-component dye comprising 2µM of calcein (Sigma-Aldrich, USA) and 4µM of ethidium bromide (Sigma-Aldrich, USA) (Bozena et al. 2001) was used.

The growth medium was drained from the wells of the plate, the cells were washed with Hanks solution (PanEco, Russia); after which 200 µl of Hanks solution (PanEco, Russia) and 100 µl of solution containing a two-component fluorescent dye were added to the cells remaining at the bottom of the wells. The cells were cultured in a CO_2 incubator (CO_2 5%) for 45 minutes at a temperature of 37° C, after which the leukocytes were washed with Hanks solution (PanEco, Russia).

Viability was assessed using a fluorescent microscope Eclipse Ti-S (Nikon, Japan) under x10 magnification. Fluorescence of living cells was recorded at a wavelength of 495 nm, that of dead cells – at a wavelength of 635 nm. To calculate the number of cells, the program EZ-C1 FreeViewer software (Version 3.90, Nikon, Japan) was used. Each experimental group consisted of 12 wells, in each of which 3 random fields of view were photographed and calculated. In total, more than 40 thousand cells were counted in the experiment. Cell viability was calculated using the formula: number of living cells/(number of living cells+number of dead cells) × 100 (Lightfoot et al. 2007).

The second stage of the study confirmed the structure of the lead compound by the physico-chemical NMR method, in order to prove the presence and place of the privileged chemical groups, which is especially important for the lead compounds selected for an in-depth study of the pharmacological properties. The ^1H NMR spectra were measured using the Agilent MR400+ spectrometer in DMSO-D_6 solutions, using the σ-chemical shift scale (ppm).

At the third stage, the cardioprotective activity was syidied in the model of coronary-occlusive myocardial infarction.

The simulation of coronary-occlusive myocardial infarction was carried out in rabbits sensu Kogan A.Kh. (Kogan and Tetelbaum 1979). The experimental protocol included 30 minutes of ischemia and 90 minutes of reperfusion. Twenty minutes before the coronary artery ligation, the studied compound was injected into the marginal vein of the rabbit's ear. Troponin I (Tn) was selected to evaluate biochemical markers of myocardial infarction. Blood sampling for its determination was performed from the right ventricle into a toss-away vacuum tube with an anticoagulant. Its concentration in plasma was determined 2 hours later, using the immunofluorescence device Triage MeterPro (Biosite, USA). The size of the necrotic zone was determined using the weight planimetry method.

All the experiments were performed in compliance with The European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [Derective 2010/63/EU].

The statistical analysis was carried out with the help of StatSoft STATISTICA 6.0 software.

Results and discussion

At the screening stage, statistically significant differences were found between the negative control group (hypoxia) when compared to the positive control group, the negative control group (DMSO), with the groups containing reference drugs and the experimental group containing a 3-oxypyride derivative under the laboratory code LHT 21–16 (Var22). All other experimental groups containing 3-oxypyridine derivatives (LHT Var6 – Var21) did not show any significant differences from the group of negative control (hypoxia), which indicates the absence of antihypoxic effect (Figure 1).

The study found that the new derivative of 3-oxypyridine under the laboratory code LHT 21–16 (cell viability 81.40±3.19%, $p<0.01$) has a more pronounced antihypoxic effect compared with the reference drugs (trimethazidine – 73.72±7.25%, $p<0.01$; Mexidol – 63.18±2.87%, $p<0.01$), which is manifested in a significant increase in the percentage of the viable cells compared with the negative control in hypoxia (38.98±5.61%).

The cell viability in the presence of hypoxia with the addition of LHT 21–16 had no significant differences in comparison with the group of positive control (79.52±3.71%), which proves a high antihypoxic effect of this compound. So, in vitro the protective antihypoxic effect of LHT 21–16 exceeds that of the reference drugs Trimetazidine and Mexidol.

At the second stage of the study, the structure of the lead compound LHT 21–16 was confirmed. Figure 4 shows the ^{1}H NMR spectrum for LHT 21–16. In the ^{1}H NMR spectrum of the compound LHT 21–16, the proton signals of the methyl and ethyl groups of 2-ethyl substituent of 2-ethyl-6-methyl-3-hydroxypyridine are shown in the form of a triplet with integral intensity of three protons and a quartet with integral intensity of two protons with chemical shifts and the spin-spin splitting constants, 1.11 (t, J = 7.5 Hz, 3H) and 2.63 (q, J = 7.5 Hz, 2H), respectively. The proton signals of the pyridine nucleus are manifested as two doublets 6.99 (d, J = 8.1 Hz, 1H), 6.84 (d, J = 8.1 Hz, 1H). The singlet of the proton of the hydroxyl group at position 3 of the pyridine nucleus is sufficiently broadened and can be poorly seen on the panoramic spectrum, but it clearly manifests itself in the analysis of integral intensities at 10 ppm. The proton signals of the pyridine nucleus of nicotinic acid appear in the region of

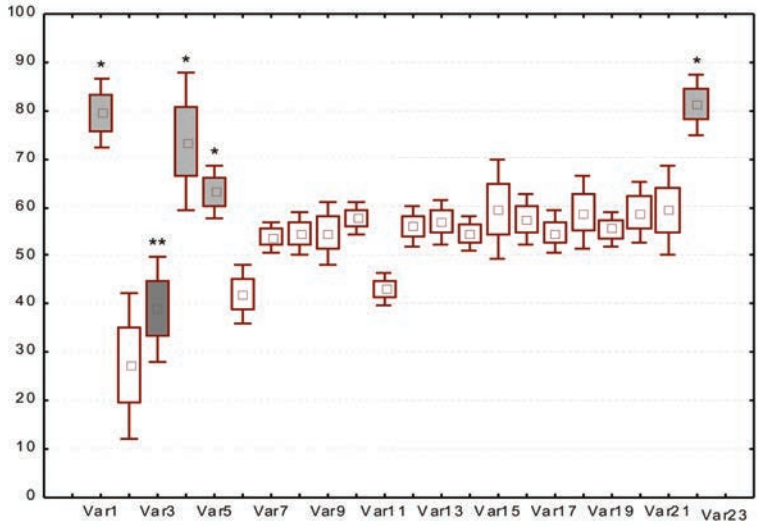

Figure 1. Results of in vitro screening in the group of 3 – oxypyridine derivatives. Var1 – positive control, Var2 – negative control (DMSO), Var3 – negative control (hypoxia), Var 4 – trimethazidine; Var5 – Mexidol; Var6 – Var22 – 3-oxypyridine derivatives (LHT). * – $p<0.01$

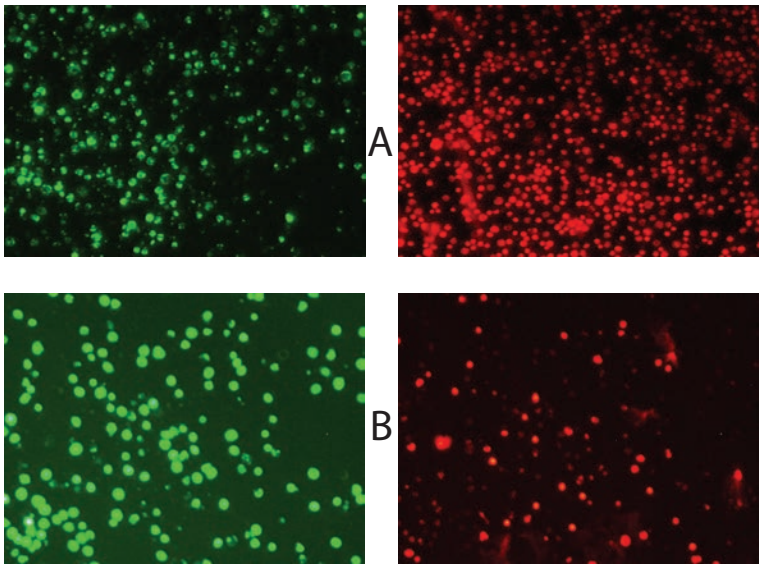

Figure 2. Fluorescence of live (green) and dead (red) leukocytes. A – hypoxia simulation (negative control); B – in the presence of the lead compounds from the group of 3-oxypyridine derivatives under laboratory code LHT 21-16. ×10 zoom

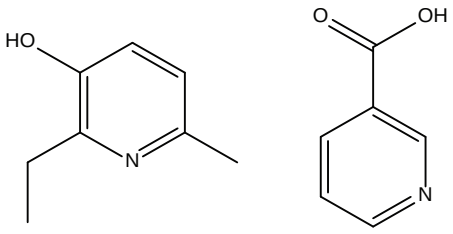

Figure 3. Chemical formula LHT 21–16

aromatic protons in the form of a singlet corresponding to the proton signal at position 2, two doublets corresponding to proton signals at positions 4 and 6, and a triplet of one proton at position 5, 8.78–8.74 (m, 1H), 8.25 (dt, J = 7.9, 1.9Hz, 1H), 7.52 (dd, J = 7.9, 4.8Hz, 1H).

Thus, the method proposed for identifying the compound of LHT 21–16 showed high sensitivity necessary to provide incoming control of the test lead compound.

In a number of experiments, the ability of Mexidol, 3-hydroxypyridine derivative, to reduce the size of the necrotic zone in the model of coronary-occlusive myocardial infarction (Mikhin et al. 2016, Danilenko and Pokrovskii 2014), therefore, it can be assumed that the lead compound LHT 21–16, has the similar properties.

The ligation of the left anterior descending artery in rabbits in the control group of animals resulted in myocardial necrosis, the size of which was 46.03±13.1%. The administration of Mexidol at a dose of 85.72 mg/kg and LHT 21–16 at a dose of 52 mg/kg resulted in a statistical-

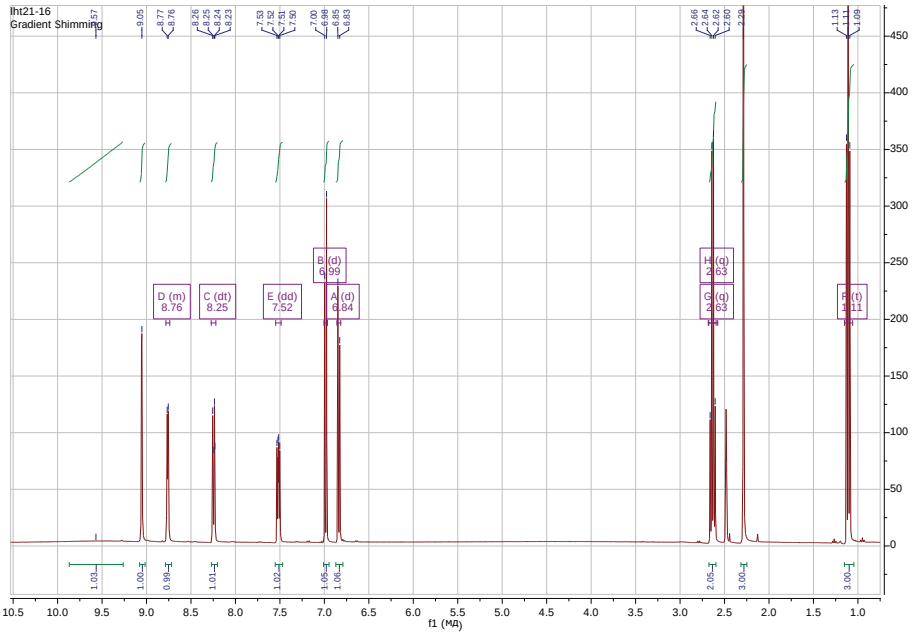

Figure 4. The ^{1}H-NMR spectrum of compound LHT 21–16.

ly significant reduction of the necrotic zone compared to that in the control group of animals.

In all the animals during ischemia/reperfusion of the left coronary artery ("control-ischemia/reperfusion", "ischemia/reperfusion+Mexidol 85.72 mg/kg", "ischemia/reperfusion+LHT-21-16 52 mg/kg"), ischemic myocardial damage was observed. In the animals, to which 20 minutes before ischemia comparison drug Mexidol 85.72 mg/kg had been intravenously administered, the ischemic zone was 31.2±4.1% (Table 1).

The lead compound LHT 21–16 (52 mg/kg) significantly reduces the necrotic zone to 26.2±2.7%, whereas in the control group it was 46.3±5.1%.

In an immunoenzyme analysis of myocardial damage in the control group of animals, the level of Tn I in blood plasma was 16.6±2.4 ng/ml, which is 4.8 times higher than that in the sham operated animals.

Reference drug Mexidol reduced the level of Tn I 1.4 times in comparison with the control group. All the animals who had been administered substance LHT 21–16 showed a 2.2-time decrease in the level of Tn I compared with the control group ($p<0.05$) and a 1.5-time decrease in comparison with reference drug Mexidol 85.72 mg/kg/day.

Thus, 3-oxypiridine derivative LHT 21–16 led to a decrease in the necrosis level under conditions of 30 minutes of ischemia followed by 90 minutes of reperfusion in rabbits, which was expressed in a decreasd size of the necrotic zone and a reduced level of Tn I, a biochemical marker of myocardial damage. It was shown in the experiment that LHT 21–16 reduces the necrotic zone 1.9 times in comparison with the control. In addition, LHT 21–16 limited an increase in myocardial damage marker Tn I, like Mexidol, but surpassing it 1.5 times in efficiency.

At the beginning of prolonged ischemia, two separate pathological processes occur. The first process is tissue damage caused by ischemia as such. The second is the biochemical changes that occur during ischemia and contribute to the surge in generating ROS and infiltrating pro-inflammatory neutrophils and other immunocytes when molecular oxygen is re-introduced into tissues during reperfusion (Popelova et al. 2008). Despite the fact that reperfusion restores the delivery of oxygen and substrates necessary for the generation of aerobic ATP, and normalizes the extracellular pH, the effect of re-oxygenation disrupts the process of oxygen reduction in the ischemic myocardium, which leads to the accumulation of significant amounts of the ROS (Kotlyarov and Smirnov 2004). It inevitably leads to the activation of the lipid peroxidation (LPO) (Štěrba 2013). LPO activation has a number of adverse consequences for cardiomyocytes caused by: postischemic myocardial hyperoxia and an influx of pro-oxidants, increased activity of membrane-bound and solubilized phospholipases, as well as to the destructive action of the resulting amphiphilic compounds (free fatty acids, lysophospholipids, LPO products) introduced into the lipid bilayer of membranes; the stretching and rupture of the sarcolemma and the membrane of the cell organelles due to the accumulation of cations in them, in Na^+, Ca^{2+}, and, as a consequence – liquid (Zhao et al. 2015). Since ROS is considered to be one of the main factors triggering the mechanism of death in ischemic/reperfusion myocardial damage, the prevention of the synthesis of ROSs or their neutralization is obviously crucial for the survival or recovery of cardiomyocyte function (Pourkhalili et al. 2009). The pharmacological group used for this purpose is antioxidants. This group includes drugs of different nature and mechanisms of action, which ultimately affect the processes of free radical oxidation of cell structures and biomolecules, primarily, peroxidation of membrane phospholipids (McClements et al. 2018).

Mexidol is one of the 3-hydroxypyridine derivatives, which is a universal drug of antioxidant pharmacotherapy, as it affects the different stages of the oxidative stress: inhibits free-radical oxidation of membrane lipids; reacts with peroxide radicals of lipids, primary and hydroxyl radicals of peptides; reduces the level of NO, increases the activity of superoxide dismutase and other antioxidant enzymes. Due to its mechanism of action, Mexidol has a wide range of pharmacological effects, implemented at least at two levels – neuronal and vascular (Volchegorskii et al. 2014).

Thus, the group of 3-oxypyridine derivatives continues to be replenished with new compounds in order to improve the pharmacological properties of the molecule. The previous studies have proved that a number of 3-oxypyridine derivatives have a pronounced antihypoxic, anti-ischemic, neuroprotective, endothelioprotective properties. However, in order to expand the spectrum of action and to improve the efficacy, new compositions with different chemical groups are being created.

The prerequisites for the high efficacy of the compound may be the presence of privileged chemical groups, in particular, hydroxyl, capable of increasing the lipophilicity and anti-radical activity of the compound. Using ^1H

Table 1. Impact of compound LHT 21-16, Mexidol on the size of myocardial necrotic zones and troponin I content in blood plasma under conditions of ischemia/reperfusion (M±m)

N	Group	Size of the necrotic zone %	The level of Troponin I (ng/ml)
1.	Sham operated animals n = 8	0.00±0.00	$3.4\pm1.1^{*}$
2.	Ischemia/reperfusion, Control n = 8	$46.3\pm5.1^{\#}$	$16.6\pm2.4^{\#*}$
3.	Ischemia/reperfusion + LHT 21–16 (26.0 mg/kg/day) n = 8	$26.2\pm2.7^{\#*}$	$7.5\pm1.2^{\#*}$
4.	Ischemia/reperfusion + Mexidol (85.72 mg/kg) n = 8	$31.2\pm3.9^{\#*}$	$11.3\pm1.7^{\#*}$

Note: the differences were statistically significant ($p<0.05$); $^{\#}$ – in comparison with group of sham operated animals; * – in comparison with group of "ischemia/reperfusion". All the test compounds were administered once intravenously 30 minutes before reperfusion.

NMR, it was found that compound of LHT 21–16 at position 3 of the pyridine nucleus has such a group.

Further, to test the high efficacy of lead compound LHT 21–16 (52 mg/kg), an evaluation of its cardioprotective activity was performed when modeling a 30-minute coronary occlusion followed by a 90-minute reperfusion, where it was shown that a 3-oxypyridine derivative LHT 21–16 (52 mg/kg) had a cardioprotective effect reducing the severity of myocardial ischemic damage, as evidenced by a decreased necrotic zone and Tn I level.

In this regard, it is worth continuing search for potential cardiotropic agents in the group of 3-oxypyridine derivatives.

Conclusions

1. By intensity of the antihypoxic effect, lead-compound LHT 21–16 from the group of 3-oxypyrine derivatives in in vitro conditions is superior to reference drugs Trimetazidine and Mexidol.
2. The physico-chemical method of NMR spectroscopy confirmed the chemical structure of lead compound LHT 21–16.
3. LHT 21–16 (52 mg/kg) have a cardioprotective effect on the model of coronary-occlusive myocardial infarction, as evidenced by a decreased necrotic zone and level of Tn I. The data obtained on the pharmacological activity of test compound LHT 21–16 allows recommending it for further in-depth study.

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