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Antihypertensive and cardioprotective effects of new compound 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide (Hypertril)

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ABSTRACT

Despite the success which was achieved in the treatment of arterial hypertension, the problem remains actual. At the departments of pharmaceutical chemistry and pharmacology of the Zaporozhye State Medical Institute (Ukraine), our research team isolated the compound 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide (Hypertril) as derivative of 4-amino-1,2,4-triazole. The objectives of this investigation were the study of cardioprotective and antihypertensive activities of this new compound Hypertril and we used the Spontaneously hypertensive rats (SHR) as an experimental model. We discovered that Hypertril has a reliable dose-dependent antihypertensive effect in the dose range 5–20 mg/kg after 30-day administration and this antihypertensive effect of Hypetril competes or significantly exceeds Metoprolol (20 mg/kg). Our studies obtained evidence of a dose-dependent improvement of myocardial energy metabolism. Hypertril reduces the manifestations of secondary mitochondrial dysfunction due to arterial hypertension. Hypertril can prevent oxidative modification of the protein; also Hypertril reduces the insufficiency of mitochondrial pores. As a result, Hypertril increases the content of ATP in the myocardium of SHR, normalizes the activity of mitochondrial enzymes, decreases lactate production and increases pyruvate. Hypertril enhances the cardioprotective effects of NO and increases the resistance of the cardiomyocytes to ischemia. The use of Hypertril leads to a dose-dependent increase of the density of cardiomyocyte nuclei, significant increase RNA content in nuclei and the cytoplasm of cardiomyocytes, and an increase of the nuclear-cytoplasmic index. These changes indicate a decrease of myocardial hypertrophy.

1. Introduction

According to WHO, diseases of the cardiovascular system take first place among the causes of death and disability of the population in the European Union, the United States, and Canada. Myocardial infarction and stroke have the highest mortality rate and arterial hypertension is the major risk factor of these diseases. The world prevalence of arterial hypertension in 2018 is up to 40%.

Despite the success which was achieved in the treatment of arterial hypertension, the problem remains actual. Most patients with arterial hypertension have a lot of other problems like atherosclerosis, diabetes mellitus etc. It is necessary to understand how these changes interact and to identify common pathways in their pathogenesis. Additionally, vascular pathology usually manifests on late stages of arterial hypertension, when the damage of the target organs (heart, brain, kidneys) has been going on for many years. Therefore, understanding of the molecular and biochemical mechanisms of damage of the target organs due to arterial hypertension and additional pharmacological regulation these processes are the central tasks of experimental and clinical medicine (Whelton and Williams, 2018; Qamar and Braunwald, 2018; Hwang et al., 2018).

According to the guidelines of the European Society of Cardiology and European Society of Hypertension, β-blockers are an important component of complex antihypertensive therapy. In the past decades, cardioselective β_1-adrenoceptor blockers have been actively used in the therapy of arterial hypertension, which remove excessive sympathetic

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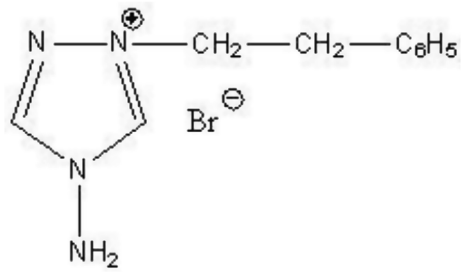

Fig. 1. The structural formula of 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide.

myocardial stimulation, have a beneficial effect on ventricular relaxation and intracardiac hemodynamics, prevent myocardial remodeling, reduce the need for oxygen in the heart, have an antiarrhythmic effect. Make allowance for above-mentioned, the development of the new drugs with selective β_1-blocking action and with additional positive effects, e.g. cardioprotective and antioxidative effects, is important current scientific interest.

At the departments of pharmaceutical chemistry and pharmacology of the Zaporozhye State Medical Institute and at the Scientific Association "Farmatron" (Ukraine) our research team isolated the compound 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide (Hypertril) as derivative of 4-amino-1,2,4-triazole. According to preliminary data which we have received, Hypertril can be attributed to β-blockers with some antioxidant activities.

So, the objectives of this investigation were the study of the cardioprotective and antioxidative effects, and antihypertensive activities of this new compound Hypertril.

The investigated substance is a crystalline powder, white or nearly white with the weak specific smell, hygroscopic. The substance is very soluble in water, easily soluble in 96% alcohol and chloroform (Fig. 1). Morphometric studies of Hypertril were conducted by microscope Ulab XY-B2BLED to detect the size and shape of powder particles (Fig. 2). Studies of 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide in the infrared spectrum were done by using PerkinElmer Spectrum One (Fig. 3). Study of Hypertril pharmacokinetics was conducted and it was established that the compound has high concentrations in the serum, heart, and urine. Hypertril very quickly distributes from the blood to the heart muscle. The drug is characterized by a very high tropism to heart muscle: the tissue distribution coefficient for the heart is 10.55%. Daily renal excretion of Hypertril is 17.2%. The acute toxicity of Hypertril was investigated in mature male Wistar-Kyoto rats in oral (p.o.) route. According to the probit analysis for which we used AnalystSoft and StatPlus, TD_{50} is 683.4 mg/kg (Mazur et al., 1999, 2010, Mazur et al., 2013a,b).

Previous studies conducted in an isolated heart have shown that Hypertril reduces heart rate. In vitro, Hypertril demonstrated the activities of a scavenger of the reactive oxygen species. Our earlier preclinical studies in the models of izadrin-induced myocardial necrosis and doxorubicin-induced heart failure showed that Hypertril administration decreases mortality, myocardial hypertrophy, myocardial fibrosis, reduces necrosis and apoptosis, improves ECG (normalization of heart rate, the amplitude of the ST interval and R wave, QRS complex, T wave). During these preclinical studies of the Hypertrile activity, no side effects were identified. However, it was found that in a subtoxic dose Hypertrile causes increase concentration of the aspartate transaminase, alanine transaminase, and bilirubin in the blood, and urea in the urine. However, these changes disappeared one month after discontinuation of the drug (Chekman et al., 2013; Belenichev et al., 2012, 2014).

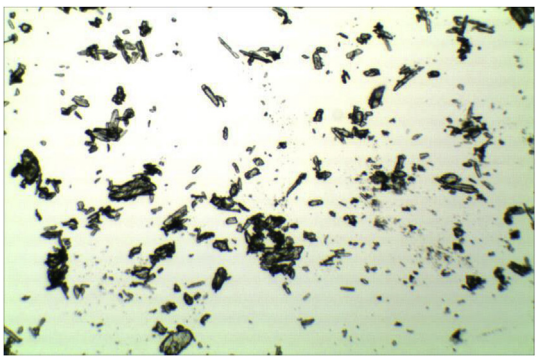

Fig. 2. The particles of 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide.

2. Materials and methods

2.1. Animal models

All experimental studies were conducted in accordance with the "Methodological recommendations for conducting preclinical studies of potential drugs of the State Expert Center of the Ministry of Health of Ukraine". The studies were performed on a sufficient number of experimental animals. All manipulations were carried out in accordance with the regulation using of the animals in biomedical experiments. The protocols of experimental studies were approved by the decision of the Ethical Committee of Zaporozhye State Medical Institute (protocol No. 33 of June 26, 2015).

10 mature male Wistar-Kyoto rats with body weight of 200–220 g and 60 mature Spontaneously hypertensive rats (SHR) with a body weight of 250–270 g were treated in common laboratory environment (12 h light cycle, T = +22 °C) with free access to water and food. All rats were 8 months old. Animals were obtained from the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine. The duration of the quarantine (acclimatization period) for all animals was 14 days. During the quarantine, every animal was inspected daily (behavior and general condition). Before the beginning of the study, animals that met the criteria for inclusion in the experiment were divided into groups by using the randomization method. Animals that did not meet the criteria were excluded from the study during the quarantine. Cells with animals were placed in separate rooms.

The tested medications were administered intragastrically by probe once per day at 10am during 30 days as a suspension in 1% starch mucus. We used Hypertril in doses 5 mg/kg, 7.5 mg/kg, 15 mg/kg, 20 mg/kg and Metoprolol (Astra Zeneca, UK Ltd.) in dose 20 mg/kg. As a reference drug, we select that is close to the test substance by chemical structure and/or has a similar mechanism of action. So we have chosen metoprolol as a control drug since we suspect that Hypertrital has some β-blocker activity additionally to its cardioprotective and antioxidative effects.

Rodents were allocated into 7 experimental groups: 1st group – 10 Wistar-Kyoto rats treated with 1% starch mucus (intact); 2nd group – 10 SHR treated with 1% starch mucus (control); 3rd – 10 SHR treated with Hypertril in dose 5 mg/kg; 4th – 10 SHR treated with Hypertril in dose 7.5 mg/kg; 5th – 10 SHR treated with Hypertril in dose 15 mg/kg; 6th – 10 SHR treated with Hypertril in dose of 20 mg/kg; 7th – 10 SHR treated with Metoprolol in dose of 20 mg/kg.

Antihypertensive activity of Hypertril and Metoprolol was assessed by lowering blood pressure (BP). For all rats, the systolic blood pressure was measured on the tale artery by the non-invasive system BP-2000 (Visitech Systems, USA). The first measurement was done a day before the beginning of the experiment and then on the 15th and 30th days of the experiment. The BP measurement was carried out in silence with the exclusion of loud noises and the human voice. The animals were accustomed to the restrainer for 5 days before the procedure. In the day of measurement animals were put in the individual restrainer, then 10 measurements of BP were performed for each rat. Animals were in restrainer no more than 30 min.

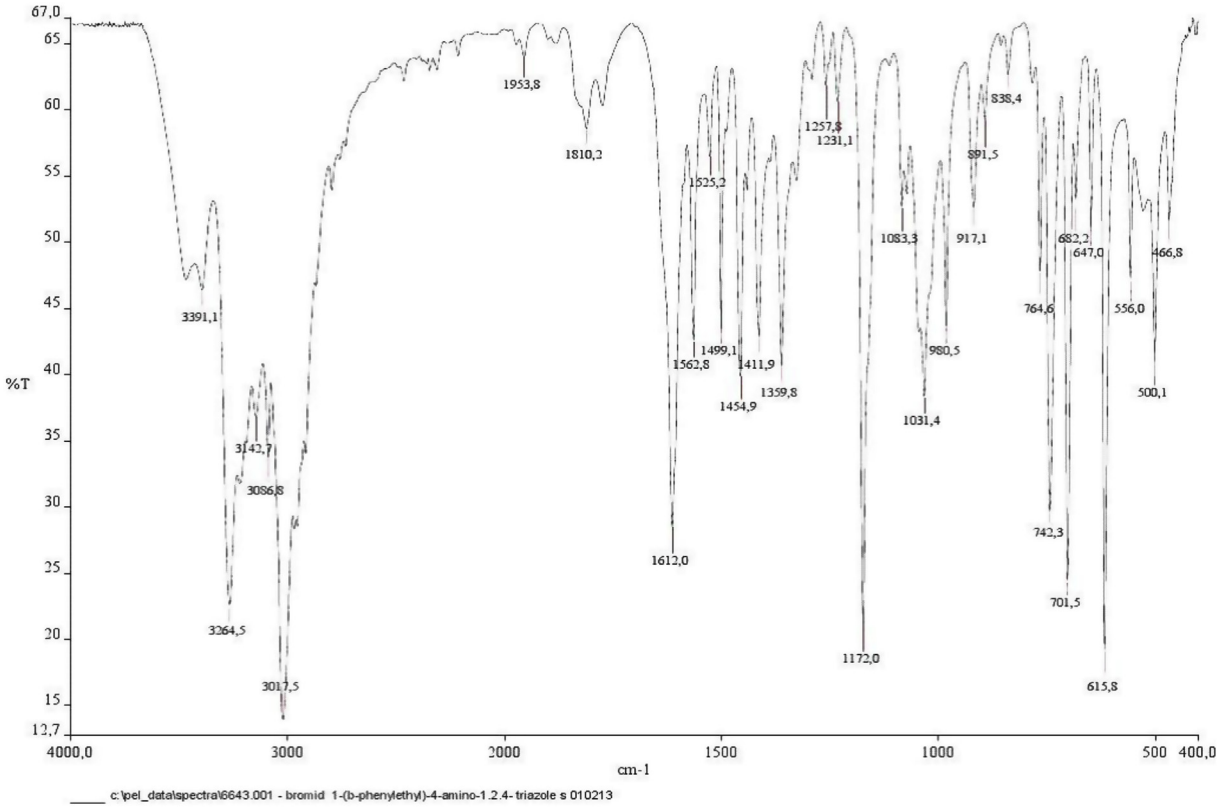

Fig. 3. Infrared spectroscopy of 1-(β-phenylethyl)-4-amino-1,2,4-triazolium bromide.

On the last day of the experiment after the 16 h of starvation, rats were anesthetized with 40 mg/kg of pentobarbitone intraperitoneally. Then animals were devitalized.

We used Sigma-Aldrich (USA) for buying all the necessary chemical compounds.

2.2. Biochemical analysis

The heart was washed with cold 0.15 M KCl (T = +4 °C) 1:10. The washed heart was cleansed from fat, connective tissue. Blood clots were removed from the internal cavities and the heart was washed once more with 0.15 M KCl (T = +4 °C) 1:10. Then we homogenized the heart in a 10-fold volume of medium (sucrose-250, tris-HCl buffer-20, EDTA-1 pH 7.4). A mitochondrial fraction was isolated by a differential centrifugation method at a Sigma 3-30k reefer centrifuge (Germany). Centrifugation was carried out for 7 min at 1000 g, then the supernatant was re-centrifuged for 20 min at 17000 g. For long-term storage, the mitochondria were frozen at −80 °C.

The markers of oxidative damage of the proteins – aldehyde phenylhydrazone (APG) and ketone phenylhydrazone (KPG) were determined for assessing the intensity of oxidative stress in the myocardium. The state of the antioxidant system was assessed by the activity of superoxide dismutase (SOD), glutathione peroxidase (GP) and catalase. The state of energy metabolism was determined by the level of the ATP, ADP, AMP, lactate, pyruvate, malate, malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities. Since the signs of mitochondrial dysfunction are a violation of the barrier functions of mitochondrial membranes, we conducted studies of the opening of giant mitochondrial pores.

The indices of oxidative damage of the proteins were determined by the interaction of oxidized amino acid residues with 2,4-dinitrophenylhydrazine (2,4-DNPH) and the formation of APG and KPG, which have an absorption spectrum 274 nm and 363 nm, respectively.

The determination of the activity of SOD was carried out according to the procedure by using phenazine methane sulfate and nitrosine tetrazolium. The activity of GP was determined by the test with tret-butyl hydroperoxide and reduced glutathione. Catalase activity was determined by the hydrogen peroxide and ammonium molybdate tests.

The amount of malate was determined by the Hohhorst method. The pyruvate content was determined by the Tsokh-Lompreht method. The lactate content was determined by the Hohhorst method. The activity of MDH was determined spectrophotometrically. Adenyl nucleotides were determined by thin-layer chromatography. The content of total SH groups was determined spectrophotometrically by reaction with 5,5-dithio-bis-7-nitrobenzoic acid.

The opening of the mitochondrial pores was determined spectrophotometrically. The protein concentration was evaluated by the Bradford method.

2.3. Morphometrical assay

The apical part of the heart was separated and was placed in the Carnua fixator for 24 h. After the standard procedure of dehydration of the tissue and its impregnation with chloroform and paraffin, the myocardium was embedded with paraplast. On a Microm-325 rotary microtome (Microm Corp., Germany), 5-μm histological sections were prepared. These sections were then rehydrated by xylene and descending ethanol concentrations (100%, 96%, 70%), washed with saline. For specific detection of RNA, histological sections were stained for 24 h with gallocyanin-chromic alum and put into a polymer medium for subsequent microscopy.

The myocardium was studied with the microscope AxioScope (Carl Zeiss), immersion lens F-Fluar 40x/1,30 Oil (Carl Zeiss) and immersion oil Immersol 518F (Carl Zeiss). All images were obtained with 8-bit camera AxioCam-ERc 5s (Carl Zeiss). All images were done with the same brightness, exposition and correction settings. Image analysis was performed in ImageJ (NIH, USA).

The following values were assessed:

- area of cardiomyocyte nuclei (μm^2);
- the concentration of RNA in the nuclei and cytoplasm of cardiomyocytes in units of optical density, which were calculated as the logarithm of the ratio of the optical density of the cell nucleus to the optical density of the intercellular substance;
- the density of cardiomyocyte nuclei as an indicator of the number of cell nuclei per 1 mm^2 of myocardial tissue;
- nuclear-cytoplasmic coefficient as a ratio of the total area of cardiomyocyte nuclei per 1 mm^2 of myocardial tissue.

2.4. Immunoblotting

Metabolites of NO were determined by the level of nitrates in the Griess reaction. The activity of total NOS was determined by the difference between the rate of NADPH oxidation. Nitrotyrosine was determined by solid-state immunosorbent ELISA Kit.

We analyzed the cytoplasmic fraction of NOS and a mitochondrial fraction of NOS in the heart homogenate. Proteins were separated in 10% polyacrylamide gel and separation was carried out by electrophoresis at a voltage of 100 V. The proteins from the gel were transferred to a nitrocellulose membrane at a voltage of 100 V and a 0.35 A for 1 h. After transfer, the membrane was placed in a buffer 1% bovine serum albumin for 20 h. After the washing in the shaker for 5 min with a solution of 0.1 M PBS, the membrane was placed in an antibody's solution to iNOS or eNOS (1:500) and incubated for 2 h at room temperature, then was washed in the shaker 4 times for 5 min with 0.1 M PBS. The membrane was placed in a solution of secondary antibodies (1:1000) and incubated for 2 h, then washed in a shaker 4 times for 5 min with 0.1 M PBS. The membrane was placed in a solution of ExtraAvidin-peroxidase + 1% solution of bovine serum albumin (1:1000), incubated for 1 h and washed. For visualization, the membrane was treated with a solution of AEK (1 tablet of 3-amino-9-ethylcarbazole was dissolved in 2.5 ml of DMF containing 47.5 ml of 0.05 M acetate buffer pH 5.0, 25 μl 30% H_2O_2). The membrane was incubated in the substrate mixture for 5–10 min, then washed with distilled water several times and dried between sheets of filter paper under a stream of cold air. We performed the assessment of the heart slides in ultraviolet excitation spectrum with the wavelength 390 nm by using a light filter with high emission 38HE (Carl Zeiss), microscope AxioScope (Carl Zeiss), immersion lens F-Fluar 40x/1,30 Oil (Carl Zeiss) and immersion oil Immersol 518F (Carl Zeiss). All images were obtained with 8-bit camera AxioCam-ERc 5s (Carl Zeiss). All images were done with the same brightness, exposition and correction settings. Image analysis was performed in ImageJ (NIH, USA). We assessed the density of the immunopositive cells.

2.5. Statistical analysis

All statistical calculations were done by «STATISTICA® for Windows». The evaluation of differences between groups was done by using one-way ANOVA or ANOVA for repeated measurements with post-hoc Bonferroni correction or Kruskal-Wallis criterion with post-hoc Dunn correction. A significant difference was considered at $P < 0.05$.

3. Results

After 30 days of the Hypetril and metoprolol management, the body weight of SHR was significantly increased compared with the weight at the beginning of the experiment (Table 1).

An analysis of the conducted study showed that intragastric administration of Hypertril at all investigated doses decreases BP (Table 2). The antihypertensive effect of Hypertril at dose 20 mg/kg was the most significant compared with other dosage regimes and exceeded significantly the effect of metoprolol after 30 days of administration.

The dose/response relationship is shown by a regression curve (Figs. 4 and 5). It can be noted that the maximum slope of the dose/response curve was observed in the range of Hypertril doses 7.5–15 mg/kg. Further increase of doses to maximal (20 mg/kg) and the giving of a minimal dose (5 mg/kg) led to a smoothing of the curve. It was established by using the method of mathematical analysis that the average effective dose (ED_{50}) is 12.5 mg/kg based on the constructed dose-response graph (Fig. 6). The dose interval, which includes the main part of the curve around the central point, is referred to the potency of the drug. The slope of the dose-response curve, especially near the mean, characterizes the spread of the doses that cause the effect. This value indicates how large the change of the reaction in the population due to the action of the drug's effective doses will be. A steep slope indicates that most of the population will react approximately equal, while a gentle slope indicates significant differences. The shape of the curve and its extreme points depend on the external and internal factors.

The index of Hypetril's specific therapeutic effect is 4, which indicates a safe clinical use of the Hypertril. Additionally, the therapeutic index (TD_{50}/ED_{50}) was used as a criterion of the Hypertril's safety. The therapeutic index is 54.7, which indicates the sufficient safety of the Hypertril.

The rats with spontaneous hypertension had a significant decrease in cardiomyocyte's nuclear-cytoplasmic ratio compared with normotensive animals, which reflects the presence of myocardial hypertrophy (Table 3). There was also a 9% decrease in an area of the cardiomyocyte nuclei and a decrease of RNA concentration in nuclei by 7.4% and in the cytoplasm of cardiomyocytes by 9%.

Hypertril additionally to antihypertensive effect showed cardioprotective effect as well. Administration of this drug at 7.5–20 mg/kg doses led to a significant increase the density of cardiomyocyte nuclei, RNA concentration in the nuclei and in the cytoplasm of the cardiomyocytes, the nuclear-cytoplasmic index compared with the control group (untreated SHR). The administration of Hypertril at 20 mg/kg had the strongest cardioprotective effect compared with other doses of this drug. Also, the cardioprotective effect of Hypertril exceeded significantly the cardioprotective effect of metoprolol.

Our biochemical studies revealed a significantly increased level of the markers of oxidative modification of the protein in the mitochondrial and cytosolic fractions in the heart of spontaneously hypertensive rats (Table 4). These data can indicate the formation of mitochondrial dysfunction due to activation of oxidative stress. Also, we registered an increase in the rate of spontaneous opening of the mitochondrial pore in 10.7 times and swelling of the mitochondria of the myocardium in SHR (Table 5).

Hypertril showed significant dose-dependent antioxidant effect. Administration of this drug at 7.5–20 mg/kg doses led to a significant decrease the markers of oxidative modification of the protein (APG and KPG) in the mitochondrial and cytosolic fractions of the heart compared with the control group (untreated SHR). Also, Hypertril exceeded the antioxidant effect of metoprolol.

There was a decreased level of ATP by 35.3% in the myocardium of the SHR, level of pyruvate by 45.5%, an increased level of lactate by 64.1% and inhibition of the mitochondrial enzymes (Table 6). This confirms the activation of anaerobic glycolysis and inhibition of aerobic oxidation in the myocardium of SHR.

Hypertril exerted a dose-dependent effect to decrease in the manifestations of secondary mitochondrial dysfunction and to improve the energy metabolism in the myocardium of SHR by increasing ATP and pyruvate content and by decreasing lactate in the myocardium. Also after 30 days of Hypetril administration, we revealed recovery of mitochondrial enzyme activity.

As a result of our studies, we established a depression of the NO formation in the myocardium of SHR, decreased NOS activity (Tables 7–8). The immunoblotting techniques showed decreased expression of eNOS and increased iNOS expression in the myocardium of SHR compared with the intact group.

Hypertril showed a dose-dependent strengthening of NO synthesis

Table 1
The effect of Hypertril on the weight.

Experimental groups	Weight at the beginning of the experiment, g	Weight on 15th day of the experiment, g	Weight on 30th day of the experiment, g
Normotensive rats (group №1)	221,4 ± 7,17	228,4 ± 8,20	232,8 ± 5,21
SHR (control) (group №2)	311,8 ± 7,17	324,6 ± 10,0	349,0 ± 7,34[a]
SHR + Hypertril 5.0 mg/kg (group №3)	301,6 ± 5,89	313,4 ± 6,02	335,8 ± 7,91[a]
SHR + Hypertril 7.5 mg/kg (group №4)	305,6 ± 8,50	315,6 ± 5,50	326,8 ± 1,38[a]
SHR + Hypertril 15.0 mg/kg (group №5)	298,4 ± 6,34	305,6 ± 4,72	316,6 ± 3,91[a]
SHR + Hypertril 20.0 mg/kg (group №6)	301,8 ± 6,26	310,0 ± 6,48	319,0 ± 7,34[a]
SHR + Metoprolol 20 mg/kg (group №7)	301,2 ± 7,12	309,8 ± 7,46	320,8 ± 10,5[a]

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs weight at the beginning of the experiment.

by increasing the activity of NO synthase compared with untreated animals. Also, Hypetril normalized expression of inducible NOS and reduced a level of the markers of nitrosative stress. Hypetril administration led to an increase in the activity of some component of the antioxidant system such as a thiol-disulfide system, which participates in the rescue of NO messengers.

4. Discussion

RNAs have an important role in the process of synthesis structural proteins and enzymes and it becomes the evident possibility of suppressing the synthetic function of the cardiomyocytes due to spontaneous hypertension. Also, the disruption of RNA synthesis in the hypertensive animals can serve as a reflection of energy deficiency and the metabolites of the permanent oxidative stress can initiate a cascade of metabolic disorder, e.g. mitochondrial dysfunction (Ardehali et al., 2012). These processes can be detected at the earliest stages of hypertension. Further development of the disease leads to deeper disturbances of these processes. Analysis of the correlation between the markers of oxidative stress on the one hand and the structural and functional characteristics of the myocardium, on the other hand, indicates the importance of the oxidative stress in myocardial remodeling due to hypertension (Porter et al., 2011). Identification of these markers of oxidative stress can serve as a prognostic factor of left ventricular changes. Hypertril improves the process of translational processes in cardiomyocytes and stimulation of protein synthesis, which indicates the presence of the antihypertrophic effect of Hypertril.

During the last decade, the different authors have revealed the important role of oxidative stress in the damage of the organs like a heart (Trujillo et al., 2009; Walters et al., 2012). Oxidative stress can promote the generation and maintenance of hypertension. A number of the studies have established that an important source of reactive oxygen species is NADP oxidases in the mitochondria. It is known that the interaction of NO with reactive oxygen species leads to the formation of highly reactive and cytotoxic products, such as peroxynitrite and nitrosonium ion. These highly active compounds are involved in the

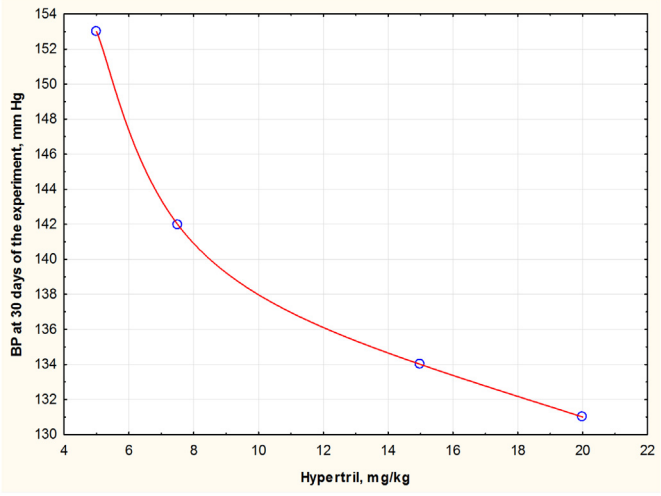

Fig. 4. Dose/response curve (polynomial).

chemical modification of the receptors and ion channels that can initiate apoptosis of the cells. In addition, reactive oxygen species can suppress eNOS activity in the endothelium as well, which makes an endothelial dysfunction and enhances the progression of arterial hypertension. Reactive oxygen species contribute to the calcium overload of the mitochondria and the disruption of their energy-producing function. The markers of oxidative modification of the protein are one of the earliest intracellular indicators of damage to macromolecules (Lesnefsky et al., 2001). The inhibition of oxidative destruction of the mitochondrial proteins decreases a degree of mitochondrial dysfunction. This was confirmed by a decreasing frequency of the mitochondrial pores opening and the swelling of myocardium mitochondria in our experimental groups. Thus, Hypertril has a dose-dependent mitoprotective effect. Metoprolol has no protective effect on myocardium mitochondria.

Table 2
The effect of Hypertril on the blood arterial pressure.

Experimental groups	BP at the beginning of the experiment, mm Hg	BP on 15th day of the experiment, mm Hg	BP on 30th day of the experiment, mm Hg
Normotensive rats (group №1)	105 ± 1,58	104 ± 1,00	106 ± 1,8
SHR (control) (group №2)	159 ± 1,00	161 ± 1,00	164 ± 1,36
SHR + Hypertril 5.0 mg/kg (group №3)	160 ± 3,53	157 ± 4,47	153 ± 5,70[a,b]
SHR + Hypertril 7.5 mg/kg (group №4)	158 ± 2,74	150 ± 5,00[b]	142 ± 2,55[a,b]
SHR + Hypertril 15.0 mg/kg (group №5)	158 ± 1,23	144 ± 2,36[a,b]	134 ± 2,36[a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	160 ± 3,56	138 ± 2,74[a,b]	131 ± 2,23[a,b,c]
SHR + Metoprolol 20 mg/kg (group №7)	161 ± 1,00	141 ± 2,23[a,b]	135 ± 1,58[a,b]

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs BP at the beginning of the experiment.
[c] $P < 0.05$ vs group №7.

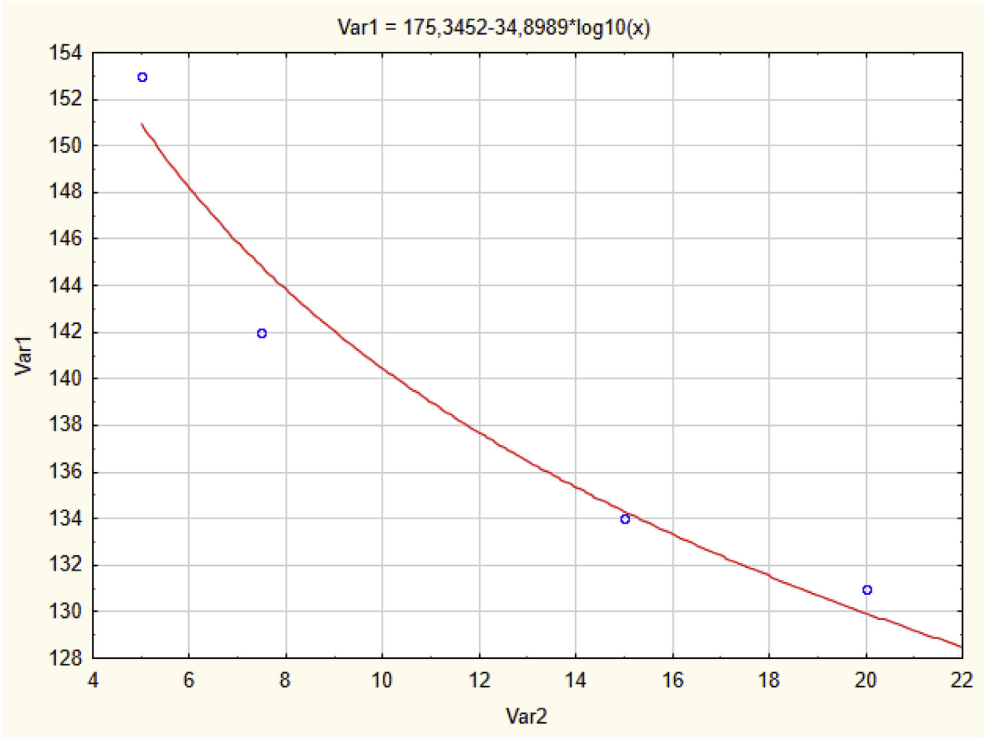

Fig. 5. Dose/response curve (logarithmic).

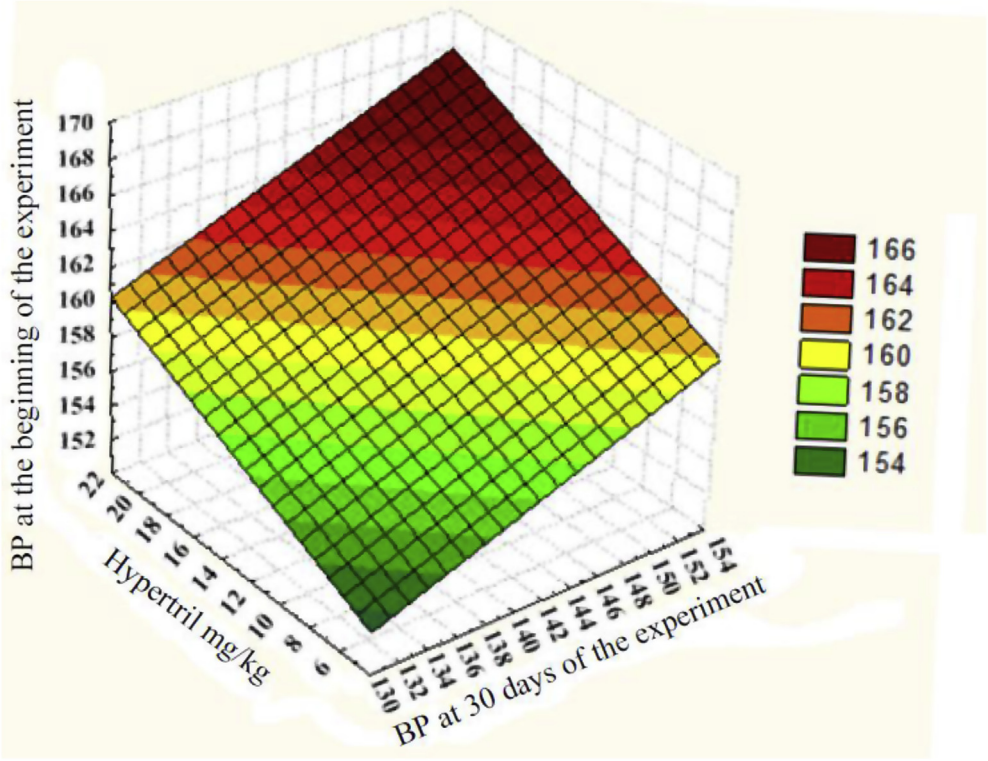

Fig. 6. Graph of dose/response surface.

During recent decades, the energy deficit at the cellular level is considered as the main reason for primary hypertension. The formation of mitochondrial dysfunction in the rats with arterial hypertension can lead to a disruption of energy production in the myocardium (Ingwall, 2009; Muller et al., 2010; Murdoch et al., 2006; Sedonn, 2007). We believe that Hypertril does not have an independent energotropic effect, and its positive effect is realized through mitoprotection. Treatment with metoprolol did not provide mitoprotective and energotropic action.

The revealed in our study disproportion between the expression of endothelial and inducible forms of NOS and the deficit of NO in the myocardium on the background of arterial hypertension can connect

Table 3
The effect of Hypertril on the morpho-functional parameters of the myocardium.

Experimental groups	Cell density,/mm^2	Cell area, μm^2	RNA concentration in core, U	RNA concentration in cytoplasm, U	Nuclear-cytoplasmic index
Normotensive rats (group №1)	3018,4 ± 59,6	13,4 ± 0,20	0,27 ± 0,001	0,022 ± 0,0002	0,044 ± 0,001
SHR (control) (group №2)	2042,8 ± 49,9[c]	12,2 ± 0,12[c]	0,25 ± 0,0009[c]	0,020 ± 0,0002[c]	0,032 ± 0,0006[c]
SHR + Hypertril 7.5 mg/kg (group №4)	2110,4 ± 55,3[a,c]	12,4 ± 0,13[c]	0,26 ± 0,003[a,b,c]	0,024 ± 0,0004[a,b,c]	0,036 ± 0,001[a,b,c]
SHR + Hypertril 15.0 mg/kg (group №5)	2401,4 ± 83,3[a,b,c]	12,4 ± 0,30[c]	0,26 ± 0,001[a,b,c]	0,026 ± 0,0003[a,b,c]	0,036 ± 0,001[a,b,c]
SHR + Hypertril 20.0 mg/kg (group №6)	2664,4 ± 49,5[a,b,c]	13,1 ± 0,25[a,b]	0,26 ± 0,001[a,b,c]	0,036 ± 0,0002[a,b,c]	0,036 ± 0,0008[a,b,c]
SHR + Metoprolol 20 mg/kg (group №7)	2228,1 ± 70,0[a,c]	11,7 ± 0,26[a,c]	0,25 ± 0,002[c]	0,021 ± 0,0005[c]	0,027 ± 0,0009[a,c]

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.
[c] $P < 0.05$ vs group №1.

Table 4
The effect of Hypertril on the indices of oxidative damage of the proteins in the heart.

Experimental groups	Mitochondrial fraction		Cytosolic fraction	
	APG, U/g protein	KPG, U/g protein	APG, U/g protein	KPG, U/g protein
Normotensive rats (group №1)	2,28 ± 0,14	1,08 ± 0,07	17,1 ± 0,75	9,52 ± 0,48
SHR (control) (group №2)	7,87 ± 0,43	5,73 ± 0,38	38,4 ± 2,7	21,8 ± 0,96
SHR + Hypertril 7.5 mg/kg (group №4)	4,35 ± 0,31[a,b]	3,88 ± 0,27 [a,b]	27,6 ± 2,1[a]	16,3 ± 1,1[a]
SHR + Hypertril 15.0 mg/kg (group №5)	3,25 ± 0,27 [a,b]	2,70 ± 0,12 [a,b]	21,2 ± 1,7 [a,b]	12,2 ± 1,1 [a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	2,45 ± 0,18 [a,b]	1,25 ± 0,06 [a,b]	18,2 ± 1,2 [a,b]	10,3 ± 0,7 [a,b]
SHR + Metoprolol 20 mg/kg (group №7)	7,21 ± 0,55	5,41 ± 0,41	31,3 ± 1,6[a]	18,8 ± 1,2[a]

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.

Table 5
The effect of Hypertril on the spontaneous opening of the mitochondrial pores of the myocardium.

Experimental groups	Δ E
Normotensive rats (group №1)	0,018 ± 0,001
SHR (control) (group №2)	0,193 ± 0,015
SHR + Hypertril 7.5 mg/kg (group №4)	0,073 ± 0,002 [a,b]
SHR + Hypertril 15.0 mg/kg (group №5)	0,048 ± 0,003 [a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	0,032 ± 0,001 [a,b]
SHR + Metoprolol 20 mg/kg (group №7)	0,18 ± 0,009

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.

with the pathological reorientation of the nitroxidergic system. In response to calcium overload, the formation of mitochondrial dysfunction and the overproduction of reactive oxygen species, the expression of eNOS and its activity are suppressed and the cytokine-dependent expression of iNOS is increased. This can promote that NO can be converted to peroxynitrite. High concentrations of peroxynitrite lead to S−, N−, O-nitrosylation of the most active regions of protein structures of the ion channels, receptors, transmembrane pores, signal molecules and development of nitrosative stress. ONOO can inhibit the electron transport chain of mitochondria, oxidize thiols and bind to iron in cytochromes, which increase the disturbances in the energy metabolism of the myocardium. This can increase the severity of cardiovascular pathology (arterial hypertension, myocardial infarction and CHF) (Vo et al., 2005; Williams et al., 2014). Hypetril showed a dose-dependent antioxidant effect, which protects the myocardium from the damage due to nitrosative stress. Compare to Hypetril, metoprolol had no protective and antioxidant effects in the myocardium of SHR. Cardioprotection of metoprolol is released only due to its β_1-blocking action and, as a result, negative chronotropic effect, reducing the preload, reducing the need for O_2. This provides a reduction in the number of cardiomyocytes with signs of necrosis and apoptosis. Metoprolol has no impact on the NO system. The low antioxidant activity of metoprolol does

Table 6
The effect of Hypertril on the indices of energy metabolism in the heart.

Experimental groups	ATP, μmol/g tissue	Lactate, μmol/g tissue	Pyruvate, μmol/g tissue	mtNAD-MDH, μmol/mg protein/min	mtSDH, nmol/mg protein/min
Normotensive rats (group №1)	3,88 ± 0,16	5,3 ± 0,27	0,167 ± 0,05	1,6 ± 0,11	5,6 ± 0,23
SHR (control) (group №2)	2,51 ± 0,07	8,7 ± 0,5	0,091 ± 0,04	0,88 ± 0,04	2,8 ± 0,12
SHR + Hypertril 7.5 mg/kg (group №4)	2,86 ± 0,07 [a,b]	6,5 ± 0,4 [a,b]	0,123 ± 0,007 [a,b]	1,10 ± 0,05 [a,b]	3,8 ± 0,21 [a,b]
SHR + Hypertril 15.0 mg/kg (group №5)	3,11 ± 0,11 [a,b]	5,7 ± 0,3 [a,b]	0,141 ± 0,005 [a,b]	1,37 ± 0,07 [a,b]	4,4 ± 0,23 [a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	3,77 ± 0,19 [a,b]	5,2 ± 0,2 [a,b]	0,161 ± 0,008 [a,b]	1,62 ± 0,04 [a,b]	5,2 ± 0,27 [a,b]
SHR + Metoprolol 20 mg/kg (group №7)	2,55 ± 0,08	8,3 ± 0,5	0,093 ± 0,04	0,90 ± 0,07	2,7 ± 0,10

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.

Table 7
The effect of Hypertril on the NO system in the mitochondrial fraction of cardiac homogenate.

Experimental groups	NOS, nmol/min/g protein	Nitrotirizin, nmol/g protein	Nitrites, μmol/g tissue	iNOS, U/g protein	eNOS, U/g protein
Normotensive rats (group №1)	4,88 ± 0,32	10,4 ± 0,63	3,8 ± 0,22	0,18 ± 0,01	10,1 ± 0,7
SHR (control) (group №2)	1,85 ± 0,10	64,5 ± 3,6	1,0 ± 0,10	1,14 ± 0,08	4,1 ± 0,3
SHR + Hypertril 7.5 mg/kg (group №4)	2,37 ± 0,18 [a,b]	51,2 ± 3,7 [a,b]	1,50 ± 0,08 [a,b]	0,87 ± 0,06 [a,b]	6,2 ± 0,4 [a,b]
SHR + Hypertril 15.0 mg/kg (group №5)	3,12 ± 0,14 [a,b]	34,2 ± 2,6 [a,b]	1,88 ± 0,11 [a,b]	0,58 ± 0,03 [a,b]	8,2 ± 0,6 [a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	3,87 ± 0,22 [a,b]	22,7 ± 1,8 [a,b]	2,54 ± 0,20 [a,b]	0,28 ± 0,011[a,b]	9,7 ± 0,7 [a,b]
SHR + Metoprolol 20 mg/kg (group №7)	1,90 ± 0,07	62,5 ± 0,18	1,1 ± 0,07	1,12 ± 0,18	4,7 ± 0,3

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.

Table 8
The effect of Hypertril on the NO system in the cytosolic fraction of cardiac homogenate.

Experimental groups	NOS, nmol/min/g protein	Nitrotirizin, nmol/g protein	eNOS, U/g protein	Regenerative thiols, mmol/g protein
Normotensive rats (group №1)	14,5 ± 1,1	5,8 ± 0,40	21,2 ± 1,0	475,2 ± 33,1
SHR (control) (group №2)	6,1 ± 0,43	2,1 ± 0,15	7,1 ± 0,21	227,7 ± 15,4
SHR + Hypertril 5.0 mg/kg (group №3)	8,1 ± 0,55 [a,b]	3,6 ± 0,21 [a,b]	10,2 ± 1,0 [a,b]	325,5 ± 21,1 [a,b]
SHR + Hypertril 7.5 mg/kg (group №4)	8,1 ± 0,55 [a,b]	4,8 ± 0,31 [a,b]	17,8 ± 1,5 [a,b]	387,5 ± 28,5 [a,b]
SHR + Hypertril 15.0 mg/kg (group №5)	12,3 ± 0,87 [a,b]	5,8 ± 0,45 [a,b]	19,2 ± 1,2 [a,b]	415,6 ± 37,3 [a,b]
SHR + Hypertril 20.0 mg/kg (group №6)	15,1 ± 1,1[a,b]	2,2 ± 0,17	6,7 ± 0,72	238,3 ± 12,4
SHR + Metoprolol 20 mg/kg (group №7)	6,4 ± 0,41	5,8 ± 0,40	21,2 ± 1,0	475,2 ± 33,1

Data are expressed as mean ± standard error.
[a] $P < 0.05$ vs group №2.
[b] $P < 0.05$ vs group №7.

not allow it to influence on the spontaneous opening of the mitochondrial pores, which has a reactive oxygen species-dependent nature. Metoprolol does not have the activities like scavenger of the reactive oxygen species and does not affect the expression of antioxidant enzymes (Lemmer et al., 2004; Hüsamettin et al., 2009).

5. Conclusion

Thus, Hypertril has a reliable dose-dependent antihypertensive effect in the dose range 5–20 mg/kg after 30-day administration. This antihypertensive effect of Hypetril competes or significantly exceeds Metoprolol (20 mg/kg).

Hypertril reduces the manifestations of secondary mitochondrial dysfunction due to arterial hypertension. Our studies obtained evidence of a dose-dependent improvement of the myocardial energy metabolism in the dose range 7.5–20 mg/kg. Hypertril can prevent one of the causes of mitochondrial dysfunction – oxidative modification of the protein, which is activated by oxidative stress reactions; also Hypertril reduces the insufficiency of mitochondrial pores. As a result, Hypertril increases the content of ATP in the myocardium of SHR, normalizes the activity of mitochondrial enzymes, decreases lactate production and increases pyruvate.

Hypertril enhances the cardioprotective effects of NO and increases the resistance of the cardiomyocytes to ischemia. Hypertril exhibits NO-mimetic properties by restoring the NO level in the cardiomyocytes and increasing the expression of endothelial NOS in both the cytosol and mitochondria. Hypertril reduces the degradation of NO and reduces its conversion into peroxynitrite as well.

The use of Hypertril in doses 7.5–20 mg/kg leads to a dose-dependent increase of the density of cardiomyocyte nuclei, significant increase RNA content in nuclei and the cytoplasm of cardiomyocytes, and an increase of the nuclear-cytoplasmic index. These changes indicate a decrease of myocardial hypertrophy.

Metoprolol shows some cardioprotective effects, but Hypertril exceeds significantly these effects of metoprolol.

Conflicts of interest

Authors have declared that no competing interests exist.

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