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Original Research

Study of antioxidant properties of 7-benzyl-8-benzylidene hydrazine xanthine derivatives *in vitro* and *in vivo*

Ludmila Yevseyeva[1], Mykolay Romanenko[2], Katherine Aleksandrova[2], Dmytro Ivanchenko[2], Sergii V. Levich[2], Sergii Pavlov[1]

[1]Department of Laboratory Diagnostics, Zaporozhye State Medical University, Zaporozhye, Ukraine, [2]Department of Biological Chemistry, Zaporozhye State Medical University, Zaporozhye, Ukraine

Address for correspondence:
Sergii V. Levich, Department of Biological Chemistry, Zaporozhye State Medical University, Mayakovsky Avenue, 26, 69035, Zaporozhye, Ukraine.
E-mail: rshlevas@gmail.com

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ABSTRACT

Objective: Acute myocardial infarction (AMI) plays a leading role in the structure of morbidity and mortality worldwide. One of the main parts of it pathogenesis is production of reactive oxygen species (ROS). The accumulation of ROS in cardiomyocytes results in lipid peroxidation, protein oxidation, DNA damage, and finally cell death. Thereby the search of novel drugs that have antioxidant activity and can be used to complex treatment of myocardial infarction is reasonable. It is known that xanthine derivatives exhibit a broad spectrum of biological activity including antioxidant so that the aim of this research was to study *in vitro* and *in vivo* antioxidant action of derivatives of 7-benzyl-8-benzylidene hydrazine xanthines. **Methods:** Investigation of antioxidant properties *in vitro* of xanthine derivatives was carried out by non-enzymatic initiation of free radical lipid peroxidation. For *in vivo* tests, we used a model of coronarogenic-metabolic AMI that was reproduced using of pituitrin and isoprenaline. This experimental part was done on 40 Wistar rats of both sexes. The test compound was injected once a day during the whole experiment at a dose of 30 mg/kg intragastrically in 30 min after pituitrin injection. For assessment of antioxidant properties *in vivo* of the most active compound superoxide dismutase (SOD) activity and markers of oxidative protein modification in the heart homogenate and lactate dehydrogenase activity in the blood were studied. **Results:** The *in vitro* study showed that almost all xanthine derivatives exhibit antioxidant properties and the most active was 3-methyl-7-p-chlorobenzyl-8-p-chlorobenzylidene hydrazinoxanthine. Obtained results also help us to establish some patterns of structure-activity relationship. Thus, combination of halogen atom (except chlor) with other types of electron donating groups (methyl and methoxy) in the structure of xanthine molecule had a positive effect on antioxidant properties. Study of antioxidant activity *in vivo* of the most active compound showed its positive influence on the activity of SOD and on oxidative modification of protein in the heart tissues. **Conclusions:** Strong antioxidant properties of 8-benzylidene hydrazine xanthine derivative were confirmed in the *in vivo* experiment. Obtained results provide experimental substantiation of search for antioxidants among xanthine derivatives.

KEY WORDS: Acute myocardial infarction, lactate dehydrogenase, protein oxidation, superoxide dismutase, xanthine derivatives

INTRODUCTION

Acute myocardial infarction (AMI) plays a leading role in the structure of morbidity and mortality worldwide. AMI occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis. Ischemia at this critical threshold level for an extended period results in irreversible myocardial cell damage or death [1]. Without probable treatment, AMI could cause necrosis of approximately one billion cardiomyocytes [2].

Oxidative stress, defined as the accumulation of reactive oxygen species (ROS), plays a pivotal role in cardiac degeneration associated with ischemia [3,4]. The major sources of ROS in the ischemic-reperfused myocardium are mitochondria, xanthine oxidase, and phagocyte nicotinamide adenine dinucleotide phosphate oxidase [5-8]. The accumulation of ROS in cardiomyocytes results in lipid peroxidation, protein oxidation, DNA damage, and finally cell death. Detrimental effects of ROS are clearly demonstrated by the findings that in the transgenic mice, in which an antioxidant protein, superoxide dismutase (SOD) is overexpressed, infarct size is markedly reduced [9,10].

Nowadays an active search for new cardioprotectors is being carried out among compounds with high antioxidant properties [11]. Substituted xanthine derivatives are an important class of pharmacologically active compounds that are

known to exhibit various pharmacological properties including antioxidant action [12-14].

In previous works, we described antioxidant and neuroprotective effects of 3-methyl- and 3-benzylxanthine derivatives [14,15]. In continuation of our research, the aim of the present work is to investigate the antioxidant properties of 8-benzylidene hydrazyna xanthines *in vitro* and in-depth studying the antioxidant action of the most active compound *in vivo*.

MATERIALS AND METHODS

Xanthine Derivatives [Figure 1]

As objects of our research we used seven xanthine derivatives:
Compound 1, 3-methyl-7-p-fluorobenzyl-8-p-methylbenzylidene hydrazynoxanthine;
Compound 2, 3-methyl-7-p-fluorobenzyl-8-p-chlorobenzylidene hydrazynoxanthine;
Compound 3, 3-methyl-7-p-chlorobenzyl-8-p-methylbenzylidene hydrazynoxanthine;
Compound 4, 3-methyl-7-p-chlorobenzyl-8-p-chlorobenzylidene hydrazynoxanthine;
Compound 5, 1,3-dimethyl-7-p-chlorobenzyl-8-p-chlorobenzylidene hydrazynoxanthine;
Compound 6, 3-methyl-7-p-chlorobenzyl-8-p-bromobenzylidene hydrazynoxanthine;
Compound 7, 3-methyl-7-p-chlorobenzyl-8-p-methoxybenzylidene hydrazynoxanthine.

Estimation of Antioxidant Activity

Suspension of egg lipoproteins was used as substrate [16]. It was prepared by homogenization of egg yolk on phosphate buffer (pH 7.4). Then, test compounds (in concentrations: 10^{-3}, 10^{-5} or 10^{-7} mol/l) were added to the suspension. Reaction of free radical oxidation was initiated by addition of 0.025 M solution of $FeSO_4 \cdot 7H_2O$ with the next incubation of obtained mixture at 37°C for 60 min. The reaction was stopped by addition of 50% solution of trichloroacetic acid with Trilon B. After centrifugation (30 min, 3000 rpm) supernatant was added to the solution of thiobarbituric acid (TBA). Then, the mixture was heated on water bath for 60 min. The colored complex of malondialdehyde (MDA) with TBA was extracted by addition of butan-1-ol. The concentration of MDA, which showed the intensity of free radical oxidation processes, was estimated by the spectroscopic measurement of the absorbance of the sample at 532 nm. Antioxidant activity was calculated by following formula:

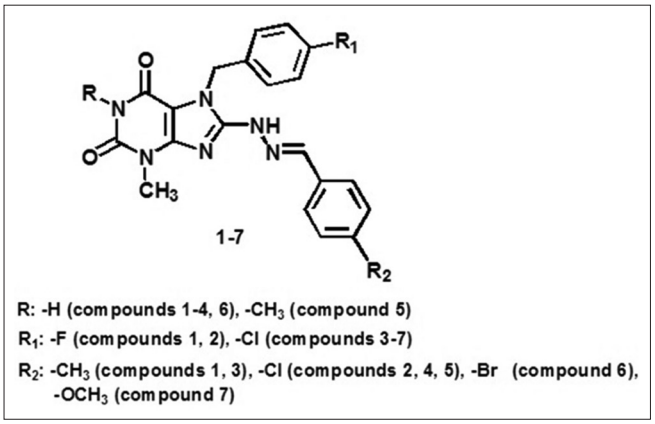

Figure 1: The structure of test compounds 1-7

Antioxidant activity (%) $= (E_c - E_t)/E_c \times 100$

E_t: Optical density of test sample
E_c: Optical density of control sample.

As reference drugs ascorbic acid (vitamin C), thiotriazoline and dibunol were used [17].

Animals

Experimental part was done on 40 albino Wistar rats of both sexes weighing 220-260 g. All animals were on standard food ration of vivarium, with natural alteration of day and night. Rats were received from nursery of Institute of Pharmacology and Toxicology of Ukraine. All experimental procedures and operative interventions were done in accordance with the World Medical Association Statement on animal use in biomedical research.

Infarction Model

For in-depth assessment of antioxidant properties of the compounds, we used a model of coronarogenic-metabolic AMI that is the most adequate in terms of clinical implications of myocardial infarction [18]. This model was reproduced by the use of pituitrin and isoprenaline [19]. They were injected according to the following scheme: Pituitrin intraperitoneally at a dose of 0.5 U/kg, 20 min later isoprenaline subcutaneously at a dose of 100 mg/kg; 6-h later isoprenaline injection was repeated and 24-h later both agents were injected at the same doses. This method caused development of small centered AMI.

The test compound was administered intragastrically with the help of a metal catheter in 30 min after pituitrin injection (once a day during the whole experiment at a dose of 30 mg/kg). Reference drug (inosine) was injected according to the same schedule at a dose of 100 mg/kg intragastrically.

Animal Grouping

All animals were divided into four equal groups (n = 10 in each group):
- Intact group that was represented by healthy animals;
- Control group that was represented by untreated animals with AMI;
- Test I group that was represented by animals with AMI and treated by xanthine derivative;
- Test II group that was represented by animals with AMI and treated by inosine.

Biochemical Analysis

For assessment of severity of ischemic injury of cardiac tissues and pharmacological correction efficiency biochemical arterial blood analysis was performed. For investigation of long-term results of pharmacological correction, the heart tissues were taken from experimental animals on the 4th day after finishing of the procedure; for biochemical investigation cardiac tissues were homogenized in cold isotonic salt solution (0.15 M KCl) at the temperature of +4°C using a glass homogenizer (tissue: salt solution ratio = 1:10). After that, the cytosolic fraction was separated by means of differential centrifugation (15000 rpm). Extract, deprived of proteins, was obtained with precise weight of cardiac tissue homogenate in 0.6 M perchloric acid ($HClO_4$) solution and further neutralized with 5 M potassium carbonate (K_2CO_3) solution [20].

The state of the antioxidant system was assessed by SOD activity. Determination of SOD activity was carried out by the method specified by Chevari *et al.* [21]. SOD activity was stated as U/mg protein/min. The index of oxidative protein modification in cardiac tissues was determined with the help of the method of Halliwell and Gutteridge [22]. For aldehyde phenylhydrazones (APH) the spectrum of absorption was detected at wavelength of 274 nm, and for carboxyl phenylhydrazones (CPH) the wavelength was 363 nm.

Activity of the cardiac specific isoenzyme of lactate dehydrogenase (LDH_1) was detected according to a method of Hohorst [23]; in brief, the creation of renovated form of NAD^+ is equal to the quantity of oxidized malate, the growth of which is indicated at 340 nm.

Statistical Analysis

The statistical data analysis was performed with the help of the software Statistica® for Windows (version 6.0) [24]. The data are presented as the sample mean ± the standard error of the mean. The fidelity of differences between experimental groups was estimated with the help of Student's *t*-test and Fisher's exact test.

RESULTS

Antioxidant Activity of Xanthine Derivatives

Almost all compounds showed relatively high antioxidant properties, and their values exceed the reference drugs in some cases [Table 1]. Their antioxidant activity was found to be within 15.79-84% (10^{-3} mol/l). 3-methyl-7-p-chlorobenzyl-8-p-chlorobenzylidene hydrazynoxanthine (compound 4) presented the best values. Activity of this compound exceeded the result of ascorbic acid, thiotriazoline and dibunol.

Compounds 2 and 3 showed medium antioxidant properties; their activity was lower than vitamin C but higher than thiotriazoline and dibunol. Compound 6 showed a low antioxidant activity and even exerted a pro-oxidant effect at concentrations of 10^{-5} and 10^{-7} mol/l.

Xanthine Derivative Activity in Conditions of AMI

For in-depth study, we chose compound 4 that showed the most pronounced antioxidant activity *in vitro*. During the simulation of AMI, we discovered increase of APH and CPH in cardiac tissues of rats [Table 2]. Injection of inosine led to decrease of toxic products of oxidative protein modification (APH level decrease on 6.97% and CPH on 33.23%). At the same time, xanthine derivative 4 showed more pronounced antioxidant effect. It decreased the level of APH for 29.7% and CPH for 47% in comparison with control group.

In the heart tissue of animals with AMI, almost two times decrease of SOD activity was defined [Table 3]. Usage of xanthine derivative for pharmacological correction led to increase of SOD activity for 26.69% in comparison with control group.

In the blood of animals with AMI five-times increased LDH_1 activity was discovered [Table 3]. Usage of antioxidants led to decrease of this enzyme activity for 38.6% (compound 4) and 5% (inosine).

Table 1: The antioxidant activities of 8-benzylidene hydrazinoxanthines derivatives 1-7

Compound	10^{-3} mol/l		10^{-5} mol/l		10^{-7} mol/l	
	M±m	%	M±m	%	M±m	%
1	0.131±0.016[2]	77.68	0.209±0.021[1]	64.21	0.188±0.022	67.79
2	0.373±0.047[2]	35.56	0.482±0.053[2]	17.78	0.507±0.081[1]	13.33
3	0.372±0.039[2]	36.36	0.533±0.07[2]	9.09	0.579±0.074	1.05
4	0.092±0.007[2]	84.15	0.116±0.013[1]	80.09	0.089±0.005[2]	84.81
5	0.107±0.012[2]	81.63	0.344±0.029[2]	41.22	0.358±0.049	38.78
6	0.493±0.052[2]	15.79	0.617±0.084[2]	−5.26	0.832±0.101	−42.11
7	0.149±0.017[2]	74.42	0.340±0.041[1]	41.86	0.334±0.035	42.89
Control			0.586±0.047			
Ascorbic acid	0.233±0.018[2]	65.18	-	-	-	-
Thiotriazoline	0.443±0.037[2]	33.94	-	-	-	-
Dibunol	0.502±0.041[2]	25.21	-	-	-	-
Control			0.671±0.074			

[1]$P<0.05$ compared to control, [2]$P<0.01$ compared to control

Table 2: Influence of 3-methyl-7-*p*-chlorobenzyl-8-*p*-chlor benzylidene hydrazynoxanthine (compound 4) on oxidative modification of protein in cardiac tissues in conditions of AMI

Animals groups	Oxidative protein modification products (u/g protein)	
	APH (270 nm)	CPH (363 nm)
Intact animals	5.4±0.33	8±0.31
Animals with AMI	17.2±1.78	32.5±2.12
Animals with AMI+compound 4	12.1±1.24*[1]	17.2±1.27*[1]
Animals with AMI+inosine	16±2.4*	21.7±1.75*

*$P<0.05$ compared to control, [1]$P<0.01$ compared to inosine group. AMI: Acute myocardial infarction, APH: Aldehyde phenylhydrazones, CPH: Carboxyl phenylhydrazones

Table 3: Influence of 3-methyl-7-*p*-chlorobenzyl-8-*p*-chlorb enzylidene hydrazynoxanthine (compound 4) on markers of ischemia and myocardial damage in conditions of AMI

Group of animals	LDH_1 (mmol/l/h)	SOD (u/mg protein/min)
Intact animals	0.32±0.02	138.1±9.5
Animals with AMI	1.58±0.17	71.4±5.5
Animals with AMI+compound 4	0.97±0.02*[1]	97.4±7.3*[1]
Animals with AMI+inosine	1.5±0.1*	77.3±3.2*

*$P<0.05$ compared to control, [1]$P<0.01$ compared to inosine group. AMI: Acute myocardial infarction, LDH: Lactate dehydrogenase, SOD: Superoxide dismutase

DISCUSSION

Antioxidant Properties *In Vitro*

In vitro study of derivatives of 8-benzylidene hydrazynoxanthines showed that almost all compounds exhibit antioxidant properties. Obtained results also help us to establish some patterns of structure-activity relationship.

In our previous work, we stated that electron donating group at position 8 of xanthine molecules had a positive effect on antioxidant activity [14]. During this experiment, we found that combinations of different electron donating groups in the xanthine molecule had different effects. Thus, the most pronounced activities were presented by compounds 4 and 5 that have two chlorine atoms in their structures. Combination of chlorine atom with other halogens led to decreasing of antioxidant properties; e.g., 3-methyl-7-*p*-chlorobenzyl-8-*p*-brombenzylidene hydrazynoxanthine (compound 6), showed pro-oxidant properties at concentrations of 10^{-5} and 10^{-7} mol/l.

Combination of halogen atom (except chlorine atom) with other types of electron donating groups (methyl and methoxy) had positive effect, and compounds 1 and 7 showed antioxidant activity higher than comparison standards (at concentration of 10^{-3} mol/l).

Antioxidant Properties *In Vivo*

The performed investigation revealed significant antioxidant features of 3-methyl-7-*p*-chlorobenzyl-8-*p*-chlorbenzylidene hydrazynoxanthine (compound 4) in conditions of experimental AMI.

It is well-known that one of the main parts of AMI pathogenesis is production of ROS by bioenergetic and chemical systems of the heart [5,6]. ROS surplus in conditions of antioxidant lack leads to oxidative modification of lipids, proteins, and nucleic acids. Under the influence of ROS, the activation of redox-sensitive genes expression begins; one of them is necessary for the protection of cells from toxic effects of oxidative stress, and others initiate apoptotic cell death in the case of surplus of ROS [5,6]. Nowadays oxidative stress and its consequence oxidative modification of proteins are one of the most considerable mechanisms of cell destruction.

Injection of compound 4 led to increased activity of the key antioxidant enzyme (SOD), that control main cardiomyocyte antioxidant safety. It should also be noted that as a result of suppressing oxidative protein modification in cardiac tissue, the stabilization of cell membrane also occurred on the background of xanthine derivative usage. The evidence of this is the positive decrease of LDH_1 hyperenzymemia of the animals that were cured by compound 4.

Summarizing the obtained results, it should be noted that the most active compound among the 8-benzylidene hydrazynoxanthines derivatives was 3-methyl-7-*p*-chlorobenzyl-8-*p*-chlorbenzylidene hydrazynoxanthine 4. Strong antioxidant properties of this compound were confirmed in an experiment *in vivo*. Obtained results gave experimental substantiation of search for antioxidants among xanthine derivatives.

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