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The study of mechanisms of anti-inflammatory activity realization of a new malonic acid derivative – maldian

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Abstract

Background: The aim of this work was to study the implementation mechanisms established earlier, the anti-inflammatory activity of a new compound – di-(2,4-dimethyl) malonic acid anilide, code name – "Maldian".

Material and methods: The study was performed on nonlinear white male rats, weighing 220-250 g. The implementation mechanisms of maldian pharmacological action studied in terms of the effect of a new substance on the level of prostaglandin E and the effect on the activity of proteolytic enzymes of the kinin system in the blood of intact animals on the background of carragenine edema. Maldian antioxidant activity was assessed by the impact on the processes of lipid peroxidation in the membranes of liver mitochondria in rats. The role of adrenal system showing maldian anti-inflammatory effect was also studied.

Results: It is found that maldian at a dose of 7.3 mg/kg has a pronounced antioxidant activity reducing free radicals oxidation processes. There is an ability to inhibit kininogens, to inhibit the activity of the leading mediator of inflammation – kallikrein in the mechanism of anti-inflammatory maldian activity. Maldian significantly decreases the level of prostaglandin E on the background of acute inflammatory reaction. Anti-inflammatory maldian activity is implemented without any effects on the adrenal cortex.

Conclusions: Considering the results obtained, maldian – (di-(2,4-dimethyl) anilide malonic acid, is a promising compound for further pharmacological study and introduction into medical practice as a new modern drug with anti-inflammatory action.

Key words: derivatives of malonic acid, anti-inflammatory action mechanisms.

Introduction

Inflammation, as a typical pathological process, is the leading link of the pathogenesis of many diseases of a modern man. An inflammatory reaction is characterized by a symptom complex of functional, structural and metabolic abnormalities at the organ and cellular levels [1]. The main humoral factors of an inflammatory reaction on the stage of secondary alteration are: the activation of free radicals oxidation (FRO) due to the increased release of aggressive oxygen and oxygen-halogen radicals, and a significant decline in the capacity of endogenous antioxidant systems; the amplification by activated macrophages and endothelial cells synthesis of nitric oxide, which has potent non-specific cytotoxic effect; the release into the blood components of the complement system, in particular the membrano-atacking complex C5-C9, which violates the integrity of the cell membrane; the activation of hydrolytic enzymes of the lysosomes releasing when cells die; the increase of the synthesis and the release by activated macrophages and T-lymphocytes of tumor necrosis factor (TNF), the last can cause apoptosis and cell death, increases the generation of inflammation focus of the nitric oxide and activates the processes of the FRO.

The cellular factors of secondary alteration are neutrophils and macrophages [2]. It should also be noted that the most important mediator element of the inflammatory response are eicosanoids, which are the main producers in the inflammation of the monocytes and macrophages [3].

The predominant eicosanoids in the inflammation focus are considered as prostaglandin – PGE1 and PGE2, leukotriene – LTB4 and 5-hydro-peroxyisobutyrate acid. Thromboxane A2, prostacyclin I2 and leukotrienes LTS4, LTD4, LTE4 are produced in a smaller number [4].

A particularly important role in the development mechanism of inflammatory reactions is about prostaglandins, which, showing a powerful vasodilatation effect, increase hyperemia and exudation. PGE1 and PGE2 increase the sensitivity of afferent receptors to bradikinin and histamine; also they induce temperature increase [5].

Considering the above the activation of the FRO and the synthesis changes and the activity of prostaglandins play the key role in the modulation of the inflammatory response, so there is an urgent search and the development of a new drug with anti-inflammatory action, in implementation of which the leading targets are: the inhibition of FRO processes and the effect on the level of inflammatory mediators – kinins and prostaglandins E [6, 7, 8].

In this aspect a promising remedy can be a new compound di-(2,4-dimethyl) anilide malonic acid, synthesized by the scientists of the National University of Pharmacy (Kharkiv, Ukraune).

The aim of this work was to study the realization mechanisms of previously shown anti-inflammatory effect of di-(2,4-dimethyl) anilide malonic acid, from now on "Maldian" [9].

Material and methods

The study was performed on nonlinear white rats. The animals were kept in standard conditions of a vivarium in accordance with Good Laboratory Practics. The experiments were carried out in order to meet the requirements of European Community Council Directive on the protec-

tion of animals used for experimental and other scientific purposes [10]. The studies were carried out on 128 nonlinear male rats weighing 220-250 g. The animals were divided into groups of 8.

The anti-inflammatory maldian activity was studied on the model of carrageenan foot swelling in rats, which was modeled by subplanetary injection of 0.1 ml of 1% solution carrageenan. In accordance with methodical recommendations on the study of anti-inflammatory activity of new drugs maldian was injected intragastrically 30 min before modeling pathology at a dose of 7.3 mg/kg [11]. The acetylsalicylic acid at a dose of 98 mg/kg and voltaren at a dose of 8 mg/kg were used as reference drugs and were injected in accordance with the scheme which was similar.

The study of mechanisms of maldian anti-inflammatory activity was conducted through 3 stages (tab. 1).

Table 1

The studying stages of realization mechanisms of maldian anti-inflammatory activity

Stages	Studying model / Conditions	The index under study
Stage 1	Acute carrageenan of foot swelling in rats	The content of PGE1 in blood plasma
		Kallikrein and kallikreinogen in serum
Stage 2	Microsomal fraction of homogenate in rats liver	The concentration of malonic dialdehyde
Stage 3	Acute carrageenan of foot swelling in rats with preadrenalectomy	Antiexudative activity index

During the first stage under the conditions of reproduced carrageenan inflammation, 4 hours after carrageenan injection, blood sampling was conducted and examined whether PGE1 is contained as well as the level of kallikrein and kallikreinogen. The rats were decapitated under ether anesthesia to sample blood.

The level of prostaglandin E1 (PGE1) was determined in radioimmunology way using «Clinical Assay» (USA) reagents. The determination of PGE1 content in blood plasma was carried out on the background of the inflammatory response. Blood for the determination of PGE1 was sampled in plastic tubes in accordance with V. D. Pomognemo and co-authors' recommendations [12]. To prevent the processes of biosynthesis and metabolism PGE1, the transition from one series to another, the test tubes while sampling and processing blood were placed in ice. As an anticoagulant ethylenediaminetetraacetate (EDTA) at a concentration of 1 mg/ml of blood was used. To block the cascade of eicosanoids transformations by platelets in the tubes, the inhibitor prostaglandinsynthase –a solution of acetylsalicylic acid (0.01 ml of 0.4% solution of 1 ml of blood) was added to the plasma samples.

The plasma was separated by centrifugation at a temperature of 4 ° C. and 2000 rpm for 30 min. until complete precipitation of platelets and stored at -20 degrees C for 2-3 days. PGE1 extraction from plasma was performed using ethyl acetate and a preliminary removal of plasma neutral lipids using petroleum ether.

Kallikrein and kallikreinogen in the serum were determined spectrophotometrically by the rate of hydrolysis of the ethyl ester of N-benzoyl – L-arginine by the method of and T. S. Paskhina and A. V. Krinskaya [13].

During the second phase of the experiment the presence of antioxidant activity in the mechanism of maldian anti-inflammatory action was being studied.

The impact of a new compound on the intensity of lipid peroxidation (LPO) was studied in vitro using microsomal fraction of homogenate of rat's liver. The intensity of Fe2+ – ascorbate -induced by LPO of microsome membranes was determined according to the standard procedures. The intensity of NADPH-dependent of LPO of microsome membranes was determined in the medium of the following composition: 100 mmole of Tris-HCL buffer (pH 7.4), 1 mmole of NADPH, 4 mmole of ADP, 14 μmol of Mohr's salt. In ascorbinsaeure LPO the medium contained 100 mmole of Tris-HCL buffer (pH 7.4), 0.5 mmol of ascorbate, 12 μmol Mohr's salt. During some experiments, 4 mmole ADP (Sigma, USA) was additionally injected into the incubating medium. The concentration of microsome protein in the incubating mixture was 0.5-0.6 mg in 1 ml. Warmed up to 37 degrees air was constantly purged through the incubating mixture, providing the saturation of the medium with oxygen and mixing. The concentration of malonic dialdehyde (MDA) was determined spectrophotometrically by the reaction of thiobarbituric acid, using SF-16, after protein precipitation by trichloroacetic acid. Maldian was injected into the incubating medium at doses of 7.3 μm and 14.6 μm. As a reference drug voltaren at concentrations of 80 μm, 500 μm and 800 μm was selected [11].

The third phase of the research was devoted to the study of a possible involvement of the adrenal system in the mechanism of realization of maldian anti-inflammatory effect.

To ascertain the mechanism of maldian anti-inflammatory action of the adrenal system, the series of experiments with carrageenan swelling in rats with previously cut out adrenal glands were carried out. The adrenalectomy was performed bilaterally on the back under ethaminal-sodium anesthesia. 5 days after the surgery, the rats were simulated carrageenan swelling of the foot in accordance with the standard method described in methodical recommendations on the study of anti-inflammatory activity of new drugs [11].

The statistical processing of experiment results was done by common pharmacology methods determining the average arithmetic meanings (X) and a standard error (SE), the reliability of differences was determined using t-Student test at the significance level of 95% (p £ of 0.05) [14].

Results and discussion

Acute carrageenan swelling is characterized by a significant inflammatory reaction (table 2). It is found out that a new compound anti-inflammatory activity is at the level of a known drug with anti-inflammatory effect of voltaren. It gives us the grounds to study the implementation mechanisms of maldian anti-inflammatory activity in details.

Table 2

Antiexudative activity of maldian and voltaren under the condition of acute carrageenan swelling (n=8)

The conditions of the experiment	Dose mg kg	Antiexudative activity, % Time of inflammation			
		1 hour	2 hours	3 hours	4 hours
Maldian	7,3	26,3±0,84	35,7±1,72	48,8±2,12	56,9±0,97
Voltaren	8,0	24,0±2,10	33,0±2,70	46,2±2,20	55,0±2,30

Today, the leading role of prostaglandins E – lipid mediators of inflammation which has potent phlogogenic and pyrogenic effect of PGE1 is a scientific fact. [15]. The first phase of the experiment was devoted to the study of maldian influence on PGE1 content in the blood plasma in the acute inflammatory response (within 4 hours after Karenin injection). The results of the experiment are presented in table 3.

Table 3

The influence of maldian and reference drugs on the content PGE1 in the blood plasma of rats with carrageenan swelling (4 hours after carragenan injection) (n=8)

The conditions of the experiment	The content PGE1 in plasma	
	Nmol/l	% to intact control
Intact control	8,0±1,58	100
Control pathology	13,8±1,18 *	172
Maldian, of 7.3 mg/kg	0,68±0,39 * #	8,5
Acetylsalicylic acid, 98 mg/kg	0,24±0,08 * #	3,0
Voltaren, 8 mg/kg	1,17±0,38 * #	15

Notes: * – statistically significant differences relatively to the intact control group – p<0.05; # – statistically significant differences relatively to the control pathology group – p<0.001.

It is found out that rats of the control pathology group have acute inflammatory reaction (4 hours after Karenin injection) which is accompanied by a significant increase up to 72% level of PGE1 in plasma, relatively of intact animals.

Maldian injection is characterized by a pronounced anti-inflammatory effect, verified by a large-scale reduction in prostaglandin fractions on the background of acute inflammatory reaction. Maldian reduces the amount of studied prostaglandin by 20 times (p<0.001) relatively to the control group pathology.

It should be noted that the effect of a new compound on the level of prostaglandin E in terms of the inflammatory response, is at the level of reference drugs – acetylsalicylic acid and voltaren and slightly higher than the activity of the latter.

Taking into account the findings of previous studies [9] it is possible to note a high degree of the correlation between the anti-inflammatory effect of a new compound and its effect on the level of PGE1 in plasma.

Kinins are important modulators of the inflammation. Regardly to that, maldian effects on the activity of proteolytic enzymes of the kinin system in terms of carrageenan inflammation were studied. The results are shown in table 4.

Table 4

Maldian and acetylsalicylic acid influence on the content of kallikrein and kallikreinogen in the blood plasma of rats in the norm and on the background of carrageenan swelling (4 hours after Karenin injection) (n=8)

The conditions of the experiment	Kallikrein MED/ml	Kallikreinogen MED/ml
Intact control	84,6±2,2	240,5±5,5
Control pathology (carrageenan swelling)	128,4±3,8 *	327,6±14,3 *
Maldian, of 7.3 mg/kg	39,5±5,1 * # $	248,8±12,4 * # $
Carrageenan swelling + Maldian, of 7.3 mg/kg	44,5±3,9 * # $	336,6±15,4 * $
Acetylsalicylic acid, 98 mg/kg	66,3±4,3 * #	421,4±13,1 * #
Carrageenan swelling + Acetylsalicylic acid, 98 mg/kg	70,3±4,8 * #	422,3±11,4 * #

Notes: * – statistically significant differences relatively to the intact control group – p<0.01; # – statistically significant differences relatively to the control pathology group, p<0.01; $ – statistically significant differences relatively to the group of acetylsalicylic acid – p<0.01.

It was found out that in animals under the conditions of acute inflammatory response, induced by carrageenan, there has been a significant increase in the level of kallikrein up to 52% and kallikreinogen by 36%, relatively to the group of intact control.

While injecting maldian it was found out a significant reduction of kallikrein up to 47% relatively to the control pathology group (table 4), meanwhile maldian has no statistically significant effect on plasma levels of kallikreinogen.

Acetylsalicylic acid contributed to the increase of kallikreinogen content in the blood plasma of healthy animals up to 75% relatively to the intact control group; meanwhile

it significantly less pronouncedly reduced the concentrations of kallikrein relatively to maldian.

The results obtained show that the studied compound inhibits the release of one of the leading mediators of kallikrein inflammation.

Due to the fact that the inflammatory reaction triggers the cascade of FRO, the next step was the studying of the potential antioxidant maldian action on the model of induced LPO in the membranes of mitochondria of rat liver. Divalent iron ions and ascorbate were used as inductors (prooxidant). The results of the experiment are shown in table 5.

Table 5

The dynamics of changes in the concentration of malondialdehyde (MDA) in liver cells of rats under the conditions of Fe2+ – ascorbate dependent LPO and when added maldian and voltaren to the incubation medium

The conditions of the experiment/ the concentration of the introduced substances, μm	The accumulation of MDA whithin 5 min of incubation	
	nmol/l	% control
The study of the maldian effect		
Control (induction LPO)	24,4±0,40	100
Maldian, 3.6 μm	26,4±0,60	108
Maldian, of 7.3 μm	15,2±0,70 * #	62
Maldian, of 16.6 μm	11,5±0,73 * #	47
The study of voltaren effects		
Control (induction LPO)	27,5±0,40	100
Voltaren, 80 μm	33,0±0,68	120
Voltaren, 500 μm	16,5±0,50 * $	60
Voltaren, 800 μm	13,6±0,32 * $	9

Notes: * – statistically significant differences relatively to the control group (induction of LPO) – p<0,001; # – statistically significant differences relatively to maldian group at a dose of 3.6 μm – p<0.001; $ – statistically significant differences relatively to voltaren group at a dose of 80 μm – p<0.001.

In the groups, which caused the intensification of LPO processes due to the injection of prooxidant, the concentration of MDA (as a marker of LPO processes) was taken as 100 %.

Dose-dependent antioxidant maldian effect was found out. So, if you increase the dose of maldian from 3.6 μm to 7.3 μm, its ability to inhibit Fe2+ – ascorbinsaeure LPO in the mitochondria of rat liver is increased by 1.7 times and the subsequent doubling of the dose reduces pathological oxidation processes by 2,3 times (table 5).

Inhibitory effect of voltaren on the oxidation of membrane lipids of mitochondria was observed at concentrations of 500 μm and 800 μm, which accounted for 60% and 49%, respectively.

Analyzing the obtained results, it should be noted that voltaren has an inhibitory influence in experimental oxidative stress only at concentrations range of toxic doses, whereas the antioxidant maldian effect is manifested in low doses which effectiveness is of middle rate and ten times lower than those of voltaren.

Thus, the ability of maldian to reduce the level of the LPO in terms of inflammation is essential in the implementation of the anti-inflammatory action and its ability to normalize the destructive processes caused by the activation of the FRO.

In our study the mechanism of maldian anti-inflammatory action of adrenal system, its antiexudative activity on the model of carrageenan swelling in adrenalectomised animals has been examined. The results of the experiment are shown in table 6.

Table 6

Maldian antiexudative activity in the experiment on adrenalectomized and non-operated rats on the models of acute carrageenan swelling (n=8)

Antiexudative activity, %	Maldian at a dose of 7,3 mg/ kg	
	Adrenalectomized rats	Non-operated rats
1 hour of the experiment	24,3 ±2,50	26,6 ±2,70
2 hours of the experiment	33,8 ±3,10	36,0 ±3,09
3 hours of the experiment	46,3 ±2,70	49,1 ±2,65
4 hours of the experiment	54,9 ±3,01	57,3 ±3,49

The results obtained confirmed previously expressed antiexudative maldian activity [9], which peaks in 3-4 hours of the inflammatory response and indicates anticyclogenesis mechanism of the anti-inflammatory action of the new compound.

Analyzing the obtained data we can conclude that the dynamics of antiexudative maldian action of adrenalectomised animals does not differ from that which takes place while injecting the substances to non-operated rats (table 6.) Statistically significant differences between these groups were not established.

Thus, in the implementation of the maldian anti-inflammatory effect it's possible to exclude its influence on the adrenal cortex and the activation of the adrenal system.

Conclusions

The mechanisms of realization of the new compound – di-(2,4-dimethyl) malonic acid anilide and anti-inflammatory activity of the new compound, under the code name – "Maldian" were studied.

Anticyclogenesis mechanism of maldian antiexudative action at a dose of 7.3 mg/kg was verified by a significant decrease of prostaglandin E1 in the blood plasma of animals on the background of carrageenan swelling.

The effectiveness of the new compound exceeds the anti-inflammatory effect of voltaren at a dose of 8 mg/kg and acetylsalicylic acid at a dose of 98 mg/kg.

Maldian has a reliable inhibitory effect on kallikrein, reducing its content in the blood plasma up to 47% in terms of carrageenan swelling, meanwhile significantly exceeds the effect of acetylsalicylic acid at a dose of 98 mg/kg.

The antioxidant maldian activity as for the effect on lipid peroxidation at the level of voltaren at a dose of 8 mg/kg was found out.

Maldian has no inducing effect on the adrenal cortex, thus eliminating the activation of the adrenal system.

Maldian is a promising compound for further study and the introduction into medical practice as an effective new anti-inflammatory drug.

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