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(54) Title: PHARMACEUTICAL COMPOSITION OF GAMMA-BUTYROBETAINE OR A PHARMACEUTICALLY ACCEPTABLE SALT AND MELDONIUM OR A PHARMACEUTICALLY ACCEPTABLE SALT

(57) Abstract: Gamma-butyrobetaine succinate and pharmaceutical composition thereof. New pharmaceutical composition relates to prevention and/or treatment of a disorder selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, chronic heart failure, transient and permanent ischemic attack including cerebrovascular accident and stroke and peripheral arterial occlusive disease.



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Description

Pharmaceutical composition of gamma-butyrobetaine or a pharmaceutically acceptable salt and Meldonium or a pharmaceutically acceptable salt

Technical Field

- 5 The present invention relates to succinate salt of gamma-butyrobetaine, method of its preparation and new pharmaceutical composition containing it. New pharmaceutical composition relates to prevention and/or treatment of a disorder selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, chronic heart failure, transient and permanent ischemic attack including
- 10 cerebrovascular accident and stroke and peripheral arterial occlusive disease.

Background Art

Gamma-butyrobetaine (GBB) is known mostly as a bio-precursor of carnitine, a key molecule in the regulation of myocardial energy metabolism.

- As it is known gamma-butyrobetaine can form salts with strong acids, such as
- 15 hydrochloric acid and sulphuric acid, but formation of salts with carbonic acids is quite complicated, while can't obtain constant molar ration between GBB and carbonic acid.

In patent EP 1103259 A (BASF AG) 30.05.2001 is disclosed use of gamma - butyrobetain salts to the preparation of preparing for the human and animal diet.

- 20 Succinate is mentioned as one of option as salt of gamma-butyrobetaine, but no particular data is occurring.

Presently atherosclerosis and stroke are among the leading causes of mortality.

Elevated levels of low density lipoprotein (LDL) cholesterol are generally regarded as high risk factors for atherosclerosis and associated cardiovascular pathologies.

- 25 Since low HDL-C is a risk factor in stroke SACCO, R., et al. High-Density Lipoprotein Cholesterol and Ischemic Stroke in the Elderly. JAMA. 2001, vol.285, p.2729-2735. WANNAMETHEE, SG, et al. HDL-Cholesterol, Total Cholesterol, and the Risk of Stroke. Stroke. 2000, vol.31, p.1882.

The use of pharmaceutical composition of Meldonium or 3-(2,2,2-

- 30 trimethylhydrazinium)-propionate (also known as Mildronate®) and gamma-butyrobetaine (GBB) for treating of cardiovascular conditions WO 97/00679 A (KALVINSH IVARS; VEVERIS MARIS) 27.02.1997 .

Meldonium is described as clinically useful anti-ischemic and stress-protective agent for treating some diseases of heart vessels and other pathologies caused by

tissue ischemization KARPOV, R., et al. Clinical Efficacy and Safety of Mildronate in Patients With Ischemic Heart Disease and Chronic Heart Failure. Kardiologiya. 2000, vol.6, p.69-74. Meldonium was shown to reduce myocardial infarct size in an experimental model of acute myocardial ischemia, without any effect on

- 5 hemodynamics SESTI, et al. Mildronate, a Novel Fatty Acid Oxidation Inhibitor and Antianginal Agent, Reduces Myocardial Infarct Size Without Affecting Hemodynamics. Journal of Cardiovascular Pharmacology. 2006, vol.47, no.3, p.493-499. Meldonium is also indicated for treating chronic cerebrovascular insufficiency DZIAK, LA, et al. Use of mildronate for the treatment of patients with
- 10 circulatory encephalopathy against a background of stenosis of major arteries of the head. Lik Sprava. 2003, vol.5-6, p.98-101. Meldonium was shown to possess antioxidant activity and offer protection against lipid peroxidation in clinic [Suslina ZA et al, Eksp Klin Farmakol (Rus) 2003;66(3);32-35]. A certain useful activity of meldonium was discovered in animal models of atherosclerosis OKUNEVICH, I,
- 15 et al. Anti-atherosclerotic action of mildronate in experiment. Patol Fiziol Eksp Ter. 2002, vol.2, p.24-27. and observed in clinics KARPOV, RS. The clinical instrumental evaluation of treatment efficacy in patients with concomitant atherosclerosis of the coronary, cerebral and peripheral arteries. Ter Arkh. . 1991, vol.63, no.4, p.90-93.

- 20 No antiatherosclerotic activity for GBB had been reported.

Disclosure of Invention

It is an object of the present invention provide, process for preparing gamma-butyrobetaine succinate and new medical use of a pharmaceutical composition, comprising Meldonium or pharmaceutically acceptable salt thereof and gamma-

25 butyrobetaine or pharmaceutically acceptable salt thereof for the prevention and/or treating of disorder selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, transient and permanent ischemic attack including cerebrovascular accident and stroke and peripheral arterial occlusive disease in a subject in need of the same.

- 30 Another object of the invention is to provide a new combination medicinal product, comprising meldonium succinate and gamma-butyrobetaine succinate.

The invention also relates to a pharmaceutical composition comprising such combination medicinal product and to the use thereof in the manufacture of a medicament.

The combination medicinal product of the present invention is anticipated to be useful in a method for treatment and/or prevention disorders, selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, coronary heart disease as chosen from the group of angina pectoris and myocardial infarction, chronic heart failure, transient and permanent ischemic attack including

cerebrovascular accident and stroke and peripheral arterial occlusive disease. It is understood that a combination pharmaceutical product as used herein means simultaneous, sequential or separate administration of the agents of the combination. An additional object of the invention is the provision of a pharmaceutical composition comprising both meldonium succinate and gamma-butyrobetaine succinate for the aforesaid purpose. Further objects of the invention will become apparent hereinafter, and still others will be obvious to one skilled in the art.

The invention comprises a medicinal product, preferably in a single-dosage unit form. The exact form in which the active ingredient is administered is not important, so long as the desired effect of the invention is obtained. The active ingredient may take the form of capsules, suspensions, dispersions, elixirs, syrups, or the like.

We have obtained gamma-butyrobetaine succinate (1:1) in high yields and as stable and non-hygroscopic at ambient conditions..

Process for preparation gamma-butyrobetaine succinate compromises: reaction of gamma-butorobetaine dihydrate with succinic acid in dry 2-propanol at elevated temperature.

We surprisingly found that the combination of meldonium or pharmaceutically acceptable salts thereof with gamma-butyrobetaine or pharmaceutically acceptable salts thereof have certain beneficial effects that are significantly superior to those of meldonium itself. It was unexpectedly discovered that meldonium in combination with gamma-butyrobetaine lowers the triglyceride and LDL-C levels and increases the HDL-C levels better than meldonium itself. We have also surprisingly found that the product of present invention ameliorates the aftereffects of experimental cerebral ischemia and stroke. This complex of more pronounced beneficial properties makes the combination of meldonium with gamma-butyrobetaine preferable to meldonium in treatment and/or prevention of disorder, selected from a group, consisting of dyslipidemia, hyperlipidemia,

atherosclerosis, chronic heart failure, transient and permanent ischemic attack including cerebrovascular accident and stroke and peripheral arterial occlusive disease. We have found that these beneficial properties are also inherent in combinations of the active components in salt form, particularly in combination of
5 meldonium succinate with gamma-butyrobetaine succinate. This combination comprises meldonium succinate WO 2005/012233 A (GRINDEKS JSC; KALVINSH IVARS; BIRMANS ANATOLIJS) 10.02.2005 and previously unknown gamma-butyrobetaine succinate, substances that were not known when the pharmacological properties of the combination of meldonium with gamma-
10 butyrobetaine were investigated and disclosed in WO 97/00679 A (KALVINSH IVARS; VEVERIS MARIS) 27.02.1997 . This new medicinal product has also outstanding activity for treating certain cardiovascular diseases, significantly surpassing that of previously disclosed combination of meldonium and gamma-butyrobetaine.

15 The medicinal product of the invention may be in a form suitable for oral use (for example as tablets, capsules, aqueous solutions or dispersible powders or granules), for parenteral administration (for example as a sterile aqueous solution for intravenous, subcutaneous, or intramuscular dosing) or as a suppository for rectal dosing. Preferably the product of the invention is provided in a form suitable
20 for oral use, for example as tablets or capsules.

The pharmaceutical composition of the invention may be obtained by conventional procedures using conventional pharmaceutically acceptable excipients or carriers and technologies.

In further aspects, the present invention provides method for prophylaxis and/or
25 treating of disorder, selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, coronary heart disease as chosen from the group of angina pectoris and myocardial infarction, chronic heart failure, transient and permanent ischemic attack including cerebrovascular accident and stroke and peripheral arterial occlusive disease, comprising the administration to a patient the
30 combination of meldonium succinate with gamma-butyrobetaine succinate.

In further aspects, the present invention provides for the use of the novel combination medicinal product of invention for the production of a medicament for prophylaxis and/or treatment of disorder, selected from a group, consisting of dyslipidemia, hyperlipidemia, atherosclerosis, coronary heart disease as chosen

from the group of angina pectoris and myocardial infarction, chronic heart failure, transient and permanent ischemic attack including cerebrovascular accident and stroke and peripheral arterial occlusive disease.

- 5 The following examples are provided to illustrate but not limit the invention.

Mode(s) for Carrying Out the Invention

Preparation of gamma-butyrobetaine succinate (1:1)

- Gamma-butyrobetaine dihydrate (1 mol) was added to stirred dry 2-propanol (1L). Succinic acid (1 mol) was added to the reaction mixture dropwise at 70°C. The
10 formed suspension was stirred and cooled at ambient temperature. The precipitates were separated by filtration and were washed on filter with 2-propanol (2x200 mL).

- The gamma-butyrobetaine succinate was dried under reduced pressure. The yield was 246.2 g (93.5%) of white crystalline gamma-butyrobetaine succinate having a
15 melting point of 86-88°C.

¹H NMR spectrum (D₂O, TMS): 1.97-2.16 (2H, m, CH₂CH₂CH₂); 2.40 (2H, t, J=7.0 Hz, CH₂CH₂CH₂COO); 2.62 (4H, s, -CH₂CH₂-(succinic acid)); 3.14 (9H, s, Me₃N⁺); 3.29-3.42 (2H, m, +NCH₂CH₂).

Found, %: C 50.18; H 8.04; N 5.32; C₁₁H₂₁NO₆

- 20 Calculated, %: C 50.35; H 8.15; N 5.26.

Crystals of gamma-butyrobetaine succinate are characterize by following powder diffraction maximum at 2 Θ angles (Cu k-alpha radiation): 6.26; 7.80; 10.38; 11.67; 12.13; 14.88; 15.74; 19.31; 20.40; 22.56; 23.29; 25.69; 27.82.

- 25 Pharmacological activity

Examples

- Tests: The pharmacological activity of tested compounds was investigated by standard methods used in the art. Animals were kept in groups of 6 in adequate cages in climatized rooms at 22 $\pm$ 1 °C, relative humidity 60 $\pm$ 5% and 12/12
30 light/darkness cycle with free access to feed and water. All experiments were carried out in accordance with the European Community Council's Directive of 24 November 1986 (86/609/EEC) relative to experimental animal care. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Substances: Cholesterol (Acros Organics), Meldonium (Grindex), animal nutrition (R 70 Lactamin), butter (commercial), Na cholate (Acros Organics). Previously unknown gamma-butyrobetaine succinate (GBBS), Meldonium succinate (MDS)
Influence on lipid levels

5 Example 1. Rat hyperlipidemia model

Method. An express-method for inducing hyperlipidemia, as proposed by Arichi et al [Chem Pharm Bull 1982;30(5):1766-1770] was used. Male Wistar rats weighing 220-250 g were used. Rats received fat-cholesterol mixture (10% cholesterol and 1% cholic acid p.o. in dose 10 ml/kg) for 10 days. Solutions of test substances or
10 water for intact or cholesterol (C) control groups were introduced one hour after the fat mixture. Blood for analysis was obtained on 11th day by cardiac puncture under ether anesthesia.

Biochemistry. Total C, HDL-C, LDL-C and TG were detected by COBAS INTEGRA 400 (Roche). Atherosclerosis index (reflecting coronary atherosclerosis and
15 discriminator for peripheral atherosclerosis) was calculated as follows: Index = LDL-C/HDL-C.

Atherosclerosis coefficient (reflecting coronary atherosclerosis) was calculated as follows: Coefficient = Total C/HDL-C.

Statistics. The data obtained were mathematically processed using Microsoft
20 Excel program and the results were expressed as mean $\pm$ standard error (SEM). Results of different groups were compared using single-factor analysis according to ANOVA and Student's t-test. Difference of the results were considered significant at $P < 0.05$.

Results. In these experiments MD was compared with combination MD+GBB or
25 MDS with GBBS. The results are given in Table 1 below. Rats that received fat mixture developed pronounced hypercholesterolemia and hyperlipidemia. Total C, LDL-C and TG indices for test animals were significantly different from those of intact controls. Combined application of MD and GBB for 10 days better than MD reduced the level of TG and atherosclerosis index in this model ($P < 0.05$ vs C
30 control in case of MD+GBB group to not significant change vs C control in case of MD group). Surprisingly the combined application of MDS+GBBS significantly reduced not only the TG levels and atherosclerotic index as MD+GBB, but also the level of LDL-C and the rate of total C/HDL-C. This fact indicates that combination MD+GBB and MDS+GBBS is useful in prophylaxis and/or treating of

hypercholesterolemia and hyperlipidemia and can find use in prophylaxis and/or treating of atherosclerosis.

Table 1

Effects of MD+GBB and MDS+GBBS on lipid levels in rat hyperlipidemia model

Group	Total C mg/dl	HDL-C mg/dl	LDL-C mg/dl	Index LDL-C/HDL-C	Coefficient Total C/HDL-C	TG mg/dl
Intact control	60±4.7**	45.5±4.5	11.2± 1.8**	0.28±0.06**	1.31±0.08**	57.9±6.5*
C control	165±14.0	50.6±1.8	108.4±12.9	2.15±0.23	3.18±0.18	102.3±11.0
MD	146±12.7	54.3±5.0	88.9±13.5	1.70±0.37	2.73±0.36	78.4±9.8
MD+GBB	131±14.2	51.0±2.6	76.4±11.1	1.47±0.17*	2.54±0.19	65.5±8.7*
MDS+GBBS	133±11.9	56.7±4.4	73.1±8.5*	1.32±0.22*	2.37±0.23*	54.3±4.3*

5 MD = MD 150 mg/kg

MD+GBB = MD 150 mg/kg + GBB 50 mg/kg

MDS+GBBS = MDS 150 mg/kg + GBBS 50 mg/kg

*P<0.05 vs C control

**P<0.005 vs C control

10 When investigating combination of MD and GBB salts it was unexpectedly discovered that succinate salts of MD and GBB have significantly higher activity, especially the combination of MDS with GBBS. Further experiments with combinations, comprising salts of MD and GBB were conducted using mice atherosclerosis model as described below.

15 Example 2. Mice atherosclerosis model

Method. A mice atherosclerosis model proposed in literature was used [Smith JD, Breslow JL, J Intern Med 1997;242: 99-109]. Cholesterol and fats were added to standard nutrition of experimental C57BL/6 female mice with average weight

20.3 g at the start of testing. According to recommendations [Paigen B et al,

20 Atherosclerosis 1987;68:231-240 and Nishina, P., et al., J Lipid Res, 1993; 34: 1413-1422] the standard nutrition was supplemented by cholesterol (1.25%), dairy butter (15%) and sodium cholate (0.5%) (excluding intact control). The test groups are listed in Table 2. After 22 weeks the extent of atherosclerotic damage was evaluated by accepted morphological, biochemical and histological methods and

25 criteria. At the end of the experiment the animals were anaesthetized

(pentobarbital, 60 mg/kg, i.p). After laparotomy the blood was collected from the abdominal vein. The blood sample was centrifuged (2000 x min⁻¹ for 15 min) and serum obtained was stored at -20°C before biochemical tests. Aortic arch,

descending and abdominal aorta were separated and preserved for further investigation in 4% buffered paraformaldehyde solution. The determination of lipids in aorta root was based on the technique developed by Paigen et al. [Paigen B et al, Atherosclerosis 1987;68: 31-240]. Cuts of aorta (10 μm) were fixed, stained with Oil red-O and hematoxylin. Damaged area (average area in μm^2 in cut) was evaluated by light microscope with a step of 80 μm beginning from aorta valve. The total area of atherosclerotic changes in the internal surface of aorta was determined by „en face” technique. The longitudinally opened aorta was stained by Sudan IV or Oil red-O for estimation of lipids. The specimens were photographed and the images analyzed by planimetric application using Image-Pro Plus 4.5.1. The atherosclerotic damage was expressed as percentage of the area of lipid infiltrate from the total area of aorta. Six to eight specimens were analyzed for each group of animals.

Biochemistry and statistics as above.

Results. After 22 weeks all animals had a lipid infiltration in aorta that comprised 5.62% from the total inner surface of aorta for untreated cholesterol controls (Table 2 below). MD treatment noticeably reduced the aorta damage area. MD and GBB combination better than MD reduced the atherosclerotic damage area in aorta root ($P < 0.05$ vs C control in case of MD+GBB group but not significant change vs C control in case of MD group). MDS and GBBS combination produced still further reduction of the aorta root damage zone that was significantly better than MD and highly significant ($P < 0.005$) vs C control.

Table 2

Levels of atherosclerotic damage in aorta root (A) and internal surface of aorta (B)

Group	A Damaged area, mkm^2	B Damaged area, %
Intact control	$36 \pm 15^{***}$	$0.03 \pm 0.02^{**}$
C control	6127 ± 1109	5.62 ± 1.01
MD 150 mg/kg	4294 ± 785	$3.49 \pm 0.42^*$
MD+GBB 150+50 mg/kg	$3474 \pm 224^*$	$3.21 \pm 0.24^*$
MDS+GBBS 150+50 mg/kg	$2868 \pm 247^{**\#}$	$2.70 \pm 0.36^*$

* $P < 0.05$ vs C control

** $P < 0.005$ vs C control

*** $P < 0.0005$ vs Intact control

$P < 0.05$ vs MD

Biochemical analysis demonstrated that combination of MD and GBB contrary to MD significantly reduced the atherosclerotic coefficient (Table 3 below). The combination of MDS and GBBS surprisingly was more efficient in improving the rate of lipid fractions and contrary to MD significantly reduced the levels of TG,

5 LDL-C and atherosclerotic coefficient (Table 3).

Table 3

Influence of MD and MD salts combination with GBB and GBB salts on lipid levels

Group	Total C, mg/dl	HDL-C mg/dl	LDL-C mg/dl	Index, LDL-C/HDL- C	Coefficient Total C/HDL- C	TG mg/dl
Intact control	66.5±3.2***	57±4.3	9.7±1.94***	0.17±0.03***	1.17±0.03***	22.3±1.98***
C control	161±8.2	50±4.3	111±6.9	2.23±0.21	3.22±0.21	48.1±2.2
MD 150 mg/kg/d	154±8.1	55±3.3	95±5.5	1.72±0.10*	2.81± 0.19	43.2± 1.95
MD+GBB 150+50 mg/kg	152±8.6	59±5.1	93±9.5	1.63±0.12*	2.63±0.12*	43.0±2.24
MDS+GBBS 150+50 mg/kg	142±8.2	59±5.0	83±4.5*	1.45±0.11***	2.45±0.11***	38.8±1.68**

*P<0.05 vs C control

**P<0.05 vs C control

10 ***P<0.0005 vs C control

#P<0.05 vs MD

Influence on heart and circulation

Example 3.

15 Rat infarction model

Method. Male Wistar rats with body weight 330 – 360 g were randomized in 3 groups:

1) I/R control (n=16), animals after infarction/reperfusion received 0.9% NaCl solution 2 ml/kg p.o. for 7 days

20 2) MD+GBB group (n=12), animals received each day p.o. 150 mg/kg MD and 50 mg/kg GBB

3) MDS+GBBS (n=12), animals received each day p.o. 150 mg/kg MDS and 50 mg/kg GBBS.

One hour after the treatment animals were narcotized (pentobarbital sodium

25 50 mg/kg i.p.) and under mechanical ventilation prepared for blocking of left coronary Experimental infarction was induced by 45 min. long occlusion of

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coronary artery with following 2 h long reperfusion. After the experiment the ischemic and necrotic area were detected by triphenyltetrazolium-Evans blue perfusion-staining method. Left ventricle was dissected and weighed and the morphological criteria calculated: percentage of the ischemic zone of the left ventricle, percentage of the necrotic zone of the left ventricle and ratio of the necrosis zone to ischemic zone (necrosis index).

Statistics as above.

Results. Miocardial ischemia with the following reperfusion caused serious heart rhythm disturbances in the control group with 6 lethalties. Combination of MD and GBB displayed a tendency to reduce the number of animals with serious heart rhythm disturbances (VT and VF), but only the combination of MDS and GBBS surprisingly significantly reduced lethality (Table 4 below).

Table 4

Effect of test substances on heart rhythm disturbance and lethality during coronary artery occlusion (45 min) and reperfusion (120 min) on rats

Group	N	VT		VF		Lethality	
		N	%	N	%	N	%
I/R control	15	14	93.3	10	67	6	43.8
MD+GBB	12	11	91.7	6	50	3	25
MDS+GBBS	12	10	83	4	40	1*	8.3

*P<0.05 vs I/R control

Morphological analysis of myocardium indicated that combination MD-GBB significantly reduced the rate of necrosis zone to both of left ventricle and ischemia zone in comparison with I/R control (Table 5). MDS+GBBS had a substantially higher influence on necrotic index and ratio of necrotic zone to left ventricle than MD+GBB (P<0.005 vs I/R control (Table 5).

Table 5

Effect of test substances on heart morphology on rats undergoing myocardial ischemia (45 min) and reperfusion (120 min); n= 8-11; Mean±SEM

Group	Left ventricle, mg	Ischemic z./left v., %	Necrotic z./left v., %	Necrotic index, ¹ %
I/R Control	803.1±25,2	48.1±1.73	36.2±1.96	75.4±3,67
MD+GBB	805.2±18.4	48.0±2.10	25.8±2.77*	54.3±5.93*
MDS+GBBS	799.5±20.5	48.0±1.57	24.8±2.23**	51.8.0±4.21**

*P<0.05 vs I/R control

**P<0.005 vs I/R control

¹ Necrotic index = Necrotic zone/Ischemic zone x 100

Thus we have surprisingly found that combination MDS+GBBS had substantially
5 better protection against the development of infarction and lethality
(cardioprotective activity) than combination MD+GBB.

Example 4. Rat chronic heart failure model

Method. Male Wistar rats weighing 300 - 330 g were used. Chronic heart failure
10 was initiated by a known method in which the heart failure developed after the
experimental myocardial infarction (MI) caused by blocking of the left coronary
artery [Sasaki H et al, J Mol Cell Cardiol 2001;33:283-294. Batista M et al, J Appl
Physiol 2007;102:2033-2039]. Animals were anesthetized by sodium pentobarbital
solution (50 mg/kg i.p.). Surgery field was prepared after the inset of anesthesia
15 and switching the animal to mechanical ventilation. Under sterile conditions the
chest and pericardium was opened and the left coronary artery occluded by
filament snare at the level of the left atrial appendage. After 45 min of ischemia,
the snare was released and the heart was allowed to reperfuse. Reperfusion was
readily confirmed by hyperemia over the surface of the previously ischemic-
20 cyanotic segment. The chest wall was closed in layers, s.c. analgesic was injected
and animal was placed on a heating pad while recovering from anesthesia. Control
animals (Sham control) underwent the same manipulations except the occlusion of
the artery. Animals that underwent the infarction were randomized in 4 groups on
the next day:

- 25 1) Infarction group (MI Control, N = 8), water p.o. once a day
 2) Sham control group (N = 8), water p.o. once a day
 3) MD+GBB group (MD+GBB, N = 8), MD 150 mg/kg and GBB 50 mg/kg
 p.o. once a day
 4) MDS+GBBS group (MDS+GBBS; N = 8), MDS 150 mg/kg and GBBS
30 50 mg/kg p.o. once a day.

On the 28th day after the induction of MI animals were repeatedly put under
anesthesia and switched to mechanical ventilation. Catheter was insert into the left
ventricle and left ventricular pressure and contractility parameters (LV dP/dt max
and dP/dt min was obtained by a differentiator EQ-601G) were recorded on

Polygraph system RM 6000 (Nihon Kohden, Japan). After recording of basal contractility parameters (LV dP/dt max and dP/dt min), left ventricular contractile functional reserve was obtained by stress test as previously described [Kaga S et al, J Mol Cell Cardiol 2006;40:138-147]. At the end of experiment, an additional anesthesia was injected and saturated KCl was introduced via the femoral vein to stop the heart in diastole. Heart was isolated, left ventricle weight and maximum (diastolic) volume measured.

Statistics. Results are expressed as mean from 6 to 8 measurements $\pm$ standard error of the mean (SEM). Differences between groups were tested for statistical significance by Student's T-test ($P < 0.05$).

Results. During 28 days after the experimental MI the animals developed heart failure, characterized by decrease in left ventricle systolic pressure (LVSP), left ventricle end diastolic pressure (LVEDP) increase and reduced left ventricle contractility (systolic +dP/dt and diastolic -dP/dt).

MDS+GBBS group displayed better protection against the deterioration of functional indices of heart after infarction than MD+GBB (MI control indices LVSP, +pP/dt and -dP/dt were with higher significance, Table 6 below). The use of MDS+GBBS better than MD+GBB protected the systolic and diastolic function of heart, retaining it at the level of sham group.

Table 6

Influence of test substances on hemodynamic indices and left ventricle contractility (+/-dP/dt); Mean $\pm$ SEM; N=6-8

Experimental group	HR ^a	LVSP ^b mmHg	LVEDP ^c mmHg	+dP/dt ^d	-dP/dt ^e
Sham Control	397 $\pm$ 6.5	148 $\pm$ 5.9*	3.9 $\pm$ 0.68**	5325 $\pm$ 201***	4435 $\pm$ 175**
MI Control	385 $\pm$ 6.2	128 $\pm$ 3.8	12.1 $\pm$ 1.70	3674 $\pm$ 189	3091 $\pm$ 221
MI+(MD+GBB)	391 $\pm$ 3.7	137 $\pm$ 2.1	6.3 $\pm$ 0.84*	4608 $\pm$ 173*	4019 $\pm$ 230*
MI+(MDS+GBBS)	389 $\pm$ 5.4	141 $\pm$ 2.8*	6.1 $\pm$ 0.82*	4705 $\pm$ 146**	4083 $\pm$ 107**

* $P < 0.05$ vs MI control

** $P < 0.005$ vs MI control

*** $P < 0.0005$ vs MI control

^a HR – heart rate, beats per min

^b LVSP – left ventricular systolic pressure, mmHg

^c LVEDP – left ventricular end-diastolic pressure, mmHg

^d + dP/dt – left ventricular contractility, systolic, mmHg/s

^e - dP/dt – left ventricular contractility, diastolic, mmHg/s

The stress test indicated that MI control group animals had substantially lower functional reserve of myocardium contractility compared to Sham control animals (Table 7 below). The average + dP/dt and –dP/dt increase during the test for MI control was about 13%, while in Sham control group it was 21%. The MDS+GBBS group had the best results in stress test that were similar to Sham control (the average increase of + dP/dt and –dP/dt during the test period was 20.9 %, but in sham group 21%); besides the functional reserve of cardiac contractility in the MDS+GBBS group is comparatively better than that of MD+GBB group both in systolic and diastolic phase (resp., $P < 0.005$ vs MI control and $P < 0.05$ vs MI control; Table 7).

Table 7

Influence of MD and MDS on contractility of left ventricle under load-stress

Group	+dP/dt; initial	+dP/dt, test	–dP/dt, initial	–dP/dt, test
Sham control	5325±201	6274±197***	4435±175**	5322±219***
MI control	3674±189	4266±243	3091±221	3386±237
MI+(MD+GBB)	4608±173*	5462±206*	4019±230*	4754±182*
MI+(MDS+GBBS)	4705±146**	5749±154**	4083±107**	4835±120**

* $P < 0.05$ vs MI control

** $P < 0.005$ vs MI control

*** $P < 0.0005$ vs MI control

The analysis of morphometric data showed that MI Control group animals within the 28 day period after the myocardial infarction had developed hypertrophy and dilatation of heart and left ventricle (Table 8).

Table 8

Influence of test substances on heart morphometric indices

Group	BW ^a , g	HW ^b , mg	LV ^c , mg	HW/BW ^d	LV/HW	LVDV ^e , ml
Sham control	467±12	1176±72	790±57	2.52±0.08*	0.68±0.01*	0.587±0.037**
MI control	447±16	1325±96	970±91	2.96±0.15	0.73±0.01	0.813±0.040
MI+(MD+GBB)	463±16	1260±71	855±62	2.71±0.08	0.68±0.01*	0.692±0.017*
MI+(MDS+GBBS)	455±13	1215±54	817±42	2.67±0.08	0.67±0.01*	0.657±0.016**

^aBW – body weight; ^bHW – heart weight

^cLV - weight of left ventricle

^dx1000

^eLVDV – left ventricular cavity volume in diastole, ml

*P<0.05 vs MI control

**P<0.005 vs MI control

Use of MDS+GBBS had more significant protection against the LV dilatation in rat chronic failure model than MD+GBB (resp., P<0.005 in case of MDS+GBBS vs MI control and P<0.05 in case of MD+GBB vs MI control). These results indicate that combination MDS+GBBS has substantial cardioprotective and adaptogenic activity in rat chronic failure model. This is evident from retaining of systolic and diastolic function and substantially higher functional reserve under stress.

These data permit to indicate MDS+GBBS for use in clinic to ameliorate and prevent complications after myocardial infarction, including therapy of heart systolic and diastolic insufficiency [Dickstein K et al, European Heart J 2008;29:2388-2442].

Example 5. Determination of antihypoxic and antiischemic effects on brain

The aim of these tests was to investigate antihypoxic and anti-ischemic effects of MD and GBB and salts thereof alone and in combination in experimental ischemia and infarction models of brain.

Mice cerebral circulatory hypoxia model

Method. ICR male mice weighing 21 - 25 g were randomized in 4 groups. Animals received per os or by intragastric catheter the test substances as aqueous solutions either once or for 7 days. The control group received the same volume of water (10 ml/kg). The last dose was given 1 hour before the experiment. The groups were as follows:

1) MD group received MD 150 mg/kg

2) MD+GBB group received MD 150 mg/kg + GBB 50 mg/kg

3) MDS+GBBS group received MDS 150 mg/kg + GBBS 50 mg/kg

4) Control group received water 10 ml/kg.

Experimental circulatory hypoxia was induced by MgCl₂ [Berga P et al, Arzneimittelforschung 1986;36(9):1314-1320] introduced during 3 s into the tail vein (2% MgCl₂, dose 200 mg/kg). Time till cessation of the last respiratory movements was registered and defined as survival time.

Statistics. Data are presented as means ± SEM of 6 to 10 separate experiments. Differences between each experimental group were compared using one-way

ANOVA with repeated comparisons (Tukey's test). $P < 0.05$ was considered as significant.

Results. MDS+GBBS combination displayed considerable higher antihypoxic activity than MD or MD+GBB as evident from the prolongation of the survival time (resp., $P < 0.005$ in case of MDS+GBBS vs Control and $P < 0.05$ in case of MD or MD+GBB vs Control; Table 9).

Table 9

Influence of test substances on survival time; $M \pm \text{SEM}$, $n=6-8$.

Group	Survival time, s
Intact control	25.3 ± 1.07
MD (150 mg/kg/d) x 7	$28.4 \pm 0.83^*$
MD+GBB (150 mg/kg/d + 50 mg/kg/d) x 7	$30.3 \pm 1.93^*$
MDS+GBBS (150 mg/kg/d + 50 mg/kg/d) x 7	$32.6 \pm 1.21^{**}$

* $P < 0.05$ vs Intact control

** $P < 0.005$ vs Intact control

Mice middle cerebral artery occlusion model

Method. Male ICR mice weighing 21 to 26 g were randomized in 5 groups. The groups were as follows:

- 1) MD group received MD 150 mg/kg
- 2) MD+GBB group received MD 150 mg/kg + GBB 50 mg/kg
- 3) MDS+GBBS group received MDS 150 mg/kg + GBBS 50 mg/kg
- 4) Control group received water 10 ml/kg
- 5) Sham group received water 10 ml/kg.

Preventive (once per day for 7 days) treatment protocol was used.

Middle cerebral artery (MCA) origin was occluded by intraluminal filament technique according to known method [Longa EZ et al, Stroke 1989;20:84-91] adapted for mice by Iwai M. et al. [Circulation 2004;110:843-848]. Animals were anesthetized by chloral hydrate (400 mg/kg i.p.). The body temperature was supported at 37 ± 0.3 °C by electronically controlled heating plate. After a midline neck incision had been made, the right common and external carotid arteries were isolated and temporally ligated. Nylon monofilament suture 6/0 (Ethicon), blunted at tip and coated with silicon resin (Xantopren; Bayer Dental), was inserted into the right internal carotid artery, and advanced approximately 10 mm distal to the

carotid bifurcation to occlude the origin of the middle cerebral artery. Sham group underwent similar surgery without occlusion. Post-operative pain was alleviated by s.c. application of tramadol (20 mg/kg) and infection prevented by ampicillin (100 mg/kg). The neurological deficit scores that reflect overall sensorimotor

5 dysfunction were determined 24 h after MCA occlusion [Wei EQ et al, Acta Physiol Sin 2003;55:742–747]: 0, no deficit; 1, flexion of contralateral forelimb upon lifting of the whole animal by the tail; 2, circling to the contralateral side; 3, falling to contralateral side; 4, no spontaneous motor activity.

After the evaluation of neurological status the animals received an overdose of sodium pentobarbital, brains were isolated and sliced in 6 layers of about 1.5 mm

10 thickness. Slices were stained with 2% triphenyltetrazolium sodium at 37°C for 15 min. and photographed. As the most adequate for calculating the brain ischemic damage the 3rd slice from cranial side on the chiasma opticum level was selected, since it was completely supplied by blood from the middle cerebral

15 artery.

Statistics. Data are presented as means $\pm$ SEM of 6 to 10 separate experiments (animals). Differences between each experimental group were compared using one-way ANOVA with repeated comparisons (Tukey's test). Differences of neurological scores between groups were analyzed by unpaired Student's t-test.

20 $P < 0.05$ was considered as significant.

Results. 24 hours after the MCA occlusion all 8 control group animals had neurological disturbances of various intensity (from 1 to 4 points) – average 2.75 points. In the sham group only one animal had slight disturbances (0.17 points, $P < 0.0005$; Table 10).

25 Table 10

Influence of test substances on neurological state 24 h after MCA occlusion; n= 7-8; Mean $\pm$ SEM

Group	Neurological state, points
Intact control	2.75 $\pm$ 1.04
Sham	0.17 $\pm$ 0.17***
MD	1.65 $\pm$ 0.26*
MD+GBB	1.43 $\pm$ 0.29*
MDS+GBBS	1.31 $\pm$ 0.21**

* $P < 0.05$ vs Intact control

**P<0.005 vs Intact control

***P<0.0005 vs Intact control

Prophylactic therapy by MD or MD+GBB prevented from the deterioration of the neurological status 24 h after MDA occlusion. Combination of MDS+GBBS displayed further increase of protective activity against neurological disturbances caused by ischemia (resp., P<0.005 in case of MDS+GBBS vs Control and P<0.05 in case of MD or MD+GBB vs Control; Table 11).

Table 11

Effect of 7 day application of test compounds on size of brain ischemic-infarction damage zone

Group	Ischemic damage zone, %
Intact control	23.7±2.35
MD	17.2±2.07
MD+GBB	14.9±1.74*
MDS+GBBS	13.7±1.70*

*P<0.05 vs Intact control

Morphological analysis revealed that MD+GBB and MDS+GBBS comparatively better than MD protected against the brain tissue damage caused by the MCA occlusion (resp., P<0.05 in case of MD+GBB or MDS+GBBS vs Control and non significant in case of MD vs Control; Table 11).

Claims

1. Gamma-butyrobetaine succinate.
2. A pharmaceutical composition, which comprises gamma-butyrobetaine succinate and Meldonium succinate.
3. The pharmaceutical composition to claim 2, wherein molar ratio of gamma-butyrobetaine to succinate is 1:1.
4. The pharmaceutical composition of claim 2, wherein the ratio of gamma-butyrobetaine succinate to Meldonium succinate is from 3:1 to 1:3.
5. Use of pharmaceutical composition according to claim 2 for the manufacture of a medicament for treatment of cardiovascular diseases.
6. Use of pharmaceutical composition according to claim 5, wherein cardiovascular disease is atherosclerosis.
7. Use of pharmaceutical composition according to claim 5, wherein cardiovascular disease is chronic heart failure.
8. Use of pharmaceutical composition according to claim 5, wherein cardiovascular disease is stroke.
9. Use of pharmaceutical composition according to claim 5, wherein cardiovascular disease is coronary heart disease.
10. Use of pharmaceutical composition according to claim 9, wherein coronary heart disease is transient ischemic attack.
11. Use of pharmaceutical composition according to claim 9, wherein coronary heart disease is permanent ischemic attack.
12. Use of pharmaceutical composition according to claim 5, wherein cardiovascular disease is peripheral arterial occlusive disease.
13. Use of pharmaceutical composition according to claim 2 for the manufacture of a medicament for treatment of lipid disorders.
14. Use of pharmaceutical composition according to claim 13, wherein lipid disorder is dyslipidemia.
15. Use of pharmaceutical composition according to claim 13, wherein lipid disorder is hyperlipidemia.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/058818

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/194 A61K31/197 A61P3/06 A61P9/00 A61P9/04
A61P9/10

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE, PASCAL, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of Box C.



See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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