EFFECTS OF THE DRUG MILDRONATE

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Summary

Mildronate (3-(2,2,2-trimethylhydrazine) propionate; THP; MET-88; meldonium, quaterine) is an anti-ischemic drug developed in the Latvian Institute of Organic Synthesis. Mildronate was designed as a promising inhibitor of carnitine biosynthesis aimed to prevent accumulation of cytotoxic intermediate products of fatty acid beta- oxidation in ischemic tissues and to block this highly oxygenconsuming process. Actually Mildronate is used in several countries in cardiology (treatment of chronic heart ischemic disease, stable angina pectoris, chronic heart failure, functional disorders of heart and vessels, cardiomyopathy, heart infarction), neurology (acute and chronic ischemic brain circulation disorders, decreased work capabilities, physical and emotional overload, recovery period after various diseases), pulmonology (bronchial asthma and obstructive bronchitis), narcology (abstinence syndrome in chronic alcoholism), ophthalmology (hemophthalm and retina haemorrhages of different aetiology, thrombosis of central vein and its branches in retina, diabetic and hypertonic retinopathies). The main mechanism of action of Mildronate is based on carnitine biosynthesis inhibition aimed to prevent accumulation of cytotoxic intermediate products of fatty acid beta- oxidation in ischemic tissues and to block this highly oxygen-consuming process. Alternatively the drug acts via stimulation of the nitric oxide production in the vascular endothelium through a modification of the γ-butyrobetaine and γ-butyrobetaine ester pools. The present review will summarize data on the Mildronate on different indications of the drug and its mechanisms of action.

Key words: *Mildronate -γ-butyrobetaine hydroxylase inhibitor – Antiischemic drug – Heart failure – Heart infarction – Brain circulation disorders – Diabetes mellitus*

I. Introduction

Adequate and uninterrupted energy supply of the cells is a necessary prerequisite for normal functions of the whole organism and separate organs, brain and heart being the most vulnerable from this point of view. Fatty acids and glucose are main fuels in the human organism. Oxidation of fatty acids supplies about 80% of energy in human heart, however this process is highly oxygen-consuming, in the case of ischemia intermediate products of fatty acids accumulate in the cell and become cytotoxic [1, 2, 3]. Protection of the cells against cytotoxic metabolites or cytoprotection is one of the possible strategies for treatment of the harmful consequences of ischemia. Mildronate (3-(2,2,2-trimethylhydrazine) propionate; THP; meldonium; MET-88; quaterin) is an anti-ischemic drug developed in the Latvian Institute of Organic Synthesis by I. Kalvinsh and collaborators following this strategy, actually it is widely used in some countries [4, 5, 6]. It is admitted that the pharmaceutical effects of mildronate on ischemic tissues are produced by the inhibition of γ-butyrobetaine (GBB) hydroxylase and reduction of the fatty acid β-oxidation [5, 7]. Via this mechanism, Mildronate inhibits the biosynthesis of carnitine and prevents the accumulation of toxic acylcarnitines in ischemic tissue. The cell metabolism shifts to increased glucose consumption [7, 4]. Mildronate appears to be effective for treatment of the

circulation disorders of CNS, heart ischemia and other pathologies. Several data indicate possible existence of an alternative mechanism of action of this drug. The present review will summarize data on Mildronate pharmacology and mechanisms of action. Data on pharmacokinetics and toxicology of the drug have been reviewed previously [6].

II. Pharmacology and indications of Mildronate

II.1. Mildronate in cardiology

II.1.1. Experimental studies. The earliest tests of the mildronate pharmacological action revealed its antiischemic effect in the myocardium. When administered orally to the rats in doses of 50 and 150 mg/kg for 10 days the drug prevented isoproterenol-induced increase of the activity of the hepatic isoform of lactate dehydrogenase in blood serum, and in a dose of 150 mg/kg prevented an increase of creatine phosphokinase activity. Against a background of the course administration of Mildronate isoproterenol failed to cause the accumulation of acyl-insoluble acylcarnitine in the myocardium. The cardioprotective effect the Mildronate manifested itself in prevention of a decrease of ATP and ADP concentrations, accumulation of AMP and a reduction of energy charge under the influence of

isoproterenol [8]. Hanaki et al. [9] studied the Mildronate effects on activities of the mitochondrial enzymes (NADH-cytochrome c reductase, succinatecytochrome c reductase, and cytochrome c oxidase) in the same model of heart lesion. Isoproterenol caused a drop of the NADH-cytochrome c reductase and cytochrome c activities; this effect was prevented by the Mildronate course (100 mg/kg) for 7 days before the isoproterenol administration. Mildronate pre-treatment protects the myocardium also from the hydrogen peroxide-induced lesions, mechanical dysfunction of the H2O2-treated rat heart as well as the fall of the ATP and ADP levels therein was prevented by the preceding Mildronate administration (10 days; 100 mg/kg/day)

Midronate restored the contractility of isolated atria [11] and the whole heart in the Langendorf system [12] after hypoxic exposure. The hearts isolated from rats pre-treated with Mildronate manifested decreased extent of the depression of the cardiac contractility and decrease of ATP and ADP in hypoxic conditions as compared to control rats [13]. Dhar et al. [14] have performed similar study on guinea-pigs treated with Mildronate. They report a slower decline in the rate of development of intraventricular pressure in hypoxic conditions in Mildronate-treated guinea-pigs as compared to the control group. Recovery of the heart functions after return to normoxia was complete in the Mildronate-treated animals, but incomplete in the untreated group. Mitochondria isolated from myocardium of the Mildronate treated animals manifested better respiratory function after hypoxia-reperfusion. It was concluded that Mildronate attenuated the hypoxic and reperfusion injury in the heart. The same conclusion was made after study of ischemia/reperfusion injuries in rat myocardium and ability of Mildronate to protect the heart against these injuries [15]. Ianson et al. [16] have studied effect of Mildronate in rats with experimental infarction of myocardium following occlusion of the left anterior descending coronary artery. The infarcted area was diminished from 29.8% down to 18.7% in rats treated with Mildronate as compared to the untreated control group. The drug affected favourably the activities of malate dehydrogenase, lactate dehydrogenase and of their isoenzymes as well as the aminotransferase activities in blood plasma. Beneficial effect on energy metabolism in ischemic dog hearts was described by Kirimoto et al. [17]. Pretreatment with the drug for 10 days (50, 100 or 200 mg/kg/day) attenuated the decrease of ATP, ADP and creatine phosphate in the ischemic area. Ability of Mildronate to improve the heart recovery after the infarction was studied in detail by Hayashi et al [18]. Congestive heart failure was produced by left coronary artery ligation in rats. Mildronate at 100 mg/kg/day was orally administered from the 2nd day after surgery. A survival study was performed for 181 days. Ventricular remodeling, cardiac function, and myocardial high-energy phosphate levels were measured for 20 days after treatment. Mildronate

prolonged survival with a median 50% survival of 103 days compared to 79 days for the heartfailure control rats. The expansion of the left ventricular cavity (ventricular remodeling) in heartfailure rats was prevented by treatment with Mildronate. The drug attenuated the rise in right atrial pressure in heart-failure rats and augmented cardiac functional adaptability against an increased load. Also, Mildronate improved the myocardial energy state in heart-failure rats. Treatment with mildronate can prevent development of left ventricular hypertrophy in rats with aortocaval shunt. This effect is attributed to improvement of the myocardial energy metabolism [19].

II.1.2. Clinical studies. In the study by Sakharchuk et al. [20] Mildronate was used for the treatment of cardiac insufficiency developed due to ischemic heart disease. It was established that mildronate produced a positive effect on the hemodynamics and gaseous composition of the blood. The patients also showed normalization of the nitrogen metabolism and activity of the membrane-bound erythrocytic enzymes. Dudko et al. [21] studied the Mildronate effect against placebo background in 50 patients with effort *angina pectoris*. Spirometric bicycle ergometry was used to evaluate the drug effects. It was established that monotherapy with mildronate is accompanied by an antianginal effect and an increase of the physical work capacity of patients. Ol'binskaia and Golokolenova [22] have reported antiarrhythmic action of Mildronate and it's the ability to prevent ventricular extrasystoles, to reduce asthenia, to enhance physical performance and adaptive reactions. The heart rate decrease appeared to be insignificant. Improvement of coronary circulation in patients with atherosclerotic lesions of heart blood vessels were reported in several clinical trials [23, 24]. In the recent trial by Teplyakov et al. [25] Mildronate manifested marked anti-ischemic effect in patients with postinfarction left ventricular dysfunction. In patients with chronic hypoxemia developed due to chronic pulmonary heart Mildronate was shown to improve the parameters of the acid-base state and gases in blood of examinees. It was more effective in patients with pronounced hypoxemia [26]. Mildronate treatment improves the oxygen circulation parameters in erythrocytes of patients with heart failure. It decreases the level of methemoglobin in the patients' blood and improves the phosphate balance, especially that of 2,3-diphosphoglycerate to control oxygen transport by hemoglobin, which is of paramount importance in hypoxia caused by coronary heart disease [27]. Daily mildronate administration (1 g/day) improves quality of life of patients with chronic heart failure [28]. Actually Mildronate is recommended as a prospective drug for pre-conditioning of the myocardium in order to prevent infarction in high-risk patient groups [5]. Recent clinical trials performed in Russia [29], Ukraine [30] and Georgia [31] confirm efficiency of Mildronate in treatment of chronic heart failure and *angina pectoris*.

Mildronate is an officially recommended drug for treatment of chronic heart ischemic disease and stable *angina pectoris,* cardiomyopathy in Latvia, Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia, Uzbekistan, Moldova, Kyrgyzstan, chronic heart failure (above countries and Lithuania), functional disorders of heart and vessels (Latvia), heart infarction (Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia, Uzbekistan, Moldova, Kyrgyzstan).

II.2. Mildronate in neurology

Probable beneficial effects of the Midronate on CNS were studied soon after synthesis of the substance, it manifested a beneficial effect on cerebral circulation disorders and CNS functions [32, 33]. The drug is applied in neurological clinics for treating brain circulation disorders [34].

The drug is officially recommended for treatment of acute and chronic ischemic disorders of brain circulation in Latvia, Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia,

Uzbekistan, Moldova and Kyrgyzstan. The drug shows mood improving effects in patients; they become more active, motor dysfunctions decrease, asthenia, dizziness and nausea are less pronounced [33]. Therefore Mildronate is officially recommended for improvement of reduced work capabilities, physical and psycho-emotional overload in all above countries. In Latvia this indication covers also patients with different diagnoses undergoing recovery period. Moreover, in

Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia, Uzbekistan, Moldova and Kyrgyzstan it is recommended for treatment of abstinence syndrome in patients with chronic alcoholism. Data on the Mildronate use in neurology have been reviewed previously [6].

II. 3 Mildronate as immunomodulator

The drug is an active interferon inducer in mice when administrated simultaneously with antigen and shows a protective effect against influenza virus when used according to therapeutic and preventive schedules. We suppose that the drug acts as a strong stressing signal dissipating from the site of vaccine injection [35]. This property of Mildronate enabled to propose its use as immunoadjuvant, especially during vaccination against influenza [36, 37]. It was also reported that Mildronate enhances the immune response in fasting animals [38] and in patients after surgery

[39]. Improvement of immunological indices after treatment with Mildronate was observed even in patients with seroresistant syphilis [40].

II.4. Mildronate in pulmonology

Immunomodulating activity of Mildronate enables its use in pulmonology for treatment of bronchopulmonary diseases. Orlov et al. [41] evaluated the immunity status of 35 patients with chronic bronchitis and infectious and allergic bronchial asthma. Defects in the humoral immunity were revealed. To correct the immunity status, all the patients were treated with Mildronate. The immunomodulatory effect of the drug was found in all the groups of the patients. Mildronate was shown to increase the activity of a secondary immune response and the bronchial potency in the persons with infectious and allergic asthma. Karashurov et al. [42] confirmed that Mildronate stimulated the cellulat immunity and decreased concentration of immunoglobulins in patients with bronchiala asthma. The drug improves the cardiac functions in patients with chronic bronchitis [43]. Mildronate produced also a membrane-stabilizing action on erythrocytes of rats with experimental chronic non-specific lung disease [44].

Mildronate is recommended for treatment of bronchial asthma and obstructive bronchitis in Ukraine and Uzbekistan.

II.5. Mildronate in gastroenterology

In experiments with modeling of gastric ulcers in rats Mildronate manifested protective effect and accelerated ulcer healing [45, 46]. Given together with extracts of meadow hay Mildronate normalized the lipid metabolism in rabbits with experimental cholelithiasis [47].

II.6. Mildronate in otolaryngology

Inadequacy between high level of metabolism in the cell and instability of energy supply followed by intensive lipolysis in mitochondria and accumulation in the cells of fatty acid metabolites is considered to be a cause of pathological processes in the ear apparatus. This consideration provides background for use of Mildronate in otolaryngology [48]. The study by

Luchikhin et al. [49] on evaluation of effectiveness of treatment of cochleovestibular disorders with Mildronate revealed that the drug appears to be efficient in 86.9% cases of acute cochleovestibular dysfunction and in 75.0% of cases of chronic disease. Thus the drug can be effectively used to treat patients with

cochleovestibular disorders. Ramazashvili et al. [50] have studied the applicability of endaural phonoelectrophoresis of 5% mildronate for the treatment of peripheral vestibular disorders. The study was carried out on 25 patients with vestibular disorders that accompanied neck osteochondrosis and neurocirculatory dystonia. The treatment gave good results in 18 patients as measured by electronystagmography and subjective sensations.

Improvement of the vestibular function was paralleled by improvement of the hearing level. As a result, Mildronate was recommended for the local treatment of vestibular problems produced by cerebral circulation disorders.

II.7 Mildronate in ophthalmology

Mildronate appears to be effective for treatment of eye trauma and burns [51, 52]. Mildronate is officially recommended for use in ophthalmology for treatment of hemophthalm and retina haemorrhages of different aetiology, thrombosis of central vein and its branches in retina, diabetic and hypertonic retinopathies in CIS countries [53]. The drug is also efficient for treatment of the optic neuropathy in glaucoma [54].

II.8 Mildronate in treatment of the diabetes complications

The idea about eventual beneficial effect of Mildronate on metabolic parameters in patients with diabetes mellitus arose from the fact that the drug had a marked antiketogenic activity in fasting experimental animals [55]. Mildronate in complex with isoptin improves the heart functions in patients with non-insulin dependent diabetes mellitus [56]. The drug also improves cerebral circulation in patients with discirculatory encephlopathy on the backgrouond of non-insulin dependent diabetes mellitus. The effect is achieved via decrease of the lipid peroxidation rate [57]. Mildronate appears to be effective also for treatment of diabetic retinopathy [58].

II.9. Mildronate in obstetrics

Benevolent effects of Mildronate on development and metabolic parameters in rabbit embryos were detected in early experimental studies [59]. In the study by Tsirkin and collaborators (cited after Podtetenev and Bratchikova [61]) beta-adrenosensibilizing effect of the drug was revealed in rat uterus myocytes. A pioneering clinical trial confirmed possibility to use the drug in practical obstetrics in order to decrease the intrauterine pressure. Bolus intravenous injection of 1 g of the

drug [61] triggered decrease of the pressure in 15 minutes, thus the effect should be considered as a "fast" one (see below). The drug can be also used for preventive treatment of perinatal pathologies in women with fetoplacental insufficiency [62].

III. Mechanisms of action of Mildronate

III.1 Carnitine-dependent mechanism

The very design of the drug was aimed on interference with the carnitine metabolism. Profound effects of the drug on carnitine level and intensity of the fatty acid beta-oxidation was revealed in the earliest investigations. This pioneering work was performed in the Institute of Organic Synthesis under guidance of B. Simkhovich. It was shown that when administered to rats the drug caused a decrease of carnitine and long chain acylacarnitine in the myocardium, oxidation of 14C-palmitate was also decreased [63]. The effect was reproduced in animals maintained on a fat-rich diet, in the isoproterenol heart failure model [8]. In rabbits the drug produced similar effects on the carnitine level [11]. More profound molecular study indicated that the enzyme γ- bytyrobetaine hydroxylase, which catalyses the last step of the carnitine biosynthesis is the main target of the drug. The drug inhibited partially purified enzyme as non-competitive inhibitor [4].

Inhibition of the rate of beta-oxidation caused increase of fatty acid concentration in blood serum of Mildronate treated rats [64] and carnitine-independent fatty acid oxidation in mitochondria [65]. Interestingly, although the drug causes increase of fatty acids in animals with normal lipid metabolism, it appeared to be capable to normalize plasma lipids in rats with hyperlipidemia induced by triton WR-1339 [66]. Efficient inhibition of the carnitine biosynthesis after Mildronate administration was reported also by other researcher groups. Besides reduction of carnitine concentration in the heart, skeletal muscles and kidneys of mildonate-treated rats Tsoko et al. [67] reported compensatory increases of acyl-CoA synthetase and carnitine palmotoyltransferase I in liver mitochondria and increase of peroxysomal fatty acid oxidation.

High affinity of the Mildronate to the γ-butyrobetaine hydroxylase is used for purification of the enzyme by means of the affinity chromatography [68]. Mildronate appears to be capable to normalize the carnitine content in liver induced by fenofibrate [69]. A thorough study of the enzyme activities in Mildronate tretad rats by Spaniol et al. [70] also indicated efficient inhibition of the γ-☐butyrobetaine hydroxylase, however the authors insist on competitive mechanism.

Decrease of carnitine concentration in blood plasma, liver and muscles was also reported. An interesting study illustarting potency of Mildronate as a γ-butyrobetaine hydroxylase inhibitor was recently conducted in Italy [71]. The aim of the study was to evaluate the effect of

Mildronate administration to pregnant and lactating rats on tissue carnitine concentrations in 4-and 13-day-old rat pups. At 14 days of gestation female rats began to receive mildronate in the diet and this continued for entire lactation period. Mildronate treatment determined a large reduction of carnitine levels in the milk of lactating dams. Because organ carnitine concentrations in neonatal rats are directly related to dietary supply, pups from mildronate group had significantly depleted levels of total carnitine in serum, heart, liver, muscle, brain and pancreas relative to controls, at 4 and 13 days of age. All functional and biochemical modifications were compatible with a carnitine deficiency status.

Besides the inhibitory effect on the carnitine biosynthesis the drug appeared to be able block carnitine transport inside mitochondria by inhibiting the carnitine acyltransferase [72]. Later it was shown that mildronate inhibits the Na+-dependent carnitine transport into the cultured myotubes [73] and isolated myocytes [74]. Increased renal excretion of the carnitine and competitive inhibition of the carnitine transport by rat renal brush-border membrane are considered to be as important in the drug mechanism of action as the block of carnitine biosynthesis [70]. Some authors consider the increase of renal clearance of carnitine to be the main mechanism of the decrease of carnitine concentration [74]. Interference of Midlronate with carnitine metabolism and transport on the organism and cellular levels are summarized in Figs 1
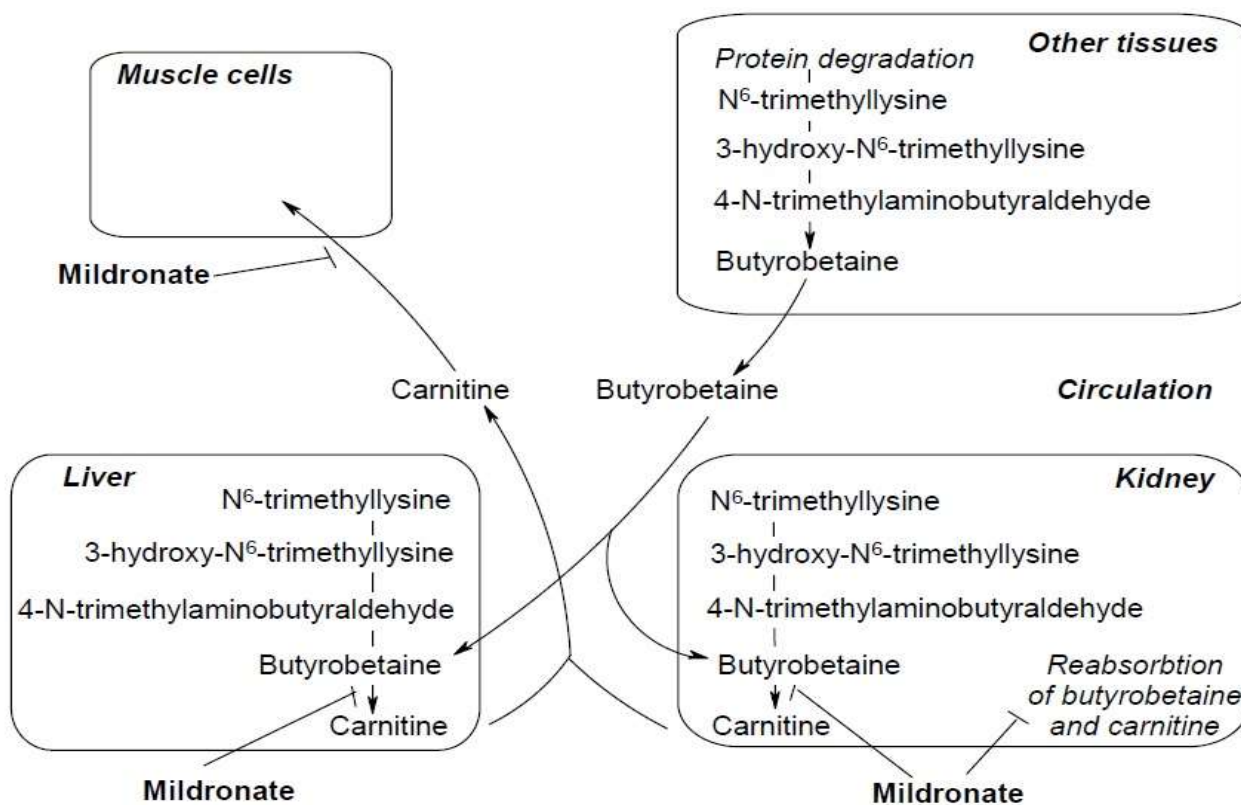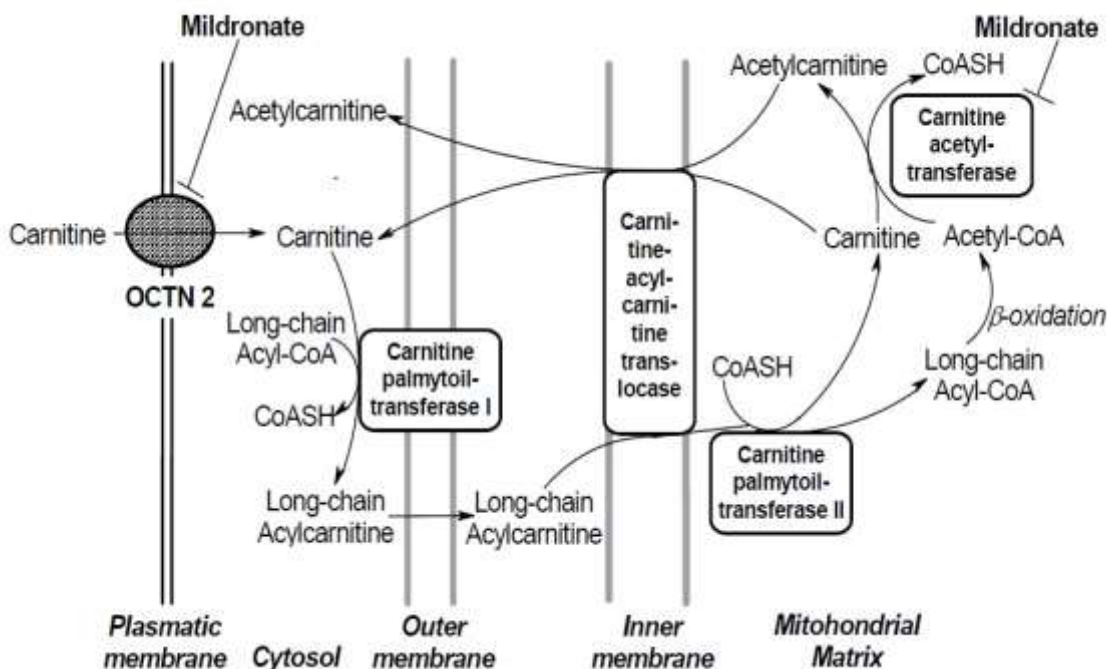
and 2.

Figure 1. Scheme of carnitine metabolism on the organism level with the indication of Mildronate action sites.

Figure 2. Transport of fatty acids and fatty acid beta-oxidation in mitochondria with the sites of the Mildronate inhibitory action.

Inhibition of carnitine biosynthesis by Mildronate alters the gene and protein expression pattern in the myocardium. It was reported that improvement of the ventricular diastolic dysfunction induced by congestive heart failure is achieved by improvement of the Ca2+ ion uptake in the sarcoplasmic reticulum [76]. It was also demonstrated that Mildronate treatment prevents the infarction-induced drop of the sarcoplasmic reticulum Ca2+ - ATPase and hexokinase I protein contents in the myocardium. This effect is attributed to the suppression of carnitine biosynthesis and compensatory increase of expression of the enzymes involved in glucose metabolism [76].

Promoter of sarcoplasmic reticulum Ca2+ - ATPase contains sequences that are expected to respond to transcription factors responsive to glucose metabolites [3]. Mildronate treatment is followed by compensatory increase of expression in the myocardium of several genes encoding the enzymes of the lipid metabolism – lipoprotein lipase, fatty acid translocase, carnitine palmitoyltransferase I and enzymes of triacylglycerol synthesis [77].

Taken in general the inhibition of carnitine biosynthesis, transport and reabsorption prevents the accumulation of toxic acylcarnitines in ischemic tissue. The cell metabolism shifts to increased glucose consumption that is beneficial in ischemic conditions. This mechanism is discussed in several publications [7, 4, 3, 5].

III.2 "Fast mechanisms"

The above Mildronate mechanism of action, based on inhibition of the fatty acid beta-oxidation is understandable if it describes the drug action in heart, muscles and other fatty acid-consuming organs, however, this mechanism can hardly explain Mildronate pharmacological effects in the brain as the brain cells normally use glucose as their sole source of energy. However, carnitine is synthesized in the brain cells, the GBB hydroxylase gene is expressed in brain [78]. This probably indicates a function different from the fatty acid transport of carnitine in the brain tissue. We will explain our point of view on this matter below. Moreover, the Mildronate effects produced by inhibition of the fatty acid beta-oxidation can be achieved only after several daylong treatments. Meanwhile, several observations indicate that Mildronate elicits several fast effects related to vasorelaxation [79, 7]. It was reported that bolus administration of Mildronate increased the animal survival after experimental myocardium infarction and improved the bioenergetic parameters of ischemic myocardium in rats [79]. Bolus intravenous injection of Mildronate increased the blood flow in the aortal arch and

decreased the peripheral resistance in blood vessels of anaesthetized cats. In dogs, it increased blood flow in carotid, mesentery and femoral arteries. In isolated vessels of rabbit ears, it reduced epinephrine-induced spasms. It also prevented the evolution of heart insufficiency symptoms in cats caused by stenosing of the lung artery (M. Veveris, personal communication). In the clinical study by Enina et al. [33] a transient decrease in arterial blood pressure was detected 30 minutes after the drug administration, and several parameters of the cerebral circulation changed 1 hour after bolus intravenous injection of Mildronate. Under clinical conditions, Mildronate single administration normalized cerebrovascular reactivity for 60-90 minutes [80]. In addition, it has been found that administration of Mildronate and γ-butyrobetaine (GBB) mixture eliminated physiological effects of nitric oxide synthase (NOS) inhibitors [81]. Mildronate appeared to be able to increase betaadrenoreactivity of smooth muscles in aortic rings in *ex vivo* systems [82]. Similarly, Mildronate produced negative chronotropic and inotropic effects when perfused in high concentrations through dog atrial and ventricular preparations [83]. Mildronate administration favors wound and ulcer healing [45, 46, 51, 52], although carnitine biosynthesis and transport block should rather inhibit cell proliferation [73].

Mildronate interferes with membrane receptor and secondary messenger activity [84]; in a few hours it triggers DNA replication, repair and methylation (Blium et al, [85, 86], reviewed in Shutenko et al., [7]). It is capable of triggering RNA-polymerase activity in isolated neurons in vitro [87]. Increase in the pre-mRNA synthesis was observed in the rat liver, spleen, heart and intestine six hours after the drug administration [88]. Decrease in the ADP-ribosylation of loosely bound chromatin non-hostone proteins can be also observed soon after the drug administration [89]. None of the above effects could be explained by inhibition of carnitine biosynthesis, thus a novel, "fast", and probably receptor-dependent mechanism needs to be researched for.

The idea of existence of a non-conventional function of carnitine and its precursor GBB and the likelihood of pharmacological interference with this function triggered the design of Mildronate by I. Kalvinsh. It was reported that besides being a carnitine precursor, GBB could undergo etherification in mammal brain tissues [90]. The structure of gamma-butyrobetaine ethyl ester strikingly resembles that of acetylcholine, the distance between positively and negatively charged poles in both molecules being almost identical. I. Kalvinsh proposed a hypothesis about existence of a specific signal transfer system based of GBB esters [53]. A possibility for the existence of such a system is suggested by the observation of an increase in the GBB concentration in stressed animals [91] and the cholinergic activity of GBB esters [92].

The proposed hypothetical mechanism could consist of the following steps: 1. Mildronate administration shifts the equilibrium between GBB hydroxylation to carnitine and GBB esterification towards GBB esters. Trace amounts of GBB esters are physiologically active, besides other organs the process proceeds also in the brain, thus the effect should be rather fast. 2. GBB ester binds its specific receptor; GBB esterase acting like acetylcholine esterase performs ester hydrolysis. 3. GBB ester hydrolysis triggers the signal transduction. Secondary messengers can be involved in the process
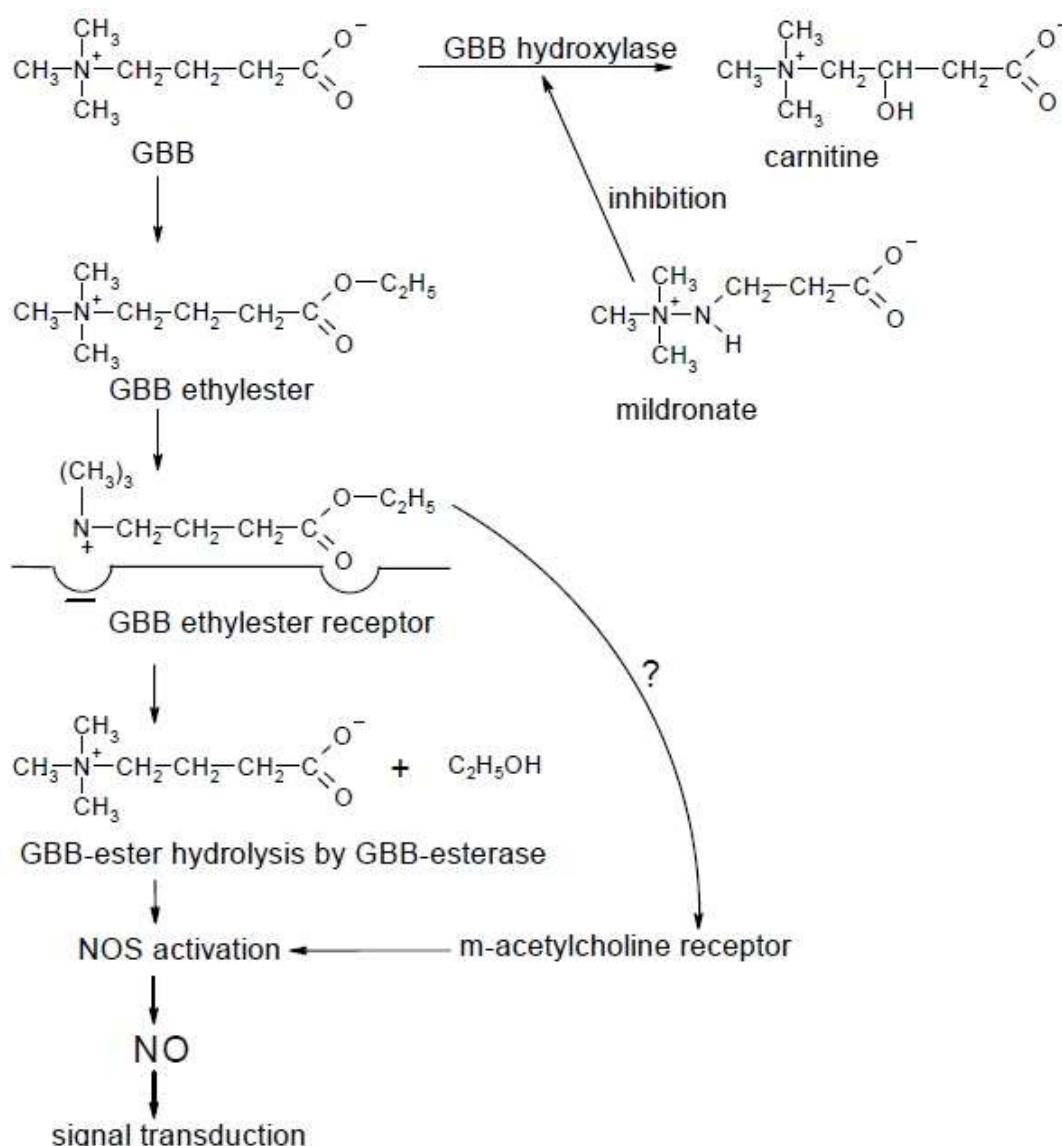(Fig 3).

Figure 3. Scheme of eventual mechanism of the fast and NO-dependent Mildronate effects.

We have performed several studies to find support for the above hypothesis. Identification of the GBB esterase activity in mammals was the first objective of the studies. Surprisingly, we have managed to reveal the existence of this enzymatic

activity in the rat blood serum. An in-chain substituted derivative of GBB ester that can be detected spectrophotometrically for the HPLC purposes was synthesized and used in the study. The substance was stable in aqueous solutions, but if incubated with the rat blood serum the HPLC revealed a peak corresponding to the GBB analogue. Up to 60% of the initial 3. 10-4 M ester were hydrolysed in an hour. Acetylcholine did not compete with the reaction; it was insensitive to acetylcholine inhibitors. Moreover, purified acetylcholinesterase could not catalyze the reaction, butirylcholinesterase was also inactive.

Thus, we concluded that a specific enzyme GBB-esterase is active in mammals. The study is in progress [93]. Existence of the GBB esterase cannot exclude the action of GBB esters via acetylcholine receptors, as the substance can bind them. Recent in vitro data obtained by Dambrova et al. [94] showed that GBB methyl ester is a potent agonist for m-type of acetylcholine receptors; GBB affinity to these receptors is much lower. A computer model of the molecular interactions between the GBB ethyl and methyl esters and the active centre of acetylcholine indicates that acetylcholine and GBB ethyl ester have the same binding modes [6]. This is a clear indication of a possibility for hydrolysis of GBB esters by this enzyme.

Concerning the following transducers of the GBB esterase signaling pathway, nitric oxide appeared to be the probable, as it was reported that Mildronate and γ-butyrobetaine (GBB) composition eliminated vasoconstriction produced by nitric oxide synthase (NOS) inhibitors [81]. We hypothesized that Mildronate might act also via a nitric oxide-dependent mechanism. In a preliminary study [95] we tried to reveal Mildronate possible effects on nitric oxide (NO) concentration in rat organs. Changes in the NO content in different rat tissues (brain cortex, cerebellum, liver, heart, kidneys) were evaluated after Mildronate administration by the electron paramagnetic resonance method (EPR). It was revealed that Mildronate (50 mg/kg, i/p) triggered a slight but reproducible wave-like increase in the NO level in the brain cortex and cerebellum 30 minutes after the drug administration. Administering the NOS inhibitor Nω-nitro L-arginine (50 mg/kg; i.p.), at the same time caused a pronounced decrease of the NO concentration that indicates the necessity of NOS activation to produce the Mildronate effect. This was the first indication of a putative NO-dependent mechanism of the drug action. Interestingly, the effect was pronounced in the brain, where "the not understandable" carnitine biosythesis takes place.

Moreover, the time course of the effect resembled that for of Mildronate vascular effects described by Enina et al. [33]. In our later studies the NO-producing effects of Mildronate were studied in comparison with γ-butyrobetaine and GBB esters. We observed an induced transient increase in nitric oxide (NO) concentrations in the rat

blood and myocardium, produced by Mildronate, GBB and GBB methyl ester [96]. The latter produced a similar effect to GBB and Mildronate at 100- times lower concentration. *In vitro*, these compounds neither modified the activities of purified neuronal and endothelial recombinant nitric oxide synthases (NOSs), nor were they able to interact with their active sites. GBB induced vasodilatation at high concentrations only (EC50 = 5 x 10-5 M), Mildronate alone displayed no vasodilating effect, however enhanced the GBB vasodilating activity. GBB methyl and ethyl esters were found to be more potent vasodilators (EC50 = 2.5 x 10-6 M). Pre-treatment of aortic rings with NOS inhibitor Nω-nitro-L-arginine methyl ester abolished the vasodilating effects of the compounds [96]. The above results provide evidence that GBB methyl and ethyl esters are potent NO- and endothelium-dependent vasodilators. While Mildronate alone elicits no activity, it sharply enhances the activity of GBB in endothelium- and NOS-dependent responses. These data suggest that fast anti-ischemic effects of Mildronate may be in part related to the stimulation of NO formation by endothelium. As none of the studied compounds could modify the NOS activity in vitro, we think that our results suggest that some receptor-mediated mechanisms are participate in activation of NO formation in the blood vessels. Both still hypothetic GBB ester–dependent receptors (GBB esterase is not hypothetic any more!) and acetylcholine receptors are possible candidates for this role. Cholinomimetic activity of GBB esters and compounds of a similar structure is known for some period of time [90]. Mildronate ethyl ester EDIHYP is even considered to be a synthetic analogue of acetylcholine [97]. The ability of GBB methyl ester to bind *m*-type of acetylcholine receptors also supports this possibility [94]. The synergistic effects of Mildronate on GBB activity described above may involve the GBB esterification, as GBB esters trigger their vasorelaxing effects at much lower concentrations than GBB itself. These results suggest to us that Mildronate fast anti-ischemic action could be mediated, at least in part, by stimulation of NO production in the vascular endothelium through a modification of the GBB/GBB ester pools. This stimulation of NO formation might be rationalized in the following ways (Fig. 3): (i) Mildronate administration inhibits GBB hydroxylation and increases the GBB intracellular pool; (ii) a part of GBB is released from cells, and, after esterification, forms potent cholinomimetic GBB esters; (iii) GBB esters, via acetylcholine receptors on endothelial cells could activate (endothelial nitrite oxide synthase) eNOS. We can still speculate that a specific GBB-ester receptor pathway can exist alternatively or in parallel to the cholinomimetic pathway.

Our data provide evidence for most steps of this hypothetical mechanism, the increase in GBB esterification after Mildronate administration remaining the missing

link. In this regard, the presence of GBB esters in living tissues was described more than thirty years ago, although their physiological significance still remains poorly known [90]. As they decrease systemic blood pressure, they display analogies with acetylcholine and activation of eNOS activity might be one of the functions of these compounds. It is of interest that carnitine has been found to produce endothelium-dependent vasorelaxation in aortic rings [98], an activity that might be mediated by esterification because carnitine esters also possess cholinergic activity [90]. An interesting observation about Mildronate interference with NO metabolism in humans was recently reported by Geichenko et al. [99]. In patients with chronic heart failure they observed decrease of NO metabolite (nitrites and nitrates, NOx) concentration in blood serum as compared to healthy persons (102.47 ± 4.64 vs 122.00 ± 12.02 nmol/ml). Complex treatment of the disease (angiotensin-converting enzyme inhibitors, beta-adrenoblockers, vasodilatators, and diuretics) provoked a drastic increase of NOx concentration (119.39 ± 6.39 nmol/ml). Addition of Mildronate to the treatment scheme (0.5 g per day, 30 days) made this increase more mild (108.83 ± 3.40 nmol/ml). The authors interpret their data as "improvement of endothelial vasoregulating function". The link between this observation and our data obtained in experiments is to be established.

Conclusion. Above data provide evidence of effectiveness of Mildronate in treatment of various diseases. With no doubt the drug should be tested for clinical use in other countries.

ACKNOWLEDGEMENTS

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