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Mildronate: An Antiischemic Drug for Neurological Indications

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ABSTRACT

Mildronate (3-(2,2,2-trimethylhydrazinium)propionate; MET-88; meldonium, quaterine) is an antiischemic drug developed at the Latvian Institute of Organic Synthesis. Mildronate was designed to inhibit carnitine biosynthesis in order to prevent accumulation of cytotoxic intermediate products of fatty acid β-oxidation in ischemic tissues and to block this highly oxygen-consuming process. Mildronate is efficient in the treatment of heart ischemia and its consequences. Extensive evaluation of pharmacological activities of mildronate revealed its beneficial effect on cerebral circulation disorders and central nervous system (CNS) functions. The drug is used in neurological clinics for the treatment of brain circulation disorders. It appears to improve patients' mood; they become more active, their motor dysfunction decreases, and asthenia, dizziness and nausea become less pronounced. Since the brain does not utilize fatty acids as fuel other mechanisms of action of mildronate in CNS should be considered. Several reports indicate the possible existence of an alternative, non-carnitine dependent mechanism of action of mildronate. Our recent findings suggest that CNS effects of mildronate could be mediated by stimulation of the nitric oxide production in the vascular endothelium by modification of the γ-butyrobetaine and its esters pools. It is hypothesized that mildronate may increase the formation of the γ-butyrobetaine esters. The latter are potent cholinomimetics and may activate eNOS via acetylcholine receptors or specific γ-butyrobetaine ester receptors. This article summarizes known pharmacological effects of mildronate, its pharmacokinetics, toxicology, as well as the proposed mechanisms of action.

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INTRODUCTION

Mildronate (3-(2,2,2-trimethylhydrazinium) propionate; MET-88; quaterine) is an anti-ischemic drug developed by I. Kalvinsh and his associates at the Latvian Institute of Organic Synthesis (OSI). The drug is widely used in several countries (10,50,56). Mildronate was designed as an inhibitor of carnitine biosynthesis aimed to prevent accumulation of cytotoxic intermediate products of fatty acid β-oxidation in ischemic tissues and to block this highly oxygen-consuming process (10,28,36,48,50,56). Initially the heart and skeletal muscles were considered to be the main targets of the drug action in the organism, as these organs are the sites of the most intensive β-oxidation of fatty acids. Indeed, mildronate has been found to be effective in the treatment of myocardial ischemia and its consequences; It is widely used for this purpose by cardiologists (10,16,50). Extensive pharmacological evaluation of mildronate revealed its beneficial effect on cerebral circulatory disorders and CNS functions (15,69). It is used for this purpose in neurological clinics (1,12) and is approved and marketed for the treatment of acute and chronic ischemic disorders of brain circulation in Latvia, Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia, Uzbekistan, Moldova, and Kyrgyzstan. Midronate appears to improve mood in patients; they become more active, their motor dysfunction decreases; asthenia, dizziness and nausea become less pronounced (15). Mildronate is, therefore, also recommended for improvement of reduced work capacity, as well as for physical and psycho-emotional overexertion. In Latvia this indication covers also patients during recovery from various diseases. Moreover, in Russia, Ukraine, Georgia, Kazakhstan, Azerbaijan, Byelorussia, Uzbekistan, Moldova, and Kyrgyzstan mildronate is used in the treatment of abstinence syndrome in patients with chronic alcoholism. Since the brain does not utilize fatty acids as fuel, the CNS effects of mildronate suggest an alternative, a carnitine-independent mechanism of action. This article reviews CNS effects, pharmacokinetics (71), toxicology (45,62) and the proposed carinitine-dependent and -independent mechanisms of action of mildronate. As stated above, mildronate is used mainly in cardiology. The experimental basis for its use in cardiovascular diseases has been recently reviewed by Dambrova et al. (10). This article reviews neuropharmacological and clinical studies with mildronate.

NEUROPHARMACOLOGY

Numerous experimental studies focused on the effects of mildronate on cerebral circulation in the ischemic brain. Mildronate, 25 mg/kg, i.v., administered daily during 14 days after induction of local brain lesions and ischemia in rabbits, facilitated the restoration of cerebral blood flow and vascular reactivity. This effect involved an improvement of restorative processes due to a more rapid normalization of reactivity of cerebral blood vessels (17). Pretreatment with mildronate appears to prepare the brain for unfavorable conditions. Hydrogen clearance, polarographic, and electric impedance measurements on conscious rabbits with electrodes implanted in the brain cortex, thalamus, and hypothalamus were used to evaluate the state of hemodynamics, oxygen and water-electrolyte balance in a model of the zero-gravity-induced cerebrovascular disorder (2). Pretreatment with mildronate (10 mg/kg, p.o.) improved hemodynamics and optimized oxygen balance

and markedly decreased brain edema. Inosine enhanced the antihypoxic and antiedematous effects of mildronate.

S. Germane (20) performed a complex neurophysiological study on the effects of mildronate on behavior of mice and rats. S. Germane demonstrated that at 20 mg/kg i.p. or 2000 mg/kg p.o. the drug exhibited peculiar effects on the central nervous system. Activating components prevailed in the activity spectrum of the drug. Mildronate increased vertical and horizontal mobility of the animals, activated exploratory behavior in the open-field test, and increased tolerance to prolonged forced swimming or hypoxia. In stressed animals mildronate affected the sympatho-adrenal system, favoring accumulation of catecholamines in the brain and adrenal glands and attenuated stress-induced somatic changes (21). In a recent study we measured the effect of mildronate on the production of nitric oxide in lipopolysaccharide (10 mg/kg)-treated rats using the electron paramagnetic resonance method (59). Lipopolysaccharide treatment increased nitric oxide (NO) level in the brain cortex from 46.0 ± 3.4 in control animals to 227 ± 27 and in cerebellum from 27.7 ± 2.6 to 218 ± 30 ng/g tissue. Mildronate (120 mg/kg) caused a significant two-fold decrease of the nitric oxide level in the brain cortex and cerebellum at one hour after drug administration, but failed to inhibit inducible nitric oxide synthase *in vitro*. We proposed that the drug reduces NO levels by reacting directly with the radical. Thus, mildronate appears to be a promising drug for the treatment of cerebral circulatory complications of sepsis (59). Furthermore, the drug increased [^{3}H]uridine incorporation into isolated rat neurons *in vitro*, probably by augmenting the overall RNA-polymerase activity in neurons (61).

CLINICAL STUDIES

It is generally accepted that chronic cerebrovascular ischemia is an important disorder leading to progression of functional neurological deficiency and cognitive impairment. The main cause of this condition is stenosis of the cerebral arteries. In the pioneering clinical trial performed by Vinnichuk (69) the clinical and hemodynamic efficacy of mildronate was evaluated in 38 patients with ischemic stroke. Mildronate (10 mL of 5% solution) was administered intravenously once a day. The effects of mildronate were compared with those of placebo (sodium chloride solution) and of two reference drugs. Hemodynamic parameters were studied by means of tetrapolar thoracic rheography, tetra- and bi-polar rheoencephalography, and xenon inhalation method. The drug was found to improve cerebral hemodynamics in patients with stroke and post-ischemic cerebral hypo- or hyper-perfusion. On the basis of these findings mildronate was recommended for the treatment of ischemic disorders of cerebral circulation.

In another trial (15) mildronate was given p.o. or i.v. to 52 patients with an early form of cerebral insufficiency and stage II–III encephalopathy. Cerebral circulation parameters were evaluated by means of echopulsography. Mildronate tended to normalize these parameters. After mildronate administration patients became more active, headaches and asthenia diminished, while efficiency increased. Placebo or reference drugs were not used in this trial. Dziak and Golik (12) used mildronate (10% infusion solution, 10 mL, for up to 10 days followed by tablets, 750 mg/day for 20 days) in patients with chronic cerebrovascular ischemia. Drug effects were compared with those of placebo. The study

was performed using computer tomography approach, ultrasonic dopplerography and computer encephalography. Thewe was a positive effect on neurological symptoms, hemodynamic, electrophysiological, and neuropsychological characteristics of the patients. In the trial by Abeuov et al., (1) attention was focused on the beneficial effects of mildronate on the higher nervous system functions distorted in patients with dyscirculatory encephalopathy (DEP). Mildronate (250 mg i.v., twice a day for 10 days) decreased the incidence of headaches, dizziness, vestibular dysfunction, and insomnia. The authors also noted improvement in memory, attention, and cognition. In this trial mildronate produced beneficial effects also in combination with other drugs. The trial was not placebo-controlled and no reference drugs were used. Antioxidant properties of mildronate were studied in diabetic patients with acute lacunar stroke and DEP (63). Administered as add-on therapy, mildronate (at a daily dose of 500 mg) increased the resistance of blood serum lipoproteins to peroxidation. Latvian neurologists stress the efficacy of the drug in improving the quality of life during rehabilitation of patients with cerebrovascular disorders, or after cerebral trauma or encephalitis. Improvements in the quality of life were judged on the basis of objective and subjective criteria in a double-blind, randomized and placebo controlled trial. It has been also reported that mildronate accelerates regression of brain lesions (68) possibly by modulating brain oxygen consumption. Cerebral oxymetry studies indicate, however, that mildronate does not change parameters of oxygen saturation of the brain tissues, but prevents decreases in these parameters caused by alcohol (39).

TOXICOLOGY

Mildronate acute and chronic toxicities were studied in different animal species using various administration routes. By bolus administration mildronate's toxicity in rats or mice is very low (LD_{50} = 20,000 mg/kg; p.o.). By repeated oral daily administration of the drug for 6 months no stable changes of hemopoiesis, functional state of liver and kidneys, or any alterations of the tissue structure in inner organs were observed (45). Other authors reported liver steatosis in rats treated with mildronate (20 mg/100 g) during the 3rd or 6th weeks of administration (62). In another report mildronate at a suprapharmacological dose (400 mg/kg/day for 60 days) was found to induce lipid accumulation in the rat liver. However liver function tests were not altered and there was no lipid accumulation in the heart (23). Mildronate had no mutagenic effects in studies with *Salmonella typhimurium* or *Drosophila* and no carcinogenic effects in mice (3). It slows the development of N-nitrosoethylamine-induced liver tumors (47), but does not interfere with the carcinogenic effects of methylcholantrene (34).

PHARMACOKINETICS

In the studies performed at the Latvian Institute of Organic Synthesis (53) the maximal mildronate blood levels in humans were observed at 2 h after oral administration of the drug; its excretion half-life was nearly 18 h. The pharmacokinetic study performed in rats by D. Meirena, V. Parinov, and A. Gilev (summarized in 53) using p.o. administration of radioactive drug labelled with ^{14}C in the α and β positions (52 mg/kg), revealed that 24 h

after administration highest levels of the label were found in liver, kidneys, spleen, lungs, and heart. In these organs the radioactivity exceeded that of the blood plasma level; in skeletal muscles, brain and adipose tissue it was close to the plasma level. Seventy three percent of the unchanged drug was excreted in the urine during the first day of administration. Urinary excretion is the main route for elimination of mildronate and its metabolites: 30% of the label was detected in the exhaled CO_2, and only 4% of the label was excreted in the feces.

The study performed at the Taiho Pharmaceuticals Co showed that disposition of mildronate after oral or i.v. administration of the drug to rats at either 2, 20, or 60 mg/kg is nonlinear (71). After 20 or 60 mg/kg doses of mildronate the levels of the radioactive drug (labeled with ^{14}C in two positions next to the carboxylic group) and total radioactivity in plasma were similar. At 2 mg/kg the plasma mildronate levels were lower than the total radioactivity. The excretion of radioactivity after oral administration of mildronate indicated that with the increase in the dose of the drug the major excretion routes of radioactivity shifted from pulmonary (as exhaled CO_2) to urinary. Major metabolites in plasma after oral administration of mildronate were glucose, succinic acid, and 3-hydroxypropionic acid. *In vitro* studies revealed that mildronate was converted to 3-hydroxypropionic acid by γ-butyrobetaine hydroxylase. 3-Hydroxypropionic acid was converted to glucose and metabolized to CO_2 by the glycolytic pathway and tricarboxylic acid cycle. The drug is metabolized mainly in the liver. No information on the metabolism of mildronate in humans has been published.

MECHANISM OF ACTION STUDIES

Carnitine-Dependent Mechanism of Action

The very design of the drug was aimed at interference with carnitine metabolism. In humans and other mammals carnitine is synthesized from amino acids, lysine and methionine. Methylation of lysine proceeds as a post-translation modification of several proteins. Trimethyllysine (TML) by trimethyllysine aldolase (TMLD) is released from degraded proteins and hydroxylated to 3-hydroxytrimethyllysine (HTML) in mitochondria in the presence of Fe^{2+} ions and ascorbic acid. Aldolytic cleavage of HTML by 3-hydroxytrimethyllysine aldolase (HTMLA) with release of glycine and trimethyl aminobutyraldehyde is followed by oxidation of the latter by trimethylaminobutyrate dehydrogenase (TMABA) in the presence of NAD to give rise to γ-butyrobetaine (GBB). GBB is hydroxylated by GBB hydroxylase [cynonym: butyrobetaine dehydrogenase (BBD)] to yield carnitine (Fig. 1). The last step of the carnitine biosynthesis in humans takes place in the liver, kidney, brain and testes. Expression of GBB hydroxylase protein and its mRNA has been detected exclusively in these organs. Carnitine is transported across the cell membranes by organ-specific Na^+-dependent organic cation transporters, OCTN-2 (expressed in the kidney, myocardium, placenta, brain cortex, and hippocampus) and OCTN-1. The latter has lower affinity to carnitine and is expressed in the small intestines, liver and kidney. Neutral and basic amino acid transporter ATB0+, the third transporter, with low affinity to carnitine, is expressed in liver and brain. In myocardium, muscle and other cells carnitine is involved in the fatty acid transport into mitochondria (Fig. 2). Cytosolic long-chain fatty acids, which are present as CoA esters are trans-esterified to carnitine in

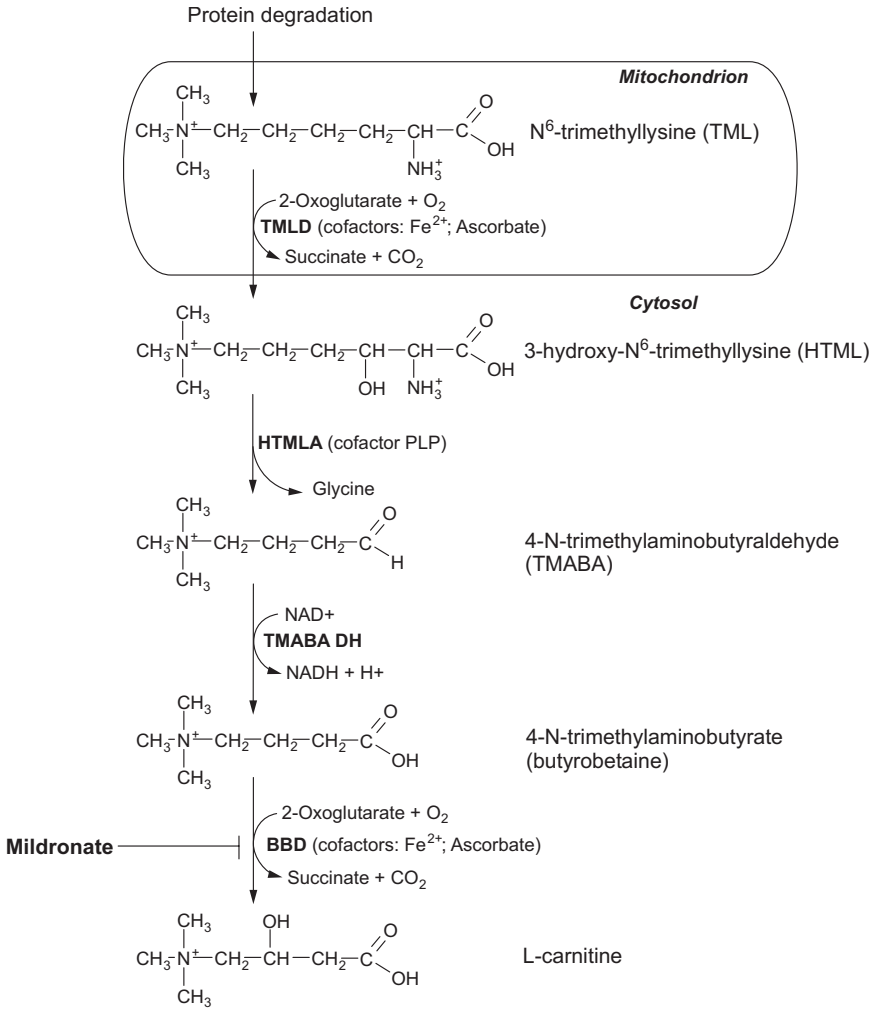

Fig. 1. Carnitine biosynthesis with the site of the inhibitory action of mildronate.

the reaction, and catalyzed by the carnitine palmytoiltransferase I (CPT I). The resulting long-chain acylcarnitine esters are transported over the inner mitochondrial membrane by carnitine-acylcarnitine translocase (CACT). On the matrix side of the mitochondrial membrane the long-chain fatty acids are transesterified to intramitochondrial CoA; this reaction is catalyzed by carnitine palmitoyltransferase II (CPT II). The long-chain CoA complexes are involved in the β-oxidation cycle. The released carnitine forms a complex with acetyl residue in the reaction catalyzed by carnitine acetyltransferase (CAT). This complex can leave mitochondria via carnitine-acylcarnitine translocase (Fig. 3) (67).

Profound effects of the drug on carnitine levels and intensity of fatty acid β-oxidation have been reviewed elsewhere (10). The studies were performed to elucidate the mechanism of cardioprotective action of mildronate. Because of the general importance of this mechanism these studies are briefly summarized here as well. Simkhovich and colleagues

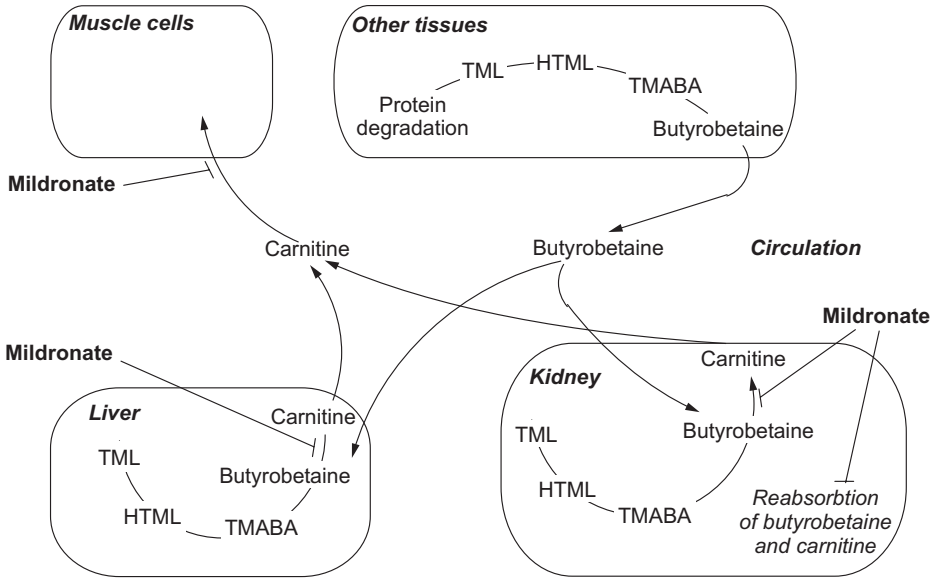

Fig. 2. Scheme of carnitine metabolism on the organism level with the indication of mildronate's action sites.

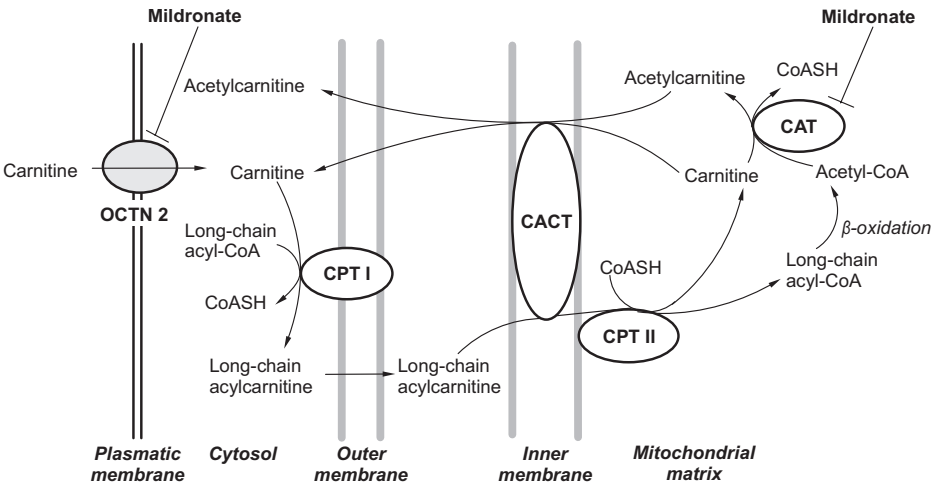

Fig. 3. Transport of fatty acids and fatty acid β-oxidation in mitochondria with the sites of the inhibitory action of mildronate.

performed pioneering studies on this mechanism. They demonstrated that in rats mildronate decreases carnitine and long-chain acylcarnitine levels, as well as oxidation of [^{14}C]palmitate (54). This effect was reproduced in the animals maintained on a fat-rich diet and in the isoproterenol-induced heart failure model (54,55). In rabbits the drug produced similar effects on carnitine level (57). More detailed molecular studies indicated that the enzyme γ-butyrobetaine hydroxylase, which catalyzes the last step of carnitine

biosynthesis, is the main target of mildronate. The drug partially and non-competitively inhibited the purified enzyme (56). The inhibition of β-oxidation led to an increase in fatty acid levels in the serum of mildronate-treated rats (51), as well as to carnitine-independent fatty acid oxidation in mitochondria (29). It is of interest that, although mildronate increases fatty acids in animals with normal lipid metabolism, it appears to normalize plasma lipids in rats with hyperlipidemia induced by triton WR-1339 (42). Other investigators also reported efficient inhibition of carnitine biosynthesis by mildronate. Tsoko et al. (66) reported that in addition to reducing carnitine concentration in the heart, skeletal muscles and kidneys of rats, mildronate produces a compensatory increase in acyl-CoA synthetase and carnitine palmotoyltransferase I in liver mitochondria and in peroxysomal fatty acid oxidation. The high affinity of mildronate to γ-butyrobetaine hydroxylase is utilized in purification of the enzyme by affinity chromatography (18). Mildronate appears to antagonize fenofibrate-induced increase in liver carnitine levels (65). In a thorough study of enzyme activities in mildronate-treated rats Spaniol et al. (62) found that the drug inhibits γ-butyrobetaine hydroxylase competitively, and decreases carnitine levels in plasma, liver and muscles.

In addition to inhibiting carnitine biosynthesis, mildronate appears to block carnitine transport inside mitochondria by inhibiting carnitine acyltransferase (52). It has been also shown that mildronate inhibits Na^+-dependent carnitine transport into the cultured myotubules (19) and isolated myocytes (35). In addition to the blockade of carnitine biosynthesis, an increased renal carnitine excretion and competitive inhibition of carnitine transport by rat renal brush-border membrane are considered to be important in the mechanism of action of mildronate (62). Some authors consider the increase in the renal clearance of carnitine to be mainly responsible for the mildronate-induced decrease in plasma levels of carnitine (35) (Fig. 3).

Inhibition of carnitine biosynthesis by mildronate alters the gene and protein expression pattern in the myocardium. It has been reported that improvement of the ventricular diastolic dysfunction induced by congestive heart failure can be achieved by improving the uptake of Ca^{2+} ions in the sarcoplasmic reticulum (23) It has been also demonstrated that mildronate prevents reduction in the sarcoplasmic reticulum Ca^{2+}-ATPase and hexokinase I protein levels following myocardial infarction. This effect is attributed to the suppression of carnitine biosynthesis and compensatory increase in the expression of enzymes involved in glucose metabolism (70). A promoter of sarcoplasmic reticulum Ca^{2+}-ATPase contains sequences that are expected to respond to transcription factors that are responsive to glucose metabolites (48). Following mildronate treatment there is a compensatory increase in myocardium of expression of several genes encoding the enzymes of lipid metabolism: lipoprotein lipase, fatty acid translocase, carnitine palmitoyltransferase I, and of enzymes involved in the synthesis of triacylglycerols (11).

In general, inhibition of carnitine biosynthesis, transport, and reabsorption reduces 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 (10,48,50,56).

Possible Mechanisms of a Rapid Response to Mildronate

The above proposed mechanism of mildronate action that involves inhibition of the fatty acid β-oxidation can hardly explain the pharmacological effects of mildronate in the

central nervous system, since brain cells utilize normally glucose as their sole source of energy. However, carnitine is synthesized in brain cells, and GBB hydroxylase gene is expressed in the brain (67). This may indicate that carnitine has a function in the brain that is unrelated to fatty acid transport. Moreover, inhibition of β-oxidation can be achieved only after repeated (for several days) administration of mildronate. Several other findings indicate that mildronate elicits several acute cardiovascular effects following single administration of the drug (46,50). It has been reported that a bolus dose of mildronate increased the survival of animals after experimental myocardium infarction and improved bioenergetics of ischemic myocardium in rats (46). A single intravenous injection of mildronate increased blood flow in the aortic arch and decreased peripheral vascular resistance in anesthetized cats. In dogs, it increased blood flow in carotid, mesenteric and femoral arteries. In isolated rabbit ear arteries mildronate reduced epinephrine-induced spasms. It also prevented cardiac insufficiency symptoms caused by stenosis of pulmonary artery in cats (M. Veveris, personal communication).

In the clinical study by Enina et al. (15) i.v. mildronate produced a transient decrease in arterial pressure and modified several parameters of cerebral circulation. In another clinical study a single dose of mildronate normalized cerebrovascular reactivity for 60 to 90 min (41). It has been also reported that mildronate and γ-butyrobetaine (GBB) combined eliminated physiological effects of nitric oxide synthase (NOS) inhibitors (31).

Sizova et al. (58) studied effects of mildronate in *ex vivo* system and found that it increases sensitivity of β-adrenoceptors in aortic rings. Similarly, Chiba et al. (8) reported that mildronate, *ex vivo* and at high concentrations, produced negative chronotropic and inotropic effects in canine atrial or ventricular preparations. At 10^{-9}–10^{-6} M mildronate had no chronotropic or inotropic effects in spontaneously beating isolated right atria, but decreased the rate and force of atrial contractions at 10^{-4}–10^{-5} M. These effects were not antagonized by atropine, suggesting that they do not involve muscarinic mechanisms. Also, in electrically paced, isolated left ventricular preparations mildronate had slight negative inotropic effect, but only at extremely high concentrations (8). Mildronate has been reported to promote wound and ulcer healing (37–39,44), although inhibition of carnitine biosynthesis or blockade of its transport should be expected to inhibit cell proliferation (19).

Mildronate interferes with the activity of membrane receptors and secondary messengers (24); triggers DNA replication, repair and methylation (5,6,50), and is capable of triggering RNA-polymerase activity in isolated neurons *in vitro* (61). An increase in the pre-mRNA synthesis and a decrease in the ADP-ribosylation of loosely bound chromatin non-histone proteins have been observed in the rat liver, spleen, heart and intestines at 6 h after administration of mildronate (7). None of the above effects could be explained by inhibition of carnitine biosynthesis, so that mildronate may have an additional, rapid and probably receptor-dependent mechanism of action.

The hypothetical existence of a non-conventional function of carnitine and its precursor GBB as well as the likelihood of pharmacological interference with this function triggered the design of mildronate by I. Kalvinsh. It has been reported that in addition to being a carnitine precursor, GBB can undergo esterification in mammalian brain tissue (26). The structure of γ-butyrobetaine ethyl ester strikingly resembles that of acetylcholine. The distance between positively and negatively charged poles in both molecules are almost identical. I. Kalvinsh suggested the existence of a specific signal transfer system based on GBB esters (30). The possibility of the existence of such a system is sug-

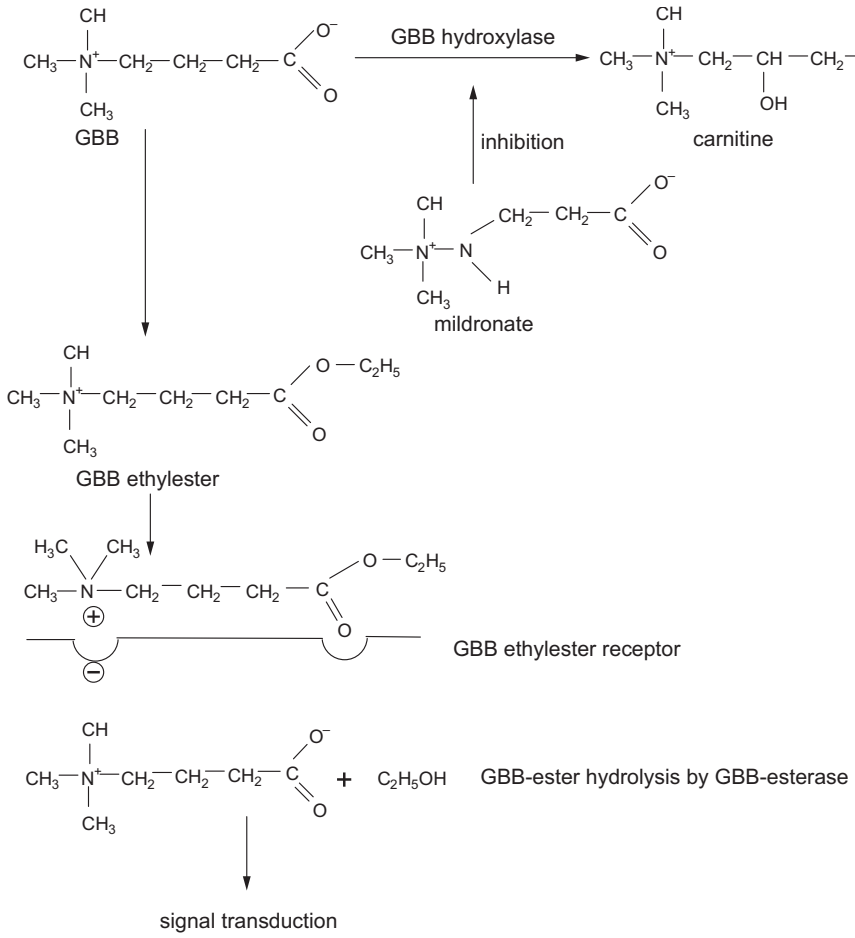

Fig. 4. Hypothetical GBB-esterase mechanism.

gested by a reported increase in GBB levels in stressed animals (64), and the cholinergic activity of GBB esters (27). The proposed hypothetical mechanism could consist of the following steps: a) Mildronate shifts the equilibrium between GBB hydroxylation to carnitine and GBB esterification towards GBB esters. Trace amounts of GBB esters are physiologically active, and the same reaction may take place in other organs, so that the onset of action of mildronate should be rapid; b) GBB ester binds to its specific receptor; GBB esterase (acting like acetylcholineesterase) hydrolyzes esters; and c) GBB ester hydrolysis triggers signal transduction. Secondary messengers can be involved in the process (Fig. 4).

We performed several studies to support the above hypothesis. The first objective was to identify GBB esterase activity in mammals. We detected the existence of its enzymatic activity in rat blood serum. A chain substituted derivative of GBB ester that can be detected spectrophotometrically was synthesized. This substance was stable in aqueous solution. After its incubation with rat blood serum HPLC revealed a peak corresponding to

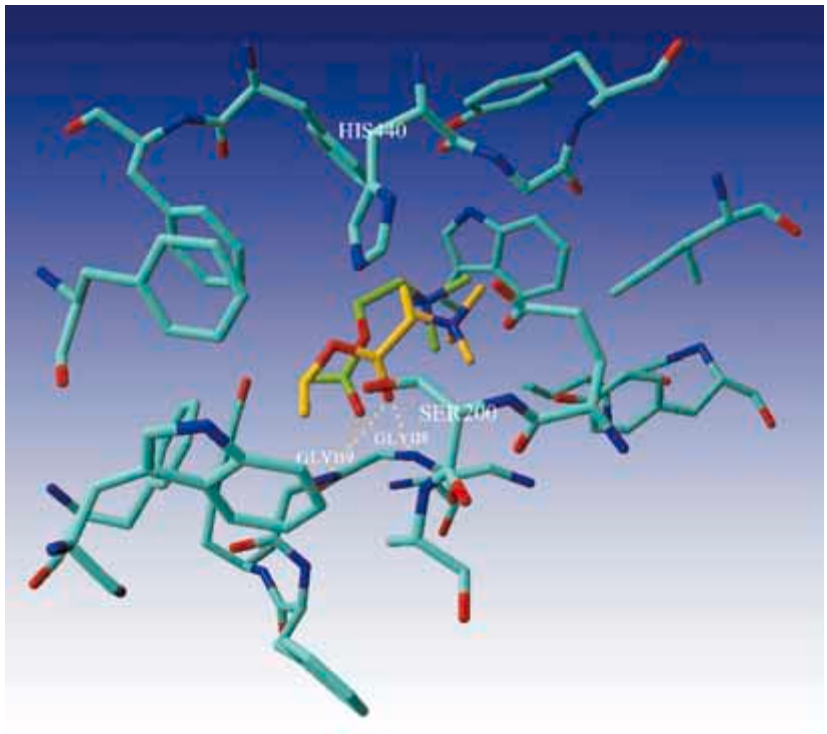

Fig. 5. Docking results of GBB ethyl ester (yellow) and acetylcholine (green) into the active center of native acetylcholinesterase from *Torpedo californica* (Protein Data Bank Code is 1ea5). Missing residues were added using software Jackal (Honig, Columbia University). For docking experiments residue His-440 was taken in non charged form with a proton on the Nδ atom. All hydrogens and electron lone pairs were added, and the resulting structure was minimized using Amber94 force field on MOE software (Chemical Computing Group, Montreal, Quebec, Canada). Structures of GBB ethyl ester and acetylcholine were built on MOE software with subsequent minimization using MMFF94s force field. Docking experiments were performed on a Grid software package (Molecular Discovery Ltd., United Kingdom) with full ligand flexibility. Image was created on Yasara software (YASARA Biosciences, Graz, Austria).

the GBB analog. Up to 60% of the initial ester was hydrolyzed in an hour. Acetylcholine did not compete with this reaction, and the reaction was insensitive to acetylcholine inhibitors. Moreover, purified acetylcholinesterase could not catalyze the reaction; butyrylcholinesterase was also inactive. We concluded that a specific enzyme GBB-esterase is likely to be present in mammals and are continuing its evaluation (43). The existence of the GBB esterase does not exclude the action of GBB esters via acetylcholine receptors. Recently published *in vitro* data (9) showed that GBB methyl ester is a potent agonist at muscarinic acetylcholine receptors; GBB affinity to these receptors is much lower. A computer model of molecular interactions between the GBB ethyl and methyl esters and the active center of acetylcholine esterase (Fig. 5) indicates that acetylcholine and GBB ethyl ester have the same binding modes and suggests a possible hydrolysis of GBB esters by this enzyme.

Since mildronate and γ-butyrobetaine (GBB) combination has been reported to eliminate vasoconstriction produced by nitric oxide synthase (NOS) inhibitors (31), it is con-

ceivable that nitric oxide is a transmitter in the GBB esterase signaling pathway. Mildronate might act also via a nitric oxide-dependent mechanism. In preliminary experiments we attempted to study possible effects of mildronate on NO levels 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). Mildronate (50 mg/kg, i.p.) triggered a slight but reproducible wave-like increase in NO level in the brain cortex and cerebellum at 30 min after drug administration If NOS inhibitor, N^{ω}-nitro L-arginine (50 mg/kg; i.p.), was administered together with mildronate, there was a pronounced decrease in NO levels indicating the dependence of the effect of mildronate on the activity of NOS. This was the first indication of a putative NO-dependent mechanism of mildronate action. Interestingly, this effect was pronounced in the brain, where no carnitine biosynthesis is expected to take place. Moreover, the time course of the effect resembled that of vascular effects of mildronate described by Enina et al. (15). In our later studies the NO-producing effects of mildronate were studied in comparison with those of γ-butyrobetaine and GBB esters. We observed a transient mildronate- and GBB and GBB methyl ester-induced increase in NO levels in the rat blood and myocardium (60). The effect of GBB methyl ester was similar to those of GBB or mildronate, but it was effective at much lower concentrations. The effects of the GBB and mildronate combination and GBB ester on NO levels in the cortex and cerebellum are presented in Fig. 6 (13). Combination of GBB (30 mg/kg i.p.) and mildronate (120 mg/kg i.p.) induces a short-term increase of NO level in the brain cortex that is detectable only at 15 min after the injection (Fig. 6A). A similar effect is produced by the GBB methyl ester administered at thousand times lower concentration (150 ng/kg). The ester increases NO levels also in the cerebellum (Fig. 6B). Changes in NO levels in the rat brain and cerebellum after administration of GBB ethyl ester at different doses are shown in Fig. 6C. The ethyl ester appears to increase NO synthesis in the brain cortex as well as cerebellum; at 0.3 mg/kg it doubles NO levels. *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 ($EC_{50} = 5 \times 10^{-5}$ M), mildronate alone had no vasodilator effect; it enhanced, however, the vasodilator activity of GBB. GBB methyl and ethyl esters were found to be more potent vasodilators ($EC_{50} = 2.5 \times 10^{-6}$ M). Pretreatment of aortic rings with NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester, abolished the vasodilator effects of both esters (60). 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 GBB-induced endothelium- and NOS-dependent responses. These data suggest that the acute antiischemic effects of mildronate may be in part due to enhancement of NO formation by endothelium. As none of the studied compounds could modify the NOS activity *in vitro*, our results suggest that some receptor-mediated mechanisms may participate in the activation of NO formation in the blood vessels. Both, still hypothetic GBB esterase-dependent receptors (GBB esterase is not hypothetical any more!), and acetylcholine receptors could be involved. Cholinomimetic activity of GBB esters and similar compounds has been known for some time (26). Mildronate ethyl ester is even considered to be a synthetic analog of acetylcholine (40). The binding of GBB methyl ester to muscarinic acetylcholine receptors also supports this possibility (10). The synergistic effect of mildronate and GBB may involve esterification

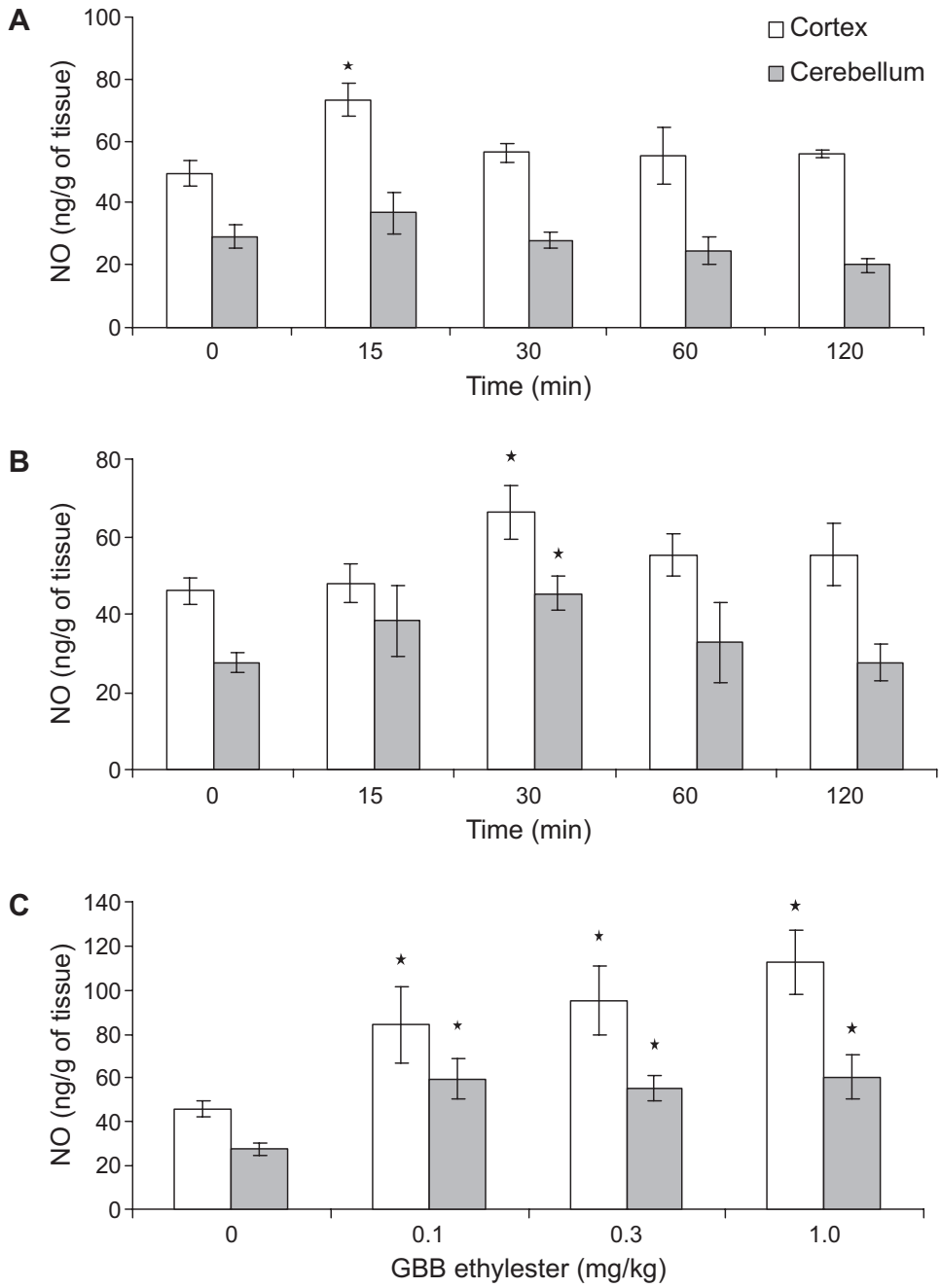

Fig. 6. Effects of mildronate: GBB composition and GBB esters on NO production rate in rat brain. **A.** Time course of changes in NO level after intraperitoneal administration of GBB (30 mg/kg) + Mildronate (120 mg/kg). Abscissa, time; ordinate, NO concentration, ng/g tissue. **B.** Time course of changes in NO level after intraperitoneal administration of GBB methyl ester (150 ng/kg). Abscissa, time; ordinate, NO concentration, ng/g tissue. **C.** Changes in NO concentration 30 min after intraperitoneal administration of GBB ethyl ester at different concentrations. White boxes, cortex; gray boxes, cerebellum. Stars indicate statistically significant difference vs. the "0" group ($p < 0.05$). Vertical bracket-line lines indicate S.E.M.

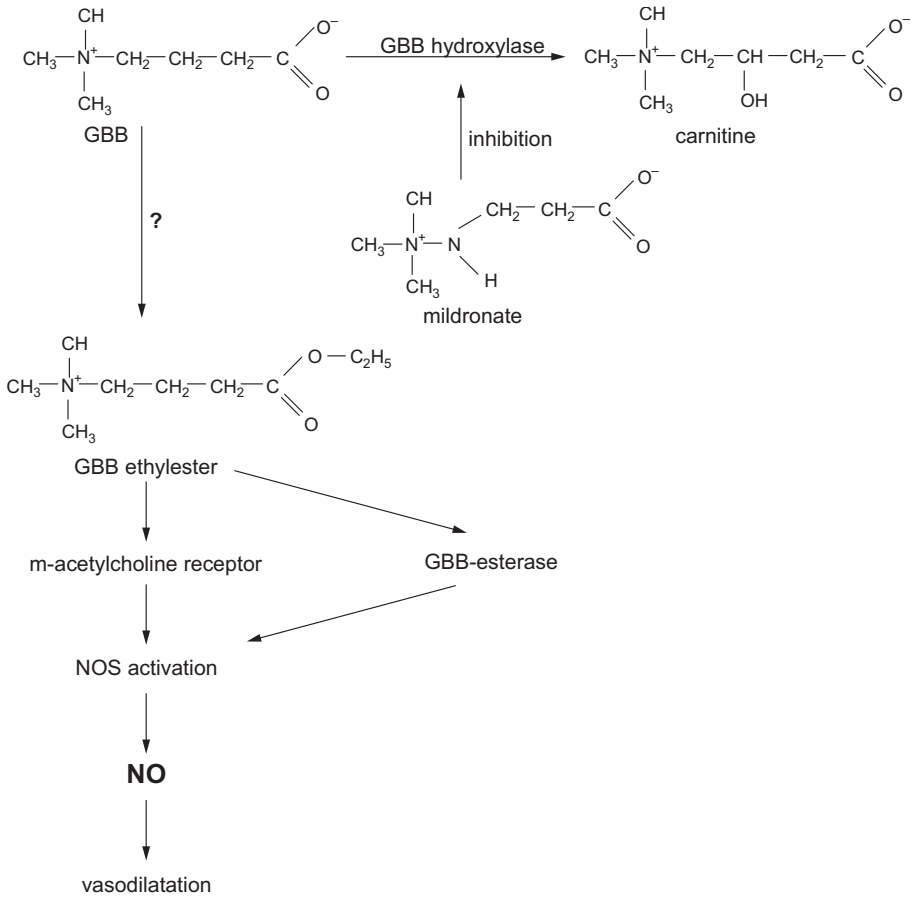

Fig. 7. Scheme of NO-dependent mechanism of acute action of mildronate.

of GBB, as GBB esters produce their vasorelaxing effects at much lower concentrations than GBB itself. These results suggest that mildronate's antiischemic action could be mediated, at least in part, by stimulation of NO production in the vascular endothelium through modification of the GBB/GBB ester pools. This stimulation of NO formation might be rationalized in the following ways (Fig. 7): (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 eNOS (endothelial nitrite oxide synthase). We can still speculate that a specific GBB-esterase 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. The presence of GBB esters in living tissues was described more than thirty years ago, although their physiological significance is still poorly understood (26). As GBB esters 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 (25), an activity that might be mediated by esterification because carnitine esters also possess cholinergic activity (26).

Thus, it appears that the mechanism of acute action of mildronate is close to being elucidated. The existence of the CNS-specific mechanism of action of the drug validates the conclusions of the experimental studies and of clinical trials, which demonstrated the efficacy of mildronate in the treatment of cerebrovascular disorders.

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