REVIEW

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Mildronate and its Neuroregulatory Mechanisms: Targeting the Mitochondria, Neuroinflammation, and Protein Expression

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***Key Words**: mildronate; neuroprotection; memory; mitochondria; neuroinflammation; protein expression.*

***Summary.** This review for the first time summarizes the data obtained in the neuropharmacological studies of mildronate, a drug previously known as a cardioprotective agent. In different animal models of neurotoxicity and neurodegenerative diseases, we demonstrated its neuroprotecting activity. By the use of immunohistochemical methods and Western blot analysis, as well as some selected behavioral tests, the new mechanisms of mildronate have been demonstrated: a regulatory effect on mitochondrial processes and on the expression of nerve cell proteins, which are involved in cell survival, functioning, and inflammation processes. Particular attention is paid to the capability of mildronate to stimulate learning and memory and to the expression of neuronal proteins involved in synaptic plasticity and adult neurogenesis. These properties can be useful in neurological practice to protect and treat neurological disorders, particularly those associated with neurodegeneration and a decline in cognitive functions.*

Introduction

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate dihydrate] was designed in the early 1980s at the Latvian Institute of Organic Synthesis, Riga, Latvia, and indicated as a cardioprotective drug, particularly for the treatment of angina and myocardial infarction (1–4). Traditionally, the mechanism of action of this drug is postulated as the inhibition of gamma-butyrobetaine hydroxylase leading to the suppression of carnitine biosynthesis and, hence, to the lowering of carnitine concentration in the heart tissue and accumulation of gamma-butyrobetaine, a carnitine precursor (1, 5, 6). Therefore, it has been suggested that the beneficial effect of mildronate is provided by a lowered level of carnitine that, in turn, results in reduced fatty acid oxidation and shifted energy metabolism from free fatty acids toward predominantly glucose utilization and increased effectiveness of ATP generation.

Thus, the postulated carnitine-lowering mechanism of mildronate contradicts the well-known notion about the vital role of carnitine in energy production and fatty acid metabolism. Carnitine deficiency occurs in the aberrations of carnitine regulation in disorders such as diabetes, sepsis, cardiomyopathy, malnutrition, cirrhosis, and endocrine disorders and with aging. Moreover, the clinical application of carnitine holds much promise in a range of neural disorders, such as Alzheimer's disease, hepatic encephalopathy, and painful neuropathies (7). Carnitine is found as a neuroprotector in the mitochondrial dysfunction model (8), and its metabolite acetyl-L-carnitine has been shown to protect dopamine-producing cells in the Parkinson's disease model (9). This metabolite maintains cellular membrane stability and restores age-related membrane changes through antioxidant effects, normalization of the levels of the nerve growth factor in the brain tissue, and stimulation of cerebral blood flow and biosynthesis of acetylcholine (10–13).

Our concept of the mechanism of the action of mildronate essential for its influence on the central nervous system (CNS) differs from the traditional explanation. We consider mildronate as a molecule with its similar structure to that of carnitine (Fig.), taking mildronate as the carnitine-mimicking substance or "false carnitine." Hence, the overload of the cell with mildronate leads to a negative feedback and a compensatory reduction of intracellular carnitine. There are a lot of arguments that encourage us to think like that. One of them is the data (14) demonstrating dose-dependent increasing concentrations of mildronate in the brain cerebellar tissue and a concomitant reduction of the carnitine level after 2-week intraperitoneal injections of mildronate (100 and 200 mg/kg) in rats. When the concentrations of L-carnitine in the control group and

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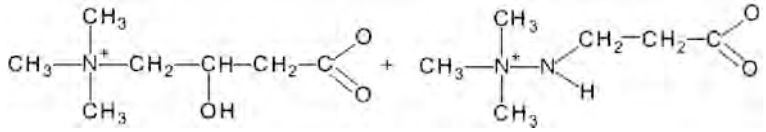

Fig. Structures of carnitine and mildronate

the sham-operated rat cerebellum were 166±8 and 161±5 nmol/g, respectively, mildronate administration reduced the L-carnitine concentration by 2- and 3-fold, respectively (to 56±3 nmol/g at 200 mg/kg); at the same time, mildronate occurred in the brain at the concentrations of 104±12 nmol/g (100 mg/kg) and 154±9 nmol/g (200 mg/kg). Similarly, cotreatment with L-carnitine and mildronate that leads to a diminished cardioprotective effect (15) may be explained by cell "saturation" with both substances that switches on the inhibitory self-protecting mechanisms to avoid this overload. This explanation is in good line with the data demonstrating that mildronate in like manner to carnitine is transported to intracellular targets of different tissues by a carnitine transporter, organic cation transporter type 2 (OCTN2), and the efficiency of transport of mildronate is even higher than that of carnitine (16). In addition, a kinetic analysis (17) revealed that mildronate interacted with the substrate-binding site for carnitine and competitively and strongly inhibited the mitochondrial carnitine/carnitine antiport. Thus, in vivo, the antiport reaction between cytosolic (administered) mildronate and matrix carnitine may cause intramitochondrial carnitine depletion (17). Mildronate was also found to inhibit carnitine acetyltransferase in a competitive manner through binding to the carnitine-binding site (18).

Putting together these data, we suggest that mildronate may fulfill the assignment of "replacement therapy" during the deficiency of carnitine. One cannot also ignore the similarity of the mildronate structure to another natural substance – betaine (glycine betaine) – which is known as a molecular chaperone capable of stabilizing the protein conformations (19–21). In this context, mildronate is considered as a synthetic aza-analogue of betaine. Nowadays, small molecules that have optimal pharmacophores (e.g., positively charged moieties) are suggested as capable of targeting the protein-protein interactions, affecting protein conformations (acting as chemical chaperons), and at least in part, acting as multiprotein coactivators of gene transcription (22, 23). Hence, small molecules might play a role of multivalent regulators of intracellular processes and have beneficial protective effects to halt neurodegenerative processes.

Our studies carried out in the Department of Pharmacology, Faculty of Medicine, University of Latvia, Riga, Latvia, during the last 5–7 years, were mostly focused on revealing the protein-stabilizing properties of mildronate in different neurotoxicity/neurodegeneration models in rodents. The present review summarizes our previously obtained data (24–31), which demonstrated the influence of mildronate on mitochondrial processes and the expression of nerve cell proteins involved in the essential pathways for cell survival and functioning. Besides, the effectiveness of mildronate at much lower doses of 20 and 50 mg/kg in comparison with the traditionally recommended doses typical for cardioprotection (100 and 200 mg/kg) has been demonstrated.

Neuroprotective Effects in Neurotoxicity/Neuroregeneration Models

Azidothymidine Neurotoxicity Model in Mice

In our studies (28), we used azidothymidine (AZT), a well-known anti-HIV drug of the nucleoside reverse transcriptase class, possessing a toxic influence on mitochondria. It depleted mitochondrial DNA and caused the dysfunction of the mitochondrial respiratory chain leading to anaerobic ATP synthesis and accumulation of lactic acid, ultrastructural changes of mitochondria, and oxidative stress by the activation of reactive oxygen species (32, 33). Particularly, the toxic influence of AZT is manifested on mitochondrial complex I (34). In turn, mitochondria play an essential role in the cell by providing energy and controlling cell survival; therefore, compromised mitochondria are considered as a key component implicated in the etiopathogenesis and development of neurodegenerative diseases, such as Parkinson's disease (35). The protection or repair mechanisms, particularly at the level of complex I, may be a promising strategy to limit mitochondrial damage and ensure cellular integrity (36).

In our experiments (28), AZT injected intraperitoneally in mice (50 mg/kg for 2 weeks) induced changes in the expression of cerebral tissue biomarker proteins (assessed immunohistochemically and by Western blot analysis), which are involved in mitochondrial functioning and apoptotic events.

Cytochrome c Oxidase, Caspase-3, Cellular Apoptosis Susceptibility Protein, Glial Fibrillary Acidic Protein, Inducible Nitric Oxide Synthase. Firstly, cytochrome c oxidase, known as a marker of the

mitochondrial respiratory chain function (37), was slightly but statistically significantly increased in comparison with the control values, while caspase-3, a biomarker protein of apoptosis (38, 39), was overexpressed by more than 2-fold (5.00±1.00 vs. 2.00±1.00, cells/mm^2, P=0.004) and cellular apoptosis susceptibility protein (CAS) (37) by about 10-fold (51.0±8.0 vs. 5.0±1.4, cells/mm^2, P=0.0007). Moreover, AZT caused a greater expression of neuroinflammation biomarkers, such as glial fibrillary acidic protein (GFAP) and inducible nitric oxide synthase (iNOS), which were related to key proteins usually overexpressed during the manifestation of inflammation (40, 41). Mildronate administration at the doses of 50 and 100 mg/kg intraperitoneally for 2 weeks acted against AZT-induced alterations in the brain tissue: it normalized cytochrome c oxidase and GFAP overexpression and protected against an increase in caspase-3, CAS, and partially iNOS expression in the brain tissue. Mildronate also reduced lymphocyte infiltration in the brain tissue caused by AZT. Thus, these data demonstrated a capability of mildronate to normalize the expression of enzymes, which were involved in mitochondrial functioning and inflammation and which were altered by a mitochondria-compromising agent. Therefore, the antineuroinflammatory activity of mildronate can be put forward to explain its action.

These data are in good agreement with our previous data obtained in the experiments with mitochondria isolated from the rat liver (24), which demonstrated that mildronate (10 μmol mg^{-1} protein) significantly protected mitochondria compromised by AZT. Mildronate reduced the generation of hydrogen peroxide, which was dramatically increased by AZT, and protected the AZT-caused inhibition of uncoupled respiration, ADP/oxygen ratio, and transmembrane potential. These data confirm the action of mildronate, which to a great extent can be targeted at mitochondria, particularly complex I.

Rat Model of Parkinson's Disease

Parkinson's disease is clearly defined as a neurodegenerative disorder that involves multifactorial mechanisms. Besides mitochondrial dysfunction, neuronal apoptosis, neuroinflammation, and microglial activation, numerous mutations in genes encoding a series of proteins, particularly α-synuclein (SNCA), Parkin, DJ-1, PTEN-induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), and 5-hydroxytryptamine receptor 2A (HTR2A), etc., lead to a progressive loss of dopamine-producing cells of the nigrostriatal system (42, 43). Unfortunately, the conventional therapy for Parkinson's disease is focused on the consequences caused by neuronal death, when the typical symptoms of Parkinson's disease are manifested. This means that the treatment is directed to stimulate the already degenerating dopamine cells and is eloquently named as "beating a dead horse" (44). Therefore, a revision of the effectiveness and usefulness of traditional drugs has been started. For instance, dopamine neurotoxicity in the early stages of Parkinson's disease has demonstrated the unsuitability of levodopa for the treatment of Parkinson's disease in this stage (45), which indicates a new nondopaminergic pathogenesis of Parkinson's disease and dopamine-replacement possibilities (44). Recently, novel pharmacotherapeutic strategies in Parkinson's disease have been developed with a focus on cellular targets for the prevention of cell death at the earlier stages of neurodegeneration cascades, related to mitochondria, inflammation, and protein pathologies (46).

Influence on the Expression of Proteins Involved in Cell Integrity and Regeneration

Our studies (25, 26) were carried out in the rat Parkinson's disease model, induced by a unilateral (right) intrastriatal injection of 20 μg/3 μL 6-hydroxydopamine (6-OHDA), a neurotoxin that induces the destruction of dopamine-producing cells. Mildronate (50 or 100 mg/kg) was intraperitoneally administered for 2 weeks before the injection of 6-OHDA. Artificial cerebrospinal fluid (aCSF) injected intrastriatally and intraperitoneal saline served as the controls. Mildronate, mostly at a dose of 50 mg/kg, significantly reduced apomorphine-induced rotations on day 21 after surgery (78.8±13.5 vs. 6-OHDA control, 147.0±20.9, P=0.003), indicating its capability to attenuate behavioral disturbances in a Parkinson's disease model by restoring the balance in the dopaminergic nigrostriatal pathways. At the end of the testing of behavioral responses, rats were sacrificed, and the expression of different proteins in the 6-OHDA-lesioned striatum and in the substantia nigra was assessed.

Tyrosine Hydroxylase. First of all, we examined (25) the expression of tyrosine hydroxylase (TH), a key enzyme associated with dopamine synthesis. We found that 6-OHDA-induced lesions caused a dramatic (5-fold) decrease in TH-positive nerve endings in the lesioned striatum (5±2 vs. 21±10 nerve endings/per mm^2, P=0.03) and approximately a 2.5-fold decrease in the substantia nigra (44±14 vs. 95±30 neurons/mm^2, P=0.01) in comparison with the aCSF control levels. Mildronate (50 and 100 mg/kg) provided a complete protection against the 6-OHDA-induced degeneration of TH-positive nerve endings in the lesioned striatum in comparison with the 6-OHDA control (25±4 vs. 5±2, nerve endings/mm^2, P=0.001; 31±3 vs. 5±2 nerve endings/mm^2, P=0.0002, respectively), as well as protected against the loss of TH-positive dopaminergic

neurons in the substantia nigra (100 mg/kg, 78±16 vs. 44±14 neurons/mm^2, P=0.04). Therefore, our data demonstrated the neuroprotective properties of mildronate against a 6-OHDA-induced loss of TH expression. These findings raise the following question: what are the crucial mechanisms responsible for this protective action of mildronate? Therefore, we paid attention to different proteins that are essentially involved in cell survival and integrity.

Heat Shock 70 kDa Protein. One of very important protein groups is molecular chaperones, which comprise several highly conserved families of proteins, which recognize and selectively bind nonnative proteins to form stable complexes, hence, preventing misfolding and aggregation of folding intermediates (47–49). The heat shock protein (HSP) family is the most commonly studied group, which also exhibits a variety of cytoprotective functions and inhibition of apoptosis (50). It has been demonstrated that the decreasing levels of HSP and SNCA aggregation in Lewy bodies coincide with the progression of Parkinson's disease (51). Logically, HSP70 overexpression has been found to reduce SNCA accumulation and toxicity in both mouse and drosophila models of Parkinson's disease (52, 53). Nowadays, a design of chemical or pharmacological chaperones (pharmacoperons) that can unfold misfolded proteins to endogenous molecules in a similar manner is an attractive approach for the future therapies of Parkinson's disease (49, 54).

In our study (26), the injection of 6-OHDA considerably decreased HSP-70 immunostaining in both the striatum and the substantia nigra. These findings are in agreement with literature data, which showed that a loss of HSP70 activity is associated with neurodegeneration (52). Mildronate per se did not influence HSP70 expression, whereas its pretreatment at the doses of 20 mg/kg and, particularly, 50 mg/kg, increased the number of HSP70-positive cells in both examined structures. For instance, in the 6-OHDA-lesioned striatum, the expression of HSP70 in the presence of mildronate at a dose of 50 mg/kg reached about 10-fold higher values than those in the 6-OHDA control (23±2 vs. 2±1 cells/mm^2), i.e., about 5-fold higher in comparison with the aCSF control (23±2 vs. 7±2 cells/mm^2). These data showed that mildronate in neurodegenerative conditions may regulate (normalize) the expression of endogenous molecular chaperone HSP70, thus, acting as a chemical chaperone molecule.

Ubiquitin. Another protein we considered worthwhile to assess was ubiquitin since the ubiquitin/proteasome system (UPS) is the major proteolytic quality control system in cells, and ubiquitin-enriched Lewy bodies in dopaminergic neurons are a hallmark of Parkinson's disease (55). A high level of ubiquitin in Lewy bodies indicates that protein degradation by proteasomes is impaired (56). Normally, proteins that are conjugated to ubiquitin are targeted at the UPS where they undergo proteolytic degradation; thus, ubiquitination pathways may play a central role in the pathogenesis of Parkinson's disease (57) and may serve as a drug target. Our experiments (25) showed that mildronate at the doses of 50 and 100 mg/kg completely prevented (to the control level) an OHDA-induced increase in the level of unconjugated ubiquitin and the formation of intracellular ubiquitin inclusions in the striatum vs. the 6-OHDA control (11±3 vs. 35±4 cells/mm^2, P=0.001, and 6±2 vs. 35±4 cells/mm^2, P=0.0002, respectively) and substantia nigra (8±2 vs. 21±2 cells/mm^2, P=0.003, and 10±3 vs. 21±2 cells/mm^2, P=0.01, respectively), leading us to the suggestion that mildronate is capable of regulating the ubiquitin proteasome pathway.

Notch-3. Another aspect related to cell survival processes is adult neurogenesis, which has a potential tool for the restoration of lost dopamine neurons and might open new vistas for the development of novel drugs in Parkinson's disease (58). In Parkinson's disesase brain structures, a decrease in progenitor cell proliferation has been demonstrated (59); however, there are also contrary data obtained in a 6-OHDA-lesioned Parkinson's disease model in rats, i.e., an increase in progenitor cell proliferation in nigrostriatal structures (60).

To assess the influence of mildronate on neurogenesis processes, we used Notch-3, a progenitor cell biomarker. The Notch family of receptors and ligands is known to play an important role in the determination of cell survival, vasculogenesis, and organogenesis, and the activation of Notch receptors has multiple roles during the CNS development, particularly during gliogenesis (61). To date, we have not found any data showing Notch-3 expression in Parkinson's disease or Parkinson's disease animal models. Our data (25) showed that the administration of 6-OHDA decreased the number of Notch-3-positive cells in both examined structures in comparison with the control group data. Mildronate per se at a dose of 50 mg/kg tended to increase the number of Notch-3-positive cells, while in the 6-OHDA-lesioned striatum and also in the substantia nigra, this dose caused a significant increase in the number of Notch-3-positive cells (9±3 vs. 3±1 cells/mm^2, P=0.045; 11±2 vs. 5±2 cells/mm^2, P=0.03, respectively), and these values were comparable with the control ones. Although the effect of mildronate on Notch-3 expression is difficult to explain, we suggest that mildronate stimulates the adult striatal progenitor cell population.

Cell-Derived Neurotrophic Factor. Very essential molecules involved in cell survival are nerve growth factors. For instance, glial cell-derived neurotrophic

factor (GDNF) is known as a substance that promotes the survival and differentiation of dopamine neurons (62, 63). It has been recently demonstrated that GDNF significantly protected against a loss of dopamine in striatal and nigral tissues in the 6-OHDA-induced rat model of Parkinson's disease (64). Furthermore, it has been confirmed that GDNF has potent dopaminotrophic effects leading to a reliable recovery of the phenotypic expression of tyrosine hydroxylase in surviving nigral neurons (65).

Our study (26) demonstrated that 6-OHDA dramatically decreased the expression of GDNF in the striatum (but not in the substantia nigra) in comparison with the control group (5±2 vs. 16±1 cells/mm^2, P=0.0004). Interestingly, mildronate per se at the doses of 20 and 50 mg/kg increased the GDNF expression in the striatum (23±2 vs. 16±1, P=0.005, and 21±2 vs. 16±1 cells/mm^2, P<0.05, respectively), while in the substantia nigra, only a tendency at a dose of 50 mg/kg was observed. Mildronate pretreatment at the doses of 20 and 50 mg/kg in the 6-OHDA group completely normalized the number of striatal GDNF-positive cells up to the aCSF control in comparison with the 6-OHDA group (18±2 vs. 5±2, P=0.001 and 24±3 vs. 5±2 cells/mm^2, P<0.001). The capability of mildronate to increase GDNF expression is considered as beneficial in comparison with that of high-molecular-weight antigenic trophic factors because mildronate is a small molecule that lacks antigenicity. We suggest that the loss of TH in our experimental PD model of Parkinson's disease can be protected at least in part by the stimulatory effect of mildronate on GDNF expression.

Nerve Cell Adhesion Molecule. Cell-cell adhesion, neurite outgrowth, synaptic plasticity, learning, and memory are also to a great extent regulated by nerve cell adhesion molecule (NCAM) (66). Our data (26) showed that mildronate per se did not influence NCAM expression in the striatum, while in the substantia nigra at a dose of 20 mg/kg, it caused a statistically significant increase in NCAM expression as compared with control (12±2 vs. 7±2 cells/mm^2, P=0.049), and this is in good agreement with some observations that showed NCAM upregulation in the 6-OHDA-lesioned substantia nigra (67). Mildronate pretreatment at a dose of 50 mg/kg increased the number of NCAM-positive cells in the 6-OHDA-lesioned striatum as compared with the 6-OHDA group (17±5 vs. 3±2 cells/mm^2, P=0.008), and these values were even higher than those of the control group (17±5 vs. 9±3 cells/mm^2, P=0.01). In the substantia nigra, mildronate pretreatment at the doses of 10, 20, and 50 mg/kg normalized the number of NCAM-positive cells increased by 6-OHDA to the control levels.

Our results demonstrating the influence of mildronate on NCAM expression indicate the capability of the drug to be involved in synaptic plasticity processes. In addition, it is known that NCAM has been identified as an alternative signaling receptor for GDNF (68).

Influence on the Expression of Proteins Involved in Neuroinflammation

It is well established that neuroinflammation plays a very essential role in the pathogenesis of Parkinson's disease (69). Moreover, glial cells are emphasized as a target for the treatment of Parkinson's disease, particularly because microglial activation precedes dopaminergic cell death and is found in the early stages of Parkinson's disease (70). If short-term gliosis promotes the formation of synapses and stimulates neurogenesis by producing energy-producing compounds and neurotrophins (71, 72), a sustained inflammatory activation of micro- and macroglia (reactive astrogliosis) induces degeneration or demyelination, which has been suggested to impede neuronal survival (72). Activated micro- and astroglia lead to the overexpression of iNOS and many proinflammatory cytokines, such as IL-1, IL-6, and TNFα, which promote neurodestruction. These neuroinflammatory events have been also observed in the Parkinson's disease model (60, 73–75).

In our previous studies, we found that the anti-inflammatory action of mildronate plays an important role in cardioprotection (76). Therefore, mildronate demonstrated a high ability of preventing AZT-induced overexpression of the nuclear factor κBp65 (NF-κBp65) in cardiomyocytes. Moreover, the anti-inflammatory action was also clearly demonstrated in the CNS in the AZT neurotoxicity model in mice (28).

Inducible Nitric Oxide Synthase. In our Parkinson's disease experiments (25), we detected a considerable increase in the expression of iNOS in the 6-OHDA-lesioned striatum and the substantia nigra, which indicated a manifestation of neuroinflammatory processes. Mildronate alone did not influence the iNOS expression, while at a dose of 50 mg/kg, it decreased the expression of iNOS in the substantia nigra by approximately 2-fold in comparison with the 6-OHDA effect (17±3 vs. 33±9 cells/mm^2, P=0.03), thus, showing that mildronate possesses anti-inflammatory activity in the Parkinson's disease model.

Glial Fibrillary Acidic Protein. Our data (25) also demonstrated that 6-OHDA induced the increased number of GFAP-positive astrocytes in the striatum (42±6 vs. 11±3 cells/mm^2, P=0.001) and in the substantia nigra (35±5 vs. 11±3 cells/mm^2, P=0.002) compared with the control group. These findings are consistent with the data of other authors who demonstrated an increase in the levels of GFAP in 6-OHDA-lesioned structures (77). The administra-

tion of mildronate alone at a dose of 50 mg/kg did not alter the GFAP level, while in the Parkinson's disease model, it reduced the number of GFAP-positive astrocytes increased by 6-OHDA in the striatum (20±4 vs. 42±6 cells/mm^2, P=0.01), and these values were comparable with those of the control group, indicating the capability of mildronate to reduce reactive astrogliosis.

Ionized Calcium-Binding Adapter Molecule 1. In our studies (25), we assessed ionized calcium-binding adapter molecule 1 (IBA-1), a microglial marker, which is specifically expressed in microglia and is upregulated when these cells are activated (74). We found that the expression of IBA-1 was increased by 2-fold in the 6-OHDA-lesioned striatum group (23±3 vs. 12±2 cells/mm^2, P=0.03) and by 2.5-fold in the substantia nigra group (32±8 vs. 11±2 cells/mm^2, P=0.04) in comparison with the control group. These findings are consistent with the results described in the literature, which showed that 6-OHDA caused neuroinflammation and microglial activation (70). Our studies showed that mildronate per se did not affect the level of IBA-1 in the 6-OHDA-lesioned striatum and in the substantia nigra; however, at the doses of 50 and 100 mg/kg, it showed a tendency to decrease the number of IBA-1-positive cells in the substantia nigra.

Endothelin 1-Induced Ischemic Stroke Model in Rats

In our experiments (29), male Wistar rats were pretreated with mildronate (100 mg/kg per os) for 7 days, followed by an intracerebral injection of endothelin 1 (ET-1) at a dose of 240 pmol/3 μL. ET-1 is a widely used substance for producing transient focal cerebral ischemia. The control group received aCSF instead of ET-1. We found that mildronate normalized (to the control levels) the poststroke functional neurological reactions, i.e., the drug reduced hemiplegias and improved walking and motor ability. Mildronate also normalized behavior in the rota-rod test by increasing the time spent on the rotating rod in the ET-1-treated animals, which was 5- to 6-fold lower in comparison with the data from the aCSF and nonoperated groups. Mildronate successfully (about by 2.5-fold) protected ET-1-induced degenerative alterations in cortical neurons assessed by the number of degenerated neurons (stained with cresyl violet). Moreover, mildronate also significantly reduced the infarction size. One may suggest that the antistroke activity of mildronate is provided via its mitochondrial, anti-inflammatory, and neuroregenerating processes.

The improvement of functional responses obtained in our experiment are in good line with other authors' data demonstrating the effects of mildronate in the stroke model caused by middle cerebral artery occlusion; however, in this model, mildronate did not influence the infarction size (14). Other authors have found that the anti-ischemic ability of mildronate is explained by its stimulatory effect on nitric oxide production in the vascular endothelium (78).

Streptozocin-Induced Neuropathic Pain Model in Rats

Streptozocin (STZ)-induced pancreatic injury is commonly used for creating the rodent models of type 1 diabetes. STZ injection to rats produces metabolic abnormalities manifesting as diabetic neuropathic pain. STZ was administered intraperitoneally at a dosage of 60 mg/kg for 2 days (a total dose of 120 mg/kg). The development of neuropathic pain was studied (79) in STZ-treated rats and assessed using the algesimeter (Ugo Basile, Italy, Model 17181) by measuring a pain threshold response to mechanical pressure in g (paw pressure test). STZ neuropathy manifested as a significant lowering of the pain threshold by about 20%–30%. The treatment with mildronate (100 mg/kg daily, intraperitoneally for 6 weeks) completely protected the development of STZ-induced neuropathic pain from the first administration week up to the end of the experiment (6 weeks), indicating the usefulness of the drug for the treatment of diabetic neuropathies.

Influence on Behavior

Anticonvulsant Activity

There are very few studies on the influence of mildronate on behavior. We found the data (80) demonstrating its anticonvulsant activity in the pentylenetetrazol seizure test, demonstrating the involvement of the alpha2-adrenergic receptor and nitric oxide-dependent mechanisms in the anticonvulsant effects of mildronate.

Memory-Enhancing Activity

Before our studies, there were no data about the influence of mildronate on memory. Recently, we have demonstrated (27) the efficacy of mildronate to improve memory after 2-hour immobilization stress-induced impairment of task performance in the passive avoidance response test (retention test after 48 hours) in rats pretreated with mildronate (50 mg/kg intraperitoneally) for 2 weeks. The Western blot analysis performed ex vivo in the hippocampal tissue demonstrated the normalization of the expression of brain-derived neurotrophic factor (BDNF), which was overexpressed after immobilization. These data are in good agreement with those obtained by other authors who demonstrated that a stress-induced increase in the expression of BDNF correlated with a decrease in memory (81). One may suggest that a BDNF-restoring action of mil-

dronate at least in part lies in its learning/memory-improving activity.

More detailed studies (31) for the first time revealed that mildronate improved learning and memory in the passive avoidance response test and the active conditioned avoidance response (CAR) test in rats. The CAR test employed bromodeoxyuridine (BrdU)-treated animals and non-BrdU-treated controls. Hippocampal BrdU incorporation was then immunohistochemically assessed in BrdU-treated, CAR-trained rats to identify proliferating cells. These results showed that mildronate administration (50 mg/kg) increased the number of BrdU/nestin-positive cells by about 4-fold in the CAR-trained rats compared with the saline-pretreated control group (21±6 vs. 5±2 cells/mm^2, P=0.03). These data indicated that both CAR training and mildronate administration increased the mitosis of neuronal progenitor cells in the hippocampus, which is an essential step for neurogenesis. To elucidate at least in part the molecular mechanisms by which mildronate improves learning and memory, the expression of a number of pertinent biomarkers, such as glutamic acid decarboxylase 65/67 (GAD65/67), acetylcholine esterase (AChE), growth-associated protein 43 (GAP-43), the transcription factors c-jun, and activator protein 1 (c-jun/AP-1/AP-1), was assessed. Mildronate pretreatment reduced the GAD65/67 immunoreactivity in the hippocampal dentate gyrus of CAR-trained rats (8±2 vs. 39±11 cells/mm^2, P=0.02), which indirectly indicated the capability of mildronate to intensify glutamatergic processes by inhibiting the conversion of glutamate to GABA. That allows suggesting that the glutamatergic system may be one of the neurochemical mechanisms of the memory-enhancing action of mildronate. Another – cholinergic – component also can be included in this action because mildronate considerably decreased the number of AChE-positive nerve fibers in the CAR-trained rats (8±3 vs. 28±6 fibers/mm^2, P=0.02). That is in good line with the traditionally accepted use of AChE inhibitors for the treatment of dementia, particularly in Alzheimer's disease. In addition, mildronate significantly increased c-jun/AP-1 immunoreactivity by more than 2-fold in the CAR-trained rats compared with the nontrained control rats that received saline. c-Jun is critical for the control of progenitor cell viability and differentiation (82).

The obtained data indicate that mildronate may stimulate learning and memory by a mechanism related to alterations in the expression of hippocampal proteins, which are involved in synaptic plasticity.

Concluding Remarks

The obtained data give a new insight into the influence of mildronate on the central nervous system. This drug shows beneficial effects in the regulation of cell processes necessary for cell integrity and survival, particularly by targeting mitochondria and by stabilizing the expression of proteins involved in neuroinflammation and neuroregeneration. These properties can be useful in neurological practice to protect and treat neurological disorders, such as Parkinson's disease, diabetic neuropathies, and ischemic stroke. Moreover, because mildronate improves learning and memory, one may suggest mildronate as a multitargeted neuroprotective/neurorestorative drug with its therapeutic utility as a memory enhancer in cognitive impairment conditions, such as neurodegenerative diseases, schizophrenia, and other pathologies associated with a decline in awareness.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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