JĀNIS KŪKA

THE REGULATION OF CARNITINE SYSTEM FOR CARDIOPROTECTION

SUMMARY OF DOCTORAL THESIS

Submitted for the degree of Doctor of Pharmacy
(Pharmaceutical chemistry)

Riga, 2011

Supervisors:

Dr. Pharm. **Maija Dambrova**

Head of Laboratory of Pharmaceutical Pharmacology, Latvian Institute of Organic Synthesis

Dr. Chem. **Renāte Medne**

Head of Department of Pharmaceutical Chemistry, Riga Stradins University

The doctoral studies were supported by the ESF Project "Support for PhD Program Studies and Earning of Scientific Degree at RSU".

The doctoral thesis was elaborated in Latvian Institute of Organic Synthesis in Laboratory of Pharmaceutical Pharmacology.

Content

Topicality of the problem

Cardiovascular diseases are the leading cause of death in Latvia and European Union. The highest mortality rates are observed in Central and East European countries, while the lowest rates are characteristic for Western and Northern European countries (Müller-Nordhorn et al., 2008). Therefore, the investigations of novel treatments to improve the survival and quality of life of cardiovascular patients are of great importance worldwide.

In order to produce adenosine triphosphate, muscle cells utilize both glucose and fatty acids as energy substrates. Under normal conditions the main energy supply for the heart is generally provided by the oxidation of fatty acids. However, under ischemic conditions a shift from fatty acid oxidation to glucose oxidation is supposed to lead to lower oxygen consumption (Beadle & Frenneaux, 2010). Carnitine regulates the intensity of long chain fatty acid utilization, and plays an important role in the regulation of glucose and fatty acid energy metabolism.

It is well-known, that carnitine facilitates the transport of fatty acids into the mitochondrial matrix, so they could be metabolized through β-oxidation to obtain energy (Strijbis et al., 2010). Physiological effects of carnitine in the muscle metabolism were discovered more than 50 years ago. Nowadays carnitine is widely used as a nutritional supplement; however pharmacological effects of carnitine, including cardioprotective activity, are not well described and studied in details. Experimental evidence suggests that different effects can be induced through changing carnitine concentration. While severe carnitine deficiency leads to conditions similar to muscular dystrophy (Georges et al., 2000), a moderate decrease in heart tissue carnitine concentration adapts the cellular metabolism to ischemic stress conditions (Dambrova et al., 2002). The investigations of the pharmacological and physiological consequences of the regulation of carnitine system could provide evidence for novel treatment possibilities for clinical treatment of cardiovascular diseases.

After summarizing the available theoretical and experimental evidence, the **hypothesis** of the present doctoral thesis was set as follows: the molecular mechanism of the cardioprotective effect of mildronate is based on mildronate-induced decrease in carnitine concentration and subsequent inhibition of fatty acid metabolism.

Aim of the study

To test the hypothesis that the cardioprotective effect can be achieved by altering carnitine concentration and inducing changes in the carnitine-related energy metabolism pathways of cardiac cell.

Objectives of the study

- To study the effects of long-term decrease in carnitine concentration on heart and liver function and changes in plasma and tissue lipid profile.
- To study the effects of changed carnitine concentration on fatty acid metabolism related enzymes carnitine palmitoyltransferase I (CPT I) and carnitine acetyltransferase (CrAT) activity and glucose metabolism related pyruvate dehydrogenase complex activity in heart mitochondria.
- To characterize the interactions of mildronate and CrAT *in vitro* and effect of mildronate on enzyme activity *in vivo*.
- To study the effects of mildronate in an isolated rat heart infarction model as well as the angioprotective effect of mildronate in a *Dahl* salt-sensitive rat hypertension model.

Novelty of the study

The differential roles of changed concentrations of carnitine and its biosynthetic precursor gamma-butyrobetaine for the cardio- and angioprotective activity of mildronate were studied.

- Obtained results support the safety of long-term administration of mildronate; it was shown that 3 month mildronate treatment at doses up to 400 mg/kg is not associated with any cardiac impairments or disturbances of liver functions and lipid profile.
- The long-term decrease in carnitine concentration induces a dose-dependent compensatory increase in carnitine palmitoyltransferase I activity.
- For the first time the binding of mildronate to CrAT active site is characterized and it is established that mildronate is a weak inhibitor *in vitro* and it does not influence CrAT activity *in vivo*.
- Evidence is provided that the cardioprotective effect of mildronate depends on decrease in carnitine heart concentration, while the angioprotective effect in *Dahl* salt-sensitive rat hypertension model depends on changes in carnitine and gamma-butyrobetaine concentrations.

1. Approbation of the study

Doctoral thesis is based on following SCI publications:

1. Jaudzems K., **Kuka J.,** Gutsaits A., Zinovjevs K., Kalvinsh I., Liepinsh E., Liepinsh E., Dambrova M. Inhibition of carnitine acetyltransferase by mildronate, a regulator of energy metabolism. *J Enzyme Inhib Med Chem*, 2009; 24(6):1269-75.
2. Liepinsh E., **Kuka J.,** Svalbe B., Vilskersts R., Skapare E., Cirule H., Pugovics O., Kalvinsh I., Dambrova M. Effects of long-term mildronate treatment on cardiac and liver functions in rats. *Basic Clin Pharmacol Toxicol*, 2009; 105(6):387-94.
3. Vilskersts R., Liepinsh E., **Kuka J.,** Cirule H., Veveris M., Kalvinsh I., Dambrova M. Myocardial infarct size-limiting and anti-arrhythmic effects of mildronate orotate in the rat heart. *Cardiovasc Drug Ther*, 2009; 23(4):281-8.
4. Vilskersts R., **Kuka J.,** Svalbe B., Cirule H., Liepinsh E., Grinberga S., Kalvinsh I., Dambrova M. Administration of L-carnitine and mildronate improves endothelial function and decreases mortality in hypertensive Dahl rats. Accepted for publication (*Pharmacological Reports*, 2011; 63(3)).
5. **Kuka J.**, Vilskersts R., Cirule H., Makrecka M., Pugovics O., Kalvinsh I., Dambrova M., Liepinsh E. Treatment with L-carnitine diminishes the cardioprotective effect of mildronate in an isolated rat heart infarction model. Iesniegts manuskripts žurnālā *International Journal of Cardiology.*

Non SCI publications:

1. **Kūka J.,** Škapare E., Makrecka M., Medne R., Dambrova M. Polinepiesātinātās taukskābes organisma funkcionēšanā. *Latvijas ārsts*, 2010; 358(3), 54.-58. lpp.
2. Dambrova M., Liepinš E., **Kūka J.,** Medne R. Karnitīna 100 gadi. No atklāšanas līdz klīniskajiem pielietojumiem. *Doctus*, 2007; 10, 4.-8. lpp.

Results are reported in following international conferences:

1. **Kuka J.,** Vilskersts R., Vavers E., Liepinsh E., Dambrova M. Effects of regulation of L-carnitine concentration in experimental heart infarction model, *Frontiers in CardioVascular Biology*, Berlin, Germany, July 16-19, **2010**. Abstracts, S134.
2. Vilskersts R., **Kuka J.,** Svalbe B., Liepinsh E., Dambrova M. Protective effects of L-carnitine and mildronate in salt-induced hypertension, *Frontiers in CardioVascular Biology*, Berlin, Germany, July 16-19, **2010**. Abstracts, S54.
3. **Kuka J.,** Skapare E., Liepinsh E., Dambrova M. Effects of L-carnitine availability on carnitine palmitoyltransferase I activity in vivo,

MiPsummer School on Mitochondrial Physiology 2010, Druskininkai, Lithuania, June 10-16, **2010**. Abstracts P2-05, P.9.

4. **Kuka J.,** Zinovjevs K., Skapare E., Liepinsh E. Characterization of mildronate as an inhibitor of carnitine acetyltransferase. *FEBS 2009 Congress: Life`s Molecular Interactions*, Prague, Czech Republic, July 4-9, **2009**. Abstracts, P.322.
5. Vilskersts R., **Kuka J.,** Svalbe B., Liepinsh E., Dambrova M. Mildronate orotate: its cardioprotective and antiarrhythmic effects and mechanism of action. *FEBS 2009 Congress: Life`s Molecular Interactions*, Prague, Czech Republic, July 4-9, **2009**. Abstracts, P.351.
6. Vilskersts R., **Kuka J.,** Liepinsh E., Dambrova M. Effects of mildronate on arrhythmias induced by coronary artery ligation, calcium chloride and aconitine. *Baltic Summer School 2008: Basic and clinical aspects of cardiac arrhythmia's*, Copenhagen, Denmark, August 17 – September 6, **2008**.
7. **Kuka J.,** Skapare E., Liepinsh E., Dambrova M. Interactions of cardioprotective drug mildronate with mitochondrial carnitine acyltransferases, *New Frontiers in Cardiovascular Research, 8th Meeting of France - New EU Members/16th Symposium of Jagiellonian Medical Research Centre*, Krakow, Poland, June 5-7. Abstracts, **2008,** P.88.

Results are reported in following local conferences:

1. Vāvers E., **Kūka J.,** Karnitīna-palmitoiltransferāzes I aktivitātes regulācija, *RSU 59. Medicīnas nozares studentu zinātniskā konference*. Bāzes priekšmetu sekcija, Rīga, Latvija, 17. marts, **2010**. Tēzes, 12.-13.lpp.
2. **Kūka J.,** Škapare E., Liepiņš E., Medne R., Dambrova M. Mildronāta ietekme uz karnitīna aciltransferāzēm. *RSU 2008. gada zinātniskā konference*, Rīga, Latvija, 13.-14. marts, **2008**. Tēzes, lpp. 30.

2. Materials and methods

Male *Wistar* (Laboratory of Experimental Animals, Riga Stradins University, Latvia) and *Dahl* salt-sensitive (*Charles River*, Germany) rats were used. The experimental procedures were carried out in accordance with the guidelines of the European Community (86/609/EEC) and local laws and policies, and the procedures were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service. *Wistar* rats were perorally treated daily with a mildronate dose of 100, 200 or 400 mg/kg to study the effects of long-term decrease in carnitine concentration. *Wistar* rats were perorally treated daily with

carnitine 100 mg/kg, mildronate or combination of both (100+100 mg/kg) to study the cardioprotective effect. *Wistar* rats were perorally treated daily with mildronate 100 mg/kg, orotic acid 100 mg/kg or mildronate orotate 200 mg/kg to study the antiarrhythmic effect. *Dahl* salt-sensitive rats received carnitine 100 mg/kg, mildronate or combination of both (100+100 mg/kg) with drinking water.

2.1. *In vitro* methods

2.1.1. Determination of carnitine, GBB and mildronate in tissues and blood plasma

The determination of carnitine, GBB and mildronate concentrations in the blood plasma and liver and heart tissues was performed by UPLC/MS/MS (Dambrova et al., 2008).

2.1.2. Biochemical measurements

Free fatty acids, triglycerides, glucose, ketone bodies, LDL-C, HDL-C, lactate, total bilirubin and activity of ALAT, ASAT and ALP were determined using kits from *Wako* and *Instrumentation Laboratory* according to manufacturers' instructions.

CrAT activity was determined in both isolated *Wistar* rat heart mitochondria and using purified pigeon breast muscle enzyme (*Sigma*, USA). Mitochondria were isolated as previously described (Wilcke et al., 1995). Assay conditions were DTNB (0.675 mM), HEPES (125 mM), EDTA (2.5 mM), acetyl-coenzyme A (0.1 mM), carnitine (0.0625 to 0.5 mM). Mildronate (10 μM to 1 mM) was used only to characterize interplay of mildronate and purified CrAT *in vitro*. CPT I activity was determined in isolated mitochondria and whole liver and heart tissue homogenates. Assay conditions were DTNB (0.675 mM), HEPES (125 mM), EDTA (2.5 mM), palmitoyl-coenzyme A and corresponding carnitine concentrations. Mitochondrial respiration was measured as previously described (Baliutyte et al., 2010). Respiration buffer solution contained 40 μM palmitoyl-coenzyme A, 36 μM palmitoyl-carnitine or 5 mM pyruvate/5 mM malate.

2.1.3. Molecular docking to characterize binding of mildronate to CrAT

Mildronate docking in the active site of CrAT was performed with the MOE2007.09 software *(Chemical Computing Group, Inc*., Canada).

2.2. Ex vivo and *in vivo* methods

2.2.1. Haemodynamic measurements *in vivo*

Systolic blood pressure and heart rate were measured in DS rats at the beginning of the experiment and after 4 and 8 weeks of administration of the compounds using a Non-Invasive Blood Pressure Controller MLT125 connected to *ADInstruments* PowerLab8/30 system.

2.2.2. Isolated rat heart infarction model according to Langendorff

The infarction study was performed as described previously (Liepinsh et al., 2006). The infarct size was calculated as a percentage of the risk area. The control values were normalized to 100 %, and all of the results are expressed as values compared to the controls

2.2.3. Experimental models of arrhythmia in rats

To examine effect of mildronate on ischemia-reperfusion induced arrhythmia, *Wistar* rats were anesthetized with sodium pentobarbital (60 mg/kg i/p). Tracheotomy was performed, and the animals were ventilated with ambient air (15 ml/kg, 55 strokes per minute) using a rodent ventilator 7025, *Ugo Basile*). The chest was opened and a sling (5/0 Surgipro II) was placed around the left coronary artery. Rats were allowed to stabilize for 20 min and then the coronary artery was occluded for 10 minutes, followed by 30 min reperfusion. To examine effect of mildronate on calcium chloride induced arrhythmias, *Wistar* rats were anesthetized with sodium pentobarbital (60 mg/kg i/p). The left femoral vein was cannulated for the administration of 10 % calcium chloride solution. Experimental heart rhythm disturbances were induced by intravenously infusing (0.01 ml/s) 10% calcium chloride solution in an arrhythmogenic dose (180 mg/kg). ECG was recorded from II standard lead using Chart 5.5 software during calcium chloride administration and 10 min after discontinuation. The following parameters were monitored: mortality, time to onset of arrhythmias and time to normal sinus rhythm and incidence of ventricular tachycardia and fibrillation.

2.2.4. Salt induced hypertension in *Dahl* rats

Endothelial function was examined in aortic rings using an organ chamber bath as described previously (Bartuś et al., 2008). DS rat heart anatomy and function was examined using iE33 ultrasonograph (*Philips Ultrasound, Inc.*). Rats were lightly anesthetized with an intraperitoneal injection of ketamine and xylazine (50 and 10 $mg \times kg^{-1}$) and M-mode tracings of the left ventricle were recorded.

2.2.5. Data analysis

Established statistical methods were used and calculations were performed with MS Excel 2003 and GraphPad Prism 3.0 (*GraphPad Software, Inc.*). p-value < 0.05 was considered to be significant. Mann–Whitney or Student's t-test, analysis of variance followed by Tukey`s or Bonferroni test were used to compare effects. Survival curves for DS rats were constructed for each group using the Kaplan-Meier method. Differences in the incidence of arrhythmias among groups were analyzed by Chi-Square test.

3. Results

3.1. Effects of mildronate and carnitine treatment on GBB, mildronate and carnitine concentrations in plasma and tissues

4, 8 and 12 week treatment with 100 (M 100), 200 (M 200) and 400 mg⁄kg (M 400) of mildronate induced dose-dependent decrease in the carnitine concentration in *Wistar* rat blood plasma. After 4 weeks of treatment with mildronate significant decreases of 3-, 5- and 13-fold, respectively, in the carnitine concentrations were observed. Time-dependent decrease in carnitine concentration was observed only in the rats treated at the 400 mg⁄kg dose, reaching a decrease of 35-fold at the end of the 12-week treatment (Figure 1A). Mildronate treatment induced up to a 14-fold increase in the GBB concentration, but this effect was dose and time independent (Figure 1B).

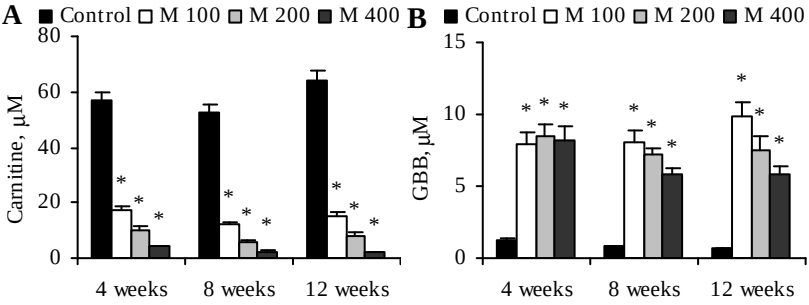

Figure 1. *Effect of long-term mildronate treatment on changes in carnitine (A) and GBB (B) concentrations in Wistar rat blood plasma. Values are represented as average ± SEM of 8–10 animals. * - p<0.05, vs. control group (Tukey`s test).*

Long-term mildronate treatment induced a dose-dependent decrease in the carnitine concentration in *Wistar* rat heart and liver tissue. Time-dependent decrease in carnitine concentration was observed only in the rats treated at the 200 mg/kg dose (Figure 2).

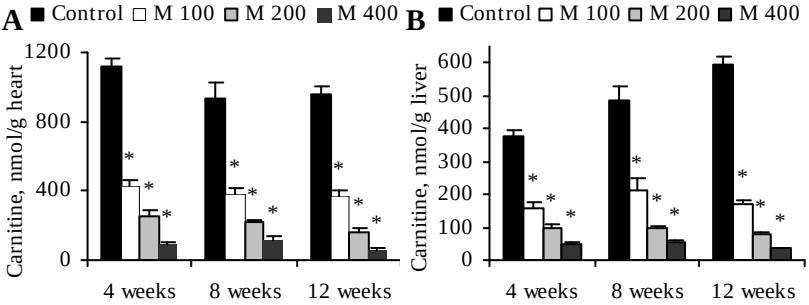

Figure 2. *Effect of long-term mildronate treatment on changes in carnitine concentration in Wistar rat heart (A) and liver (B) tissue. Values are represented as average ± SEM of 8–10 animals. * - p<0.05, vs. control group (Tukey`s test).*

GBB concentration increased up to 10-fold in heart tissue (Figure 3A), and up to 66-fold in liver tissue (Figure 3B), however none of these changes were dose or time-dependent.

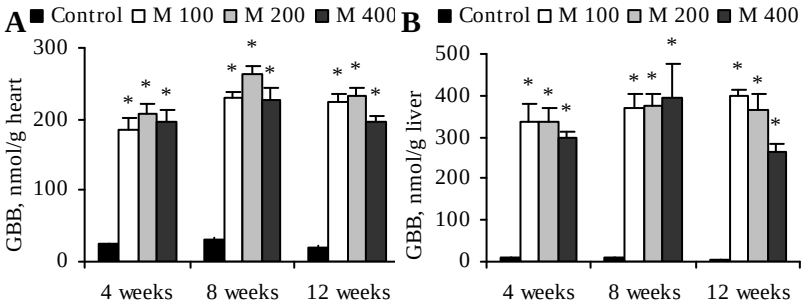

Figure 3. *Effect of long-term mildronate treatment on changes in GBB concentration in Wistar rat heart (A) and liver (B) tissue. Values are represented as average ± SEM of 8–10 animals. * - p <0.05, vs. control group (Tukey`s test).*

Effects of 14-day treatment with carnitine (100 mg/kg; C 100), mildronate (100 mg/kg; M 100) or both (100+100 mg/kg; C+M) on

carnitine and GBB concentrations in *Wistar* rat blood plasma are shown in figure 4. Treatment with carnitine induced a significant increase in its blood plasma concentration by 24 % as compared to control; mildronate treatment induced a 3-fold decrease in carnitine concentration. Treatment with a combination of both substances decreased carnitine concentration by 18 % (Figure 4A). Treatment with carnitine induced only a slight 2-fold increase in GBB concentration, whereas treatment with mildronate or the combination of both substances increased GBB concentration by up to 10- and 20-fold, respectively (Figure 4B).

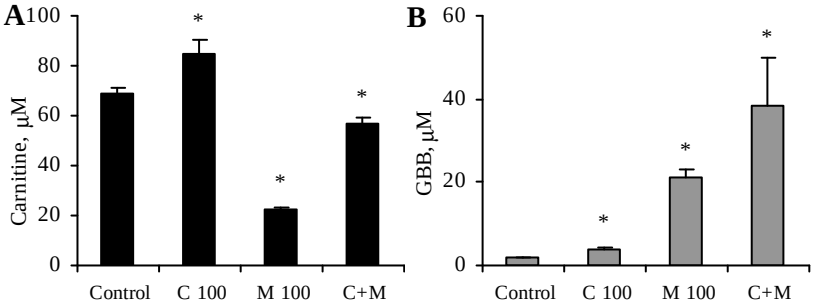

Figure 4. *Effects of 14-day treatment with carnitine, mildronate or both on carnitine (A) and GBB (B) concentrations in Wistar rat blood plasma. Values are represented as average ± SEM of 10 animals. * - $p<0.05$, vs. control group (Student's t-test).*

As in blood plasma, treatment with carnitine increased its heart tissue concentration (by 31 %). After treatment with mildronate, the carnitine concentration in heart tissue was decreased by 69 %. Treatment with carnitine and mildronate combination had no significant impact of carnitine concentration in heart tissue as compared to control (Figure 5A). Similar to the findings in blood plasma, treatment with carnitine induced only a slight 2-fold increase in GBB concentration in heart tissue, whereas treatment with mildronate or the combination of both substances increased the GBB concentration in heart tissue by 5- and 7-fold, respectively.

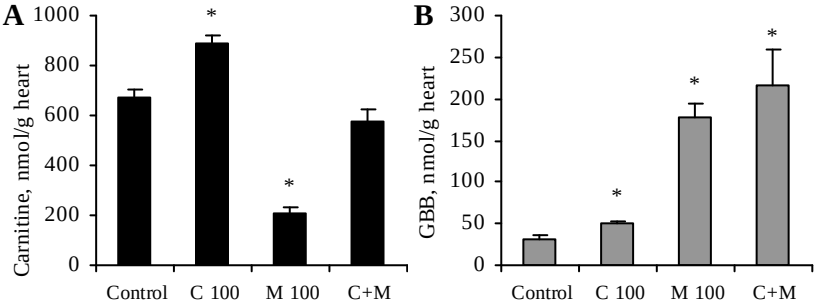

Figure 5. *Effects of 14-day treatment with carnitine, mildronate or both on carnitine (A) and GBB (B) concentrations in Wistar rat heart tissue. Values are represented as average ± SEM of 10 animals. * - p<0.05, vs. control group (Student's t-test).*

3.2. Effects of changes in carnitine concentration on enzyme activity and biochemical measures in blood plasma and tissues

3.2.1. Effect on CPT I activity

The effect of mildronate treatment on CPT I activity in *Wistar* rat heart tissues is shown in figure 6.

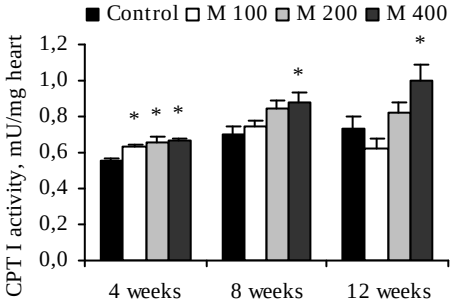

Figure 6. *Effect of long-term mildronate treatment on Wistar rat heart CPT I activity. Values are represented as average ± SEM of 6 animals. * - p<0.05, vs. control group (Tukey`s test).*

CPT I activity was determined in tissue homogenates after 4, 8 and 12 weeks of treatment with 100, 200 and 400 mg⁄kg of mildronate. In heart tissue homogenates after 4 weeks mildronate treatment, the basal CPT I activity was significantly increased by up to 21 % in all of the

mildronate treated groups, whereas after 8 and 12 weeks of treatment, the CPT I activity was significantly increased by 25 % and 35 %, respectively, only in the rats treated with a 400 mg⁄kg dose of mildronate. In liver tissue homogenates, no significant changes in the basal CPT I activity were observed.

3.2.2. Effect on mitochondrial respiration

Mitochondrial respiration was measured in isolated *Wistar* rat heart mitochondria after 14 day treatment with carnitine (100 mg/kg), mildronate (100 mg/kg) or both (100+100 mg/kg), using palmitoyl-coenzyme A, palmitoyl-carnitine and pyruvate/malate as a substrates. Mitochondrial respiration results are shown in table 1. Treatment with mildronate decreased the mitochondrial respiration on palmitoyl-coenzyme A, but did not have any statistically significant impact on mitochondrial respiration on pyruvate/malate or palmitoyl-carnitine. Treatment with combination of mildronate and carnitine increased mitochondrial respiration on palmitoyl-carnitine and pyruvate/malate.

Table 1

Effects of 14-day treatment with carnitine, mildronate or both on isolated *Wistar* rat heart mitochondria respiration on different energy substrates

Substrate	Control	C 100	M 100	C+M
	O_2 consumption, nmol/min/mg protein			
40 µM palmitoyl-coenzyme A	36.9±3.8	41.0±4.9	26.8±0.8*	36.8±1.4
36 µM palmitoyl-carnitine	16.4±1.2	21.4±2.8	19.2±0.9	21.3±1.2*
5 mM pyruvate/5 mM malate	41.3±2.7	44.7±6.0	42.6±4.1	52.4±2.4*

*Values are represented as average ± SEM of 6 animals. * - $p<0.05$, vs. control group (Student's t-test).*

3.2.3. Effect on liver functionality markers

Effects of long-term (4-12 weeks) decrease in carnitine concentration induced by treatment with 100, 200 and 400 mg⁄ kg of mildronate on the levels of *Wistar* rat liver function markers are shown in table 2.

Table 2

The effect of decreased carnitine concentration on liver function markers in *Wistar* rat blood plasma

	Control	M 100	M 200	M 400
		4 weeks		
ASAT, U/l	49.0±3.8	46.5±6.2	48.2±5.8	43.1±4.4
ALAT, U/l	20.6±1.6	19.5±0.6	19.4±1.7	19.9±1.0
ALP, U/l	33.5±3.2	29.8±3.2	34.0±2.9	35.7±3.1
Total bilirubine, mg/dl	0.39±0.17	0.27±0.09	0.17±0.02	0.29±0.05
		8 weeks		
ASAT, U/l	39.7±3.2	38.2±3.9	41.7±5.1	37.3±3.3
ALAT, U/l	17.2±2.1	17.1±1.8	18.3±1.5	17.2±1.1
ALP, U/l	16.7±1.5	21.3±1.0*	18.9±1.1	17.5±2.1
Total bilirubine, mg/dl	0.31±0.04	0.33±0.05	0.30±0.04	0.30±0.03
		12 weeks		
ASAT, U/l	39.9±1.0	42.5±1.8	36.6±2.1	44.4±1.1*
ALAT, U/l	15.8±1.1	17.8±1.4	15.8±0.9	19.0±1.8
ALP, U/l	18.8±0.5	20.2±1.2	21.6±1.6	23.0±1.9*
Total bilirubine, mg/dl	0.23±0.04	0.20±0.03	0.24±0.02	0.19±0.02

*Values are represented as average ± SEM of 8-10 animals. * - p<0.05, vs. control group (Tukey`s test).*

Mildronate treatment did not affect the total bilirubine, ASAT and ALAT values. The ALP level was significantly increased by 28 % after 8 weeks of treatment with 100 mg⁄kg of mildronate and by 23 % after 12 weeks of treatment with 400 mg⁄kg of mildronate.

3.2.4. Effect on lipid profile in liver tissue

Effects of long-term decrease in carnitine concentration on triglyceride and fatty acid concentrations in *Wistar* rat liver tissues are shown in figure 7. The triglyceride concentration in the livers of fed rats that received the 400 mg⁄kg dose of mildronate was significantly increased after 4, 8 and 12 weeks of treatment by 21 %, 20 % and 12 %, respectively (Figure 7A). The fatty acid concentration in the liver was significantly increased by 24 % and 14 % only after 12 weeks of treatment with 200 and 400 mg⁄kg of mildronate, respectively (Figure 7B).

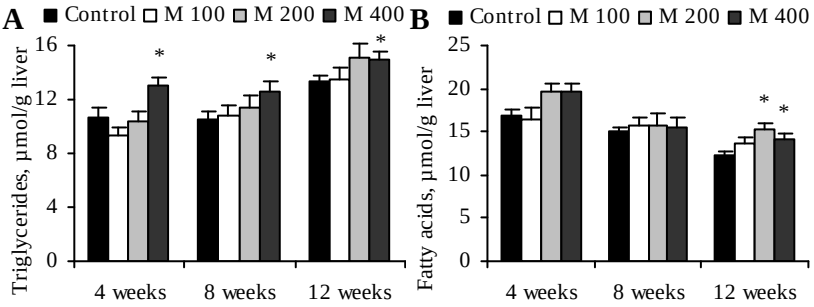

Figure 7. *Effects of long-term mildronate treatment on triglyceride and fatty acid concentrations in Wistar rat liver tissues. Values are represented as average ± SEM of 8-10 animals. * - p <0.05, vs. control group (Tukey`s test).*

3.3. Characterization of mildronate binding to CrAT

Binding of mildronate to CrAT was determined *in vitro* using purified enzyme (*Columba sp.*). Mildronate inhibits CrAT in a competitive manner (Figure 8); however it is weak inhibitor with a K_i value of 5.2 ± 0.6 mM (Figure 9).

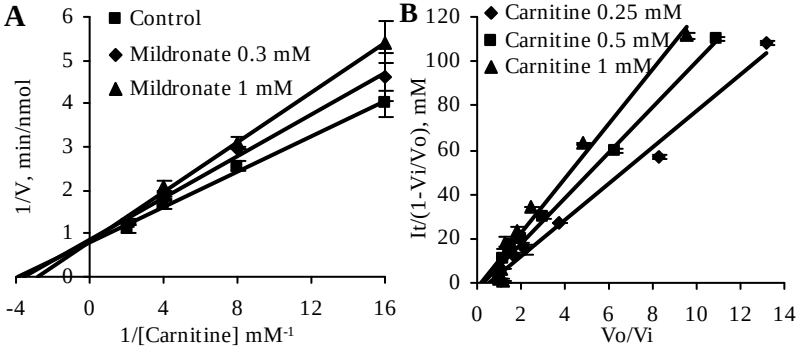

Figure 9. *Lineweaver–Burk (A) and Henderson (B) plots to characterize binding of mildronate to CrAT. Points represent average ± SEM of 3 measures. I_t-total concentration of inhibitor; V_0-control velocity; V_i-velocity in presence of inhibitor.*

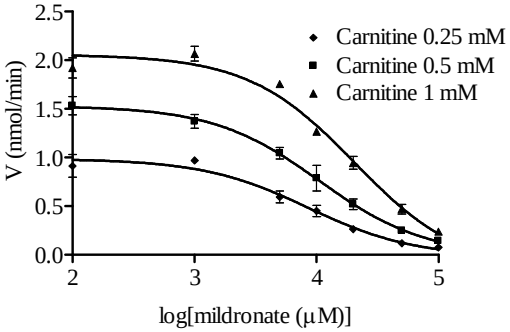

Figure 9. *Dose–response curves for the inhibition of CrAT by mildronate at carnitine concentrations 0.25, 0.5, and 1 mM. Points represent average ± SEM of 3 measures.*

For detailed characterization of the mildronate molecule on CrAT, molecular docking was used. Docking results (Figure 10) showed the conformation, where the trimethylammonium group of the mildronate was exposed to the solvent. However, the carboxyl and α-CH_2 groups of mildronate and carnitine were bound to CrAT very similarly. The catalytic His343 is hydrogen-bonded to the NH group of mildronate, instead of the OH moiety in carnitine. This caused differences in binding of both ligands. The trimethylammonium group of mildronate was situated in a channel approximately 4 Å away from the protein surface and was exposed to the solvent, whereas the trimethylammonium group of carnitine was buried in a hydrophobic pocket.

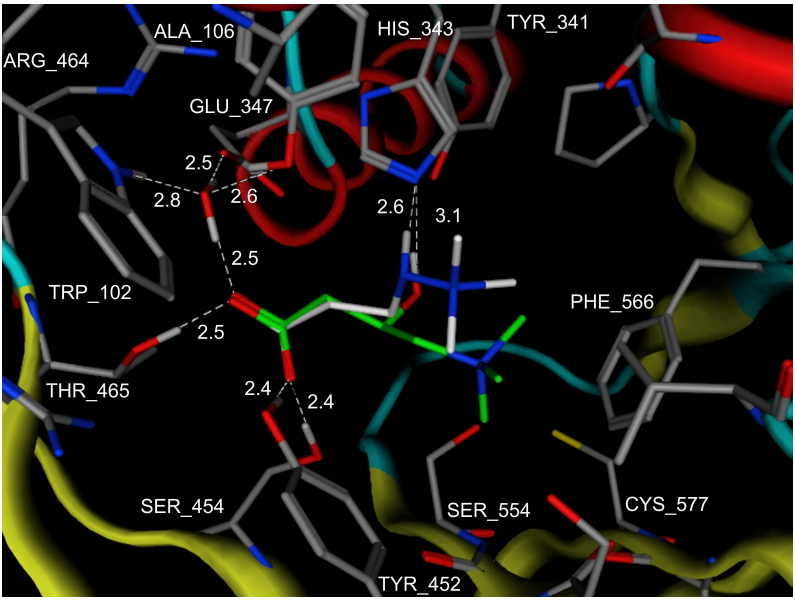

Figure 10. *Proposed model for the positioning of carnitine and mildronate in CrAT enzyme. Carnitine carbons are shown in green and mildronate carbons are shown in light gray. All hydrogen atoms, except those involved in hydrogen bonds, are omitted. Hydrogen bonds are shown as white dashed lines. Produced with MOE 2007.09.*

14-day treatment with carnitine (100 mg/kg; C 100), mildronate (100 mg/kg; M 100) or both (100+100 mg/kg) did not affect CrAT activity in heart mitochondria *in vivo*.

3.4. Effect of changes in carnitine concentration on infarct size

Effects of 14-day treatment with carnitine (100 mg/kg), mildronate (100 mg/kg) or both (100+100 mg/kg) on infarct size and carnitine concentrations in the *Wistar* rat heart tissues are shown in figure 11. Mildronate decreased carnitine concentration in heart tissue and reduced infarct size by 34 % compared to control group. Carnitine treatment reduced infarct size by 28 %, but this effect was not statistically significant. Mildronate and carnitine combination had no significant impact on infarct size or carnitine concentration.

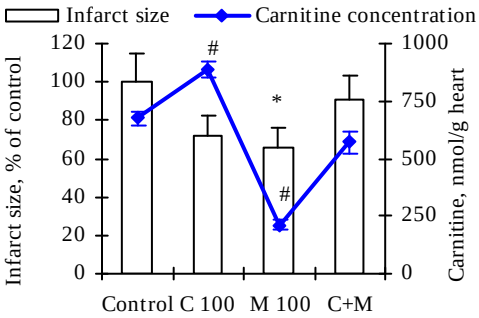

Figure 11. *Effects of 14-day treatment with carnitine, mildronate or both on carnitine concentration and infarct size in Wistar rat heart tissue. Values are represented as average ± SEM of 10 animals. #,* - p<0.05, vs. control group (Student's t-test).*

Effects of changes in carnitine concentration (Figure 5A) induced by 14-day treatment with carnitine, mildronate or both on CPT I activity in mitochondria isolated from *Wistar* rat hearts are shown in figure 12. Treatment with mildronate decreased CPT I activity by 26 %.

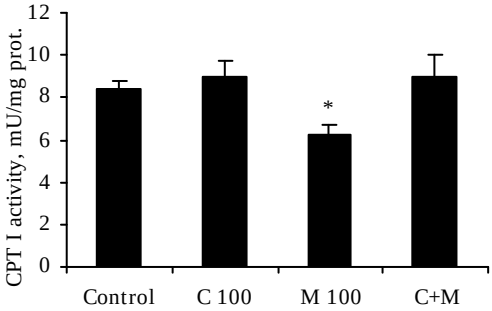

Figure 12. *Effects of treatment with carnitine, mildronate or both on CPT I activity in the presence of carnitine concentrations found in the heart tissue after the treatment (700 μM for control, 900 μM for C 100, 200 μM for M 100 and 600 μM for C+M group). Values are represented as average ± SEM of 5 animals. * - p<0.05, vs. control group (Student's t-test).*

3.5. Effects of changes in carnitine concentrations on *Wistar* rat haemodynamic measurements

Decrease in carnitine concentration after treatment with mildronate (100, 200 and 400 mg⁄kg) for 4, 8 and 12 weeks did not induce any changes in isolated heart parameters (heart rate, coronary flow, LVDP, left ventricle contractility and relaxation, cardiac work).

3.6. Anti-arrhythmic effects of mildronate and mildronate orotate

3.6.1. Effects on ischemia-reperfusion induced arrhythmia

Effects of mildronate (100 mg/kg) and mildronate orotate (200 mg/kg) on time to onset of arrhythmias during occlusion and time to onset of normal sinus rhythm during reperfusion are shown in figure 13. In all the experimental groups the first arrhythmia episodes started approximately 320 s after the start of coronary artery occlusion, however both drugs significantly reduced the time elapsed prior to onset of normal sinus rhythm during reperfusion (175±36 s in the control group, 73±21 s in the mildronate group and 60±8 s in the mildronate orotate group).

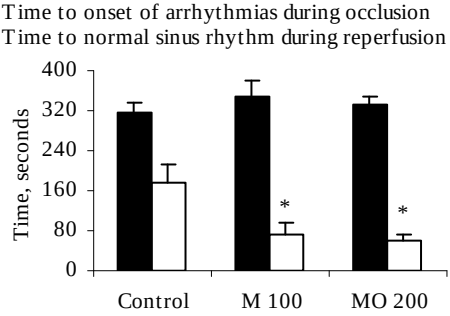

Figure 13. *Effects of 14-day treatment with mildronate and mildronate orotate on time to onset of arrhythmias during occlusion and time to normal sinus rhythm during reperfusion in ischemia-reperfusion induced experimental arrhythmia model in Wistar rats. Values are represented as average ± SEM of at least 6 rats. * - p<0.05, vs. control group (Tukey`s test).*

Treatment with mildronate and mildronate orotate had no effect on ventricular tachycardia during occlusion and reperfusion, but protected rat hearts against ventricular fibrillation during occlusion (only mildronate orotate) and reperfusion (both drugs) (Figure 14).

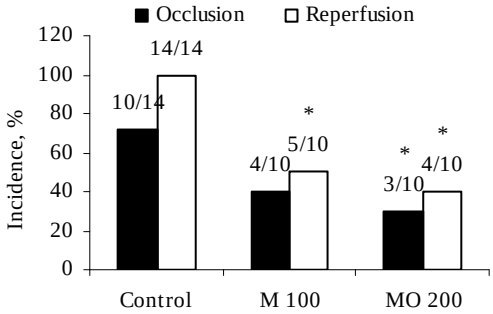

Figure 14. *Effects of 14-day treatment with mildronate and mildronate orotate on incidence of ventricular fibrillation during occlusion and reperfusion in Wistar rats. * - p<0.05 vs. control group (Chi-Square test).*

Mildronate and mildronate orotate significantly decreased the cumulative duration of fibrillation during reperfusion (38±14 s in the control group, 3±2 s in the mildronate group and 2±2 s in mildronate orotate group), but had no significant effect on fibrillation duration during occlusion (Figure 15).

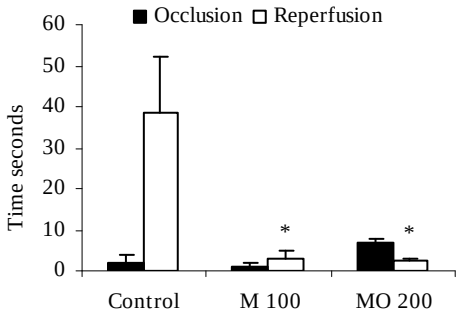

Figure 15. *Effects of 14-day treatment with mildronate and mildronate orotate on duration of fibrillation during occlusion and reperfusion in Wistar rats. Values are represented as average ± SEM of at least 6 rats. * - p<0.05, vs. control group (Tukey`s test).*

3.6.2. Effect on calcium chloride induced arrhythmia

Treatment with mildronate and mildronate orotate had no effect on time to the onset of arrhythmias after infusion of calcium chloride

(26±4 and 25±4 s, respectively as compared to 30±4 s in the control group) and on times to normal sinus rhythm (119±20 and 97±12 s, respectively as compared to 159±39 s in the control group). Mildronate and mildronate orotate treatment decreased the incidence of ventricular tachycardia induced by calcium chloride in *Wistar* rats (Figure 16).

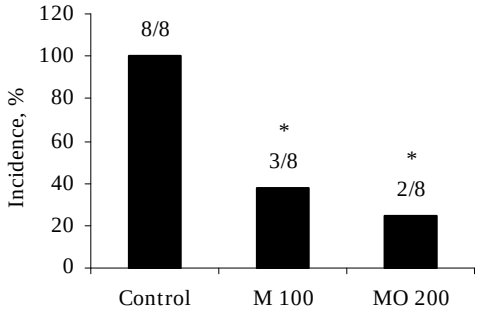

Figure 16. *Effects of 14-day treatment with mildronate and mildronate orotate on the incidence of ventricular tachycardia in Wistar rats. * - p<0.05, vs. control group (Chi-Square test).*

3.7. Effects of treatment with mildronate and carnitine on hypertension in *Dahl* salt-sensitive rats

3.7.1. Effects on carnitine and GBB concentration in blood plasma

Effects of 14-week treatment with carnitine (100 mg/kg), mildronate (100 mg/kg) or both (100+100 mg/kg) on carnitine and GBB concentrations in *Dahl* rat blood plasma are shown in table 3. Consumption of diet with high salt (PS, 8 % NaCl) load decreased concentration of carnitine nearly 2-fold compared with that of rats consuming diet with normal salt (NS, 0.3 % NaCl) load. Administration of carnitine (PS/C 100) or combination (PS/C+M) significantly increased the concentration of carnitine in the plasma, but the treatment with mildronate (PS/M 100) decreased it as compared to PS control group animals. Consumption of high or normal salt load did not affect GBB concentration, but administration of mildronate, carnitine or their combination significantly increased the concentration of GBB in blood plasma samples 6-, 1.5- and 12-fold.

Table 3

Effects of treatment with mildronate, carnitine and their combination on carnitine and GBB concentrations in *Dahl* rat blood plasma

	NS group	High salt diet (PS)			
		Control	M 100	C 100	C+M
Carnitine, μM	$55\pm6^{*,\#}$	$23\pm3^{\#}$	$5\pm1^{*}$	$56\pm4^{*,\#}$	$36\pm2^{*,\#}$
GBB, μM	$1.5\pm0.1^{\#}$	$1.5\pm0.1^{\#}$	$8.6\pm1.0^{*}$	$2.2\pm0.4^{*,\#}$	$18.1\pm3.4^{*,\#}$

*Values are represented as average ± SEM of 3-10 animals. * p<0.05, vs. PS Control; # p<0.05, vs. HS/M 100 group.*

3.7.2. Effect on survival rate

Effects of long-term treatment with mildronate (100 mg/kg), carnitine (100 mg/kg) and their combination (100+100 mg/kg) on *Dahl* rat survival rate are shown in figure 17.

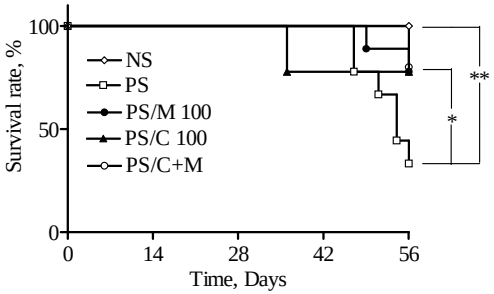

Figure 17. *Kaplan–Meier plots of the survival rate of DS rats fed a normal-salt diet or a high salt diet and treated either with vehicle, carnitine, mildronate or their combination. *, p<0.05, vs. PS Control; ** p<0.01, vs. PS Control.*

Kaplan–Meier analysis revealed that the survival rate of PS group rats was markedly reduced compared with that of animals from the NS group In the PS group after 8 weeks of treatment, the survival rate was 30 % (3 of 10 animals), while there were no lethal cases in the NS group. Administration of a combination of carnitine and mildronate significantly increased the survival rate compared with the PS group, and after 8 weeks of treatment, the PS/C+M group survival rate was 80 % (8 of 10 animals). The obtained data showed that there was no statistically significant increase in survival rate in the PS/C 100 (80 %, 8 of 10 animals) and PS/M 100 (80 %, 8 of 10 animals) groups compared

with the PS control group although the number of animals that survived was similar as in PS/C+M group.

3.7.3. Effects on systolic blood pressure and heart rate

At the beginning of the experiment in all of the experimental groups the average systolic blood pressure was about 125 mmHg. Treatment with mildronate (100 mg/kg), carnitine (100 mg/kg) and their combination (100+100 mg/kg) did not influence the systolic blood pressure, which had increased to above 170 mmHg in all the groups, reaching as high as 195 mmHg (Figure 18).

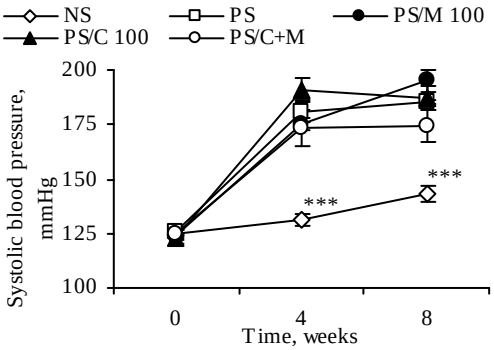

Figure 18. *Effects of 8-week treatment with mildronate, carnitine and their combination on systolic blood pressure in Dahl rats. Values are represented as average ± SEM of 3-10 animals. *** p <0.001, vs. PS Control (Mann-Witney test).*

No differences were among the heart rate of animals of all of the experimental groups before switching to the high salt diet or on the fourth week of the treatment. However, on the eighth week of the experiment, the mean heart rate in the PS control group was 510±10 bpm, but in the NS group and after administration of mildronate (PS/M 100) and a combination of mildronate and carnitine (PS/C+M) heart rate was lower by 16, 12 and 10 %, respectively (Figure 19).

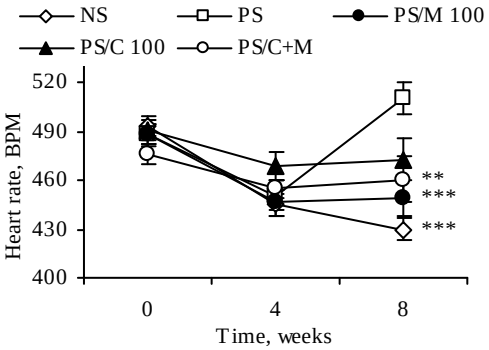

Figure 19. *Effects of 8-week treatment with mildronate, carnitine and their combination on heart rate in Dahl rats. Values are represented as average ± SEM of 3-10 animals. **, p<0.001, vs. PS Control; *** p<0.001, vs. PS Control (Mann-Witney test).*

3.7.4. Effects on endothelial function

Endothelium-dependent relaxation to acetylcholine in the aortic rings of PS group animals was significantly impaired compared with NS group animals. Treatment with a combination of mildronate and carnitine improved endothelium-dependent relaxation, but mildronate or carnitine alone had no impact on acetylcholine-induced relaxation (Figure 20).

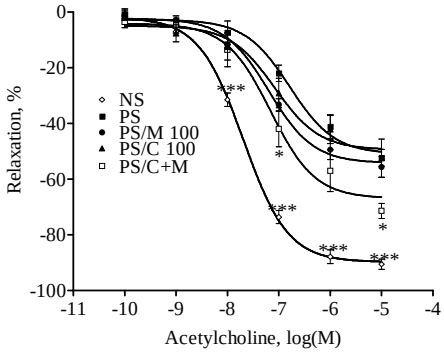

Figure 20. *Effects of 8-week treatment with mildronate, carnitine and their combination on endothelial function in Dahl rats. Values are represented as average ± SEM of 3-10 animals. *** p<0.001, vs. PS Control (Mann-Witney test).*

4. Discussion

The present work describes the effects of changes in carnitine concentration on functionality of cardiovascular system under normoxic and ischemic conditions. Treatment with carnitine and mildronate were used to induce changes in carnitine concentration. The cardioprotective effects of altered carnitine concentration during ischemia, development of hypertension and changes in carnitine-dependent enzyme activities were characterized.

4.1. Effects of decreased carnitine concentration on heart function and blood plasma and tissue biochemical parameters

Carnitine regulates the energy metabolism pathways in the heart and skeletal muscle. The physiological range of carnitine concentration in various tissues is maintained by a complex transporter system. However, the critical levels of carnitine for the function of the heart and the liver are not well-established.

Mildronate is known to decrease carnitine concentration through inhibition of biosynthesis of carnitine (Simkhovich et al., 1988) and its reabsorption in the kidneys (Spaniol et al., 2001). As a result of biosynthesis inhibition, mildronate treatment is known to elevate GBB concentrations in blood plasma and tissues (Liepinsh et al., 2006). The present study confirms that 4-12 week treatment with mildronate (100, 200 and 400 mg/kg) induces a significant decrease in the carnitine concentration simultaneously with increase in GBB concentration in blood plasma (Figure 1) and heart (Figure 2A and 3A) and liver (Figure 2B and 3B) tissues. Unlike GBB, changes in carnitine concentration were mildronate dose-dependent. This suggests that mildronate doses equally inhibit GBBH, but the dose-dependent decrease in carnitine concentration is most likely due to decreased reabsorption of carnitine in the kidneys, because of increasing inhibition of OCTN2.

Some controversy is present in literature regarding effects of mildronate induced decrease in carnitine concentration on liver functions. It has been suggested that decrease in carnitine concentration induced by 6-week treatment with mildronate (200 mg⁄kg) may develop liver steatosis (Spaniol et al, 2003). However, it was shown that steatosis is transient in fasted rats and disappears upon refeeding (Degrace et al., 2007). In the present study, to examine effects of long-term (4-12 weeks) mildronate (100, 200 and 400 mg/kg) treatment induced decrease in carnitine concentration on liver functions, we performed a histological

examination of liver cryosections, measured liver triglyceride and fatty acid contents, as well as assayed for liver damage markers and measured lipid profile in the blood plasma. In liver cryosections we did not find any signs of fat accumulation, while a statistically significant increase in the triglyceride concentration by 10-20 % was only observed after treatment with a 400 mg⁄kg dose of mildronate (Figure 7A). A statistically significant increase (of 15 %) in the liver fatty acid concentration was observed only after 12 weeks of treatment (Figure 7B).

Decrease in carnitine concentration had no significant effect on blood plasma lipid profile; the only changes that could indicate about decreased fatty acid β-oxidation were observed after treatment with 400 mg⁄kg dose of mildronate and more pronounced these changes were in fasted rats. Thus, decrease in β-hydroxybutyrate concentration that could indicate about decreased fatty acid and increased glucose metabolism (van Knegsel et al, 2005), we observed only after 12 weeks of treatment with a 400 mg⁄kg dose of mildronate.

Diverse effects of decreased carnitine concentration on liver function observed after treatment with mildronate, might be related to the origin of 3-(2,2,2-trimethyl-hydrazinium)-propionate used for studies. *Spaniol et al*. (Spaniol et al., 2003) have used custom prepared 3-(2,2,2-trimethyl-hydrazinium)-propionate, that may have been contaminated with several highly hepatotoxic impurities, such as 1,1,1-trimethylhydrazinium salts and hydrolysis products of the fully substituted hydrazinium derivative (Hmelnickis et al., 2008). In our studies and study by *Degrace et al*. (Degrace et al., 2007), only commercial 3-(2,2,2-trimethyl-hydrazinium)-propionate or mildronate from the original manufacturer JSC Grindeks was used and no toxic effects have ever been observed.

CPT I converts carnitine to form acyl-carnitine that can be transported in mitochondria. Therefore, it was important to determine how decrease in carnitine concentration affect CPT I activity. Previously it was shown that decrease in carnitine concentration after treatment with mildronate induces an increase in CPT I mRNA and protein levels in both heart and liver tissues (Degrace et al., 2004; Degrace et al., 2007; Liepinsh et al., 2008). After treatment with mildronate CPT I activity was increased in heart and liver tissues when activity measurements were performed in isolated mitochondria in the presence of fixed carnitine concentrations. Decrease in carnitine tissue concentration does not affect

the sensitivity of CPT I to malonyl-coenzyme A inhibition (Tsoko et al., 1995; Degrace et al., 2004; Degrace et al., 2007).

We determined CPT I activity in the presence of carnitine concentrations that are found in tissues (i.e. carnitine concentrations were not equalized as in previous studies). CPT I activity was increased after long-term (4-12 weeks) treatment with mildronate in the heart tissue (Figure 6), but not in liver tissues. After 2 week treatment with mildronate CPT I activity in heart tissue was significantly reduced for 26 % as compared to the control (Figure 12), but CPT I dependent fatty acid β-oxidation was significantly reduced for 27 % (Table 1). Previously, it was shown that treatment with mildronate at a doses of 200 mg/kg (21 days, mice) and 800 mg/kg (10 days, rats) induces a compensatory increase in CPT I mRNA expression (Degrace et al., 2004; Liepinsh et al., 2008). In this study we did not observe any significant changes in CPT I mRNA expression.

The obtained results indicate that changes in CPT I activity, protein and mRNA expression after treatment with mildronate depend on decrease itself and duration of decrease in carnitine concentration. This could be explained by observation that different tissues have different isoforms of CPT I, which are differentially sensitive to carnitine. For example, the rat isoforms of CPT I in the liver (CPT IA) and muscle (CPT IB) have a K_m for carnitine that is 30 and 500 μM, respectively. In the heart, both CPT I isoforms, CPT IA and CPT IB, are present and as a result, the average K_m for carnitine in the heart is about 200 μM (Brown et al., 1995). Therefore, carnitine concentrations found in heart (0.6-3 mM, depending on species) and liver tissues (0.4-3 mM, depending on species) in normal conditions are more than enough to facilitate the transport of fatty acid in mitochondria. We can conclude that only marked or long-term decrease in carnitine concentration induces a compensatory increase in CPT I protein and mRNA expression as well as an increase in CPT I enzyme activity in heart and liver tissues.

It was shown that marked decrease in carnitine concentration induces hypoglycemia or facilitates glucose oxidation in rats (Broderick, 2006), while moderate decrease in carnitine concentration after treatment with mildronate (200 mg/kg) facilitates glucose uptake and increases glucose metabolism-related gene expression in mice (Liepinsh et al., 2008). Decrease in carnitine concentration even after treatment with mildronate at a dose of 400 mg/kg did not affect glucose concentration in the fed and fasted *Wistar* rat blood. This result suggests that the effect of

decrease in carnitine concentration on glucose metabolism strongly depends on substrate availability and energy requirements. Thus, in cases of partially decreased fatty acid β-oxidation, changes in glucose metabolism could play a compensatory role. It should be noted that it is more important to facilitate glucose metabolism during ischemia-reperfusion rather than under normoxic conditions in order to achieve cardioprotection.

Previous studies have shown that mildronate inhibits CrAT activity to some extent; it was suggested that the inhibition of CrAT by mildronate and increase in acetyl-coenzyme A concentration might be an important pharmacological mechanism for maintenance of intra-mitochondrial metabolic pathways. However, recent findings of facilitation of glucose metabolism after treatment with mildronate (Liepinsh et al., 2008) contradict previous findings, because increase in acetyl-coenzyme A levels would decrease PDH activity. To give insights into the CrAT binding of mildronate we performed experiments with purified enzyme *in vitro* and we also measured the activity of the enzyme *ex vivo* in isolated mitochondria after treatment with mildronate for 2 weeks. Biochemical measurements confirmed that mildronate is a weak inhibitor of CrAT as the K_i value in the presence of carnitine was 5.2 ± 0.6 mM (Figure 8 and 9). Molecular docking suggested that mildronate competes with carnitine for binding in the active site of CrAT. The bound conformation of mildronate closely resembles that of carnitine except for the orientation of the trimethylammonium group, which in the mildronate molecule is exposed to the solvent (Figure 10). This might explain why binding of mildronate to CrAT declines so much in the presence of carnitine. Although it has been suggested that the active sites of all acyltransferazes are similar (Jogl et al., 2004), it was demonstrated that mildronate does not inhibit CPT I (Tsoko et al., 1995); this indirectly suggests that mildronate and carnitine bind differently to the catalytic sites of these enzymes.

To give additional insights about effects of mildronate on CrAT, we determined the activity of enzyme in isolated *Wistar* rat heart mitochondria after treatment with mildronate at a dose of 100 mg/kg. Although carnitine concentration is decreased in cytosol, it was shown that treatment with mildronate increases carnitine concentration in heart mitochondria (Degrace et al., 2004). Activity measurements of CrAT *ex*

vivo confirmed *in vivo* data, because no changes in enzyme activity as compared to the control group were observed. These results suggest that neither mildronate, nor changes in carnitine concentration do affect CrAT activity directly under normal conditions *in vivo*. However, the changes in carnitine concentration might play an important role in the regulation of CrAT activity during ischemia-reperfusion. Thus, increase in the mitochondrial carnitine concentration and decrease in long-chain acyl-coenzyme A concentrations could increase CrAT activity and facilitate glucose metabolism.

Altogether present findings indicate that the decreased carnitine concentration after treatment with mildronate induces changes in energy metabolism-related enzyme activity that lays grounds for cardioprotective effects under ischemic conditions.

4.2. Effect of changes in carnitine concentration on infarct size

Both increase and decrease in carnitine concentration are known to reduce ischemia induced cardiac cell injury. Treatment with carnitine decreased the myocardial infarct size after permanent occlusion of the left coronary artery by ligation (Mouhieddine & de Leiris, 1993), but it did not influence the infarct size after ischemia-reperfusion injury (Briet et al., 2008). Decrease in carnitine concentration after treatment with mildronate was shown to be cardioprotective (Dambrova et al., 2002; Liepinsh et al., 2006). Previously it was shown that the cardioprotective effect of mildronate correlates with the increase in carnitine biosynthesis precursor GBB concentration (Liepinsh et al., 2006).

This study evaluated the anti-infarction effects of mildronate (100 mg/kg), carnitine (100 mg/kg) and, in particular, a combination of both substances (100+100 mg/kg) in an isolated rat heart ischemia-reperfusion injury model; the cardioprotective effects were analyzed with respect to the observed changes in carnitine and particularly GBB levels in blood plasma and heart tissue.

14-day treatment with mildronate resulted in a marked decrease in carnitine concentration in heart tissues by 69 % as well as a significant decrease in infarct size by 34 %. Treatment with mildronate plus carnitine diminished both the mildronate-induced decrease in carnitine tissue concentration and the cardioprotective effect of mildronate. Treatment with carnitine alone increased its heart tissue concentration by 28 %, but the decrease in the infarct size was not significant (Figure 11). No significant differences were observed in the haemodynamic

parameters during ischemia-reperfusion in any of the treatment groups when compared to the control group, indicating that the observed effects of mildronate and carnitine on infarct size are not related to changes in cardiac workload.

Treatment with carnitine or mildronate alone or with the combination of mildronate and carnitine induced an increase in GBB blood plasma (Figure 4B) and heart tissue (Figure 5B) concentrations. The highest GBB concentration was observed in the combination treatment group; however, the combination treatment did not reduce the infarct size (Figure 11).

Obtained results confirm that the mildronate-induced long-term decrease in carnitine concentration, as opposed to an increase in GBB concentration, in heart tissue is a key mechanism of action leading to the cardioprotective effects of mildronate. Decrease in carnitine concentration not only leads to inhibited CPT I dependant fatty acid metabolism, but it also compensatory facilitates glucose metabolism by increasing glucose metabolism related gene expression and enzyme activity (Broderick, 2006; Liepinsh et al., 2008).

4.3. Effects of changes in carnitine concentration on the development of hypertension

Hypertension is a well established risk factor for myocardial infarction and the development of endothelial dysfunction (Hirooka et al., 2008) and atherosclerosis (Biswas et al., 2003). Meanwhile treatment with carnitine has a certain impact on the development of hypertension-related complications (de Sotomayor et al., 2007). Mildronate also possess anti-atherosclerotic and angioprotective effects (Vilskersts et al., 2009). The aim of the present study was to investigate whether the administration of carnitine, mildronate or their combination for 8 weeks is protective against hypertension-induced complications in *Dahl* salt-sensitive (DS) rats.

DS rats fed with a high salt diet (PS, 8 % NaCl) developed marked hypertension already after 4 weeks of treatment (Figure 18). After 8 weeks of treatment elevated systolic blood pressure lead to the development of cardiac hypertrophy, but we did not observe the development of heart failure. Thus, in our study, we investigated the effects of the test compounds on hypertension-induced complications.

Experimental animals from PS group had decreased plasma concentration of carnitine compared with NS group animals (Table 3).

Differences between both groups could be result of an increased renal loss of carnitine due to development of renal injuries (Zhu et al., 2009). Treatment with carnitine (PS/C 100) completely restored, while combination treatment (PS/C+M) partially restored blood plasma concentration of carnitine (Table 3). Treatment with mildronate significantly decreased carnitine concentration, so it was possible to study effects of different carnitine availability on the development of hypertension-induced complications. Similarly to effects in *Wistar* rats, test compounds increased GBB concentration also in DS rat blood plasma (Table 3), especially in the combination treatment group.

Mortality in the PS control group was significantly increased compared to the NS group and administration of a combination of carnitine and mildronate significantly decreased mortality by 80 % (Figure 17). Because there were no differences between the functional parameters of the heart and lung to body weight indexes between groups, the possible cause of death could be the left ventricular hypertrophy, a recognized risk factor for myocardial infarction and lethal ventricular arrhythmias (Messerli, 1999).

Our results obtained in *Wistar* rat model suggest that mildronate possess an anti-arrhythmic effect which is presented as a decreased incidence of different types of arrhythmias (Figure 14 and 16), decreased time elapsed prior to onset of normal sinus rhythm (Figure 13), decreased duration of arrhythmias (Figure 15). It was hypothesized that increase in acyl-carnitines could provoke arrhythmias (Bonnet et al., 1999), therefore the anti-arrhythmic effect of mildronate could be related to decrease in CPT I activity (Figure 12) induced by decreased carnitine concentration in heart tissue (Figure 5A). As a result, also the concentration of acyl-carnitine should be decreased in mildronate treated heart tissues. In addition, some anti-arrhythmic effects of carnitine have been described (Najafi et al., 2008). Thus, the increased survival of DS rats after treatment with a combination of mildronate and carnitine could be result of both mechanisms (Figure 17).

It has been shown that resting heart rate is an independent predictor of cardiovascular morbidity and mortality, irrespective of the presence of co-morbidities (Palatini, 2008). Our results are in agreement with this observation, because after 8 week treatment the highest heart rate was found in PS group animals (Figure 19), along with the highest mortality (Figure 17) compared to NS group animals. Treatment with a combination of mildronate and carnitine decreased heart rate and also

significantly reduced mortality (Figure 17). Although mildronate treatment for 8 weeks also decreased heart rate, the protective effect against mortality was not so pronounced ($p<0.06$ vs. PS group) as in the combination group. These findings indicate that reduction of mortality in the PS/C+M group (Figure 17) could be related to the previously described cardioprotective effects of the tested compounds in the heart.

Similar to resting heart rate, endothelial dysfunction has been shown to be associated with the occurrence of cardiovascular events and hypertension-induced organ damage (Xu et al., 2009). In our study, the effects of treatment with carnitine, mildronate or their combination on the development of endothelial dysfunction were assessed in isolated DS rat aortic rings. Angioprotective effects of mildronate and carnitine have been described before (Vilskersts et al., 2009), however in the present study only administration of their combination attenuated the development of endothelial dysfunction (Figure 20). It has been suggested that the mechanism of attenuation of the development of endothelial dysfunction is based on direct influence on endothelium and vascular tissues, and not on the anti-hypertensive activity (Tzemos et al., 2001). Our results support this theory, as endothelial function was improved (Figure 20) without significant effect on arterial blood pressure (Figure 18).

Although changes in GBB concentration in the heart tissue correlate with the cardioprotective effect of mildronate in an experimental ischemia-reperfusion model in rats (Liepinsh et al., 2006), our results suggest that this effect depends on decrease in carnitine concentration (Figure 11). Meanwhile the angioprotective effect of combination of mildronate and carnitine most likely is related to increase in GBB concentration. Altogether, mildronate, carnitine and especially their combination increased GBB concentration in blood plasma. The most pronounced increase in GBB concentration and protection against hypertension-induced complications were observed in the combination group, thus suggesting the importance of GBB. These data suggest that the angioprotective action of GBB could improve the cardioprotective effects related to decrease in carnitine concentration, however experimental proof for such hypothesis remains to be found.

5. Conclusions

- Obtained results support the safety of long-term administration of mildronate. Treatment with mildronate for up to 3 month at doses that in the heart tissue decrease carnitine concentration 8- to 16-fold and increase GBB concentration 8- to 10-fold, but in the liver tissue decrease carnitine concentration 8- to 17-fold and increase GBB concentration 30- to 66-fold is not associated with cardiac impairment or disturbances in liver function and does not induce significant changes in lipid profile in tissues and blood plasma.
- Long-term treatment with mildronate inhibits CPT I dependent fatty acid β-oxidation. Decrease in carnitine concentration induced by 14-day mildronate treatment inhibits CPT I activity, therefore, mitochondrial respiration on palmitoyl-coenzyme A is decreased by 27 %. A compensatory increase in enzyme activity in the heart tissue is observed only after at least a month-long decrease in carnitine concentration.
- Under normal conditions mildronate does not affect pyruvate metabolism, since CrAT and pyruvate dehydrogenase complex activities in heart mitochondria are unchanged. Although mildronate is weak inhibitor of CrAT *in vitro* and competes with carnitine for binding to the catalytic site of an enzyme, *in vivo* it does not affect the enzyme activity.
- A mildronate-induced decrease in carnitine concentration in the heart tissue is a key mechanism of action for the cardioprotective effects of mildronate during ischemia. When mildronate induced decrease in carnitine tissue concentration is diminished, no cardioprotective effect can be observed.
- Angioprotective effect of mildronate and its combination with carnitine in the salt-induced hypertension model is based on the increase in GBB concentration in the blood plasma of *Dahl* rats.

- **Molecular mechanism of the cardioprotective effect of mildronate is based on the decreased carnitine concentration that decreases CPT I activity and inhibits fatty acid metabolism.**
- **Carnitine system regulation is a new tool for pharmaceutical industry to develop new medicines for the treatment of cardiovascular diseases.**

6. References

Baliutyte G, Baniene R, Trumbeckaite S, Borutaite V, Toleikis A. Effects of Ginkgo biloba extract on heart and liver mitochondrial functions: mechanism(s) of action. *J Bioenerg Biomembr*, 2010; 42(2):165-172.

Bartuś M, Łomnicka M, Kostogrys RB, Kaźmierczak P, Watała C, Słominska EM, Smoleński RT, Pisulewski PM, Adamus J, Gebicki J, Chlopicki S. 1-Methylnicotinamide (MNA) prevents endothelial dysfunction in hypertriglyceridemic and diabetic rats. *Pharmacol Rep*, 2008; 60(1):127-138.

Beadle RM, Frenneaux M. Modification of myocardial substrate utilisation: a new therapeutic paradigm in cardiovascular disease. *Heart*, 2010; 96(11): 824-830.

Biswas S, Dastidar DG, Roy KS, Pal SK, Biswas TK, Ganguly SB. Complications of hypertension as encountered by primary care physician. *J Indian Med Assoc*, 2003; 101(4):257-259.

Bonnet D, Martin D, Pascale De Lonlay, Villain E, Jouvet P, Rabier D, Brivet M, Saudubray JM. Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children. *Circulation*, 1999; 100(22):2248-53.

Briet F, Keith M, Leong-Poi H, Kadakia A, Aba-Alkhail K, Giliberto JP, Stewart D, Errett L, Mazer CD. Triple nutrient supplementation improves survival, infarct size and cardiac function following myocardial infarction in rats. *Nutr Metab Cardiovasc Dis*, 2008; 18(10):691-699.

Broderick TL. Hypocarnitinaemia induced by sodium pivalate in the rat is associated with left ventricular dysfunction and impaired energy metabolism. *Drugs R D*, 2006; 7(3):153-161.

Brown NF, Weis BC, Husti JE, Foster DW, McGarry JD. Mitochondrial carnitine palmitoyltransferase I isoform switching in the developing rat heart. *J Biol Chem*, 1995; 270(15):8952-8957.

Dambrova M, Cirule H, Svalbe B, Zvejniece L, Pugovichs O, Zorenko T, Kalvinsh I, Liepinsh E, Belozertseva I. Effect of inhibiting carnitine biosynthesis on male rat sexual performance. *Physiol Behav*, 2008; 95(3):341-347.

Dambrova M, Liepinsh E, Kalvinsh I. Mildronate: cardioprotective action through carnitine-lowering effect. *Trends Cardiovasc Med*, 2002; 12(6):275-279.

de Sotomayor MA, Mingorance C, Rodriguez-Rodriguez R, Marhuenda E, Herrera MD. L-carnitine and its propionate: improvement of endothelial function in SHR through superoxide dismutase-dependent mechanisms. *Free Radic Res*, 2007; 41(8):884-891.

Degrace P, Demizieux L, Du ZY, Gresti J, Caverot L, Djaouti L, Jourdan T, Moindrot B, Guilland JC, Hocquette JF, Clouet P. Regulation of lipid flux between liver and adipose tissue during transient hepatic steatosis in carnitine-depleted rats. *J Biol Chem*, 2007; 282(29):20816-20826.

Degrace P, Demizieux L, Gresti J, Tsoko M, André A, Demaison L, Clouet P. Fatty acid oxidation and related gene expression in heart depleted of carnitine by mildronate treatment in the rat. *Mol Cell Biochem*, 2004; 258(1-2):171-182.

Georges B, Le Borgne F, Galland S, Isoir M, Ecosse D, Grand-Jean F, Demarquoy J. Carnitine transport into muscular cells. Inhibition of transport and cell growth by mildronate. *Biochem Pharmacol*, 2000; 59(11):1357-1363.

Hirooka Y, Kimura Y, Sagara Y, Ito K, Sunagawa K. Effects of valsartan or amlodipine on endothelial function and oxidative stress after one year follow-up in patients with essential hypertension. *Clin Exp Hypertens*, 2008; 30(3):267-276.

Hmelnickis J, Pugovics O, Kazoka H, Viksna A, Susinskis I, Kokums K. Application of hydrophilic interaction chromatography for simultaneous separation of six impurities of mildronate substance. *J Pharm Biomed Anal*, 2008; 48(3):649-656.

Jogl G, Hsiao YS, Tong L. Structure and function of carnitine acyltransferases. *Ann N Y Acad Sci*, 2004; 1033:17-29.

Liepinsh E, Vilskersts R, Loca D, Kirjanova O, Pugovichs O, Kalvinsh I, Dambrova M. Mildronate, an inhibitor of carnitine biosynthesis, induces an increase in gamma-butyrobetaine contents and cardioprotection in isolated rat heart infarction. *J Cardiovasc Pharmacol*, 2006; 48(6):314-319.

Liepinsh E, Vilskersts R, Skapare E, Svalbe B, Kuka J, Cirule H, Pugovics O, Kalvinsh I, Dambrova M. Mildronate decreases carnitine availability and up-regulates glucose uptake and related gene expression in the mouse heart. *Life Sci*, 2008; 83(17-18):613-619.

Messerli FH. Hypertension and sudden cardiac death. *Am J Hypertens*, 1999; 12(12 Pt 3):181S-188S.

Mouhieddine S, de Leiris J. Cardioprotective effect of L-carnitine in rats submitted to permanent left coronary artery ligation. *Arch Int Physiol Biochim Biophys*, 1993; 101(6):411-416.

Müller-Nordhorn J, Binting S, Roll S, Willich SN. An update on regional variation in cardiovascular mortality within Europe. *Eur Heart J*, 2008; 29(10):1316-1326.

Najafi M, Garjani A, Maleki N, Eteraf Oskouei T. Antiarrhythmic and arrhythmogenic effects of L-carnitine in ischemia and reperfusion. *Bull Exp Biol Med*, 2008; 146(2):210-213.

Palatini P. Heart rate as predictor of outcome. *Blood Press Monit*, 2008; 13(3):167-168.

Simkhovich BZ, Shutenko ZV, Meirena DV, Khagi KB, Mezapuķe RJ, Molodchina TN, Kalviņs IJ, Lukevics E. 3-(2,2,2-Trimethylhydrazinium) propionate (THP)--a novel gamma-butyrobetaine hydroxylase inhibitor with cardioprotective properties. *Biochem Pharmacol*, 1988; 37(2):195-202.

Spaniol M, Brooks H, Auer L, Zimmermann A, Solioz M, Stieger B, Krähenbühl S. Development and characterization of an animal model of carnitine deficiency. *Eur J Biochem*, 2001; 268(6):1876-1887.

Spaniol M, Kaufmann P, Beier K, Wüthrich J, Török M, Scharnagl H, März W, Krähenbühl S. Mechanisms of liver steatosis in rats with systemic carnitine deficiency due to treatment with trimethylhydraziniumpropionate. *J Lipid Res*, 2003; 44(1):144-153.

Strijbis K, Vaz FM, Distel B. Enzymology of the carnitine biosynthesis pathway. *IUBMB Life*, 2010; 62(5):357-362.

Tsoko M, Beauseigneur F, Gresti J, Niot I, Demarquoy J, Boichot J, Bezard J, Rochette L, Clouet P. Enhancement of activities relative to fatty acid oxidation in the liver of rats depleted of L-carnitine by D-carnitine and a gamma-butyrobetaine hydroxylase inhibitor. *Biochem Pharmacol*, 1995; 49(10): 1403-1410.

Tzemos N, Lim PO, MacDonald TM: Nebivolol reverses endothelial dysfunction in essential hypertension: a randomized, double-blind, crossover study. *Circulation*, 2001; 104(5):511-514.

van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod Nutr Dev*, 2005; 45(6):665-688.

Vilskersts R, Liepinsh E, Mateuszuk L, Grinberga S, Kalvinsh I, Chlopicki S, Dambrova M. Mildronate, a regulator of energy metabolism, reduces atherosclerosis in apoE/LDLR-/- mice. *Pharmacology*, 2009; 83(5):287-293.

Wilcke M, Hultenby K, Alexson SE. Novel peroxisomal populations in subcellular fractions from rat liver. Implications for peroxisome structure and biogenesis. *J Biol Chem*, 1995; 270(12):6949-6958.

Xu JZ, Zhang Y, Wu SN, Niu WQ, Zhu DL, Gao PJ. Impaired endothelial function in hypertensive patients with target organ damage. *J Hum Hypertens*, 2009; 23(11):751-757.

Zhu A, Yoneda T, Demura M, Karashima S, Usukura M, Yamagishi M, Takeda Y. Effect of mineralocorticoid receptor blockade on the renal renin-angiotensin system in Dahl salt-sensitive hypertensive rats. *J Hypertens*, 2009; 27(4):800-805.

Acknowledgements

I would like to thank my supervisors Maija Dambrova and Renāte Medne who coordinated and supported my work.

Thanks to my colleagues without whom this work would not be possible: theoreticians and practitioners Edgars Liepiņš and Reinis Vilšķērsts, brain specialist Līga Zvejniece, animal caretakers Helēna Cīrule and Ligita Kariņa, masters in biochemistry Elīna Škapare and Baiba Švalbe, assistants Rūdolfs Mežapuķe and Olga Žarkova, document organizer Raita Brikmane and talented students Marina Makrecka and Edijs Vāvers.

I would like to say thanks to direction of Latvian Institute of Organic Synthesis, in person to Ivars Kalviņš.

I would like to say thanks to Solveiga Grīnberga and Osvalds Pugovičs, Edvards Liepiņš and Kristaps Jaudzems, Aleksandrs Gutsaits and Kirils Zinovjevs for their help in obtaining results.

Special thanks to my family.

Thanks for financial support to JSC “Grindeks” and JSC “Grindeks” foundation “Support for science and education”. Materials were obtained with the help of Latvian State Research Program grant No. 2010.10-4/VPP-4.

The doctoral studies were supported by the ESF Project "Support for PhD Program Studies and Earning of Scientific Degree at RSU”.