Dihydropyridines’ metabolites-induced early apoptosis after myocardial infarction in rats; new outlook on preclinical study with M-2 and M-3

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Abstract Our previous studies established cardio-protective effects of furnidipine and its active metabolites called M-2 and M-3. The aim of current research was to compare the effects of single oral pretreatment with 20 mg kg⁻¹ of M-2 and M-3 on mortality, different forms of arrhythmias, blood pressures parameters and ST-segment changes during occlusion (for 90 min) and reperfusion in the model of myocardial infarction in rats evoked by left anterior descending coronary artery occlusion. Additionally, the development of programmed cell death and biochemical parameters in blood serum were studied at 4th day after infarction. Furnidipines’ metabolites effectively reduced mortality index while did not markedly influence on blood pressures parameters, arrhythmias, ST-segment changes as well as biochemical parameters. Intriguingly, programmed cell death study (TUNEL) showed distinct increase in the amount of apoptotic nuclei in post-infarcted myocardium, granulation tissue and what is more in arteriolar walls after M-2 and M-3 application. Moreover, M-2 turned out to be more powerful in stimulation of apoptosis in granulation tissue surrounding infarcted area whereas M-3 presented balanced profile in this matter. Taking into account that programmed cell death plays positive role in post-infarcted heart healing, M-2 presents itself as more attractive agent for oral pre-treatment in early stages of ischemia by non-stable individuals due to its more specific action in stimulation repairing processes in granulation tissue as well as in arteriolar walls. While M-2 and M-3 are common metabolites present in degradation pathways of many widely used dihydropyridines in clinic, this key fact put the new outlook on understanding additional mechanism and effects of not only furnidipines’ metabolites but also other dihydropyridines.

Keywords Furnidipines’ metabolites · Reperfused myocardial infarction · Programmed cell death · Rat · Hemodynamic · Arrhythmias

Introduction

Although reperfusion of the myocardium following coronary occlusion reduces infarct size and improves its function [1, 2], numerous studies point to potential detrimental effect of reperfusion on endothelium, myocardial muscle structure, coronary vascular reactivity and potentially lethal rhythm disturbances [3–5] as well as for apoptosis [6, 7]. In addition, observations made during therapeutic revascularization procedures in infarct patients strongly suggest that reperfusion might facilitate remobilization of small vessels [8–10]. Despite considerable efforts, results in protection or therapeutic management of reperfusion-triggered pathologies and ischemic coronary artery diseases still are far from being satisfactory.

The dihydropyridine derivatives (DHPs) currently used for therapeutic purposes possess L-type calcium channel blocking properties, and treatment of hypertension and certain specific forms of angina pectoris remain to be their main therapeutic indications [11]. In addition, it has been shown that DHPs can protect the heart from stunning, ischemia as well as ventricular arrhythmias, and that they

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possess beneficial effects against experimental atherosclerosis [9, 10, 12–19]. Furthermore, many studies revealed that calcium channel blockers can reduce infarct size in experimental animals [5, 20–22]. It is unclear though, whether these therapeutically interesting pharmacological properties demonstrated for many DHPs are only due to their blocking effects on the L-type calcium channel. In fact, it is well established that some of them can enhance opening probability of L-type channels [23] and yet others can effectively modulate various ion channels and pharmacological targets as well. Due to their different activities, DHPs are sometimes referred to be pharmacologically “privileged structures” [24, 25].

Attempts to reach such goals have led to the identification of furnidipine (FUR) [26, 27]. Initially, FUR (for structure see Fig. 1) was identified structurally and functionally as a DHP with potent L-type calcium channel blocking activity, good oral bio-availability and exceptionally high safety margin in pre-clinical studies [28, 29]. Later efforts to define its pharmacological activity profile revealed though, that unlike most therapeutically used calcium antagonists, FUR is highly selective in relaxing both venous capacitance and arterial vessels resistance. Although it has no influence on heart conduction system, it revealed antiarrhythmic activity in the aconitine-induced arrhythmias model in rats [26, 27, 29]. In addition, FUR pretreatment afforded protection against norepinephrine-induced cardiomyocyte necrosis in rats and its effective dose range in this model was much broader than that of nitrendipine. What is more, after oral as well as intravenous pretreatment, FUR can not only dose-dependently influence on arterial blood pressure through the vessels relaxation and reduction in myocardial oxygen consumption, but also can afford protection against reperfusion-triggered myocardial damages and especially rats’ mortality decrease evoked by lethal arrhythmias [30]. Interestingly, its protective effects on myocardial tissue damage, judged by the creatine kinase reduction in blood, were observed as well [30]. Although antihypertensive effects of FUR were apparent in several animal models [26, 31] results of various controlled clinical trials indicated that in comparison to several other calcium antagonists, its antihypertensive efficacy is negligible.

At least two FUR metabolites lacking calcium channel blocking activity with prolonged plasma half-life have already been identified in human volunteers and called as M-2 and M-3 [31]. In addition, plasma levels of FUR oxidative metabolites were always higher than that of the parent molecule after oral administration. Consequently, both metabolites were screened for their typical blocking activity and cardio-protective potential in several in vitro models commonly used for such purposes [31]. Early data in our laboratories have revealed similar efficacy of M-2 in the same experimental model as used for FUR. Unlike furnidipine, M-2 did not modulate blood pressure parameters or heart rate [30]. Furthermore, other authors proved that M-2 itself did not influence significantly the guinea pig cardiomyocyte action potential. However, it antagonized dose-dependently ($1 \times 10^{-7}$–$3 \times 10^{-7}$ M) and markedly the veratridine-induced action potential lengthening as well as the anoxia-induced action potential shortening and additionally, prevented from the cellular shape changes [31].

Comparing the influence of continuous infusion of pro-drug to M-2 and M-3 in working rats’ heart model [32–34]. We established that FUR evoked significantly weaker influence on coronary and aortic flow, whereas both metabolites caused a significant coronary flow increase. What is more, M-3 caused the aortic systolic and diastolic pressures decrease. Due to clear differences found between all three agents, we concluded that the cardio-depressant potency of both metabolites is overcome by advantageous vasodilatatory effect. In addition, studying the effects of

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**Fig. 1** Structure of parent drug, furnidipine, and its metabolites M-2 and M-3
M-2 in two different models of working heart ischemia (low-flow and regional), we confirmed that it improved coronary flow in both models, while favorably maintaining aortic pressure parameters [35]. Moreover, it provided outstanding protection against deleterious effects of calcium overload (induced by veratridine in the Langendorff heart) by significant prevention of the left ventricular diastolic pressure rise and coronary flow decrease [36, 37].

Concluding these results, it allowed us to propose the working hypothesis that the protective effects against cellular damage evoked by FUR could be mainly attributed to its metabolites and moreover, indicate other sites of action different from the L-type calcium channel suggesting pleiotropic effects on the ischemic heart by imparting protection in various ways [34, 35, 38].

Since in vitro results do not always correspond to in vivo outcomes, we studied the influence of M-2 on hemodynamic parameters and ischemia- and reperfusion-induced arrhythmias in rats [15, 30, 39] for further testing its potential value as a therapeutic agent in infarcted hearts. Dose- and time-response curves were obtained after oral administration of M-2 in order to establish its pharmacokinetic properties and pharmacodynamic half-life. We proved that it significantly reduced mortality, incidence and duration of severe arrhythmias with differential influence on blood pressure, which depended on dose and time of administration [35]. The optimal oral dose of M-2 and M-3 was 20 mg kg$^{-1}$ and those has been used in current study as well.

Considering the all promising results, we decided to perform present study in order to compare the effects of M-2 as well as M-3 pretreatment on different forms of arrhythmias, blood pressures and ST-segment changes during occlusion (for 90 min) and reperfusion in the model of myocardial infarction in rats evoked by permanent left anterior descending coronary artery (LAD) occlusion [40]. Furthermore, the development of programmed cells death and biochemical parameters in blood serum for the heart damage were studied at 4th day after myocardial infarction.

Materials and methods

Experimental animals

Male Sprague–Dawley rats ($n = 54$; Central Animal Farm, Medical University of Silesia, Katowice, Poland) weighing approx. $320 \pm 25$ g and maintained under standard condition (ambient temperature 21–23 °C; with 12 h dark/light cycle) with ad libitum access to food (Altromin 1220, Altromin GmbH, Lage, Germany) and tap water, served as experimental animals. The animals were fasted overnight before the experiment. The study was performed with the approval of the Local Bioethical Committee and all experiments were conducted in accordance with NIH regulations of animals care described in the “Guide for the Care and Use of Laboratory Animals” (NIH publication, p. 2–107, revised 1996).

Drugs and reagents used

Two metabolites of furnidipine were used: M-2 [2,6-dimethyl-5methoxy-carbonyl-4-(2’-nitrophenyl)-pyridine-3-carboxylique acid, MW 330.29] and M-3 [3-methyl 5-(tetrahydrofuran-2-yl) methyl 2,6-dimethyl-4-(2’-nitrophenyl) pyridine-3,5-dicarboxylate, MW 414.45] (Fig. 1). Both metabolites were supplied by Cermol S.A. (Geneva, Switzerland) and were primarily dissolved in diluted dimethylsulfoxide (DMSO) and later in water. For oral administration, M-2 or M-3 solutions were prepared in 0.4 % aqueous dimethylsulfoxide and given in a volume of 5 mL kg$^{-1}$ through inserted stomach-tube. Water was used in the control group, nevertheless, in order to consider the influence of DMSO itself, additional control group was added (0.4 % DMSO). Unless otherwise stated, all other reagents were of the highest purity and were supplied by Sigma Chemical Co. (Deisenhofen, Germany).

Experimental infarction in rats

For this study an improved preparation previously described by others [41, 42] with own modifications described in details elsewhere [32, 40, 43–46] was used. The Lambeth Conventions were used also as a guideline for this research [47].

The rats were anesthetized with pentobarbital (60 mg kg$^{-1}$/at the beginning/+30 mg kg$^{-1}$ i.p./10 min before reperfusion/pentobarbital sodium salt Sigma, Deisenhofen, Germany). To compare the depth of anesthesia, reflex response to noise and pain induced by the pinching of the limbs and distal portion of the tail, were tested in each rat at the beginning and the end of the experiment as prescribed [48, 49]. Rectal temperature was maintained at approximately 38 °C.

The left common carotid artery was cannulated with a filled catheter (saline with 2 IU mL$^{-1}$ heparin) for systolic and diastolic blood pressures (BPs, BPD) measurement using an ISOTEC transducer (Hugo Sachs Elektronik, March-Hugstetten, Germany).

The myocardial infarction was induced by permanent left anterior descending coronary artery occlusion for 90 min and followed by 15 min of reperfusion. Only rats that survived until 4 day after infarction were taken into account during our investigations.

In brief, the trachea was incised longitudinally and cannulated to allow artificial ventilation. The chest was
opened under ventilation with room air (55–60 % humidity, 23 °C, stroke volume 0.8 mL 100 g⁻¹ of body weight; rate 54 strokes min⁻¹ with the positive end-respiratory pressure of 1 cm H₂O; Rodent VENTILATOR-UB 7025, Hugo Sachs Elektronik, March-Hugstetten, Germany) [50] by left thoracotomy at the fifth intercostal space and the fifth and fourth ribs were sectioned approximately 2 mm from the left margin of the sternum. After opening the pericardium the heart was not exteriorized and a sling (6/0 Prolene 0.7 suture attached to 3/8 circled BV-1 a 9.3 mm atraumatic, reverse cutting needle, EH 7406H, Ethicon GmbH, Norderstedt, Germany) was placed around LAD close to its origin (2 mm below). Then the ligature was passed through a plastic pad (polyethylene, 2 mm OD/0.5 ID, thickness 0.2 mm). The left coronary artery was reopened for 15 min. The mortality index (MI) was calculated for the rats that survived this period (Table 1).

Experimental groups, design and measured parameters

The rats were randomly divided into four groups. Same doses of M-2 and M-3 (20 mg kg⁻¹ each) or the vehicles (water or 0.4 % DMSO in the volume of 5 mL kg⁻¹ each) were orally administered 1 h before LAD occlusion. At the beginning of our study, each experimental group consisted of: water (n = 7), DMSO (n = 13), M-2 (n = 12) and M-3 (n = 12) rats. The LAD was occluded for 90 min and then reopened for 15 min. The mortality index was calculated for the rats that survived this period (Table 1).

The continuous blood pressure parameters (BPs and BPD) measurements were performed and recorded every 10 min during the occlusion and reperfusion (from 5th to 105th min; see Table 1). The ST-segment changes (in mm) were estimated every 5 min during the occlusion and reperfusion periods (see Table 2).

The number of the premature ventricular beats (PVBs) during occlusion and reperfusion was counted as well as the incidence (in %) and duration (in seconds) of the spontaneously reversible ventricular fibrillation (VF), ventricular tachycardia (VT), salvos, bigeminy or trigeminy that occurred during both periods were measured from the continuous ECG recordings using own software (off-line) (see Table 3). All rhythms’ disturbances in rats were distinguished due to rules described in details elsewhere [47, 51] (Fig. 2).

Biochemical estimation in blood serum

At the 4th day of experiment, 1 mL of rats’ blood was collected directly from aortic arch and without heparinizing dissolved in saline (1/1 vol/vol.) to analyze creatine kinase (CK, U/L; wavelength 340 nm, Reagent-test, Gilford, Ciba-Cornig, Cambridge, MA, USA) [52], glutamate-pyruvate transaminase (GTP, U/L, 340 nm) and glutamate-oxaloacetate transaminase activity (GOT, U/L, 340 nm) in order to estimate heart muscle damage as well as the level of glucose (mg dL⁻¹, 340 nm), urea (mg dL⁻¹, 530 nm), bilirubin (mg dL⁻¹), creatinine (mg dL⁻¹, 340 nm) and α-amylase (U/L, 578 nm) spectrophotometrically (Specol 220, VEB Carl Zeiss, Jena, Germany) [53] (Table 4). In order to obtain the physiological values of biochemical parameters mentioned above, the intact group (n = 10) was added to the trial.
Effects of single oral pretreatment with 20 mg kg⁻¹ M-2 or M-3 on blood pressure during 90 min of the left anterior coronary occlusion and 15 min of reperfusion in rats

|                   | Continuous blood pressure measurement (mmHg) (mean ± SD) |
|-------------------|----------------------------------------------------------|
|                   | Occlusion (90 min) Reperfusion (15 min)                  |
| 5 min             | 95 min Br/Bd                                              |
|                   | 105 min Br/Bd                                             |
|                   | 115 min Br/Bd                                             |
|                   | 125 min Br/Bd                                             |
|                   | 135 min Br/Bd                                             |
|                   | 145 min Br/Bd                                             |
|                   | 155 min Br/Bd                                             |
|                   | 165 min Br/Bd                                             |
|                   | 175 min Br/Bd                                             |
|                   | 185 min Br/Bd                                             |
|                   | 195 min Br/Bd                                             |
Table 2  Effects of single oral pretreatment with 20 mg kg\(^{-1}\) of M-2 or M-3 on ST-segment changes during 90 min of left anterior coronary occlusion and 15 min of reperfusion in rats

| Experimental group | ST-segment estimation (J point (mm)) (mean ± SD) | Before  | Oclusion |
|-------------------|--------------------------------------------------|---------|----------|
|                   |                                                  | 1 min   | 5 min    | 10 min   | 20 min   | 30 min   | 40 min   | 50 min   | 60 min   | 70 min   | 80 min   | 90 min   |
| Control n = 4/7   | -0.05 ± 0.06                                    | -0.027 ± 0.105 | -0.022 ± 0.021 | 0.132 ± 0.204 | 0.092 ± 0.147 | 0.082 ± 0.159 | 0.07 ± 0.120 | 0.01 ± 0.145 | 0.055 ± 0.151 | 0.085 ± 0.179 | 0.11 ± 0.16 |
| DMSO n = 10/13    | -0.021 ± 0.044                                  | -0.02 ± 0.042 | -0.009 ± 0.028 | 0.082 ± 0.109 | 0.083 ± 0.101 | 0.073 ± 0.026 | 0.073 ± 0.083 | 0.079 ± 0.099 | 0.079 ± 0.099 | 0.064 ± 0.091 | 0.051 ± 0.055 |
| M-2 n = 10/12     | -0.015 ± 0.03                                  | -0.006 ± 0.07 | -0.007 ± 0.01 | 0.086 ± 0.1 | 0.095 ± 0.11 | 0.09 ± 0.11 | 0.101 ± 0.13 | 0.119 ± 0.15 | 0.114 ± 0.15 | 0.096 ± 0.15 | 0.096 ± 0.12 |
| M-3 n = 10/12     | -0.041 ± 0.047                                  | -0.02 ± 0.043 | 0.0 ± 0.062 | 0.061 ± 0.125 | 0.158 ± 0.318 | 0.067 ± 0.12 | 0.076 ± 0.042 | 0.081 ± 0.134 | 0.07 ± 0.122 | 0.073 ± 0.117 | 0.088 ± 0.118 |

| Experimental group | ST-segment estimation (J point (mm)) (mean ± SD) | Reperfusion |
|-------------------|--------------------------------------------------|-------------|
|                   |                                                  | 100 min | 105 min |
| Control n = 4/7   | 0.095 ± 0.119                                   | 0.075 ± 0.116 | 0.075 ± 0.116 |
| DMSO n = 10/13    | 0.05 ± 0.073                                    | 0.047 ± 0.074 | 0.047 ± 0.074 |
| M-2 n = 10/12     | 0.045 ± 0.09                                    | 0.042 ± 0.09 | 0.042 ± 0.09 |
| M-3 n = 10/12     | 0.066 ± 0.148                                   | 0.06 ± 0.141 | 0.06 ± 0.141 |

The differences in ST-segment changes was calculated using non-parametric Kruskal–Wallis ANOVA test with appropriate post hoc test. For other details see Table 1 and text.
reaction were visualised with diaminobenzidine (DAB) procedure. Cellular nuclei were counterstained with hematoxylin and cytoplasm slightly with eosin. Finally the sides were dehydrated and mounted in Canada balsam. The control, positive slides were made from rat prostate at 3rd day after castration.

The stain in the cells labelled by TUNEL technique was visualised as dark brown precipitate (dark cells). These cells were considered as the cells during the programmed cell death (PCD) process.

After histological evaluation, the main striking or representative areas from the tissue were captured with digital camera and written as *.tif files using 24 bit color palette at fixed magnification (150 magnification) using POLYVAR (Reichert-Leica) light microscope.

The quantitative evaluation of TUNEL-positive cells was done using ImmunoRatio software as percentage of Table 3 Effects of single oral pretreatment with 20 mg kg\(^{-1}\) of M-2 or M-3 on different forms of arrhythmias occurred during 90 min of left anterior coronary occlusion and 15 min of reperfusion in rats

| Experimental group | Arhythmmas calculation (mean ± SD) |
|--------------------|----------------------------------|
|                    | Occlusion (90 min)               |
|                    | PVBs (number) | VT duration (s) | VF duration (s) | Salvos duration (s) | Bigeminy duration (s) | Trigeminy duration (s) |
| Control \(n = 4/7\) | 17 ± 18.7* | 5.3 ± 10.5 | 3.75 ± 7.5 | 2.75 ± 3.2 | 19.75 ± 25.7 | 0 |
| DMSO \(n = 10/13\) | 10.8 ± 13.3* | 8.1 ± 15.2 | 9.0 ± 28.5 | 1.2 ± 2.6 | 11.3 ± 19.5 | 2.7 ± 7.5 |
| M-2 \(n = 10/12\) | 7.6 ± 9.2* | 17.9 ± 32.64 | 3.0 ± 9.4 | 2.1 ± 3.2 | 25.2 ± 33.7* | 10.8 ± 34.15 |
| M-3 \(n = 10/12\) | 8.2 ± 11.5* | 23.1 ± 52.8 | 0.6 ± 1.89 | 2.4 ± 3.77 | 18.8 ± 29.5 | 3.0 ± 9.4 |

|                    | Reperfusion (15 min)              |
|                    | PVBs (number) | VT duration (s) | VF duration (s) | Salvos duration (s) | Bigeminy duration (s) | Trigeminy duration (s) |
| Control \(n = 4/7\) | 0.25 ± 0.5 | 0 | 0 | 0 | 0 |
| DMSO \(n = 10/13\) | 1.1 ± 3.14 | 0 | 0 | 0 | 0 |
| M-2 \(n = 10/12\) | 1.3 ± 2.75 | 0 | 0 | 0 | 0.6 ± 1.89 |
| M-3 \(n = 10/12\) | 0.6 ± 1.35 | 2.1 ± 6.64 | 0 | 0 |

The differences in arrhythmias duration [ventricular tachycardia (VT), ventricular fibrillation (VF), salvos, bigeminy or trigeminy in seconds] and the number of premature ventricular beats (PVBs) were calculated using non-parametric Kruskal–Wallis ANOVA test with appropriate post hoc test. Values marked with * \(p < 0.05\) are significantly different from the values of corresponding parameter in reperfusion. For other details see Table 1 and text.

Fig. 2 Characteristic electrocardiogram tracings (recorded from I limb lead with recorder speed 100 mm s\(^{-1}\)): a normal tracing before coronary artery (LAD) occlusion, b ventricular extrasystoly (VE), c multiple premature ventricular beats (PVBs), d ventricular fibrillation (VF), e ST-segment elevation during ischemia, f ST-segment elevation after LAD occlusion, g ejection fraction, h ECG tracing during late reperfusion.
DAB stained nuclei to all nuclei in a region of interest at 150× magnification, separately for myocardial muscle and for resorptive granulation tissue in the site of infarcts’ three consecutive areas [55]. All doubtfully stained areas were excluded from calculations and assessment.

**Statistical analysis**

Blood pressures parameters (BPs, BPd), the ST-segment changes, all forms of arrhythmias as well as biochemical parameters were measured only in the reperfusion surviving animals. Except for the mortality index, all other results are expressed as mean ± standard deviation (SD). Because the data were not normally distributed, for all comparisons non-parametric Kruskal–Wallis ANOVA test was used [56] with appropriate post hoc test. In order to estimate the significance between mortality and incidence of different forms of arrhythmias, the Chi square-test ($\chi^2$; Yates) was used in all comparisons.

The quantitative evaluation of TUNEL-positive cells were compared using Kruskal–Wallis test for equal medians and non-parametric ANOVA with Mann–Whitney pairwise test as post hoc verification of significant probability (Statistica v. 10 software). In all cases differences were considered significant at $p < 0.05$.

**Results**

**Mortality index, blood pressures parameters and ECG study in occlusion and reperfusion**

The mortality index did not differ significantly among studied groups, however, in M-2 or M-3 treated animals only two animals did not survive the experiment (16 %) while in each controls groups three rats died (42.8 and 23.1 %, respectively) (Table 1).

During 105 min of the continuous blood pressure measurement no significant changes were considered in occlusion as well as in reperfusion between studied groups (Table 1). Similarly, no significant changes have been found in ST-segment between studied groups (Table 2).

Unlikely, the number of PVBs was significantly reduced in reperfusion in compare to occlusion period in all groups ($p < 0.05$). In addition, the bigeminy duration in reperfusion was markedly reduced after M-2 pretreatment ($p < 0.05$). Similar action was observed concerning the trigeminy duration, but this effect was not significant (Table 3).

**Biochemical estimation in serum samples**

A single administration of water, 0.4 % DMSO, M-2 or M-3 did not significantly influence on the concentration of glucose, amylase, urea and GTP measured in blood serum at 4th day after infarction.

Only M-3 single pretreatment reduced significantly glutamate-oxaloacetate transaminase concentration in comparison to water control group ($p < 0.05$), however, M-2 and DMSO groups slightly diminished this parameters as well (Table 4). Similarly, M-3 strongly reduced creatine kinase concentration in comparison to M-2 and DMSO groups ($p < 0.05$). It should be mentioned that M-2 caused the highest increase of this parameter among all tested groups. The concentrations of bilirubin and creatinine in serum were below the detection limit in all groups (Table 4).

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**Table 4** Effects of single oral pretreatment with 20 mg kg$^{-1}$ of M-2 or M-3 on blood parameters measured after 90 min of left anterior coronary occlusion and 15 min of reperfusion in rats

| Experimental group | Glucose (mg/dL) | Bilirubin (mg/dL) | Creatinine (mg/dL) | Amylase (U/L) | GTP (U/L) | GOT (U/L) | Creatine kinase (U/L) | Urea (mg/DL) |
|--------------------|----------------|------------------|--------------------|--------------|-----------|-----------|----------------------|------------|
| Intact $n = 10$    | 139.6 ± 6.92   | <0.5             | <0.5               | 4 323.3 ± 241.9 | 21.35 ± 1.29 | 109.8 ± 9.3 | 216.4 ± 87.7 | 53.46 ± 2.68 |
| Control $n = 4/7$  | 137 ± 31.7     | <0.5             | <0.5               | 2 975 ± 697.6  | 24.8 ± 3.86 | 109.6 ± 10.3* | 384.2 ± 106.8 | 59.5 ± 0.7  |
| DMSO $n = 10/13$   | 137.9 ± 17.6   | <0.5             | <0.5               | 2 996 ± 512    | 23.1 ± 5.86 | 84.8 ± 13.5 | 362.7 ± 105.2* | 60.3 ± 6.7  |
| M-2 $n = 10/12$    | 128.3 ± 20.2   | <0.5             | <0.5               | 3 169 ± 1063.8 | 22 ± 6.53  | 85.5 ± 24.8 | 481.2 ± 317.5* | 54.6 ± 7.03 |
| M-3 $n = 10/12$    | 139.1 ± 12     | <0.5             | <0.5               | 2 779 ± 627.4  | 20.2 ± 4.8  | 64.1 ± 18   | 184.7 ± 88   | 60 ± 3.82   |

Bilirubin and creatinine were below detection limit. For other details see Table 1 and text. The differences in blood parameters was calculated using non-parametric Kruskal–Wallis ANOVA test with appropriate post hoc test.

Values marked with * $p < 0.05$ are significantly different from the values of M-3 group.
Morphological study

At 4th day after myocardial infarction, the histological findings in all studied slices were generally similar in treated and non-treated rats. They represented resorptive inflammatory infiltrations inside and granulation tissue (data not shown).

Programmed cell death

At 4th day after myocardial infarction, TUNEL-positive cell nuclei were present scanty in control and DMSO groups in myocardial cells as well as in granulation tissue, whereas in M-2 and M-3 groups brownish stained TUNEL-positive nuclei were easily recognized in both lesions (Figs. 3, 4). Moreover, in M-2 and M-3 treated animals, apoptotic cells were found also in arteriolar walls, whereas in control and DMSO groups this phenomenon was never exhibited (Fig. 5). Statistical analysis revealed marked increase in the amount of apoptotic nuclei after both furnidipines’ metabolites treatment in myocardial as well as granulation tissue, when compared to control and DMSO groups. Additionally, M-3 presented significantly stronger proapoptotic effect than M-2 in all cases. The statistical and quantitative results are presented in details in Figs. 3 and 4.

Discussion

In chosen rats’ model of myocardial infarction followed by 15 min of reperfusion, the single oral pretreatment with both studied metabolites effectively reduced mortality index of the animals, did not markedly influence on blood pressure as well as on the ST-segment changes (Table 1, index of the animals, did not markedly influence on blood both studied metabolites effectively reduced mortality as well as it strongly reduced CK values in comparison to M-2 and DMSO groups ($p < 0.05$). Although some beneficial effects of the tested compounds were present, in view of our previous experiments with these agents [34, 35, 38, 57] it could be concluded that the model used in present study is not suitable to quantify their optimal cardioprotective effects.

Despite the fact that no visible differences were found in routine histopathological investigation, the most intriguing results of this research concern the programmed cell death study. At 4th day after reperfused infarcted rats’ heart, TUNEL-positive cell nuclei were present scanty in myocardial cells as well as in granulation tissue in control and DMSO groups, whereas in M-2 and M-3 groups brownish stained TUNEL-positive nuclei were easily recognized in both tissues. At least in this issue, the clear difference between M-3 and M-2 due to proapoptotic influence was found. In both tissues, the M-3 increased approx. 30 % the number of TUNEL-positive nuclei, while the M-2—approx. 5 %. Moreover, several differences in apoptotic rate between ischemic myocardium and granulation tissue were noticed after application of each agent. Percentage of the total effect in both tissues (calculated as in the example of DMSO granulation: 5/negative/+3/positive/ = 8, what results 3/positive/is 37 % of total) revealed that: (a) DMSO stimulated apoptosis in granulation tissue only (37 %), (b) M-2 is more powerful in apoptosis stimulation in granulation tissue than in post-ischemic myocardium cells (52 vs 32 %), (c) M-3 is equally potent in apoptosis stimulation in both tissues (approx. 50 %).

According to these results, M-2 is more likely in promoting apoptosis in granulation tissue, while M-3 possess more balanced profile for apoptosis stimulation in both tissues.

It should be also noticed that we cannot exclude the DMSO membrane effects on apoptosis stimulation in granulation tissue caused by both furnidipines’ metabolites. The obligatory use of DMSO as a solvent for dihydropyridines derivatives (even in the concentrations lower than 0.4 %) will always mimics to some extend these agents’ action due to its ability to generate apoptosis per se [58, 59].

Interestingly, apoptotic cells were also found in arteriolar walls after both metabolites administration, whereas in control and DMSO groups this phenomenon was not observed at whole.

In view of the fact presented above, the fundamental, controversial questions return: whether the apoptosis in general is beneficial for healing, remodeling processes after myocardial infarction and whether the proapoptotic or antiapoptotic agents are wanted participants in this game?

Although programmed cell death proceeds by the same mechanism in each cell, the meaning of this process in
heart healing can be different, due to the consequences it brings. Whereas ongoing process of apoptosis may be beneficial in one condition, in other it may be linked with detrimental effects. It appears obvious that apoptosis contributed with myocyte loss as well as organising fibrotic tissue in the place of ischemia results in sustained contractile failure of myocardium [60]. Other authors reported also that myocytes apoptosis is likely to precede necrosis connected with disintegration of cells and in consequence, deterioration of heart function [61, 62]. Furthermore, the experimental study on mice proved that inhibition of apoptotic cascade by transfer of adenoviral antiapoptopic soluble Fas gene in the 3rd day after ligation-induced myocardium infarction could be potentially valuable therapeutic strategy in cardiac diseases [63]. In addition, recent data revealed as well that anoikis, defined as special type of PCD induced by cell detachment, is responsible for pathological remodeling of cardiovascular tissue in heart failure [64, 65].

On the other hand, the functional role of apoptosis in granulation tissue seems to have different meaning. According to the fact that TUNEL detection of apoptosis is not limited to the dying myocytes, but it also points out other cells undergoing this process [62], TUNEL positivity in granulation tissue may be related to the presence of inflammatory cells which accumulate in the injured myocardium area. It is well established the infiltrated leukocytes and macrophages play essential role in infarct healing
by their ability of scavenging ischemic area from death myocytes as well as stimulation of angiogenesis and myofibroblast proliferation [66]. In addition, the inflammatory cells after fulfilling their role undergo the process of PCD which is, in comparison to necrosis, a ‘safe death pathway’ which protects survived myocytes from excessive inflammation process caused by release of their cytotoxic factors such as cytokines and proteases [62].

Moreover, our finding of apoptotic cells in arteriolar walls in M-2 and M-3 treated groups sounds favourably in the light of the statement indicating that apoptosis helps in rebuilding the intima of coronary vasculatures by elimination of injured cells, what results in quicker growth of new endothelium [62].

In general, the balance between programmed cell death and regeneration processes in all kinds of tissues seems to be crucial aspect in determination of myocardium recovery after infarction. In the light of the histological results presented above, M-2 shows itself as more attractive agent for oral pretreatment in early stages of ischemia by non-stable individuals suggested elsewhere [57] due to its more specific action in stimulation resorptive processes in granulation tissue as well as in arteriolar walls. Furthermore, this working hypothesis enlarge our outcomes from previous study where M-2 positively influenced on post-infarction heart remodeling. We have proved, M-2 administration from 6th to 35th day after infarction effectively prevents cardiomyopathy development through the
revitalisation of the coronary arteries, infarct scar remodeling as well as acceleration of angiogenic events [38].

Although apoptosis can be induced by ischemia itself [62], it cannot succeed without oxygen as it is active, energy-requiring process. Accordingly, reperfusion is one of the triggers responsible for promoting the apoptotic cascade and is associated with higher TUNEL positivity [62, 67]. It has been proven the apoptotic nuclei are particularly evident in the infarcted areas where spontaneous reperfusion most often occurs [61, 68]. The higher amount of TUNEL-positive nuclei in samples collected from M-2 and M-3 groups suggests better reperfusion in myocardium cells in groups pretreated with furnidipines’ metabolites in contrast to control and DMSO groups. Clearly, this effect may be achieved due to their proven various vasodilatatory properties, which do not belong to dihydropyridines derivatives. Taking into account, that programmed cell death could be summarized: M-2 and M-3 are common metabolites present in degradation pathways of many and widely used dihydropyridines in clinic, especially in long-term secondary prevention of myocardial infarct.

In conclusion, searching for confirmation of results obtained in our previous studies concerning favourable effects of furnidipines’ metabolites (especially M-2) administration on mortality, blood pressure, arrhythmias and post-infarcted remodeling, one could consider that our expectations for next beneficial outcomes to some extent failed. Despite there are no distinguishing differences regarding FUR metabolites hemodynamic profiles as well as arrhythmias incidence and duration, fortunately, we decided to challenge the problem at the histopathological level as well. Surprising effect of M-2 and M-3 single pretreatment paired with higher apoptotic rate in post-ischemic myocardium as well as in granulation tissue and what is more, in arteriolar walls is a major interest and again makes furnidipines’ metabolites intriguing objects for future investigations.

It must be remembered, M-2 and M-3 are common metabolites present in degradation pathways of many and widely used dihydropyridines in clinic, especially in long-term secondary prevention of myocardial infarct.

This key fact put the new outlook on understanding additional mechanism and effects of not only furnidipines’ metabolites as well as other dihydropyridines.

Taking into account, that programmed cell death could play positive role in post-infarcted heart healing, it might be summarized: M-2 is more specific in stimulation of repairing processes in granulation tissue after occlusion-induced myocardium infarction. Accordingly, it may allow to introduce a novel class of agents with attractive properties, which do not belong to dihydropyridines derivatives due to their different chemical structure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Kloner RA, Ellis SG, Lange R, Braunwald E (1983) Studies of experimental coronary artery reperfusion. Effects on infarct size, myocardial function, biochemistry, ultrastructure and microvascular damage. Circulation 68:38–115
2. Stack RS, Philips HR III, Grierson DS, Behar VS, Kong Y, Peter M et al (1983) Functional improvement of jeopardized myocardium following intracoronary streptokinase in acute myocardial infarction. J Clin Invest 72:84–95
3. Selwyn AP, Welman E, Fox K, Horlock P, Pratt TP, Klein M (1979) The effects of nifedipine on acute experimental myocardial ischemia and infarction in dogs. Circ Res 44:16–23
4. Ribeiro LGT, Brandon TA, Debauche TL, Maroko PR, Miller RR (1981) Anti-arrhythmic and hemodynamic effects of calcium channel blocking agents during coronary arterial reperfusion. Comparative effects of verapamil and nifedipine. Am J Cardiol 48:69–74
5. Melin JA, Becker LC, Hutchins GM (1984) Protective effect of early and late treatment with nifedipine during myocardial infarction in the conscious dog. Circulation 69:131–141
6. Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E et al (1996) Acute myocardial infarction in human associated with activation of programmed myocyte cell death in the surviving portion of the heart. J Mol Cell Cardiol 28:2005–2016
7. Mani K (2008) Programmed cell death in cardiac myocytes: strategies to maximize post-ischemic salvage. Heart Fail Rev 13:193–209
8. Braunwald E, Kloner RA (1985) Myocardial reperfusion: a double-edged sword. J Clin Invest 76:1713–1719
9. Opie LH (1994) Myocardial stunning: are calcium antagonists useful? Cardiovasc Drugs Ther 8:533–541
10. Opie LH, Yusuf S, Kübler W (2000) Current status on safety and efficacy of calcium channel blockers in cardiovascular diseases: a critical analysis based on 100 studies. Prog Cardiovasc Dis 43:171–196
11. Roden DM (2003) Antiarrhythmic drugs: past, present and future. J Cardiovasc Electrophysiol 14:1389–1396
12. Elliott G, Chew CYC, Singh BN (1980) Therapeutic implications of slow-channel blockade in cardio-circulatory disorders. Circulation 62:669–679
13. Faria DB (1981) Calcium antagonists: their effectiveness in decreasing the occurrence of ventricular fibrillation and reducing infarct size after a coronary artery occlusion. Am J Cardiol 47:15–19
14. Thandroyen FT (1981) Protection against ventricular fibrillation by calcium antagonists. Am J Cardiol 47:15–18
15. Crome R, Hearse DJ, Manning AS (1986) Ischemia-and reperfusion-induced arrhythmias: beneficial actions of nifedipine. J Cardiovasc Pharmacol 8:1249–1256
16. Lichtlen PR, Hugenholtz PG, Raffenbeul W, Hecker H, Jost S, Nikutta P et al (1990) Retardation of coronary artery disease in man by the calcium channel blocker nifedipine. Results of INTACT (International Nifedipine Trial on Antiatherosclerotic Therapy). Cardiovasc Drugs Ther 5(Suppl):1047–1068
17. Lüscher TF, Yang Z (1993) Calcium antagonists and ACE inhibitors. Effect on endothelium and vascular smooth muscle. Drugs 46:121–132
18. Ferrari R, Cucinelli F, Bolognesi R, Bachetti T, Boraso A, Bernocchi P et al (1994) How do calcium antagonists differ in clinical practice? Cardiovasc Drugs Ther 8:565–575
19. Nikol S, Huehns TY, Hölling B (1997) Novel uses and potential for calcium antagonists in revascularization. Eur Heart J 18:105–109
20. Hamm CW, Opie LH (1983) Protection of infarction myocardium by slow channel inhibitors. Comparative effects of verapamil, nifedipine and diltiazem on the coronary ligated isolated working rat heart. Circ Res 52(Suppl 1):1129–1138
21. Crottogini AJ, Depaoli JR, Barra JG (1985) The effect of the new calcium antagonist nisoldipine (Bay K-5552) on myocardial infarct size limitation in conscious dogs. Am J Heart 110:753–760
22. Tumas J, Deth R, Kloner RA (1985) Effects of nisoldipine, a new calcium antagonist, on myocardial infarct size and cardiac dynamics following acute myocardial infarction. J Cardiovasc Pharmacol 7:361–367
23. Schramm M, Thomas G, Towart R, Frąckowiak G (1983) Novel dihydropyridines with positive inotropic action through activation of Ca"2+ channels. Nature 303:535–537
24. Evans BE, Rittle KE, Bock MG, DiPardo RM, Feiring NG, Whitter WL et al (1998) Methods for drug development. Development of potent, selective, orally effective cholecystokinin antagonists. J Med Chem 31:2235–2246
25. Triggie D (2003) 1,4-dihydropyridines as calcium channel ligands and privileged structures. Cell Mol Neurobiol 23:293–303
26. Statkov P, Chatterjee SS, Straumann D, Sunkel C, Priego J, Fau M (1990) Furnidipine (CRE 319) a new selective and long-acting calcium entry blocking agent. Eur J Pharmacol 183:1320
27. Alajarin R, Vaquero JJ, Alvarez-Builla J, Pastor M, Sunkel C, Fau-de-Casa-Juana M et al (1995) Synthesis, structure, and pharmacological evaluation of the stereoisomers of furnidipine. J Med Chem 38:2830–2841
28. Sunkel C, Fau de-Casa-Juana M, Statkov P, Straumann D (1984) 1,4-Dihydropyridines esters and drugs containing these esters. PCT Int. Appl. WO 8402,132. Chemistry Abstract 101, P1911700q
29. Alajarin R, Alvarez-Builla J, Vaquero JJ, Sunkel C, Fau-de-Casa-Juana M et al (1995) Synthesis and chromatographic separation of the stereoisomers of furnidipine. Tetrahedron Asymmetry 4:617–620
30. Krzemiński TF, Grzyb J, Porc MP, Chatterjee SS (2006) Antiarrhythmic and cardio-protective effects of furnidipine in a rat model: a dose response study. Eur J Pharmacol 549:91–97
31. Letelier CS, Munoz MFDC, Gomez JA, Ortega JM, Statkow P, Vogel HG et al (1995) Time-dependent changes of hemodynamic parameters in rat hearts of experimental animals after myocardial infarction using the ‘‘Working Heart’’ method. In: 9th freiburg focus on biomeasurement, pharmaco logical evaluation of cardioprotective substances. Biomesstechnik-Verlag March GmbH, Germany, 128–42
32. Vogel HG, Vogel HW, Schölvens BA, Sandow J, Müller G, Vogel FW (2002) Drug discovery and evaluation: pharmacological assays. Springer, Berlin, p 219
34. Krzemiński TF, Hudziak D, Sia³aœczyk AW, Porc M, Kœdziea A (2008) Differential effects of furmidipine and its active metabolites in rat isolated working heart. Vascul Pharmacol 49:91–96
35. Krzemiński TF, Mitrega K, Varghese B, Hudziak D, Porc M, Kœdziea A et al (2011) Cardio-protective effects of an active metabolite of furmidipine in two models of isolated heart and on in vivo ischemia- and re-perfusion-induced arrhythmias in rats. J Cardiovasc Pharmacol 57:183–193
36. Langendorff O (1898) Untersuchungen am überlebenden Saugetierherzen. III. Abhandlung. Vorübergehende Unregelmässigkeiten des Herzschlags und ihre Ausgleichung. Pflügers Archiv Eur J Physiol 70:473–486
37. Krzemiński TF, Kurcak A, Kapustecki J, Kowaliski J, Szo³inski Z, Brus R (1991) A new concept of the isolated perfused heart preparation with on-line computerized data evaluation. J Pharmacol Toxicol Methods 25:95–110
38. Mitrega KA, Porc M, Krzemiński TF (2013) The beneficial effects of post-myocardial infarction, long oral treatment with M-2 in preventing the development of cardiomiopathy in rats. J Clin Exp Cardiol 4:12
39. Clark C, Foreman MI, Kane KA, McDonald FM, Parratt JR (1980) Coronary artery ligation in anesthetized rats as a method for the production of experimental dysrhythmias and for the determination of infarct size. J Pharmacol Toxicol Methods 3:357–368
40. Krzemiński TF, Nożyœski JN, Grzyb J, Porc M (2008) Widespread myocardial remodeling after acute myocardial infarction in rat. Features for heart failure progression. Vascul Pharmacol 48:100–108
41. Selye H, Bajusz E, Grasso S, Mendell P (1960) Simple techniques for the surgical occlusion of coronary vessels in the rat. Angiology 11:398–407
42. Guendjev Z (1997) Experimental myocardial infarction of the rat and stimulation the revascularization by the flavonoid drug crataemon. Arzneimittelforshung 27:1576–1579
43. Dembiœiska-Kiecœ A, Dulak J, Partyka L, Krzesz R, Dudek D, Bartuœ S (1998) Induction of nitric oxide synthase (NOS) and vascular endothelial growth factor (VEGF) in experimental model of angioplasty and heart ischemia. Recent advances in prostaglandin, thromboxane and leukotriene res. In: Sinzinger H, Samuelsson B, Vane JR, Paoletti RP, Ramwell P, Wong PYK (eds) Advances in experimental medicine and biology. Plenum Press, New York, pp 163–167
44. Heba G, Krzemiński TF, Porc M, Grzyb J, Dembiœiska-Kiecœ A (2001) The time dependent TNFα, iNOS and VEGF expression in experimental model of chronic myocardial infarction in rats. J Vasc Res 38:288–299
45. Heba G, Krzemiński T, Porc M, Grzyb J, Dembiœiska-Kiecœ A (2001) Relation between expression of TNFα, iNOS and VEGF mRNA and development of heart failure after experimental myocardial infarction in rats. J Physiol Pharmacol 52:39–52
46. Krzemiński T, Nożyœski JK, Grzyb J, Porc M, Źeglœni S, Filas V et al (2005) Angiogenesis and cardioprotection after TNFα-inducer-Tolpa Peat Preparation treatment in rat’s hearts after experimental myocardial infarction in vivo. Vascul Pharmacol 43:164–170
47. Walker MJ, Curtis MJ, Hearse DJ, Cambell RWF, Janse MJ, Yellon DM et al (1988) The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. Cardiovasc Res 22:447–455
48. Stormont MF, Lampe I, Barlow OW (1930) A comparison of the premedication values of several barbituric acid derivatives in relation to nitrous oxide anesthesia. J Pharmacol Exp Ther 39:165–175
49. Weatherall J (1960) Anaesthesia in new-born animals. Br J Pharmacol Chemother 15:454–457
50. Kleinman L, Radford E (1986) Harvard apparatus bioscience catalogue, p 25–26
51. Budden R, Detweiler DK, Zbinden G (1980) The rat electrocardiogram in pharmacology and toxicology. Pergamon Press, New York
52. Gerrhardt W (1983) Creatine kinase. Routine UV-method. In: Bergmeyer HU, Bergmeyer J, Grassl M (eds) Methods of enzymatic analysis. Enzymes 1: oxidoreductases transferases. Verlag Chemie Weinheim III, Basel, pp 510–518
53. Yellon DM et al (1988) The Lambeth Conventions: guidelines for the surgical occlusion of coronary vessels in the rat. Features for heart failure progression. Vascul Pharmacol 3:357–368
54. Fryer RM, Hsu AK, Nagase H, Gross GJ (2000) Opioid-induced cardioprotection against myocardial infarction and arrhythmias: mitochondrial versus sarcolemmal ATP-sensitive potassium channels. J Pharmacol Exp Ther 294:451–457
55. Tuominen VJ, Ruotoistenmäki S, Viitanen A, Jumppanen M, Isola J (2010) ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesteron receptor (PR), and Ki-67. Breast Cancer Res 12:R56 http://breast-cancer-research.com/content/12/4/R56
56. Szanto T (1993) It is all in the numbers. J Am Coll Cardiol 21:835–837
57. Mitrega KA, Porc M, Krzemiński TF (2014) Differential effects of furmidipines’ metabolites on reperfusion-induced arrhythmias in rats. PLoS ONE 9:e114194
58. Banicõ B, Nipicõ D, Suput D, Milisav I (2011) DMSO modulates the pathway of apoptosis triggering. Cell Mol Biol Lett 16:328–341
59. Galvao J, Davis B, Tilley M, Normando D, Duchen MR, Cordeiro MF (2013) Unexpected low-dose toxicity of the universal solvent DMSO. FASEB J 28:1317–1330
60. Prech M, Marsza³ek A, Schröder J, Filas V, Lesiak M, Jemielity M et al (2010) Apoptosis as a mechanism for the elimination of cardiomyocytes after acute myocardial infarction. Am J Cardiol 105:1240–1245
61. Piro FR, di Gioia CR, Gallo P, Giordano C, d’Amati G (2000) Is apoptosis a diagnostic marker of acute myocardial infarction? Arch Pathol Lab Med 124:827–831
62. Yaoita H, Ogawa K, Maehara K, Maruyama Y (2000) Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction. Cardiovasc Res 45:630–641
63. Okada H, Takemura G, Kosai K, Tsujimoto A, Esaki M, Taka-hashi T et al (2009) Combined therapy with cardioprotective cytokine administration and antiapoptotic gene transfer in postinfarction heart failure. Am J Physiol 296:H616–H626
64. Michel J-B (2003) Anônikis in the cardiovascular system: known and unknown extracellular mediators. Arterioscler Thromb Vasc Biol 23:2146–2154
65. Taddei ML, Giannoni E, Fiaschi T, Chiarugi P (2012) Anônikis: an emerging hallmark in health and diseases. J Pathol 226:380–393
66. Abbate A, Bussani R, Biondi-Zoccai GGL, Rosiello R, Silvestri F, Baldi F et al (2002) Persistent infarct-related artery occlusion is associated with an increased myocardial apoptosis at post-mortem examination in humans late after acute myocardial infarction. Circulation 106:1051–1054
67. Gottlieb RA, Burleson KO, Kloner RA, Babiœ RL, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94:1621–1628
68. Olivetti G, Quaini F, Sala R, Lagrasta C, Corradi D, Bonacina E et al (1996) Acute myocardial infarction in human associated with activation of programmed myocyte cell death in the surviving portion of the heart. J Mol Cell Cardiol 28:2005–2016
69. Garciarena CD, Caldzic CI, Portiansky EL, Chiape de Cingolani GE, Ennis IL (2009) Chronic NHE-1 blockade induces an antiapoptotic effect in the hypertrophied heart. J Appl Physiol 106:1325–1331