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CARDIOPROTECTION IN PRECLINICAL ISCHEMIA/ REPERFUSION MODELS

PhD thesis

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List of Abbreviations

ADMETox:	Absorption, Distribution, Metabolism, Excretion and Toxicology
AMI:	Acute Myocardial Infarction
ANOVA:	Analysis of Variance
ARCM:	Adult Rat Cardiac Myocytes
DMSO:	Dimethyl sulfoxide
DNA:	Dezoxyribonucleic acid
ECG:	Electrocardiogram
ECM:	Extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
ERK:	Extracellular signal-regulated kinase
ERK:	Extracellular signal-regulated kinases
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GO:	Gene ontology
GPC:	L-alpha-glycerylphosphorylcholine
HDL:	High density lipoprotein
HF:	Heart failure
HR:	Heart rate
HRP:	Horseradish peroxidase
I/R:	Ischemia/Reperfusion
IC50:	Half maximal inhibitory concentration
IL-12:	Interleukin 12
IPC:	Ischemic Preconditioning
i.v.:	Intravenously
JNK:	c-Jun N-terminal kinase
JNK:	c-Jun N-terminal kinases
kDa:	kiloDalton
LAD:	left anterior descending
LDL:	Low density lipoprotein
MABP:	Mean arterial blood pressure

MAPK:	Mitogen-activated protein kinase
MMP:	Matrix Metalloproteinase
MMPI:	Matrix Metalloproteinase Inhibitor
MVO:	Microvascular Obstruction
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO:	Nitric oxide
NRCM:	Neonatal Rat Cardiac Myocytes
p38 MAP:	p38 mitogen-activated protein kinase
PCI:	Percutaneous Coronary Intervention
PCSK9:	Proprotein convertase subtilisin/kexin type 9
PI:	Propidium iodine
PVDF:	Polyvinylidene fluoride
QC:	Quality control
R:	Reperfusion
RIPA:	Radioimmunoprecipitation assay buffer
ROS:	Reactive oxygen species
RT:	Room temperature
SDS-PAGE:	Sodium Dodecyl Sulphate - Polyacrileamid Gel Electrophoresis
SI/R:	Simulated ischemia/reperfusion
SI:	Simulated ischemia
STEMI:	ST-elevated myocardial infarction
TBS:	Tris Buffered Saline
TG:	Triglyceride
TGF-β:	Transforming growth factor beta
TLR:	Toll-like receptor
TNFα:	Tumour necrosis factor alpha
TTC:	2,3,5-Triphenyl-tetrazolium chloride
vs:	versus

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1. Introduction

1.1. Ischemic heart diseases

Acute myocardial infarction (AMI), commonly known as heart attack, is a major consequence of ischemic heart disease and is considered as the single most common cause of death (Fig. 1.), accounting for 1.8 million annual deaths or 20% of all deaths in Europe (Ibanez et al. 2018). The European Society of Cardiology define the term AMI when there is evidence of myocardial injury with necrosis in a clinical setting consistent with myocardial ischemia. The cause could be various, including coronary artery occlusion and/or stenosis. Myocardial infarction (MI) could lead to irreversible "fibrotic" damage to cardiomyocytes as a result of prolonged (more than 20 minutes) ischemia to the myocardium. Lack of oxygen develops energy starvation and necrosis but the other influencing factor of the infarct size is reperfusion injury.

Those cells, which could tolerate the ischemic period, might could not tolerate the reperfusion. The sudden change upon reperfusion, when fresh and oxygenated blood is reentering the affected area, could lead to oxidative stress, therefore more cell death. The result of such insult to the heart muscle cells could alter not only the metabolism of the cells, but the structure or function of the involved area of myocardium significantly, depending on the location of the occlusion/stenosis and time of starvation. The direct effect of such infarction is mainly observed in the decreased cardiac muscle contractility, the manifestation of systolic dysfunction, along with the abrupt reduction of cardiac output.

MI has been well established in medical literature to be a prominent cause of heart failure (HF) - the end-stage of the cardiovascular disease continuum (Dzau et al. 2006). The underlying mechanism is complex and have been studied extensively in the past few decades (Downey and Cohen 2009).

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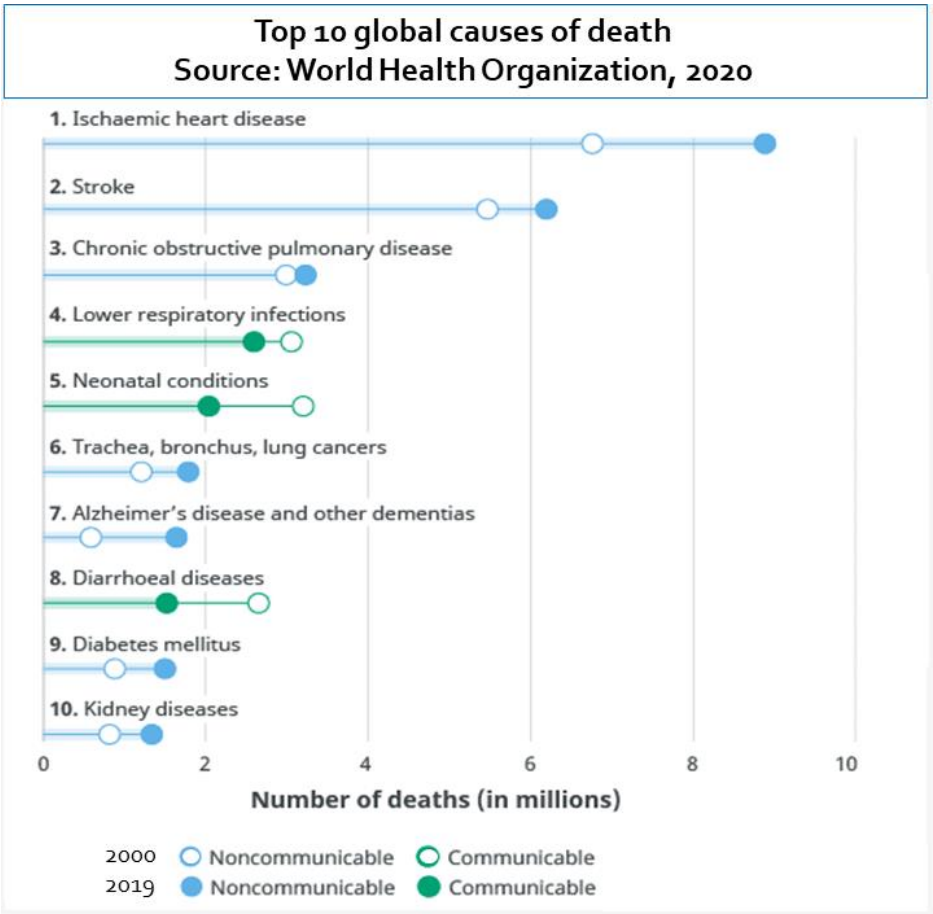

Figure 1. Leading causes of death, World Health Organization; 2020.

Reperfusion injury is an additional factor influencing the final infarct size resulted by MI. Reactive oxygen-nitrogen species (ROS) have been found to be potential mediators of factors such as intracellular and mitochondrial calcium load as well as the opening of the mitochondrial permeability transition pore. Those, in addition to the direct activation of matrix metalloproteinase-2 (MMP-2), are the events responsible for ischemia/reperfusion (I/R) injury (Zavadzkas et al. 2011). Cell surface receptors could also lead to activation of intracellular signaling pathway resulting cell death. The uncontrolled activation and release of intracellular contents due to cell lysis, such as matrix metalloproteinases (MMPs) into the extracellular matrix (ECM), could cause more damage and adverse structural changes. Such effects lead to multiple harmful consequences including damaging contractility apparatus of cardiomyocytes leading to remodeling, localized inflammatory responses, arrhythmias and hemodynamic instability.

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1.2. Cardioprotection: intervention and available treatments

For efficient treatment of AMI, the aim is to reduce ischemia and reopen the coronary vessels by unblocking them and/or preventing further clotting.

Final infarct size is one of the best predictors of long-term adverse events in ST-elevated myocardial infarction (STEMI) survivors (Reinstadler, Thiele, and Eitel 2015). The introduction of a specific infarct-limiting therapy in clinical practice has a massive clinical and socioeconomic impact. Several strategies, including pharmacological and mechanical therapies, have shown a reduction of infarct size by reducing ischaemia/reperfusion injury, including microvascular obstruction (MVO) in experimental and small-scale clinical trials, but to date no large trial has demonstrated a clinical benefit (Ferdinandy et al. 2014). Ischemic pre-, post-, remote, and pharmacological conditionings are promising, on the other hand in clinical practice, the benefit of these interventions has yet to be seen (de Miranda et al. 2020).

Percutaneous intervention is the gold standard in treating STEMI, however, it is only beneficial during a window of 90-120 minutes beginning upon contact with a medical professional. If not applicable, then fibrinolysis, with tissue plasminogen activator or streptokinase, is recommended within 30 minutes upon arrival to the hospital (Ibanez et al. 2018).

Antithrombotic therapy, antiplatelet drugs and anticoagulants are the cornerstones of the pharmacological approach in the acute phase of STEMI, including: unfractionated heparin (enoxaparin or bivalirudin may be alternatives), and loading dose of aspirin. P2Y12 receptor inhibitors (prasugrel/ticagrelor) should be considered after careful assessment of the ischemic and bleeding risk of the patient. Fibrinolytics are often used to dissolve clots and to prevent new clots from forming and existing clots from growing. The pharmacological treatment includes the immediate and simultaneous management of pain as well with morphine and nitroglycerine can be used to widen the blood vessels. Beta-blockers lower the blood pressure via relaxing the heart muscle, and it could limit the severity of the damage. Angiotensin convertase enzyme (ACE) inhibitors can also be used to lower blood pressure and decrease stress on the heart (Maxwell 1999). A recent cohort study showed association between increased plasma

ACE2 concentration and increased risk of major cardiovascular events (Narula et al. 2020).

According to the Cardiac Arrest and Reperfusion Strategy guidelines by the European Society of Cardiology, patients with ST-elevation on post-resuscitation ECG have to undergo a primary percutaneous coronary intervention (PCI) strategy. In cases without ST-segment elevation on post-resuscitation ECG but with a high suspicion of ongoing myocardial ischaemia, urgent angiography should be considered within 2 h after a quick evaluation to exclude non-coronary causes. In all cases, the decision to perform urgent coronary angiography should take into account factors associated with poor neurological outcome. Surgical intervention (coronary artery bypass graft), is advised in those cases where the stenosis is over 50% in the left main coronary artery, or over 70% in the proximal left anterior descending (LAD) and proximal circumflex arteries, or if the occlusion is in several coronary arteries (Ibanez et al. 2018).

There is an unmet clinical need for cardioprotective therapies against myocardial ischemia-reperfusion (I/R) injury (Hausenloy et al. 2013; Hausenloy et al. 2017). Therefore, novel potential cardioprotective therapies are much sought for. In line with this need, plenty of substances including natural biomolecules have been extensively tested for their cardioprotective potential. New approaches are containing therapeutical options like stem cell therapy, such as intracoronary administration of bone marrow stem cells and endocardial mesenchymal stem cells (Itier and Roncalli 2018). However, lack of efficacy showed in randomized clinical trials, where the transplantation of bone marrow stem cells resulted in limited improvement on cardiac function for MI patients (Shi et al. 2021). Another issue is the safety measures, stem cells have the ability to proliferate and differentiate, therefore there is a concern whether stem cells promote tumor growth and metastasis (Volarevic et al. 2018). Another category is mechanical interventions, such as remote ischemic conditioning. Unfortunately, the clinical efficacy proved to be lower than pre-clinical testing. This is due to the limited subgroups of patients that yet need to be defined (Le Page and Prunier 2015). Molecular targeting such as PCSK9 antibodies, which are considered for preventive therapy, have not been sufficiently developed (Sabatine et al. 2017). In the case of reperfusion injury, MMP2 inhibitors have shown promising preclinical and clinical studies (Cerisano et al. 2015).

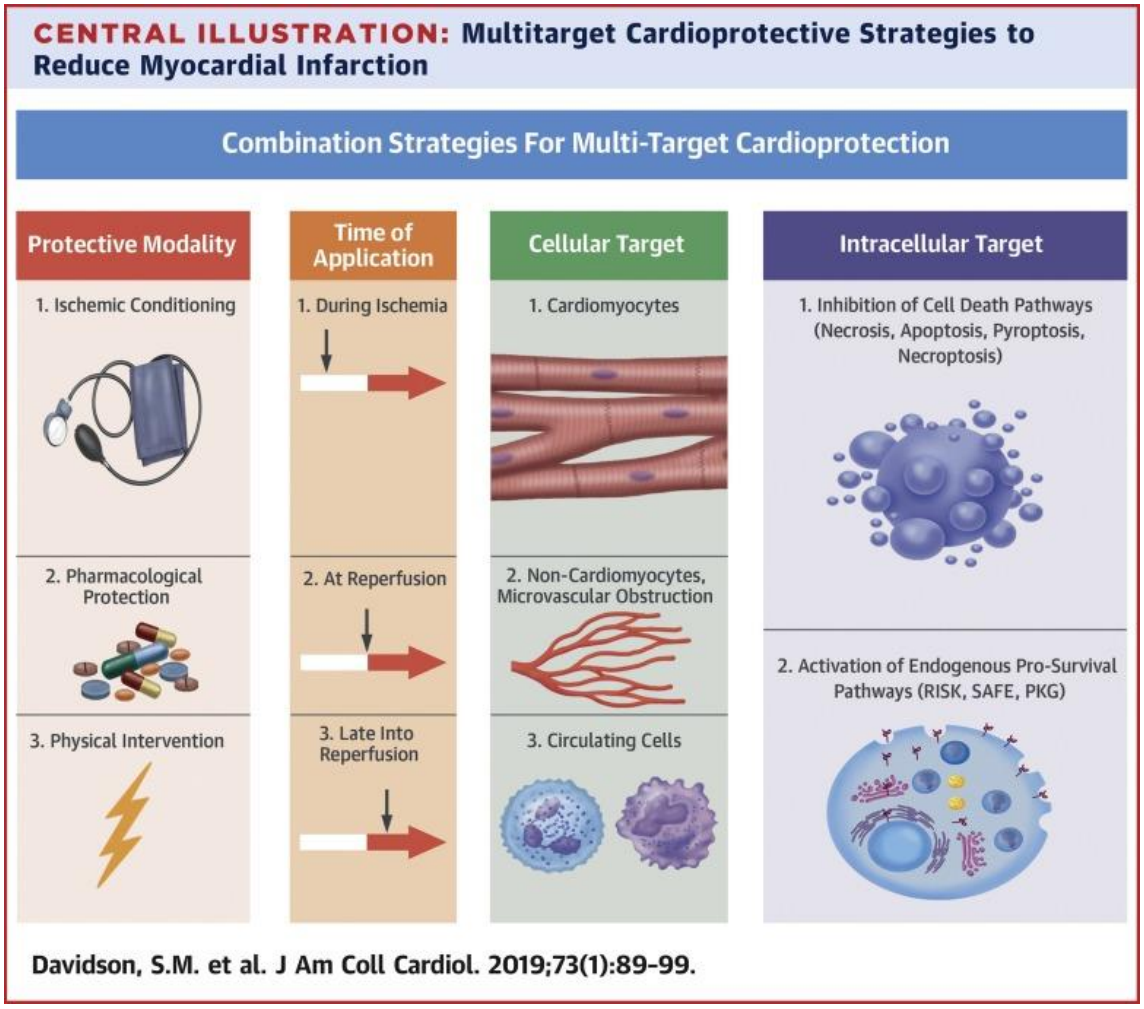

Figure 2. Original illustration from Davidson et al. J Am Coll Cardiol. 2019 (Davidson et al. 2019) about the multitarget cardioprotective strategies to reduce myocardial infarction.

In this thesis 5 different molecules were tested against AMI, **i)** a natural choline derivative L-alpha-glycerylphosphorylcholine, **ii)** a natural extracellular matrix component decorin and **iii)** three synthesized matrix metalloproteinase inhibitors. According to the latest approach for cardioprotection (Davidson et al. 2019) (Fig.2.) we considered above mentioned molecules as pharmacological protection with careful consideration of the time of application, mainly focusing on the cardiac myocytes as primary targets for protection.

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1.3. Possible cardioprotective candidates

1.3.1. L- alpha-glycerylphosphorylcholine

L-alpha-glycerylphosphorylcholine (choline alphoscerate, GPC) is a natural endogenously produced choline derivative (Fig. 3.) and acetylcholine precursor in the brain which (in the form of a synthetic compound) is widely used as a food supplement (Kawamura et al. 2012). It has been shown that GPC supplements may help substitute for insufficient dietary choline (Zeisel et al. 1991). GPC is converted metabolically to phosphatidylcholine, the active form of choline that is able to increase acetylcholine levels in the brain (Abbiati et al. 1993; Lopez et al. 1991; Brownawell, Carmines, and Montesano 2011). Choline has been recognized as an essential nutrient for humans and has made recommendations for the dietary choline intake (Institute of Medicine and National Academy of Sciences, 1998).

GPC is generally considered a safe and non-toxic compound (Brownawell, Carmines, and Montesano 2011) and it is generally recognized as safe in the USA (GRN 000419). A multicentric, clinical trial confirmed the therapeutic role of alpha-GPC on the cognitive recovery of patients with acute stroke or transient ischemic attack (Barbagallo Sangiorgi et al. 1994). In addition to its neuroprotective effects, GPC was documented to preserve mitochondrial respiration in liver mitochondria and to reduce hepatic ischemia-induced oxidative stress and inflammation in rodent models of ischemia/reperfusion (I/R) (Strifler et al. 2016; Hartmann et al. 2014) and reduced ROS production in a relevant rodent model of mesenteric I/R (Tokes et al. 2015). Therefore, we hypothesized that GPC could prevent oxidative damage following I/R insult in other tissues, including the myocardium.

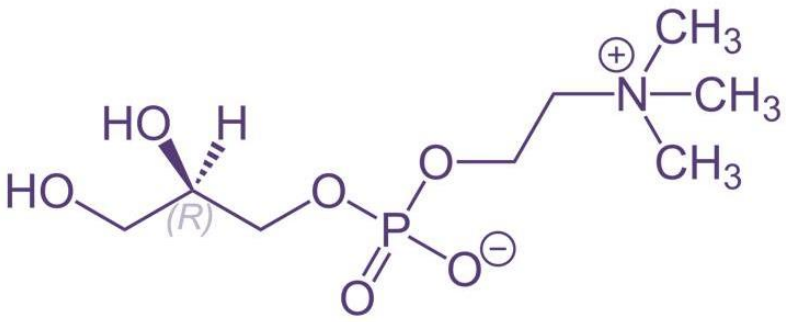

Figure 3. Chemical structure of GPC.

1.3.2. Decorin

Decorin is natural component of the connective tissue (Fig. 4.). Decorin is included in a broad range of cellular processes including collagen fibrillogenesis, wound repair, angiostasis, tumor growth, and autophagy (Gubbiotti et al. 2016). In cardiovascular system it is proposed to play a key role in the proper tissue scar formation following myocardial infarction (Weis et al. 2005). Decorin is a bi-functional proteoglycan, besides being a component of extracellular matrix (ECM), as a signaling molecule it interacts with different tyrosine kinase receptors , as well as with the innate immunity receptors Toll-like receptors-2 and -4 (TLR-2, TLR-4), leading to synthesis of the proinflammatory cytokines TNFα and IL-12 (Frey et al. 2013). Decorin interacting via cell surface receptor regulates plenty of cellular functions (reduces TGF-β dependent fibrosis, proliferation of myofibroblasts and endothelial cells, renal and neuronal protection from ischemic assaults), but these processes are dependent on cell types and pathological circumstances (Vu et al. 2018). Recently, a protective effect of decorin on acute I/R injury in rat kidneys has been documented (Alan et al. 2011).

Proteoglycans are potential cardioprotective macromolecules as Gáspár et al. have previously shown that exogenous administration of biglycan, protects myocardial cells from simulated ischemia and reperfusion (SI/R) injury via Toll-like receptor-4-mediated mechanisms involving activation of survival kinases such as ERK, JNK and p38 MAP kinases and increased nitric oxide (NO) production (Csont et al. 2010; Gaspar et al. 2016).

In line with the cardioprotective effects of biglycan we hypothetized that another proteoglycan from small leucine-rich proteoglycan family, decorin, could also exert cardioprotection. Decorin and biglycan share many common features such as structural similarities or similar size of their core proteins (40 kD); however, there are also some differences making decorin potentially more favorable for application than biglycan. Decorin is usually “decorated” with only one chondroitin or dermatan sulfate chain representing a less diverse group, while biglycan can be decorated with either one or two chains

However, it is not known if decorin exerts acute cardiocytoprotective effect.

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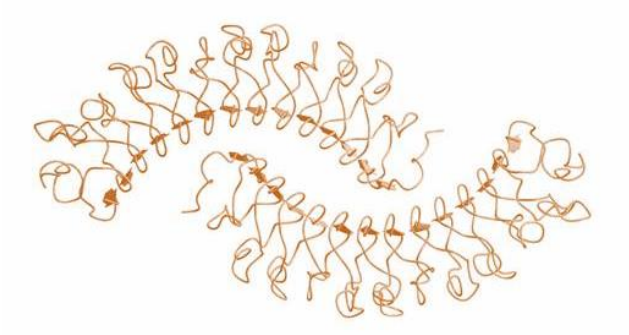

Figure 4. Ribbon protein structure of decorin

1.3.3. Matrix metalloproteinase inhibitors

MMPs are zinc-dependent endopeptidases and the gelatinase-type MMP-2 isoenzyme occurs physiologically in the heart and synthesized by cardiac myocytes, fibroblasts, and endothelial cells. MMP-2 is synthesized as inactive zymogen form and it could be activated either via limited proteolysis (Kandasamy et al. 2010) or conformational changes induced by reactive oxygen/nitrogen species (Chow, Cena, and Schulz 2007; Zavadzkas et al. 2011).

MMP-2 has a critical role as an intracellular mediator of cardiac I/R injury contributing to the acute mechanical dysfunction that occurs immediately following reperfusion (stunning injury) (Cheung et al. 2000). The main intracellular targets of MMP-2 in I/R injury are the proteins of the contractile apparatus (DeCoux et al. 2014), such as Troponin I (Wang et al. 2002), titin, myosin light chain-1 (Van Eyk et al. 1998), and α-actinin in cardiac myocytes (Sawicki et al. 2005) (Fig.5.).

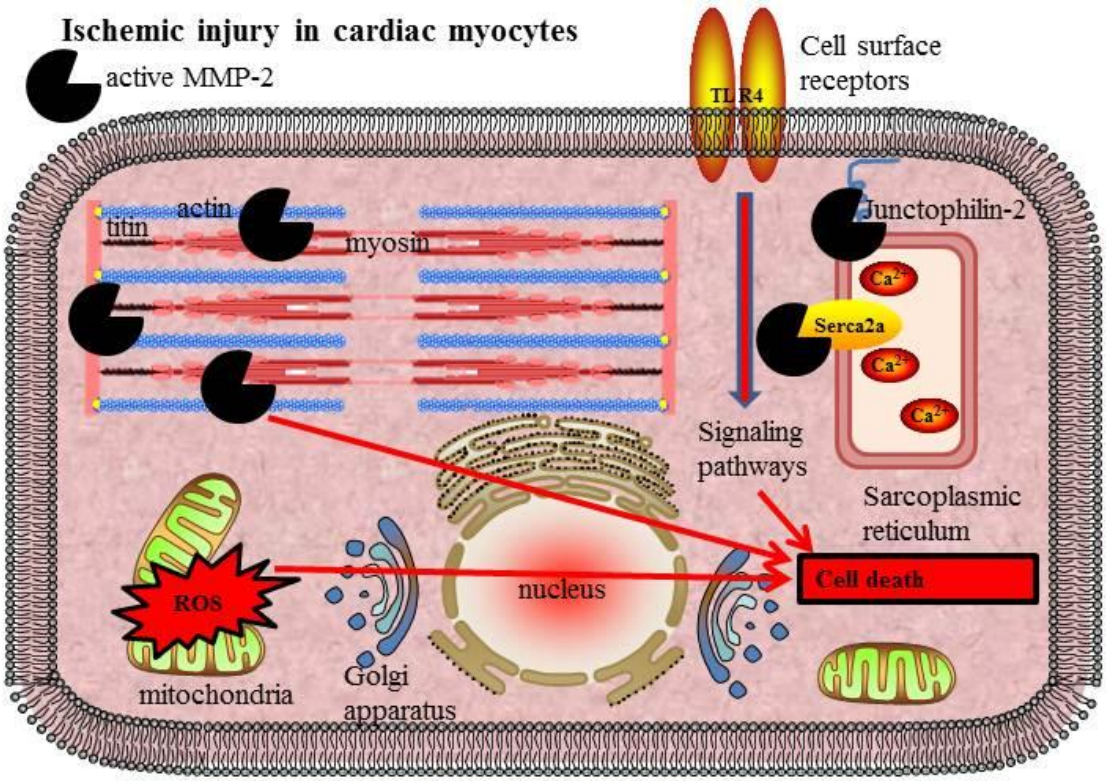

Figure 5. Ischemic injury in cardiac myocytes. Activated matrix metalloproteinase-2 (MMP-2) cleaves the proteins of the contractile apparatus and cause instability in the cells Ca^{2+} handling. Elevated reactive oxygen species (ROS) from the mitochondria and various intracellular signaling pathways are all contributors to the damage, leading to cell death.

MMP-2 has a fundamental role in I/R induced myocardial injury, therefore the inhibition of MMP-2 seems to be a promising target in therapy of AMI. Several preclinical studies showed infarct size reducing effect of MMP-inhibitors, but its translation into human clinical practices has been failed so far. The first generation of MMP inhibitors were hydroxamic acid type of molecules, which mechanism of action was based on the Zn^{2+} ion chelation (Rao 2005; Jacobsen et al. 2010). However, broad spectrum inhibition of MMPs has unwanted side effect, e.g. muscoloskeletal syndrome (Renkiewicz et al. 2003). Ilomastat, a hydroxamic acid type non-selective MMP inhibitor, were tested in ex vivo and *in vivo* mouse hearts by Bell et al for its cardioprotective effect administered upon reperfusion (Bell et al. 2013). Our group previously shown its cardioprotective effect in ex vivo isolated rat heart (Giricz et al. 2006) and in *in vivo* acute myocardial infarction rat model (Bencsik et al. 2014).

In 2006, an early clinical trial (PREMIER), a hydroxamic acid type MMP inhibitor (PG-116800) was tested in AMI patients (Hudson et al. 2006; Cerisano et al. 2015) but showed no real beneficial effect. The only exception of MMP inhibitor was doxycycline (Spaulding et al. 2018), which showed a significant cardioprotective effect in AMI patients, however, the number of enrolled patients was limited (Cerisano et al. 2015).

Therefore, it is reasonable to develop novel structures for more selective and moderate inhibitors of MMP-2 with cardioprotective indication. In the previous study of Bencsik et al.(Bencsik et al. 2018), imidazole and thiazole carboxylic acid-based compounds, novel MMP inhibitor molecules were developed (Fig. 6).

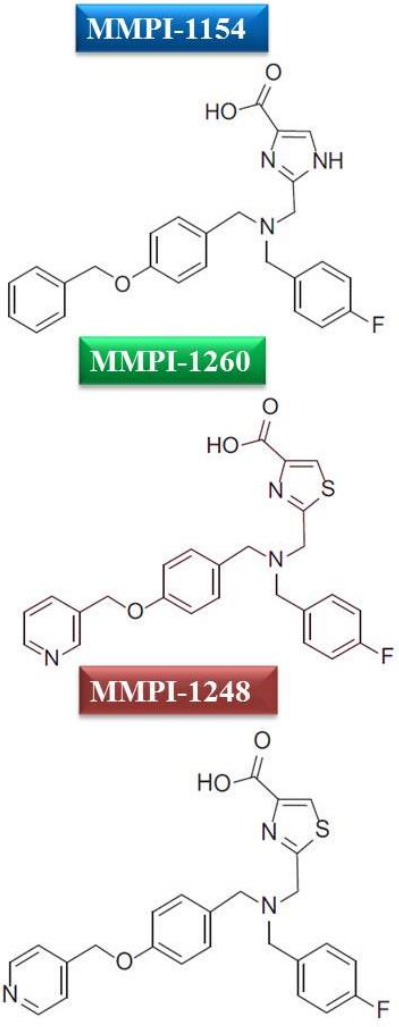

Figure 6. Chemical structures of the tested matrix metalloproteinase-2 inhibitors. (A) Imidazole-4-carboxylic acid derivate and (B and C) thiazole-4-carboxylic acid derivatives.

The imidazole and thiazole carboxylic acid-based compounds have shown superior MMP-inhibiting effects (inhibitory concentration 50%, IC_{50}) when compared to the hydroxamic acid type derivatives *in vitro* in gelatin zymography assays. Six compounds, including MMPI-1154, MMPI-1260 and MMPI-1248 were found to be protective against simulated ischemia/reperfusion induced injury in cardiac myocytes (Table 1.) (Bencsik et al. 2018).

Table 1. Most effective concentration of MMPI based on zymographic measurement. Data from Bencsik et al, *Development of Matrix Metalloproteinase-2 Inhibitors for Cardioprotection*, Front Pharmacol. 2018.

Code name	IC 50	*In vitro* cardioprotection
MMPI-1154	2.5 µM	Yes
MMPI-1157	6 µM	No
MMPI-1247	16µM	Yes
MMPI-1248	9 µM	Yes
MMPI-1253	10 µM	Yes
MMPI-1254	16 µM	Yes
MMPI-1260	2.6 µM	Yes

1.4. Cardiac comorbidities

Cardiac comorbidities could change the response of pharmacological treatment by shifting the cell's metabolic homeostasis or by exhausting the intracellular protective pathways, therefore investigating new compound against AMI is not enough to be tested in healthy young animals, but we also have to seek information from animals suffering from other diseases.

Hypercholesterolemia is one of the major modifiable risk factors for AMI (Sack and Murphy 2011). The most common hypercholesterolemia has polygenic background and manifests as raised blood cholesterol, often accompanied by metabolic syndrome and type 2 diabetes (Soran et al. 2018). Since the discovery of endogenous cardioprotective mechanisms against I/R injury (local and remote ischemic pre-, post-, and perconditioning, pharmacological conditioning) several molecular contributors of

cardioprotective maneuvers were explored. However, even after promising preclinical attempts aiming to trigger of these cardioprotective mechanisms, the translation of the results into clinical practice has remained unsolved. This probably due to the presence of several additional factors including cardiovascular comorbidities, e.g.,hyperlipidemia or diabetes mellitus (Ferdinandy et al. 2014). Many cardioprotective strategies act through common end-effectors and may be suboptimal in patients with comorbidities (Davidson et al. 2019). Therefore, to improve clinical outcomes, novel therapeutic strategies are needed against myocardial I/R injury, which may preserve cardioprotection even in the presence of comorbidities (Hausenloy et al. 2017).

Hypercholesterolemia and metabolic disease are common comorbidities resulted from a sedentary lifestyle and an increased intake of saturated and unsatured trans-fatty acids (Ibrahim and Jialal 2020). Elevated LDL-cholesterol causes endothelial and myocardial dysfunction, as well as exacerbates I/R-induced myocardial injury. Cholesterol-enriched diet-induced hyperlipidemia led to an increase of cardiac peroxynitrite formation in ex vivo rat hearts and to a decreased bioavailability of NO, which contributed to the deterioration of cardiac performance (Onody et al. 2003). Early increase in peroxynitrite after postconditioning plays a role in cardioprotection, but in hyperlipidemia the cardioprotective effect of postconditioning is blocked (Kupai et al. 2009). In mouse heart, the cholesterol-enriched diet led to alterations in preconditioning-induced gene expression and changes of oxidative/nitrosative stress signalling, which attenuated the cardioprotective effect of preconditioning (Kocsis et al. 2010). Endogenous cardioprotective mechanisms against I/R has been showed impaired in hyperlipidemic *in vivo* rat AMI model (Andreadou et al. 2017). *In vitro* primary neonatal cardiomyocytes showed increased level of total ROS and decreased level of viability after I/R injury, when receiving modified high-cholesterol medium (Makkos et al. 2019). The cardioprotective effect of ischemic preconditioning is lost in hypercholesterolemia at least partially due to peroxynitrite-induced activation of matrix metalloproteinase (MMP)-2 (Giricz et al. 2006).

Additionally, inhibition of MMP-2 showed comparable cardioprotection as ischemic preconditioning against I/R injury in the heart of normolipidemic rats and this protection was preserved even in the presence of hyperlipidemia (Giricz et al. 2006).

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2. Aims

The aim of this thesis is to achieve cardioprotection by using different potentially protective compounds (Fig.7.) in preclinical models,

1) to test the hypothesis that GPC could prevent ischemia-induced cell death and oxidative stress in cardiac myocytes subjected to simulated I/R and

2) to test if decorin exerts cardioprotective effects against simulated I/R injury in primary cultures of isolated neonatal as well as adult rat cardiomyocytes, and to reveal molecular pathways involved in these effects and

3) to test cardioprotective effect of MMP inhibitors (MMPI-1154, -1260, and -1248) in an *in vivo* rat model of acute myocardial infarction in presence or absence of hypercholesterolemia.

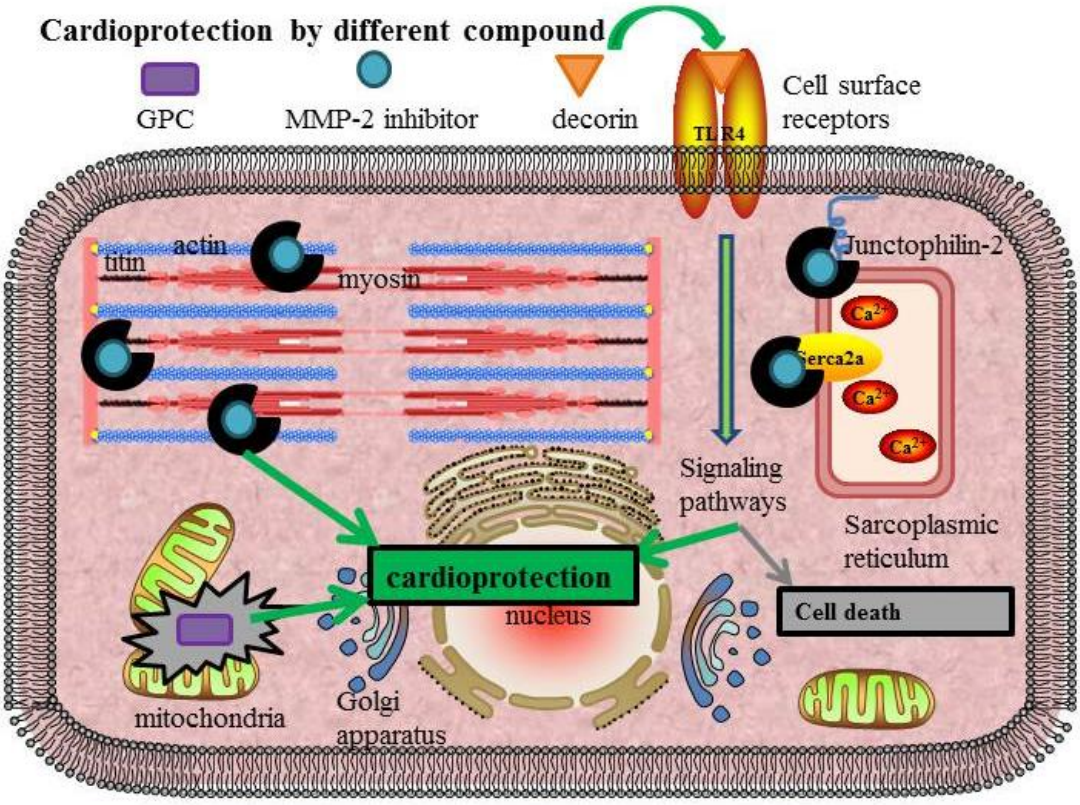

Figure 7. Visual summary about the possible targets of investigated compounds to achieve cardioprotection.

3. Materials and Methods

Our experiments were performed in accordance with the EU directive guidelines for the care and use of laboratory animals, published by the European Union (2010/63/EU). Methods were also reviewed by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority of Hungary) and were approved by the Animal Welfare Committee of the University of Szeged (XXVIII/171/2018).

3.1. Animals for experimentation

3.1.1. Animals for *in vivo* study

Wistar rats were purchased from Toxicoop and were housed in individually ventilated cages (Sealsafe IVC system, Tecniplast S.p.a., Varese, Italy) which conform the size recommendations of the abovementioned EU guidelines. Litter material (Lignocell hygienic animal bedding, from Farmermix Kft) placed beneath the cage has been changed at least three times a week. The animal room was temperature controlled (22±2 °C), and it had a 12 h light/dark cycle. The animals were acclimatized in the housing facility for 5 days prior to the start of the animal experiments. Animals were fed with standard rodent chow and filtered tap water was available ad libitum. Animals from the comorbidity groups were fed with standard rodent chow supplemented with 2 % cholesterol (04820, Molar Chemicals) and 0,25 % cholic acid (C1254, Sigma Aldrich). All animal chow was made by Farmermix Kft to assure standard homogeneity, shape and quality of the chow.

3.1.2. Culturing primary neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes (NRCMs) were isolated from newborn Wistar rats as described previously (Gorbe et al. 2010). The neonatal rats were sacrificed by cervical dislocation. The hearts were rapidly removed and placed in a cold phosphate buffered saline solution. After separation of atria, ventricles were minced with a fine forceps and digested in 0.25 % trypsin (Gibco BRL) for 25 minutes in a Falcon tube in 37°C water bath. Then the cell suspension was centrifuged (450 x g for 15 minutes at 4 °C). The cell pellet was resuspended in culture medium - Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-Glutamine, and AB/AM (Sigma). The single cell suspension was pre-plated in 6-well plates at 37 °C for 90

minutes to enrich the culture with cardiomyocytes. No cytostatic compound was added. The non-adherent myocytes were collected and plated at a density of 10^5 cells/well onto 24-wells plates or $1.8x10^4$ cells/well in case of 96-wells plates. Culture medium was changed the day after preparation to 1 % FBS containing differentiation medium. The cells were maintained at 37 °C in a standard CO_2 incubator (Humidified atmosphere of 5 % CO_2).

3.1.3. Culturing adult rat cardiac myocytes

Adult rat cardiac myocytes (ARCMs) were isolated as described previously (Gaspar et al. 2016). The hearts of 200–250 g male Wistar rats were excised after euthasol anaesthesia (50 mg/kg) and heparin injection (50 U/kg). The hearts were then stabilized by retrograde aortic perfusion on Langendorff system with solution A (In mM: $NaHCO_3$ 25, KCl 4.7, NaCl 118.5, $MgSO_4$-$7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10, Dyacetyl monoxime (BDM) 10 and $CaCl_2$ 5 μM). Collagenase Type II (8000 U) was used for gentle digestion of tissue in solution B (in mM: $NaHCO_3$ 25, KCl 4.7, NaCl 118.5, $MgSO_4$-7 H_2O 1.2, KH_2PO_4 1.2, glucose 10, BDM 10, $CaCl_2$ 50 μM, 1% BSA) for 30–45 min. The heart was further washed and minced in solution C (in mM: $NaHCO_3$ 25, KCl 4.7, NaCl 118.5, $MgSO_4$-7 H_2O 1.2, KH_2PO_4 1.2, glucose 10, $CaCl_2$ 50 μM) and filtered. After filtration, the cells were washed 3 times with solution C. The calcium concentration was increased gradually to 1.8 mM. Cells were harvested in M199 medium (5 % FBS, L-carnitine 5, taurine 5 and creatine-monohydrate 5mM). Cardiac myocytes were plated onto laminin coated (10 μg/mL) coverslips placed in 24-well plates at density of 7.5×10^3 cell/well. After 3 h, the FBS-containing M199 was replaced with serum free M199. Two-day-old cultures were used for simulated ischemia/reperfusion experiments.

3.2. *In vitro* and *in vivo* models for acute ischemic heart diseases

3.2.1. *In vitro* - Simulated ischemia/reperfusion

To simulate ischemic conditions, the culture medium was replaced with a hypoxic solution containing in mM: NaCl 119, KCl 5.4, $MgSO_4$ 1.3, NaH_2PO_4 1.2, HEPES 5, $MgCl_2$ 0.5, $CaCl_2$0.9, Na-lactate 20, BSA 0.1 % pH 6.4. To induce hypoxia the cells were then placed in a tri-gas incubator gassed through with a mixture of 95 % N_2 and 5 % CO_2 (to keep the O_2 level under 0.5%) for 240 min at 37 °C. Normoxic control cells were covered with normoxic solution containing in mM: NaCl 125, KCl

5.4, NaH_2PO_4 1.2, $MgCl_2$ 0.5, HEPES 20, $MgSO_4$, 1.3, $CaCl_2$ 1, glucose 15, taurine 5, creatine-monohydrate 2.5 and BSA 0.1 %, pH 7.4 and cells were kept in normoxic incubator. After simulated ischemia (SI) or normoxia, the cells were placed to normoxic incubator; hypoxic or normoxic medium was then replaced by culture medium (Simulated reperfusion, R) for 2 hours at 37°C. NRCMs were subjected to SI for 4 hours and 2 h R (Fig. 8.B and 9.B), while ARCMs were subjected to 0,5 h SI and 2 h R (Fig. 10.). Previously, we have shown that the experimental model of isolated cultures of heart-derived cells exposed to SI/R represents a validated tool for testing potential cardioprotective effects of different substances (Gorbe et al. 2010; Bencsik et al. 2014; Paloczi et al. 2016).

3.2.2. *In vivo* - Experimental model of acute myocardial infarction

All surgical procedures were basically performed as described previously (Kiss et al. 2016) and modified according to the present study (Figure 12). Male Wistar rats weighing 260-340 g were anesthetized by ip. injection of pentobarbital sodium (Repose 50 %, Le Vet. Pharma). The rats were weighed and stomach and chest area shaved. Maintenance of the body core temperature (37±1 °C) was assisted using a constant temperature heating pad. The trachea was intubated with a plastic cannula connected to a rodent ventilator (Model 7025, Ugo Basile SRL). The animals were ventilated with room air (6.2 ml/kg, 70±5 breath/min). Blood pressure (from right carotid artery), surface-lead ECG, and body core temperature were monitored throughout the experiments to ensure the stability of the preparation (Haemosys data acquisition system, Experimetria). The right jugular vein was cannulated for the administration of the test compounds or vehicle. A thoracotomy was performed at the 5th intercostal space and the heart was exposed through the fifth intercostal space. A 5-0 Prolene suture was placed around the left anterior descending (LAD) coronary artery. The coronary artery was then occluded for 30 min followed by 120 min reperfusion.

3.3. Experimental groups

3.3.1. Source of tested compounds

GPC was provided by our collaborator, (Molecular Weight: 257.2 g/mol, Lipoid GmbH, Ludwigshafen, Germany; dissolved in 0.5 ml of sterile saline solution at 0.064 mM concentration).

Decorin was purchased from Sigma-Aldrich (Decorin is an approx. 100 kDa proteoglycan consisting of a 40 kDa core protein and one chondroitin or dermatan sulfate glycosaminoglycan chain. 0,5 mg in the vial was dissolved in 500 ul sterile distilled water to prepare a stock solution of 10 µM).

MMPI-1154, MMPI-1260 and MMPI-1248 were synthetized by Targetex Ltd. (Molecular weight: MMPI-1154: 445 g/mol; MMPI-1260: 464 g/mol; MMPI-1248: 463 g/mol, MMPIs were dissolved freshly in DMSO to prepare a stock solution of 10µmol/kg)

Table 2 . Summary table of experimental groups.

Type of experiments	Tested compound	Experimental set up	Concentration of compound
In vitro cell culture	GPC	Normoxia	1-100 µM
		Simulated ischemia	1-100 µM
	decorin	Normoxia	1-100 nM
		Simulated ischemia	1-100 nM
In vivo rat model	MMPI-1154	Acute myocardial infarction	0.3-3 µmol/kg
	MMPI-1260	Acute myocardial infarction	0.3-3 µmol/kg
	MMPI-1248	Acute myocardial infarction	1-10 µmol/kg

3.3.2. L-alpha-glycerylphosphorylcholine treatment in neonatal rat cardiac myocytes

The concentration-dependent influence of GPC on cell viability and the degree of oxidative stress were tested in 3-day-old primary rat cardiac myocyte cultures exposed to acute (15 minutes), short-term (3-hours), and long-term (24-hours) GPC treatment in the 1-100 µM concentration range under normoxic conditions (n=8-16) (Fig. 8.A). One group of cells was pre-treated with GPC for 3 hours and then subjected to simulated ischemia/reperfusion (SI/R). During the entire protocol, the GPC concentration range (1-100 µM) was sustained (Fig. 8.B). In all experiments, GPC was

dissolved in physiological saline, while the Vehicle group was treated with physiological saline solution alone in 0.1 v/v%. 2-3 isolation rounds were performed for each experimental series and data of all individual wells were analysed.

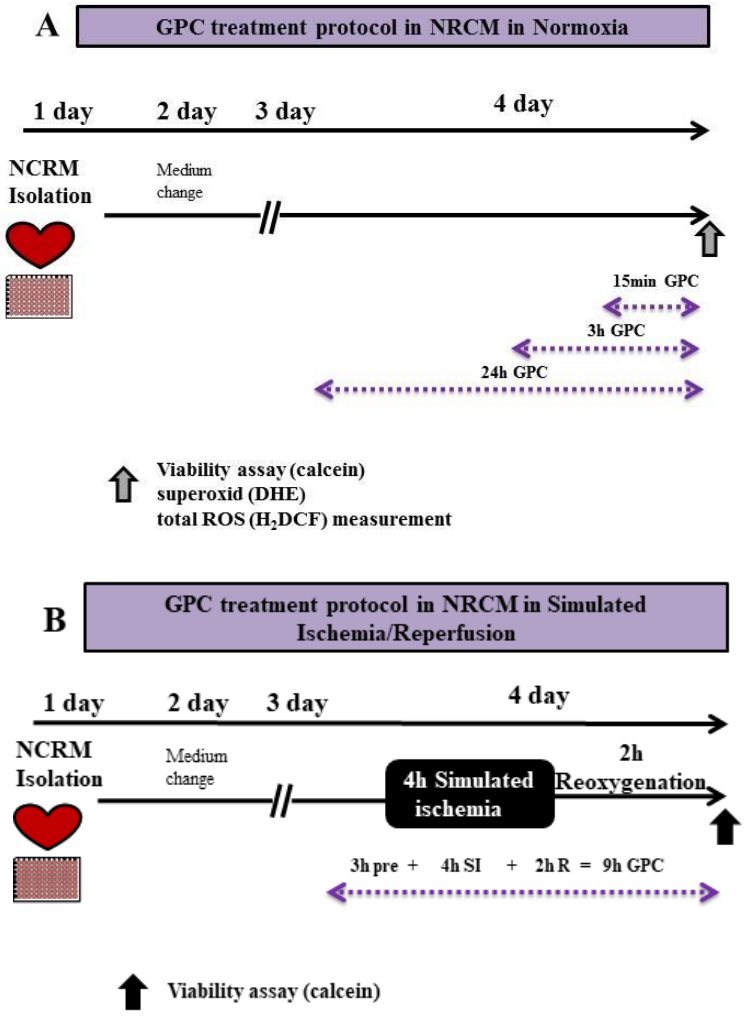

Figure 8. Experimental protocol: After the isolation of the neonatal rat cardiomyocytes (NRCMs) from newborn Wistar rats, the cells were then cultured in a 10% FBS containing medium for 24 hours. After the first day, the cells were kept in a 1% FBS containing medium. **A)** Glycerophosphorylcholine (GPC) treatment was applied under normoxic conditions. Three series of cells were treated at day 3 with GPC for 15 minutes, 3 hours or 24 hours, respectively, all prior to cell viability measurements. **B)** GPC treatment was then applied 3 hours prior to 4 hour simulated ischemia (SI), and during the 2 hour-reperfusion period.

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3.3.3. Decorin treatment in neonatal rat cardiac myocytes

NRCMs at day-3 were pretreated with 0 (Vehicle), 1, 3, 10, 30, 100 nM concentration of decorin for 20 h, respectively (Fig.9.A). Then 4 h SI and 2 h R, or normoxic control treatment was applied as described above. Normoxic controls were treated with vehicle or decorin throughout the entire investigation (20 h, 4 h, 2 h). At the end of the reperfusion, the cell viability was measured with calcein assay.

In separate experiments (Fig.9.B), we investigated the mechanism of action of decorin. For assessment of the role of NO in the protective effect of decorin, the cells were treated with 3 nM decorin in the absence or presence of 10 µM NO synthase inhibitor L-nitro-arginine methyl ester (L-NAME) during 4 h SI (Gaspar et al. 2016). Similar experimental design was performed to assess the role of protein kinase G (PKG) downstream to NO-cGMP with application of 600 nM KT-5823 (Selective PKG inhibitor) (Bencsik et al. 2014) and the role of TLR-4 signalling with application of 50 µM TAK-242 (TLR-4 inhibitor) (Gaspar et al. 2016).

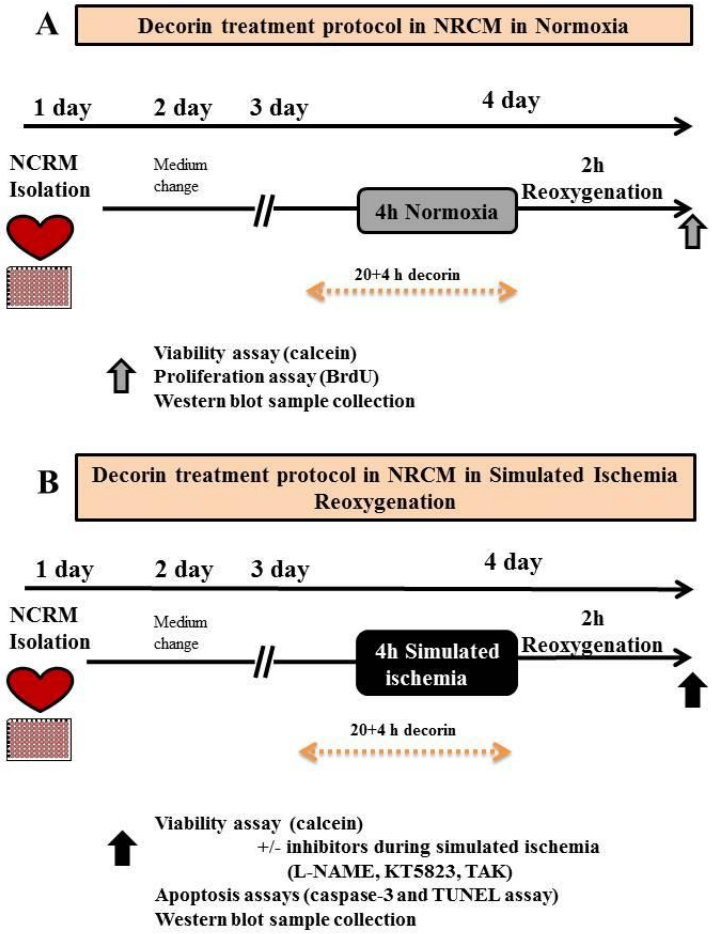

Figure 9. Experimental protocol of decorin treatment. NRCM: neonatal rat cardiac myocytes. After isolation, cells were cultivated for 3 days. A) In normoxic conditions viability and proliferation were investigated and we collected samples for western blot. B) Under simulated ischemia/reperfusion injury viability was measured after decorin treatment combined with or without different inhibitors; in separate experiments, only treating cells with decorin, apoptosis was measured and samples were collected for western blot.

3.3.4. Decorin treatment in adult rat cardiac myocytes

ARCMs are fully differentiated and sensitive cells, therefore the length of simulated ischemia was only 30 minutes. Cells were treated with 1, 3, 10 nM concentrations only during SI/R (Fig.10.). At the end of 2 hours simulated reperfusion (reoxygenation) viability measurement was done by using calcein fluorescent assay.

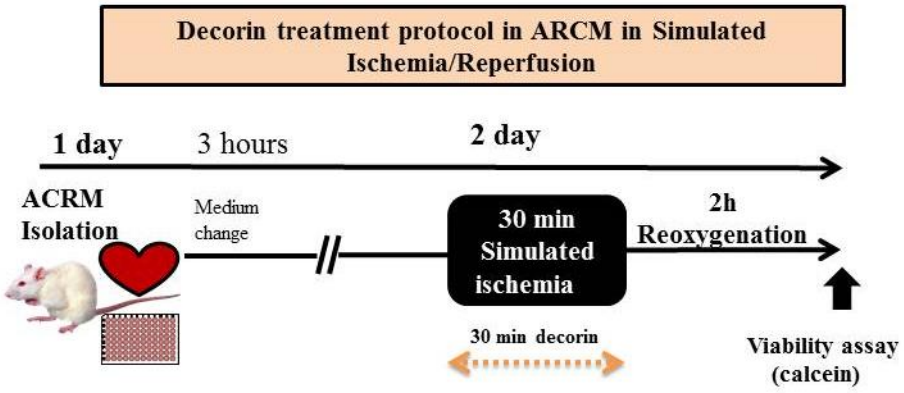

Figure 10. Experimental protocol for decorin treatment against simulated ischemia/reperfusion injury in ARCM. ARCM: adult rat cardiac myocytes.

3.3.5. Matrix metalloproteinase inhibitor tolerability test *in vivo*

Before the use of MMPI molecules in AMI model, preliminary tolerance testing was performed with each molecule (Fig.11.). For each safety testing n=8 male Wistar rats were used. After anaesthesia animals were placed on a heating pad and rectal temperature, heart rate and mean arterial blood pressure (MABP) were monitored. Rats were given 5 increasing doses of the inhibitors dissolved in DMSO (dimethyl sulfoxide; DMSO, D8418, Sigma-Aldrich). Distilled water, physiological saline, ethanol and

DMSO were tested as potential vehicle. DMSO was chosen as a vehicle after testing the solubility of the molecules. Every 20 minutes, 60 µl volume were administered iv. 0.1, 0.3, 1.0, 3.0 and 10 µmol/kg. Twenty minutes after the administration of the highest dose, animals were terminated.

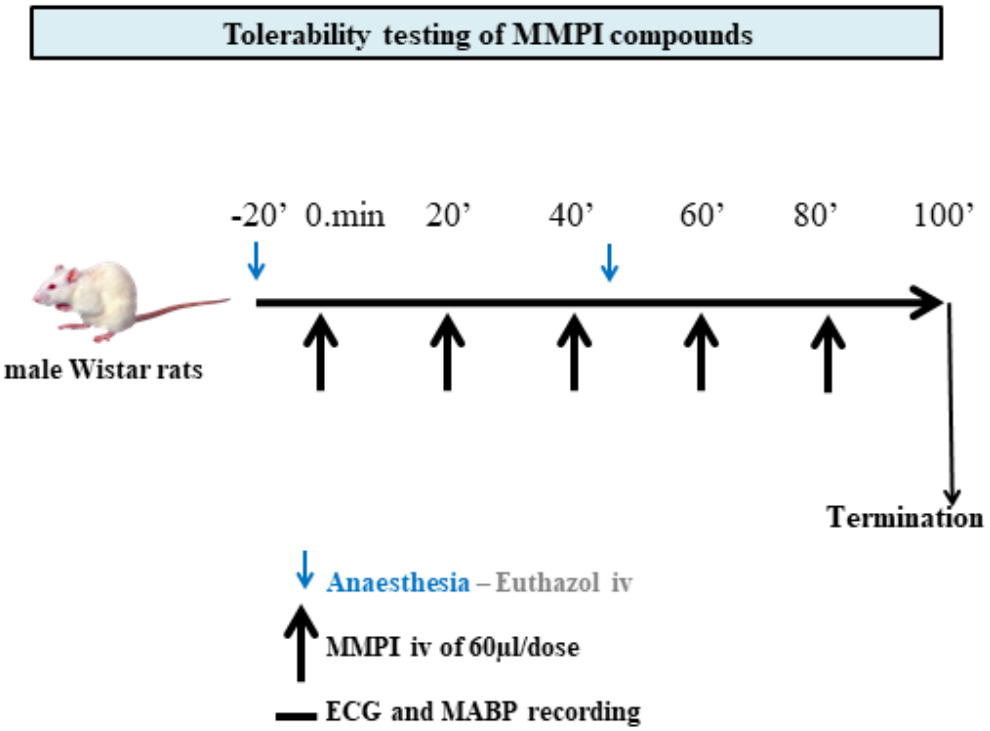

Figure 11. Protocol for tolerability testing of different MMPI compounds.

3.3.6. Matrix metalloproteinase inhibitor treatment *in vivo*

Animals were randomly assigned to the experimental groups with a total number of 15 animals/group. We performed two substudies to show the cardioprotective effects of the selected MMP inhibitor compounds in normocholesterolemic and in hypercholesterolemic animals. In the young normocholesterolemic model, three MMP inhibitor compounds (MMPI-1154, -1260, and -1248, see Figure 6) were tested against AMI in rats. Animals of the 1154 and 1260 test groups received 0.3; 1 and 3 µmol/kg of each MMP inhibitors. MMPI-1248 was administered in 1 µmol/kg, 3 µmol/kg or 10 µmol/kg based on previous IC_{50} measurement (Bencsik et al. 2018). MMP inhibitor compounds were injected intravenously (iv.) at the 25th min of ischemia, in slow bolus through the right jugular vein. Vehicle (dimethyl sulfoxide; DMSO, D8418, Sigma-Aldrich)-treated group served as negative control and DMSO was administered iv. in 60 µl as a slow bolus. In the first experimental setup, in the young normocholesterolemic

model, we used ilomastat, a non-selective, hydroxamic acid-type MMP inhibitor as a positive control to decrease myocardial infarct size after I/R injury. Ilomastat was administered iv. at the 25th min of ischemia at 6 μmol/kg dose and repeated at the 10th and 25th min of reperfusion with half doses, 3 μmol/kg, according to (Bencsik et al. 2014); also see Fig. 12.A.

In the hypercholesterolemic model, animals were divided into 2 major groups: (i) age-matched normocholesterolemic animals were fed with normal rodent chow for 12 weeks, while (ii) hypercholesterolemic animals received standard rodent chow supplemented with 2 % cholesterol and 0,25 % cholic acid. MMPI-1154 and -1260, in one efficacious dose for each (MMPI-1154 at 1 μmol/kg, MMPI-1260 at 3 μmol/kg) was tested against AMI in both age-matched normocholesterolemic and hypercholesterolemic animals as well. In both major groups the same vehicle (DMSO) was used. However, in the second substudy, ischemic preconditioning (IPC), the only-known reproducibly efficacious cardioprotective maneuver was used for positive control. Before test ischemia 3 cycles of 3 minutes ischemia and 5 minutes reperfusion was applied as preconditioning stimuli (Fig. 12.B).

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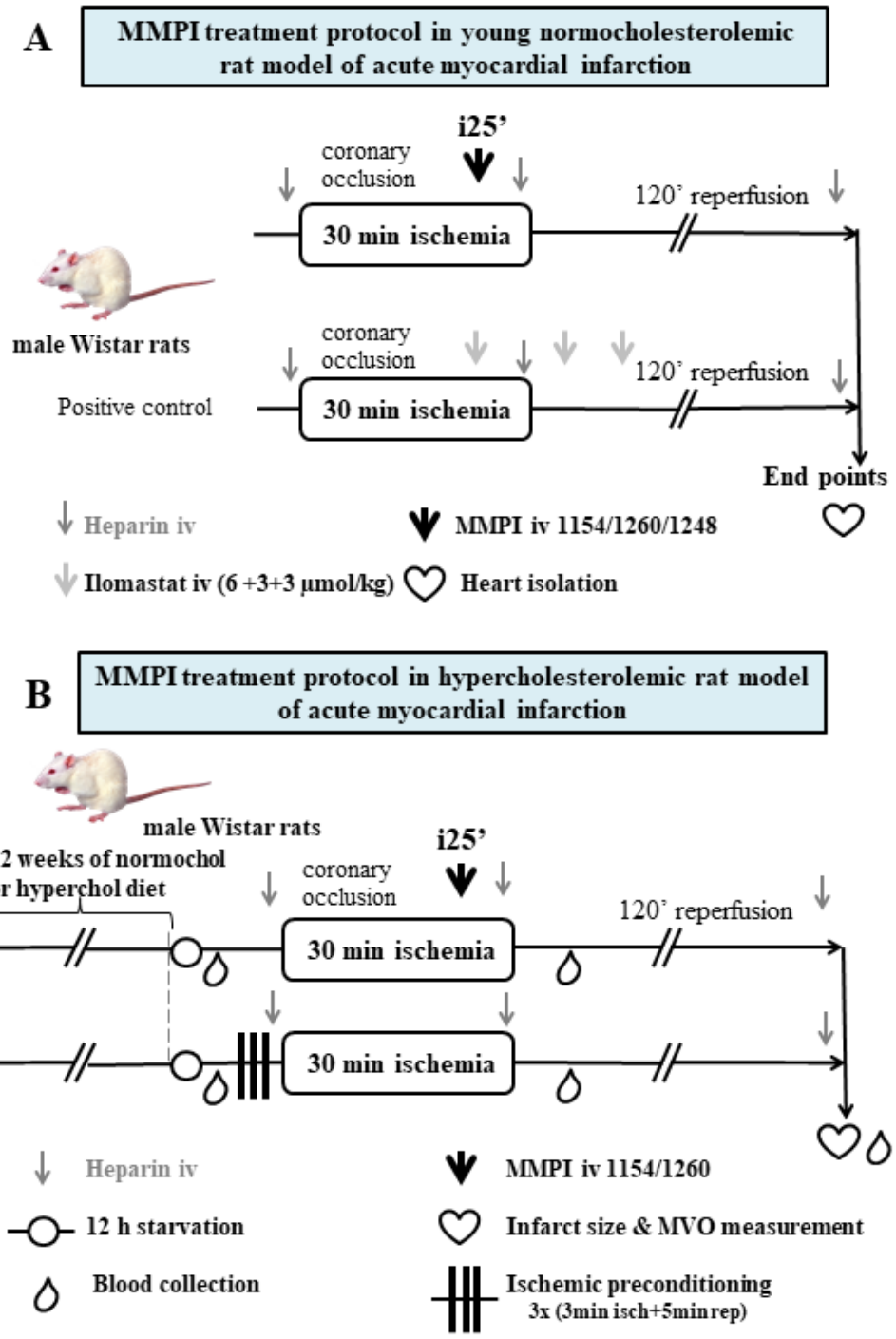

Figure 12. Experimental protocol of *in vivo* acute myocardial infarction in A) normocholesterolemic and B) hypercholesterolemic and age-matched normocholesterolemic rat model. MMPI: matrix metalloproteinase inhibitor, iv: intravenous, MVO: microvascular obstruction.

3.4. *In vitro* measurements

3.4.1. Cell Viability Assay

Cell viability was assessed by a calcein assay performed in each group after 2 h reperfusion. The cell-permeant calcein-AM dye (PromoKine) stains living cells as they convert the stain into green-fluorescent calcein by intracellular non-specific esterases (Miles, Lynch, and Sikes 2015). The growth medium was removed, then cells were washed with PBS twice and incubated with calcein (1 μM) dissolved in DMSO and further diluted in D-PBS, for 30 min in a dark chamber. Then the calcein solution was replaced with fresh PBS and the fluorescence intensity of each well was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech). Fluorescence intensity was measured in well scanning mode (scan matrix: 10x10; scan diameter: 10 mm; bottom optic; no of flashes/scan point: 3; temp: 37 °C; excitation wavelength: 490 nm; emission wavelength: 520 nm).

In the case of ARCMs the cell number varied from well-to-well, therefore living cell number was expressed in ratio of total cell count. ARCMs were incubated with propidium iodide (PI, 50μM) for 7 minutes included digitonin (10-4 M) (Sigma) to permeabilize and kill the cells. Then the PI solution was replaced with fresh PBS and fluorescence intensity of each well was detected; 544/610 nm. The cytoprotective effect of different compounds was compared to simulated ischemic control groups (Tuboly et al. 2019).

3.4.2. Oxidative stress measurements

The level of superoxide (O^{2-}) was measured at the end of the experimental protocol by dihydroethidium (DHE) staining. DHE exhibits blue fluorescence in the cytoplasm and upon oxidation, it intercalates into the DNA and switches to a bright red fluorescence. The fluorescent intensity of each well was detected by a fluorescent plate reader (FluoStar Optima, BMG Labtech) in a well scanning mode (530/620 nm).

The total reactive oxygen species (ROS) content was measured by cell-permeant 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which is a chemically reduced form of fluorescein and used as an indicator of ROS level in cells. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCFDA is converted to the highly fluorescent 2'-7'-dichlorofluorescein (DCF). The fluorescent

intensity of each well was detected later by a fluorescent plate reader (FluoStar Optima, BMG Labtech) in a well scanning mode (480/520nm).

3.4.3. Proliferation assay

Testing the proliferative effect of decorin, BrdU (5'-bromo-2'-deoxiuridine, Abcam) incorporation assay was performed using the Cell proliferation ELISA kit. The cells were pretreated for 24 hours with the BrdU-labeling solution (Final concentration 10 μM) followed by fixation and denaturation by 30 min incubation with FixDenat solution. Then, horseradish peroxidase conjugated anti-BrdU antibody was added to the wells and incubated for 90 mins at room temperature. Finally, tetramethylbenzidine substrate was added for 30 mins and the absorbance was measured using plate reader (FluoStar Optima, BMG Labtech) at 450/620 nm.

3.4.4. TUNEL assay

DeadEnd Fluorometric TUNEL assay (Promega, G3250) measures nuclear DNA fragmentation by catalytically incorporating fluorescein-12-dUTP. Detection of fragmented DNA in apoptotic NRCMs after SI/R was performed according to manufacturer instruction. Briefly, cells were fixed 4 % methanol-free formaldehyde solution in PBS (pH 7.4) for 25 mins, then washed and cells were permeabilized by 0.2 % Triton® X-100 solution in PBS for 5 minutes. After removing liquids, enzyme and fluorescent nucleotide mix were added to cells and incubated for 60 minutes at 37 °C. After washing steps cells were stained with DAPI (4′,6-diamidino-2-phenylindole, blue-fluorescent DNA stain). Green and blue fluorescence intensity of each well was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech). Fluorescence intensity was measured in well scanning mode, for TUNEL: 490/530 nm and for DAPI 435/460 nm.

3.4.5. Caspase assay

Investigation of apoptosis was assessed by CellEvent Caspase-3/7 assay (Thermofisher Scientific) performed on NRCMs after SI/R, according to manufacturer instruction. The growth medium was removed, then cells were washed with PBS twice and incubated with caspase-3/7 reagent (1 μM) dissolved in DMSO and further diluted in D-PBS, for 30 min in a dark chamber. The Green Detection Reagent is a four-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye, which is non-fluorescent until

cleaved by caspase-3/7. After activation of caspase-3/7 in apoptotic cells, the cleaved DEVD peptide is enabling the dye to bind to DNA and produce a bright green fluorogenic response. Then the caspase-3/7 reagent solution was replaced with fresh PBS and the fluorescence intensity of each well was detected by fluorescent plate reader (FluoStar Optima, BMG Labtech). Fluorescence intensity was measured in well scanning mode (503/530 nm).

3.4.6. Western blot sample collection

For western blot analysis cell lysate sample was collected from neonatal rat cardiomyocyte culture. NRCMs at day-2 were pretreated with 0 (Vehicle), 1, 3, 10, 30, 100 nM concentration of decorin for 20 h, respectively. Then 4h SI treatment was applied, at the end cells were washed twice with ice-cold D-PBS, scraped from wells, and collected in homogenization buffer (1× Radioimmunoprecipitation assay buffer (RIPA) containing protease inhibitor cocktail and phosphatase inhibitors) collected in Eppendorf tubes. The number of cells for n=1 (biological sample) was collected and pooled together from 2 wells. To reach n=4 we collected cells from 4 independent isolation. Cells were sonicated with an ultrasonic homogenizer (10 s, 4 °C). The homogenate was centrifuged (14000 × g, 10 min, 4 °C), the supernatants were further concentrated using Amicon® Ultra-4 Centrifugal Filter Units (10 kDa cut-off limit). Concentrated lysate were kept on -70°C until western blot measurement. (Gaspar et al. 2016)

3.4.7. Western blot

Protein concentration of the homogenates was determined with BCA Protein Assay Kit (Thermofisher Scientific). 40 µg of protein was loaded on 8 or 10 % polyacrylamide gels and was separated by standard SDS-PAGE followed by transfer of proteins onto PVDF membranes (90 V, 15min, 110V, 85min), transfer overnight, 200mA. After the transfer, the membranes were checked with Ponceau solution (0.05g Ponceau powder in 5% acetic acid). Membranes were blocked (2 h, RT) in 0.05% Tris buffered saline (TBS)-Tween20 containing 5% non-fat milk. Membranes were incubated with p-Akt (Cell Signaling, #9271, 60 kDa), Akt (Cell Signaling, #92972, 60 kDa) (1:500) and GAPDH (Cell Signaling, #5174, 37 kDa) (1:10000) primary antibody overnight at 4 °C in 5% milk followed by incubation with anti-rabbit-HRP secondary antibody (1:2000, in case of GAPDH 1:10000) for 2h, at room temperature. After washing, membranes were

developed with an enhanced chemiluminescence kit. Bands were quantified by Image Lab (BioRad) software.

3.4.8. Gelatin zymography

Blood samples were collected from femoral vein at 5th min of reperfusion and from abdominal aorta at 120th min of reperfusion (termination). In order to investigate MMP-2 and MMP-9 activity, gelatin zymography was performed as described previously in detail (Bencsik et al. 2017). Briefly, rat plasma samples, 50 µg protein/lane, were loaded and separated by electrophoresis on a 10% SDS-polyacrylamide gel copolymerized with 2 mg/ml gelatin from porcine skin (G1890, CAS 9000-70-8, Sigma-Aldrich; St. Louis, MO). An internal standard (selected plasma sample) was loaded into each gel to normalize activities between gels.

After electrophoresis (90 V, 90 min), gels were washed in 2.5% TritonX 100 with gentle agitation and then incubated for 20 h at 37°C in zymography development buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl2, 200 mM NaCl). Zymographic gels were stained with 0.05% Coomassie Brilliant Blue G-250 dye (20279, CAS 6104-58-1, ThermoScientfic) in a mixture of methanol-acetic acid-water [2.5:1:6.5 (v/v)] and destained in water-based 4% methanol-8% acetic acid (v/v). After this step zymograms were scanned. MMP activity was detected as a colorless, transparent zone on a blue background (Fig. 13.) and the clear bands in the gel were quantified by densitometry using the Quantity One software (Bio-Rad, Hercules, CA) and expressed as the ratio to the internal standard, and presented in arbitrary units. For the positive controls, gelatinase zymography standard was used containing human MMP-2 and -9 (Chemicon Europe Ltd., Southampton, United Kingdom). For negative control, lanes containing plasma samples were cut off after renaturation and were incubated separately in development buffer in the presence of calcium chelator EGTA [ethylene glycol-bis(2-aminoethylether)-N,N,N0,N0-tetraacetic acid; 10 mM] for 20 h at 37°C. Since no gelatinolytic activities could be seen in negative control gels, we concluded that all visible bands from the activity of MMP were suppressed (data not shown).

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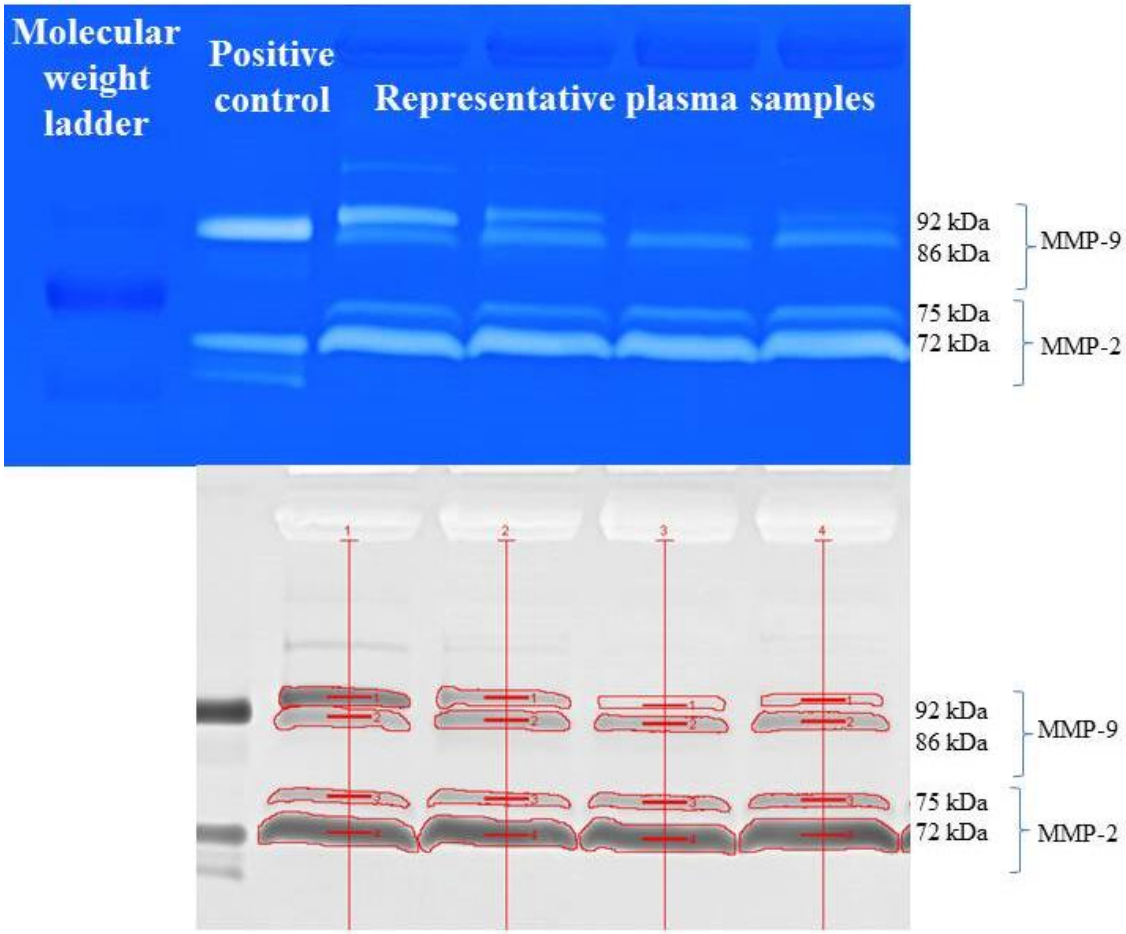

Figure 13. Representative image of gelatin zymography. Coomassie-blue stained gel with light blue bands, which occurs due to MMP enzyme activity. Black and white scanned image is used to quantify the band's pixel intensity and area via Quantity One software.

3.4.9. Lipid panel measurement

In the comorbid model, to determine the development of hypercholesterolemia (Csont et al. 2013) after the 12 weeks cholesterol enriched diet (Figure 12.B), blood samples were collected from the animals before coronary occlusion surgery. Animals had a 12 hours starvation prior to baseline blood sampling (cannulated carotid artery). Blood was taken into heparinized tubes and centrifuged at 4 °C for 15 min at 1000 g to gain plasma. Total cholesterol, triglyceride, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and glucose levels were measured by using a Hitachi Cobas8000 automated system at the Institute of Laboratory Medicine at the University of Szeged.

3.4.10. RNA isolation

In independent experiments NRCMs were treated 20 hours with 3 nM decorin or vehicle prior to 4 hours of SI. Cells were collected and lysed in 1 mL of QIAzol Lysis Reagent (QIAgen). Then Total RNA was extracted using Direct-zol™ RNA MiniPrep System (Zymo Research) according to the manufacturer's protocol. The RNA Integrity Numbers and RNA concentration were determined by RNA ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, USA) and RNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

3.4.11. RNA library construction

For Gene Expression Profiling (GEx) library construction, QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH, Wien, Austria) was applied according to the manufacturer's protocol. The quality and quantity of the library was determined by using High Sensitivity DNA1000 ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, USA) and dsDNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Pooled libraries were diluted to 1.8 pM for 1x86 bp single-end sequencing with 75-cycle High Output v2 Kit on the NextSeq 550 Sequencing System (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

For small RNA library construction, NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, MA, USA) was applied according to the manufacturer's protocol. The quality and quantity of the library QC was performed by using High Sensitivity DNA1000 ScreenTape system with 2200 Tapestation (Agilent Technologies, Santa Clara, CA, USA) and dsDNA HS Assay Kit with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Pooled libraries were diluted to 1.8 pM for 2x43 bp paired-end sequencing with 75-cycle High Output v2 Kit on the NextSeq 550 Sequencing System (Illumina, San Diego, CA, USA) at the Xenovea Ltd. according to the manufacturer's instructions. Raw and processed RNA-sequencing datasets were deposited in the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) under the accession number of E-MTAB-9325.

3.4.12. Bioinformatics evaluation of the RNA sequencing data

Adapter trimming, quality and length filtering of raw RNA sequencing reads were performed by Cutadapt (version 1.15) (Martin 2011). During quality and length filtering reads with an average Phred quality score below 30 or a length less than 19 nucleotide were excluded from further analysis. FastQC (version v0.11.8) and MultiQC (version v1.7) were used for quality control analysis (Ewels et al. 2016). Reads obtained this way were aligned to Rnor_6.0 NCBI Rattus norvegicus reference genome and were annotated using the corresponding reference annotation by HISAT2 (version 2.0.4) and featureCounts (version of Subread v2.0.0), respectively (Kim et al. 2019; Liao, Smyth, and Shi 2014). DESeq2 Bioconductor package was utilized for normalization and differential expression analysis (Love, Huber, and Anders 2014). Correction of the p-values for multiple comparisons was done by calculating the false discovery rate according (FDR) to Benjamini and Hochberg (Hochber 1995).

3.4.13. Gene Ontology enrichment analysis

The online PANTHER Overrepresentation Test (geneontology.org, version released on 7 April 2020 (Mi et al. 2019) was performed against the Rattus norvegicus reference gene list to assess Gene Ontology (database version released on 23 March 2020) biological process terms enriched among genes that were differentially expressed when considering the non-corrected p-values. For the enrichment analysis Fisher's exact test was applied with false discovery rate correction for multiple comparisons.

3.4.14. High resolution respirometry

In vitro tests were performed using cardiac mitochondria and high resolution respirometry (HRR, (Oxygraph-2k high resolution respirometer, Oroboros Instruments, Innsbruck; Austria) to analyze the effects of GPC on mitochondrial respiration. During the measurements, mitochondria undergo different “states” by the sequential addition of substrates or inhibitors and the respiratory capacity could be assessed at multiple levels of the respiratory chain. Briefly, cardiac samples from newborn Wistar rats were homogenized in 1 ml of MitOx respiration medium (120 mM KCl, 20 mM HEPES, 10 mM KH_2PO_4, 86 mM $MgCl_2$, 0,025% BSA) with a glass Potter homogenizer, and subsequently, 50 µl of homogenates were immediately placed into the detection

chambers, which were calibrated to 200 nmol/ml oxygen concentrations in room air. The respirometry data were normalized to wet weight.

In an independent experiment 1 µM - 100 µM GPC solutions were used in order to determine the effects of GPC on mitochondrial respiration (Fig. 14). First, the steady-state basal oxygen consumption of the homogenates (basal respiration) was measured. Then, the complex II-linked respiration (state II) was determined after the addition of 0.5 µM rotenone (complex I inhibitor) and 10 mM succinate (complex II substrate). Subsequently, the complex II-linked oxidative phosphorylation capacity (state III respiration) was estimated by adding saturating concentration of ADP to the medium.

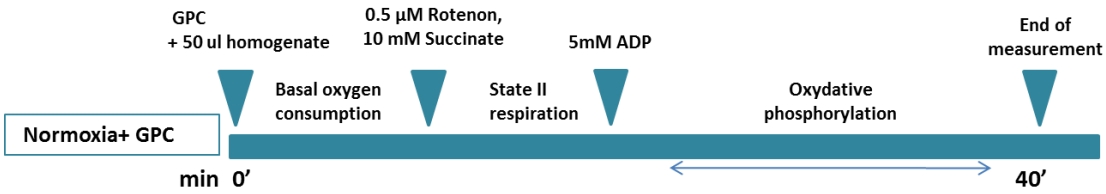

Figure 14. Experimental protocol of oxidative phosphorylation measurement on neonatal cardiac mitochondria.

3.5. Ex vivo staining of rat hearts for end-point measurements

3.5.1. Determination of myocardial infarct size

At the end of the 120 min reperfusion period, the hearts were isolated and infarct size was determined as described previously (Csonka et al. 2010). Briefly, the LAD was re-occluded and the heart was perfused with 4 ml of 0.25 % (w/v) Evans-blue dye (E2129, Sigma-Aldrich) in Langendorff mode to delineate the area at risk. Stained hearts were rapidly frozen (-20 ºC for at least 2 hours), cut into 2 mm thick slices (total of six), and each slice was incubated at 37 ºC in 1 ml of 1 % (w/v) 2,3,5-Triphenyltetrazolium-chloride (TTC, 108380, Merck Biosciences) dissolved in 50 mM phosphate buffer (pH 7.4) for 10 min. Slices were then transferred to 10 % formalin solution for 10 min, rinsed, and then placed between glass plates, finally digital photos were taken (Canon, SX60 HS) from both sides of the heart slices. The differently stained areas of the heart images (white: infarcted region, red: area at risk, blue: non-ischemic region, see Fig. 15.) were quantified by digital planimetry (Infarctsize™ 2.5,

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Pharmahungary 2000 Ltd). Evaluation of all images was carried out in a blinded manner by an experienced person throughout the study.

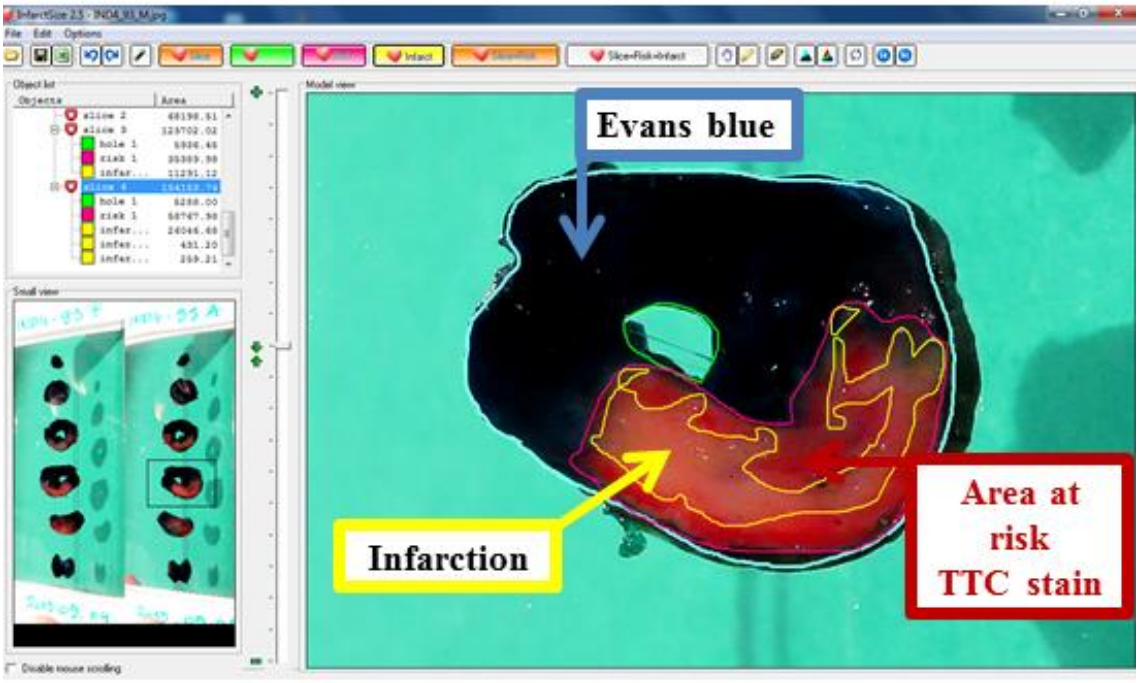

Figure 15. Representative image of infarct size measurement of rat heart slices after Evans blue and 2,3,5-Triphenyl-tetrazolium chloride (TTC) double staining. White: infarcted region, red: area at risk, blue: non-ischemic region.

3.5.2. Determination of microvascular obstruction

In the comorbid model (Figure 12.B), microvascular obstruction (Bonanad et al. 2013) was measured from the isolated hearts. At the end of reperfusion, hearts were isolated and before the reocclusion of LAD, thioflavine-S (T1892, Sigma-Aldrich) stain was perfused through the whole heart using Langendorff retrograde perfusion system. Then hearts were stained with Evans-blue as described above in detail. The hearts were freshly cut into six 2 mm-thick slices and were placed in a dark chamber under UV light. The fluorescens thioflavine-S was visible where it could penetrate the tissue through the coronary capillaries, expect the areas where microvascular obstruction occurred (see representative images at Figure 39.A).

3.6. Statistical analysis

Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post-hoc* tests were used to analyse differences in mean values of the experimental groups versus vehicle from *in vitro* cell culture based experimental data. All-cause mortality and incidence of arrhythmias were analyzed using Chi-square test. Area at risk, infarct size and MVO of test compounds were compared to vehicle using one-way ANOVA followed by Fisher LSD/Dunnett's post hoc test. Hemodynamic data of the test compounds were compared to vehicle by repeated measure of ANOVA followed by Dunnett's post hoc test. Lipid panel measurement compared the effect of 12 week diet between normo- and hypercholesterolemic animals with Student's t-test. Significance value was chosen *$p<0.05$.

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4. Results

4.1. Cardioprotection model *in vitro* and *in vivo*

To investigate the cardioprotective effect of different compounds, suitable platforms are needed. For *in vitro* cell culture models, first the SI/R model has to be validated, therefore we compared the viability of cells exposed to simulated ischemia (hypoxic solution combined with hypoxic environment) to that observed after the normoxia protocol (normoxic solution combined with normoxic environment). To see a therapeutic window we seeked a significant decrease in cell viability, therefore we tested several experimental protocol. In case of neonatal cardiac myocytes the simulated ischemia was chosen to be 12 hours, 6 hours and 4 hours (Fig.16, unpublished data).

All three SI/R protocols resulted in significant decline in cell viability in comparison to the normoxic group, 72%, 77% and 80% respectively (Fig. 16.). Based on this results, we used the 4 hour simulated ischemia model to test the cardio-cytoprotective effect of different compounds.

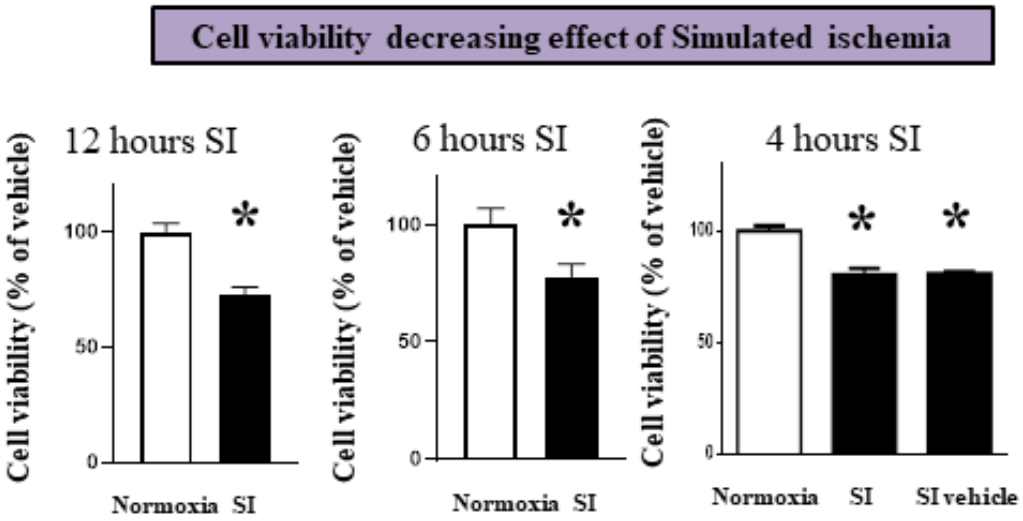

Figure 16. The effect of simulated ischemia on cell viability. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*p<0.05 vs Normoxia and #p<0.05 vs SI/R Vehicle treated cells (n=5-6). SI: simulated ischemia

In vivo AMI protocols were developed in our research group for many years. The 30 min coronary occlusion followed by 120 min reperfusion considered a standard and reliable model, vehicle-treated (dimethyl sulfoxide) ischemic group (63.7±1.9% and 55.9±3.4%) showed similar infarct size to what we measured in previous studies (66.1±4.6%) (Bencsik et al. 2014); 59.8±4.5% (Kiss et al. 2016).

4.2. Testing of potential cardioprotection in cardiac myocytes under normoxic contition

4.2.1. L- alpha-glycerylphosphorylcholine

4.2.1.1. Acute effect of L- alpha-glycerylphosphorylcholine in neonatal rat cardiac myocytes under normoxic conditions

In normoxic conditions, the acute (15 minutes) treatment with different concentration of GPC did not change the cell viability of NRCMs in comparison to the vehicle control (Fig. 17.A). Neither the superoxide production nor total ROS accumulation has changed significantly after 15 min exposure to GCP in cardiac myocytes (Fig. 17.B,C).

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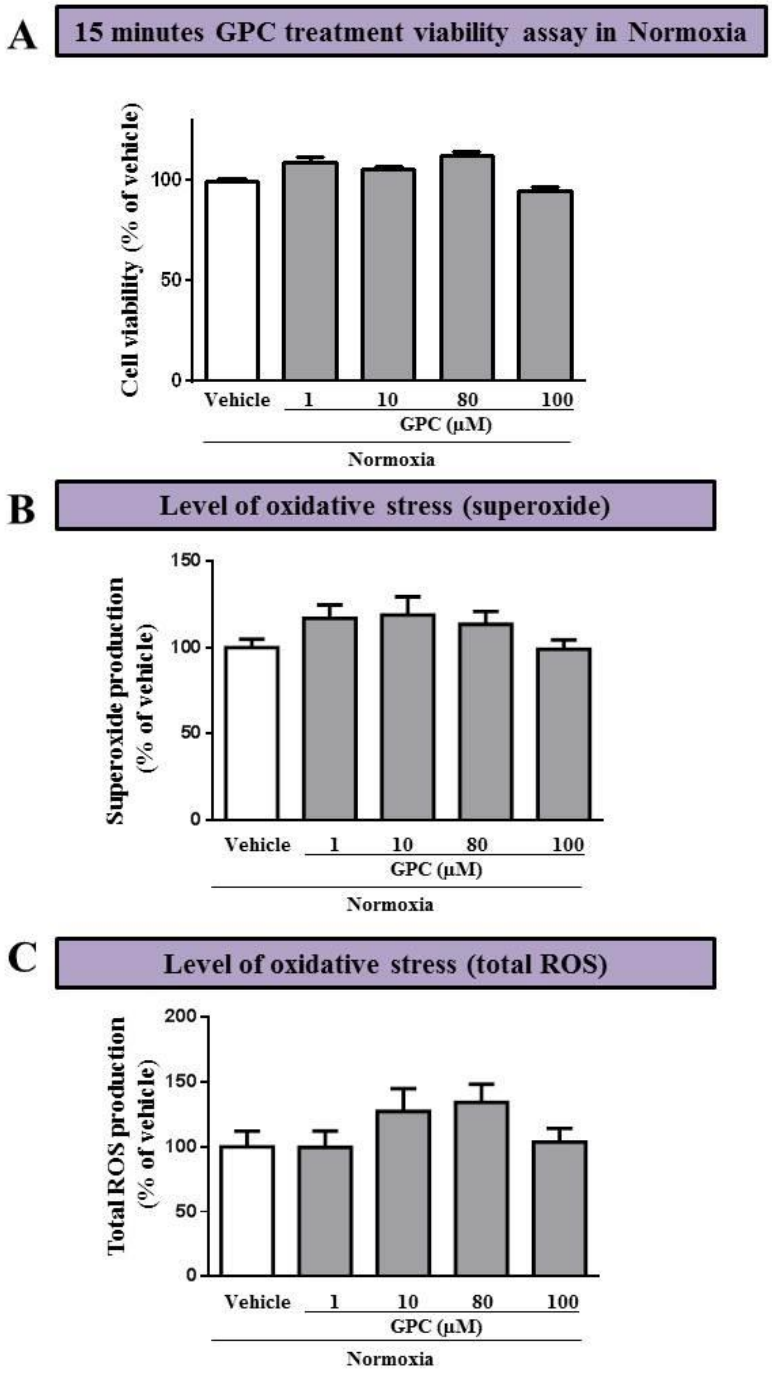

Figure 17. Results of acute GPC treatment in NRCM under normoxic conditions. The acute effect of 15 mins pre-treatment of GPC in different concentration on NRCM's **A)** viability **B)** superoxide production and **C)** total ROS production. Results are normalized to normoxic control group. Data are presented as mean ± S.E.M. Statistical analysis of data was performed by One-Way ANOVA, followed by Dunnett's multiple comparison test,*$p<0.05$ vs normoxic controls, #$p<0.05$ vs vehicle treated cells (n=8-16).

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4.2.1.2. Short-term effect of L- alpha-glycerylphosphorylcholine in neonatal rat cardiac myocytes under normoxic conditions

Similarly to those observations after acute administration under normoxic conditions, the short-term (3-hours) GPC treatments had no impact on the cell viability of NRCMs compared to the vehicle control (Fig. 18.A).

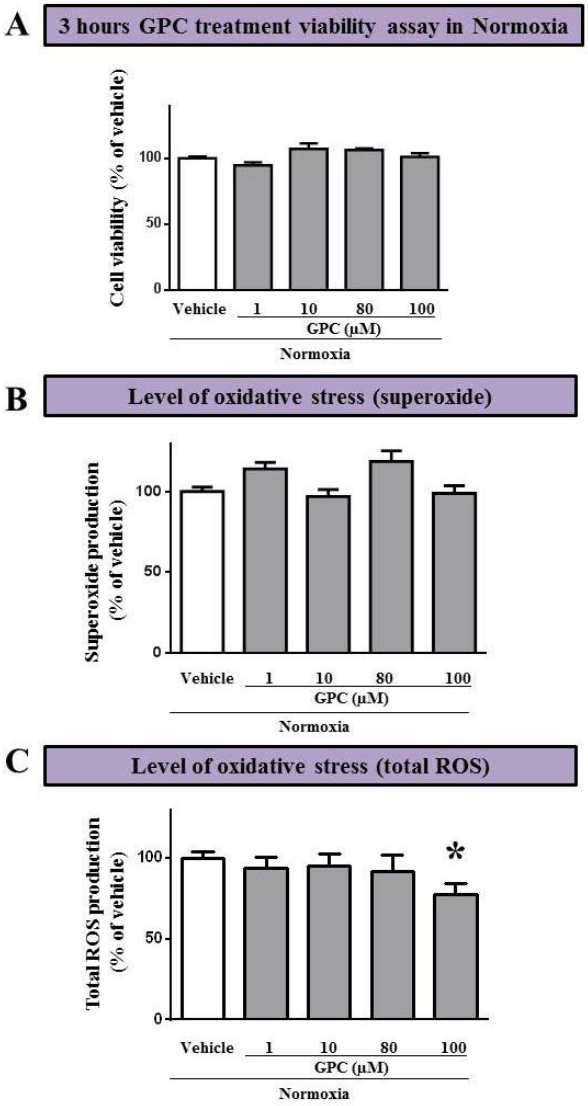

Figure 18. Results of short-term effect of different concentrations of GPC on **A)** cell viability **B)** superoxide production and **C)** total ROS production in isolated NRCMs after 3 hours of GPC treatment. Results are normalized to vehicle-treated cells. Data are presented as mean ± S.E.M. Statistical analysis of data was performed by One-Way ANOVA, followed by Dunnett's multiple comparison test,*$p<0.05$ vs vehicle treated cells (n=8-16).

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In short-term applications of GPC, none of the applied concentrations had an influence on the superoxide level in cardiomyocytes (Fig. 18.B). Nevertheless, the overall ROS production was significant reduced after 3-hour treatment with 100 µM GPC compared to the vehicle treated group (Fig. 18.C). Taken together this surprising observation with the increased oxygen consumption rate (Fig.19.), we suggest a direct intra-mitochondrial effect of exogenous GPC. It was noted in previous study by Stifler et al. (Strifler et al. 2016) that 80 and 100 µM concentration of GPC that was represented by a higher oxygen consumption rate and a subsequent physiological mitochondrial ROS-formation. It might due to a general compensatory mechanism to scavenge the extended amount of ROS which was manifested in a significant decrease at 100 µM GPC treatment.

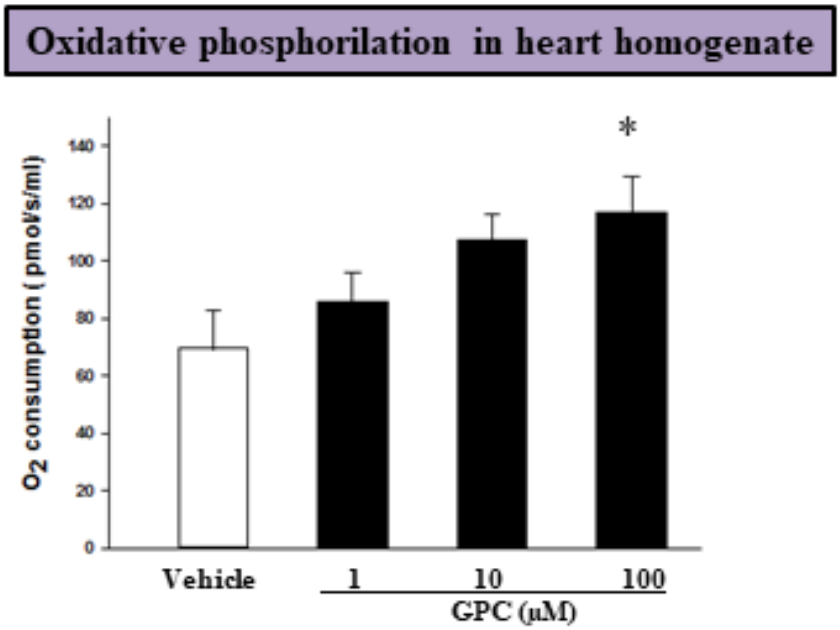

Figure 19. Oxidative phosphorylation (in pmol/s/ml) of neonatal cardiac mitochondria. Columns represent the effect of different GPC concentrations on oxidative phosphorylation of cardiac mitochondria. Data are means ± SEM. Statistical analysis of data was performed by One-Way ANOVA, followed by Dunnett's multiple comparison test, *$p<0.05$ vs vehicle.

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4.2.1.3. Long-term effect of L- alpha-glycerylphosphorylcholine in neonatal rat cardiac myocytes under normoxic conditions

The long-term GPC treatment, which was applied for 24 hours, a profound cell death was induced by each applied GPC concentration in NRCMs, as compared to the vehicle-treated group (Fig. 20.A). In accordance with these findings, the intracellular superoxide levels significantly increased at each applied concentration of GPC (Fig. 20.B) and the overall ROS concentrations also significantly increased at 1 µM and 100 µM concentration of GPC (Fig. 20.C) which might reflect some interesting crosstalk between mitochondrial function and other intracellular ROS-scavenger mechanisms.

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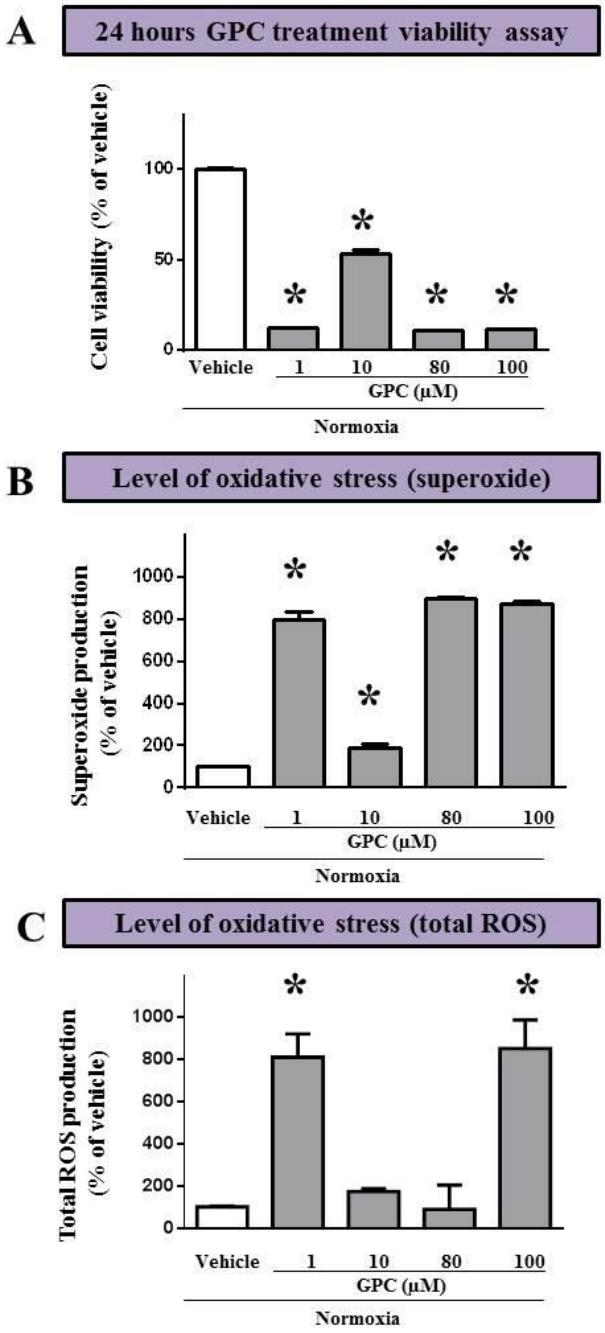

Figure 20. The long-term effect of different concentrations of GPC on **A)** cell viability **B)** superoxide production and **C)** total ROS production in isolated NRCMs after 24 hours of GPC treatment. Results are normalized to vehicle-treated cells. Data are presented as mean ± S.E.M. Statistical analysis of data was performed by One-Way ANOVA, followed by Dunnett's multiple comparison test,*$p<0.05$ vs vehicle treated cells (n=8-16).

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4.2.2. Concentration-dependent effect of decorin on cell viability and proliferation in isolated neonatal rat cardiac myocytes under normoxic conditions

Different concentrations (1-100 nM) of decorin treatment of NRCMs for 4+2 hours under normoxic conditions showed that decorin applied in 3 nM and 10 nM concentrations significantly increased cell viability, while 1 nM, 30 nM and 100 nM concentrations have not changed that when compared to the vehicle-treated normoxic group (Fig. 21.A).

Cell proliferation could distort the results of cell viability, therefore we checked the proliferative effect of decorin in isolated NRCMs under normal circumstances. The rate of cell proliferation was measured by synthetic bromodeoxyuridine (BrdU) incorporation into the cells. Only 1 nM decorin concentration increased significantly the cell proliferation in NRCMs, the other concentrations had no effect on it (Fig. 21.B).

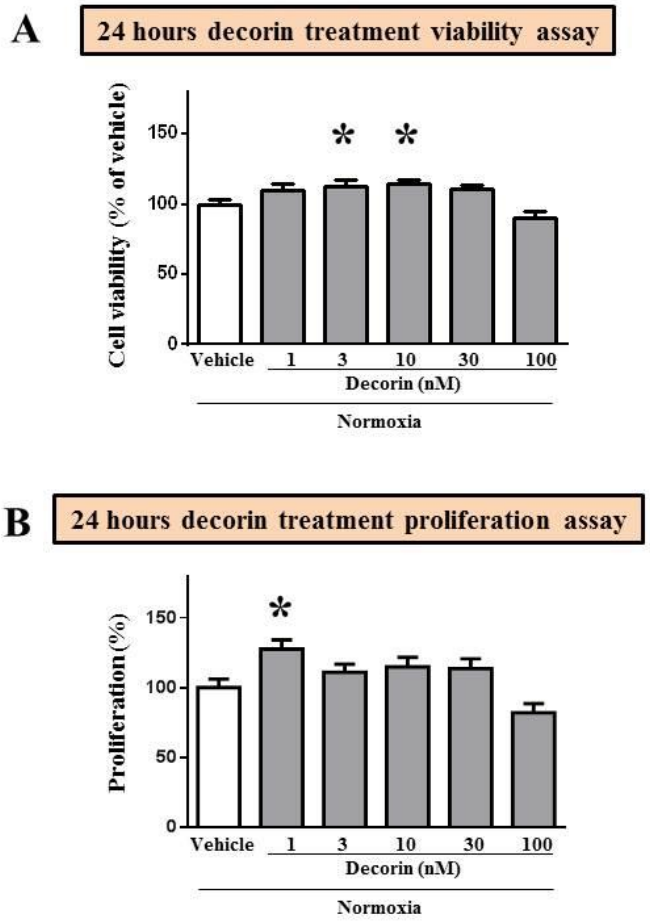

Figure 21. The effect of different concentrations of decorin on the **A)** cell viability and **B)** cell proliferation in NRCMs in normoxic conditions. Cell viability measured by

calcein staining based on intracellular esterase's enzyme activity. The rate of proliferation was evaluated by BrdU incorporation into the cells. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*$p<0.05$ vs vehicle treated cells (n=5-6).

4.3. Testing potential cardioprotection in cardiac myocytes under simulated ischemia/reperfusion

4.3.1. Effect of short-term application of L- alpha-glycerylphosphorylcholine in isolated neonatal rat cardiac myocytes exposed to simulated ischemia/reperfusion injury

The 3 hour long GPC pre-treatment was tested on NRCMs to see the degree of SI/R-caused reduction in cell viability. Most of the applied concentrations of GPC had no effect on cell death compared to the vehicle. The 80 µM GPC pre-treatment; however, significantly improved cell viability after the SI/R injury (Fig. 22.A) and therefore it is a promising cytoprotective compound.

In order to understand the anti-oxidant features of GPC in the chosen concentration range under SI/R conditions, we investigated the superoxide production and the overall ROS-formation of the cardiac cells. Interestingly, none of the applied GPC concentrations caused significant changes in these parameters (Fig 22.B,C) which suggests that the cytoprotective effect of GPC might be other than its direct ROS-scavenger capacity.

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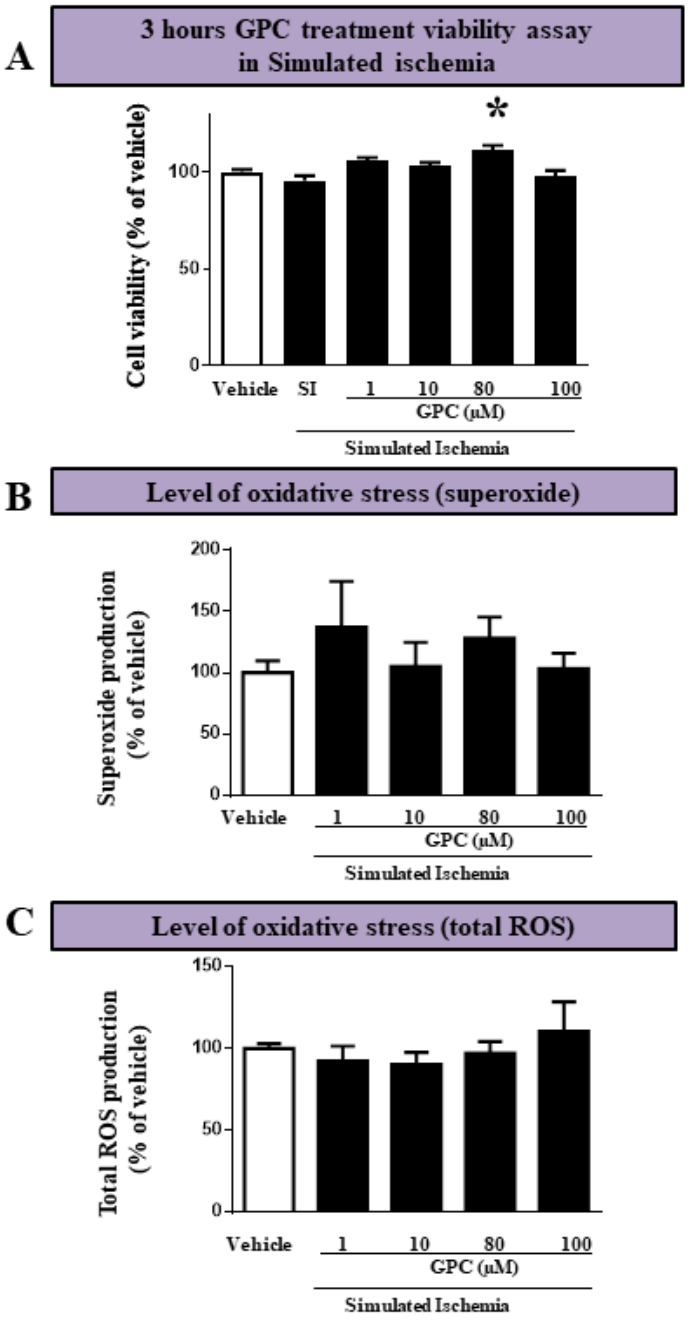

Figure 22. The effect of different concentrations of GPC on A) cell viability B) superoxide production and C) level of total ROS exposed to SI/R. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*$p<0.05$ vs Normoxia and #$p<0.05$ vs SI/R Vehicle treated cells (n=5-6).

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4.3.2. Protective effect of decorin on cell viability in isolated neonatal and adult rat cardiac myocytes exposed to simulated ischemia/reperfusion injury

In a separate experiment we investigated the direct effect of decorin on NRCMs in SI/R injury. Different concentrations (1-100 nM) of decorin treatment significantly increased the cell viability of NRCMs after 4 h/2 h of SI/R in comparison to vehicle-treated group in a concentration-dependent manner. The most effective concentrations were 1 nM, 3 nM and 10 nM (Fig. 23.A).

To further test the most effective concentrations of decorin on the cell viability, isolated adult rat cardiac myocytes (ARCMs) were exposed to 30 min/2 h of SI/R. Since ARCMs are more sensitive to hypoxia, therefore 30 minutes of ischemia created a right platform to test the most efficacious three concentrations of decorin: 1 nM, 3 nM, and 10 nM. There was a significant difference between Normoxia control group and simulated ischemia treated with only vehicle, which means that the SI/R protocol was successful. All concentrations of decorin attenuated SI/R-induced cell death as compared to the vehicle-treated group (Fig. 23.B).

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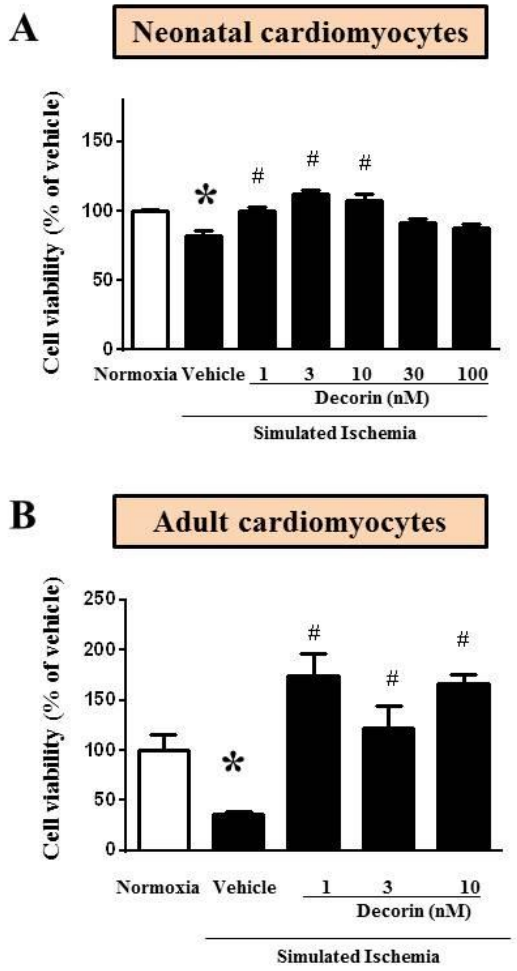

Figure 23. The effect of different concentrations of decorin on cell viability in isolated A) neonatal rat cardiomyocytes and B) adult rat cardiomyocytes exposed to SI/R. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*$p<0.05$ vs Normoxia and #$p<0.05$ vs SI/R Vehicle treated cells (n=5-6).

4.4. Possible mechanism behind cardioprotective effect

4.4.1. The effect of different inhibitors on decorin-induced cardioprotection in neonatal rat cardiac myocytes exposed to simulated ischemia/reperfusion injury

To explore potential cardioprotective signaling pathways and the involvement of NO in the mechanisms of action of decorin against SI/R injury, the NO-synthase inhibitor, L-NAME at 10 µM was used in combination with decorin in NRCMs exposed to SI/R. For the inhibitory co-treatment we only applied the most effective concentration, the 3 nM of decorin. It is significantly increased cell viability but this

effect was not affected by L-NAME co-treatment. However, cell viability was increased by L-NAME alone (Fig. 24.A).

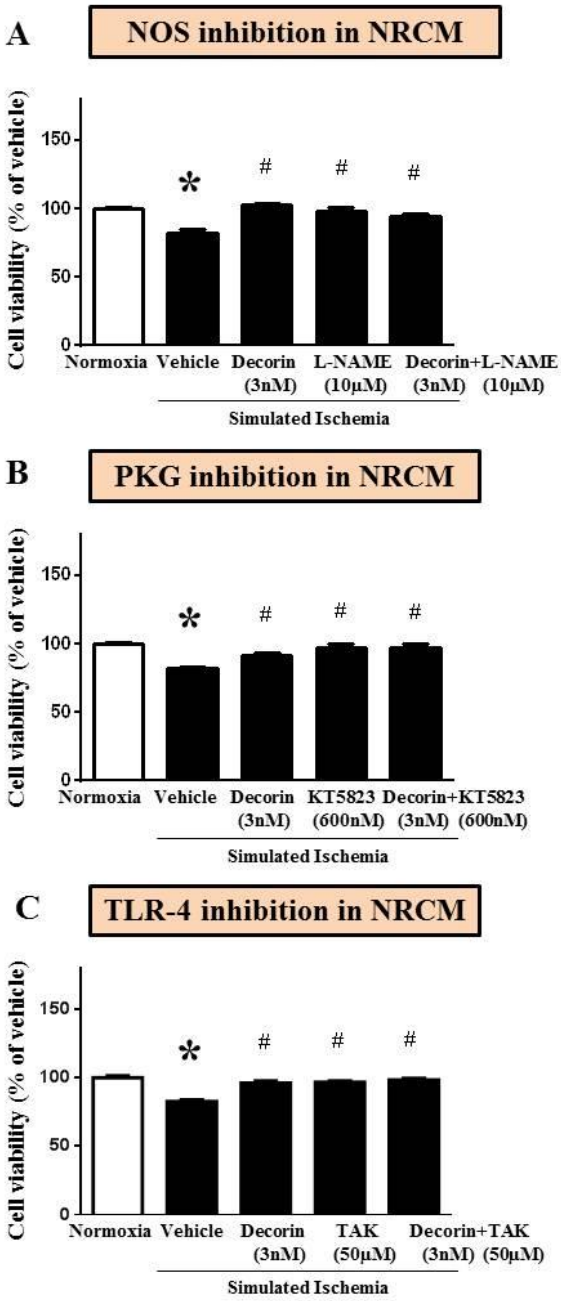

Figure 24. The effect of 3 nM decorin and following inhibitors alone or in combinations: A) 10 µM L-NAME (NOS inhibitor); B) 60 nM KT-5823 (PKG inhibitor); and C) 50 µM TAK-242 (TLR-4 inhibitor); on the cell viability in NRCMs exposed to SI/R. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*$p<0.05$ vs Normoxia and #$p<0.05$ vs Vehicle treated cells (n=5-6).

After NO-synthetase inhibition, we further investigated the potential involvement of NO/cGMP/PKG signaling pathway by using the protein kinase G (PKG) inhibitor KT-5823. Similarly to the previous set up KT-5823 (at 60 nM) was used alone and combined with decorin in NRCMs exposed to SI/R. Decorin at 3 nM significantly increased cell viability and this effect was not affected by KT-5823 co-treatment. However, cell viability was significantly improved by KT-5823 alone (Fig. 24.B).

Upon the inhibition of TLR-4 receptor via TAK-242 (at 50 µM) together with decorin in NRCMs exposed to SI/R, we investigated the involvement of decorin with the TLR-4 triggered mechanism. Decorin at 3 nM significantly increased cell viability but the protective effect was not affected by TAK-242 co-treatment. However, as it showed with the other inhibitors, cell viability was significantly improved by TAK-242 alone (Fig. 24.C). Therefore we can not be sure if those pathways are involved in the cardioprotective effect of decorin, but they seem independent from it.

4.4.2. The effect of 3 nM decorin on apoptosis in isolated neonatal rat cardiac myocytes exposed to simulated ischemia/reperfusion injury

Based on the most pronounced cardiocytoprotection at 3 nM of decorin treatment on cell viability of NRCMs exposed to SI/R, we collected data on the rate of apoptosis in a separate experiment. For this purpose we used TUNEL fluorescence staining (Fig. 25.A), and caspase-3/7 activity (Fig. 25.B) measurement after the 20+4 hours 3nM decorin treatment combined with simulated ischemia/reperfusion injury, but the decorin treated cells showed no difference compared to the vehicle-treated group.

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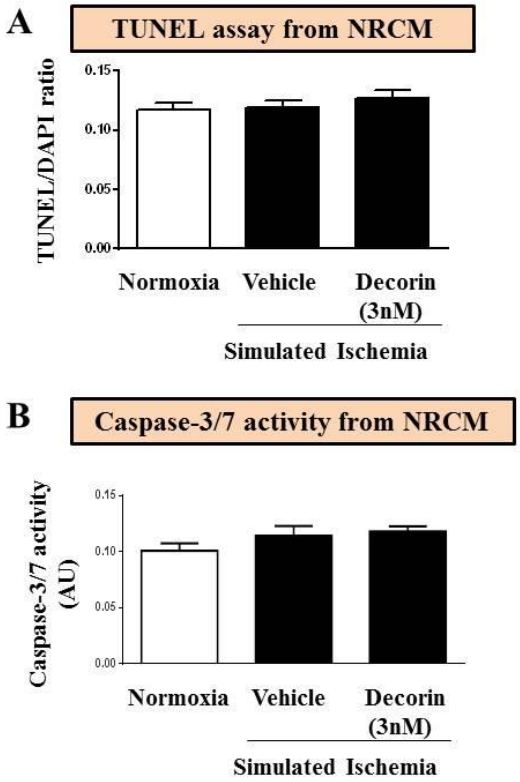

Figure 25. The effect of 3 nM decorin on apoptosis in NRCMs exposed to SI/R. Results of A) TUNEL assay and B) Caspase assay. Data are normalized to vehicle-treated Normoxia and presented as mean ± S.E.M. One-Way ANOVA, Dunnett's multiple comparison test,*p<0.05 vs normoxia (n=4). NRCM: neonatal rat cardiac myocytes, AU: arbitrary unit

4.4.3. Akt and activated phospho-Akt protein level in neonatal rat cardiac myocytes

In the western blot measurement we masured Akt and phospho-Akt (p-Akt) level from cardiac myocytes with 20+4 hours treatment of different concentrations of decorin (vehicle, 1, 3, 10, 30 or 100 nM) under normoxic conditions or in SI. Phosphorilation of Akt is very dynamic and in this experiment we were investigatin the early changes, what is changing during ischemia. The results showed no difference between groups in case of total Akt/GAPDH ratio under normoxic conditions (Fig. 26.A). Phosphorylated-Akt (p-Akt) showed concentration-dependent manner of protein expression in NRCMs, but there is no significant differences between groups (Fig. 26.A). In separate experiment NRCMs went through 4 hours of simulated ischemia without reperfusion

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and the decorin treated cells showed elevated trends in total Akt/GAPDH ratio. On the contrary, a significant decrease of p-Akt was shown in case of 3, 10, 30 nM of decorin (Fig. 26.B). Phosphorylation of Akt shows no similarity either to total Akt level or normoxic p-Akt/Akt ratio.

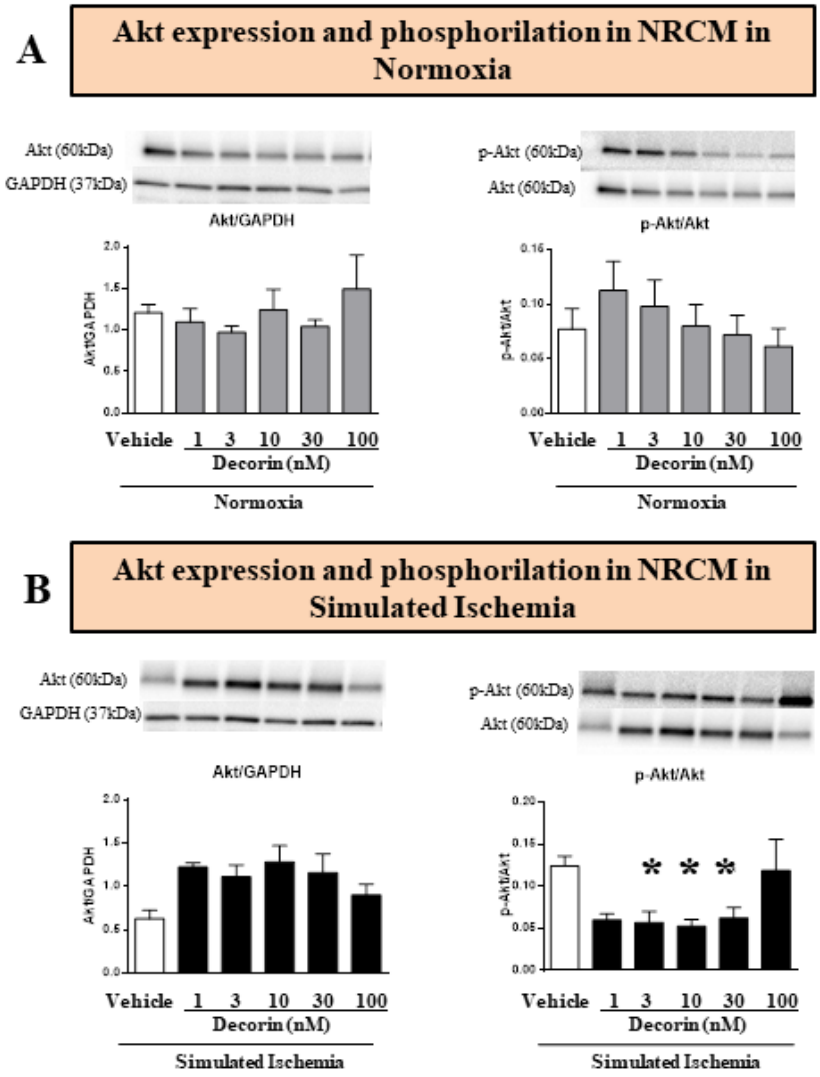

Figure 26. The effect of 1, 3, 10, 30 and 100 nM decorin on Akt, p-Akt protein level in neonatal rat cardiac myocytes (NRCMs) exposed to SI/R or Normoxia. A) Akt/GAPDH expression ratio and p-Akt/Akt ratio in Normoxic conditions. B) Akt/GAPDH expression ratio and p-Akt/Akt ratio in SI/R. Representative western blot images are included above quantified column diagram. Data are presented as mean ± S.E.M., One-Way ANOVA, Fisher post hoc test,*$p<0.05$ vs normoxia (n=2-4).

4.4.4. Differentially expressed mRNA from neonatal rat cardiac myocytes

Since no inhibitory testing, neither apoptotic assay, nor western blot analysis brought us closer to the mechanism of action of decorin's cardio-cytoprotective effect, next we approached the question in a new, unbiased, non hypothesis driven way. Therefore NRCMs were exposed to 4 hours of simulated ischemia with or without 20 hours 3 nM decorin treatment and mRNA expression changes were identified by sequencing from the cell lysate. By RNA sequencing out of the 29496 genes annotated in the reference annotation 19487 were detectable and, if no correction for multiple comparisons was applied, 419 showed different expression. After correction for multiple comparisons 2 genes, namely zinc finger, MYND-type containing 19 (Zmynd19) and eukaryotic translation initiation factor 4E nuclear import factor 1 (Eif4enif1) were observed to be significantly upregulated due to the decorin treatment (Fig. 27).

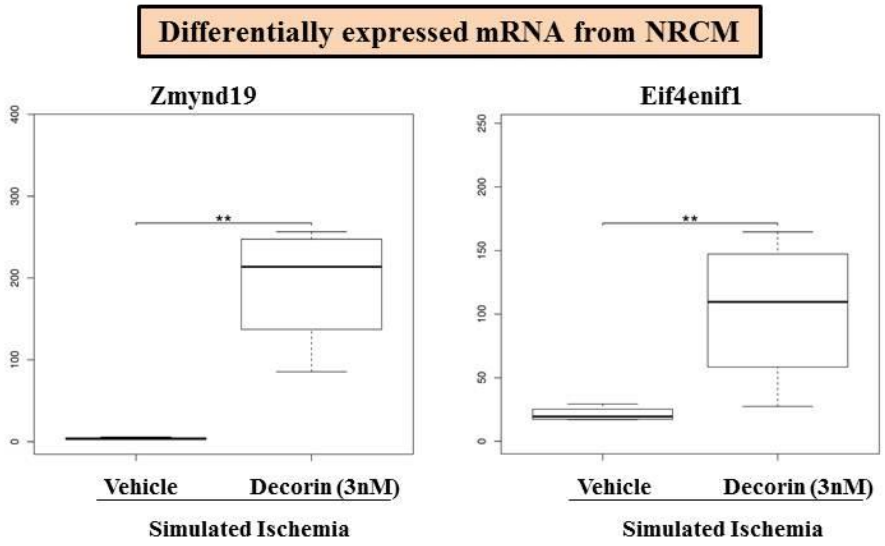

Figure 27. Significantly differentially expressed mRNAs in the decorin-treated NRCM samples compared to the vehicle treated group. p-values and corrected p-values (false-discovery rate according to Benjamini and Hochberg) were calculated by the DESeq2 software package. ** denotes corrected p-values <0.05.

4.4.5. Gene Ontology Analysis

To explore what biological processes are modified by decorin treatment, Gene Ontology (GO) enrichment analysis was performed. The result of the GO analysis clearly showed that differentially expressed mRNAs were significantly associated with

e.g., response to oxidative stress, response to antibiotic, mitotic cell cycle, cellular macromolecule metabolic process, organonitrogen compound metabolic process and nitrogen compound metabolic process (Table 3.).

Table 3. Gene ontology biological process enrichment analysis of the differentially expressed transcripts upon decorin treatment. Fold enrichment shows the ratio of the number of the uploaded genes compared to expected number according to reference list. Fold enrichment value over 1 denotes overrepresentation, meanwhile below 1 denotes underrepresentation of the particular process, respectively. Raw p-values were obtained by Fisher's exact test. Corrected p values were calculated according to Benjamini-Hochberg correction (false discovery rate).

Gene Ontology (GO) biological process name and code	Fold Enrichment	Raw p-value	Corrected p-value
response to oxidative stress (GO:0006979)	2.90	2.21E-05	1.78E-02
response to antibiotic (GO:0046677)	2.71	3.83E-05	2.95E-02
mitotic cell cycle (GO:0000278)	2.61	6.59E-05	4.83E-02
cellular macromolecule metabolic process (GO:0044260)	1.53	4.34E-06	5.00E-03
organonitrogen compound metabolic process (GO:1901564)	1.48	9.12E-06	9.20E-03
nitrogen compound metabolic process (GO:0006807)	1.48	1.25E-07	2.24E-04
cellular metabolic process (GO:0044237)	1.46	3.79E-08	8.74E-05
macromolecule metabolic process (GO:0043170)	1.44	5.80E-06	6.24E-03
organic substance metabolic process (GO:0071704)	1.42	2.76E-07	3.72E-04
primary metabolic process (GO:0044238)	1.40	2.14E-06	2.66E-03
metabolic process (GO:0008152)	1.39	2.46E-07	3.61E-04
biological_process (GO:0008150)	1.11	1.43E-05	1.28E-02
Unclassified (UNCLASSIFIED)	.54	1.43E-05	1.36E-02
sensory perception (GO:0007600)	.29	1.73E-05	1.47E-02
G protein-coupled receptor signaling pathway (GO:0007186)	.18	7.61E-09	3.07E-05
sensory perception of chemical stimulus (GO:0007606)	.10	2.42E-07	3.91E-04
sensory perception of smell (GO:0007608)	.05	8.43E-08	1.70E-04
detection of stimulus involved in sensory perception (GO:0050906)	.05	2.61E-08	7.03E-05
detection of stimulus (GO:0051606)	.04	5.29E-09	2.85E-05
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	< 0.01	8.69E-09	2.81E-05

detection of chemical stimulus involved in sensory perception (GO:0050907)	< 0.01	3.87E-09	3.12E-05
detection of chemical stimulus (GO:0009593)	< 0.01	2.68E-09	4.32E-05

4.5. Testing of cardioprotective effects of Matrix metalloproteinase inhibitor compounds against acute myocardial infarction in young normocholesterolemic rats

4.5.1. Tolerability of Matrix metalloproteinase inhibitors

To determined the safety of the MMP inhibitors preliminary tolerance testing was performed with each molecule. Mean arterial blood pressure (Table 4), heart rate (Table 5) and body temperature were not changed throughout the experiments as compared to the vehicle-treated animals. Vehicle-treated animals were given with the appropriate volume of DMSO in each treating timepoint (i.e. at 0; 20; 40; 60 and 80 min of the experiments).

Table 4. Mean arterial blood pressure (mmHg) of the animals during tolerability testing. There was no significant difference in mean arterial blood pressure between groups as compared to the vehicle-treated group as analyzed by repeated measures two-way ANOVA, n=8/group.

		0.1 µmol/kg		0.3 µmol/kg		1 µmol/kg
MABP	**baseline**	**0'**	**10'**	**20'**	**30'**	**40'**
Vehicle	132±10	129±15	120±12	113±13	113±11	120±12
MMPI-1154	151±8	146±7	133±7	134±8	138±6	133±8
MMPI-1260	138±8	137±8	121±13	120±18	123±14	114±15
MMPI-1248	105±8	98±8	103±9	99±8	90±9	82±7

		3 µmol/kg		10 µmol/kg		
MABP	**50'**	**60'**	**70'**	**80'**	**90'**	**100'**
Vehicle	112±13	117±11	107±11	110±10	113±9	113±7
MMPI-1154	140±7	137±7	124±12	128±5	134±6	137±7
MMPI-1260	122±11	119±12	116±11	123±10	126±10	129±10
MMPI-1248	88±13	91±10	89±14	79±11	110±11	99±9

Table 5. Heart rate (beats/minute) of the animals during the tolerability testing. There was no significant difference in heart rate between groups as compared to the vehicle-treated group as analyzed by repeated measures two-way ANOVA, n=8/group.

		0.1 µmol/kg		0.3 µmol/kg		1 µmol/kg
HR	**baseline**	**0'**	**10'**	**20'**	**30'**	**40'**
Vehicle	425±15	412±14	418±6	389±9	405±8	397±10
MMPI-1154	436±16	444±12	403±18	411±19	411±8	438±10
MMPI-1260	435±5	439±4	422±8	432±15	417±12	428±6
MMPI-1248	440±9	446±11	417±11	427±17	400±13	380±21

		3 µmol/kg		10 µmol/kg		
HR	**50'**	**60'**	**70'**	**80'**	**90'**	**100'**
Vehicle	379±9	384±6	387±12	378±8	376±16	385±17
MMPI-1154	393±15	413±14	389±15	405±17	390±9	406±15
MMPI-1260	413±8	416±12	411±13	420±12	413±10	416±10
MMPI-1248	386±14	403±18	377±32	373±19	384±11	385±19

At the end of the tolerability testing there was no sign of organ failure. Only the urine was discolored to dark, this phenomenon might have occurred due to known hemolytic effect of dimethyl sulfoxide (DMSO) (Montaguti, Melloni, and Cavalletti 1994), otherwise rats well tolerated the DMSO treatment (Gad et al. 2006). Overall no mortality occurred during the testing period in case of MMPI-1260, and only one animal died in the MMPI-1248 test group and two in the MMPI-1154 test group due to technical issues (Fig. 28.) and we declared the molecules safe. Even the rats tolerated the DMSO based treatment, we maximized the iv. volume at 60 µl for each animal, partly because DMSO is a strong organic solvent and on the other hand the molecules were dissolved completely in this amount.

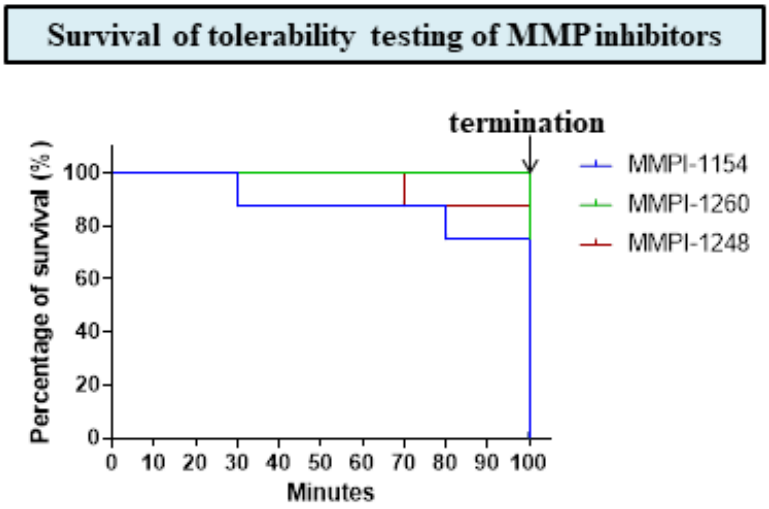

Figure 28. Kaplan-Meier curve of survival of male Wistar rats during matrix metalloproteinase inhibitor (MMPI) tolerability testing (n=8).

4.5.2. All-cause mortality

The rate of mortality was low in the *in vivo* experimental set up and the all-cause mortality did not differ among groups. In most cases, mortality occurred before the administration of MMP inhibitors (Fig. 29.) due to various reason, eg. arterial bleeding due to slipping canule or individual sensitivity to anesthesia or too great induction of myocardial infarction.

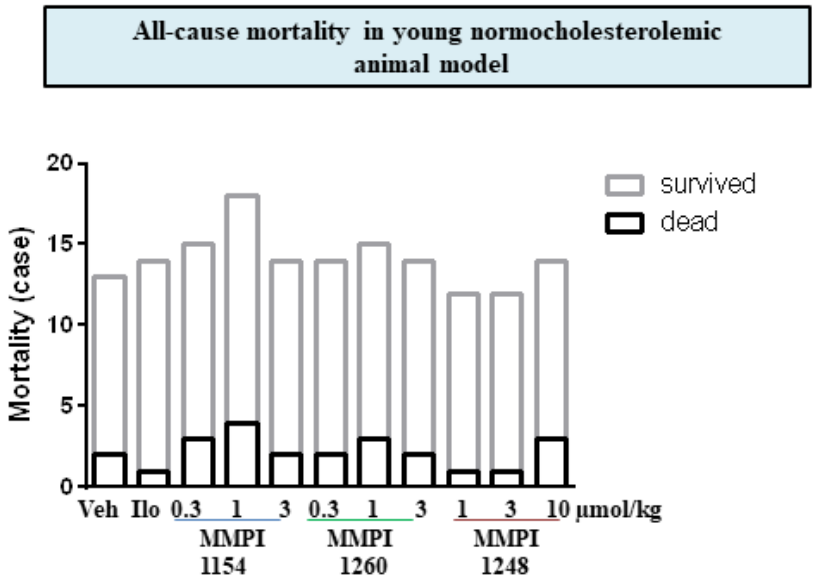

Figure 29. All-cause mortality in young normocholesterolemic animal model of acute myocardial infarction.

4.5.3. Infarct size

The three tested compounds (MMPI-1154, -1260, and -1248) previously showed cardio-cytoprotective effect, published by our research group (Bencsik et al. 2018). Area at risk data did not show any difference among experimental groups after the 30 min coronary occlusion and 120 min reperfusion. That means the surgical induction of AMI was successful and similar between animals, therefore infarct size data are comparable. Two of the MMP inhibitor compounds showed significant reduction in infarct size: MMPI-1154 at 1 µmol/kg and MMPI-1260 at 3 µmol/kg decreased infarct size significantly as compared to the vehicle group (Figure 30.A,B), from 63.68±1.91 % to 53.53±3.36 % and 56.64±2.46 %, respectively. The third inhibitor, MMPI-1248, showed no reduction of infarct size in any of the applied doses when compared to the ischemic control group (Figure 30.C). Therefore, only the two effective MMP inhibitor compounds were further studied in hypercholesterolemic conditions. Ilomastat failed to reduce infarct size (60.46±3.60 %) in the present study in spite of previous studies, which have demonstrated the infarct size-limiting ability of ilomastat in rodent model of AMI (Bell et al. 2013; Bencsik et al. 2014). This shows the problems of reproducibility of cardioprotective studies.

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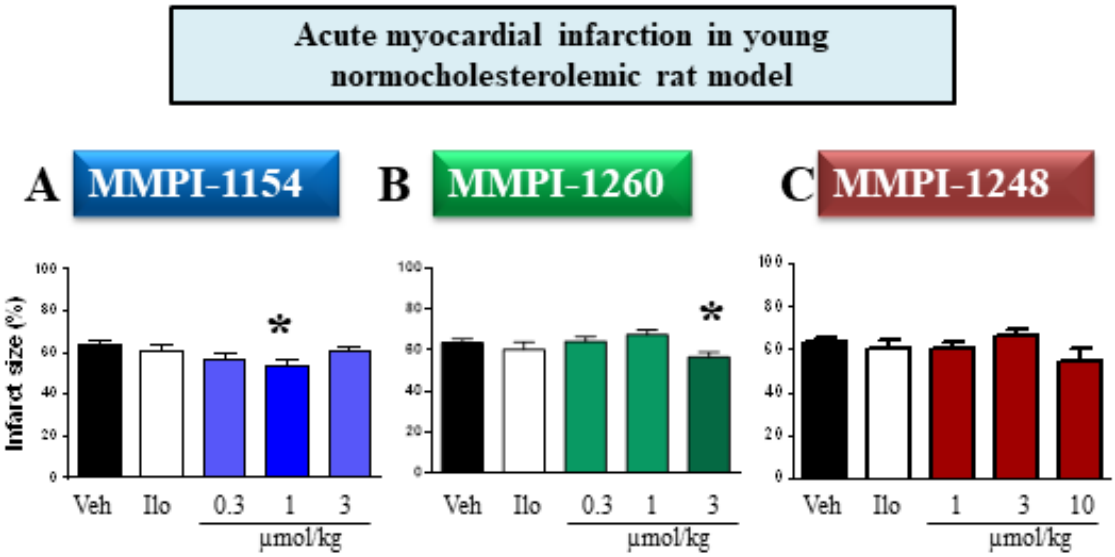

Figure 30. The effects of MMP inhibitors on infarct size in young normocholesterolemic rats subjected to *in vivo* 30 min coronary occlusion followed by 120 min reperfusion. Veh: Dimethyl sulfoxide, Ilo: ilomastat. One-way ANOVA followed by Fisher LSD post hoc test, n=12-14, data are expressed as means ± SEM, *p < 0.05.

4.5.4. Incidence of Severe Arrhythmias

LAD occlusion induced ischemia could trigger arrhythmias in the rat hearts. Therefore ventricular tachycardia (VT) and fibrillation (VF) were recorded during the whole surgical intervention. Test compounds, ilomastat or vehicle were given as slow bolus at 25th min of ischemia. Representative images show different type of tachyarrhythmias from ECG recordings (Fig. 31.). There were no significant differences in the incidence of severe arrhythmias among experimental groups (Fig. 32.).

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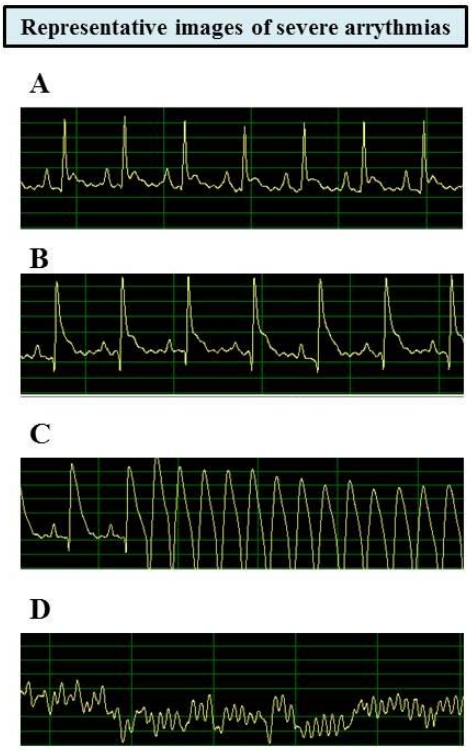

Figure 31. Representative recordings of rat ECG. A) normal baseline B) ST-elevation and negative Q wave of ischemic period C) ventricular tachyarrythmia (VT) D) ventricular fibrillation (VF). ECG: electrocardiogram

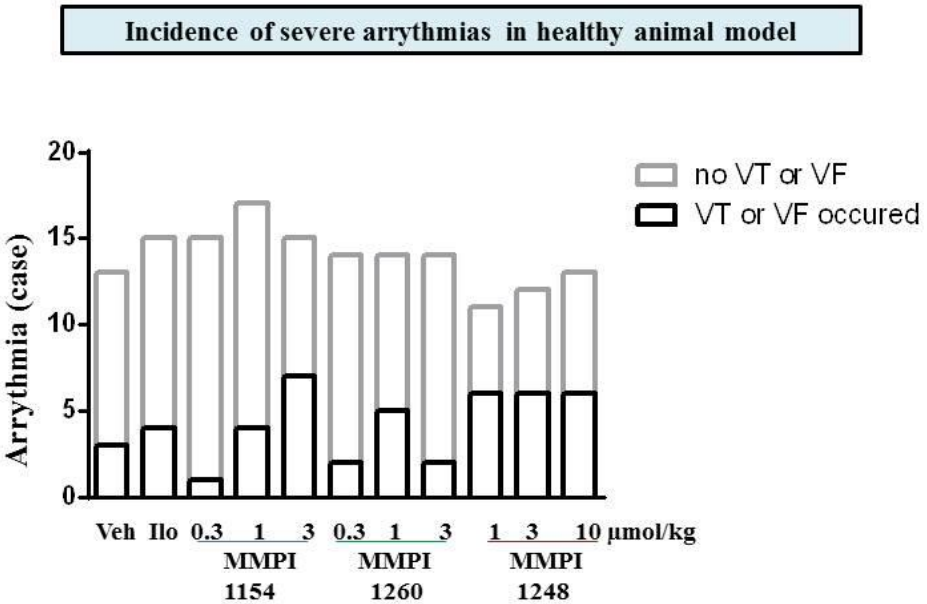

Figure 32. Incidence of severe arrhythmias during 30 min ischemia, graph shows the number of animals which experienced arrhythmias.. LAD occlusion induced ischemia

could trigger arrhythmias in the rat hearts. Test compounds as well as ilomastat or vehicle were given at 25th min of ischemia. Khi-square test, n=11-13, *p<0.05.

4.6. Testing of cardioprotective effects of Matrix metalloproteinase inhibitor compounds against acute myocardial infarction in hypercholesterolemic and age-matched normocholesterolemic rats

4.6.1. Comorbid model validation

In a separate experiment MMP inhibitors were tested in a comorbid model, AMI was combined with hypercholesterolemia. Male Wistar rats developed hypercholesterolemia due to 12 weeks cholesterol-enriched diet (Fig. 12.B). However, the body weight of the animals did not show any difference in the two major groups (Fig. 33).

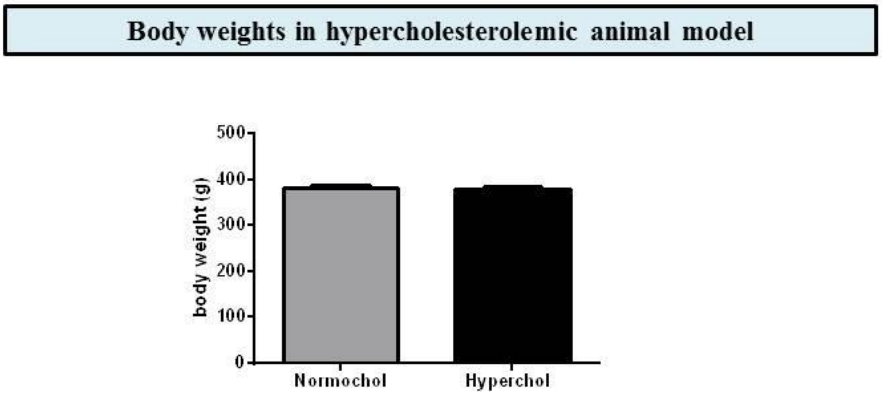

Figure 33. Body weight of normocholesterolemic and hypercholesterolemic test groups after 12 weeks of diet. Student's t-test, n=60, no significant difference between groups

At the end of 12 weeks diet, development of hypercholesterolemia was validated from baseline blood samples (after 12 hours fasting) by measuring the total cholesterol (Nchol: 2.10±0.04 mmol/L and Hchol: 8.09±0.38 mmol/L, respectively) and LDL levels (Nchol: 0.49±0.03 mmol/L, and Hchol: 6.69±0.34 mmol/L, respectively), which were significantly higher in hypercholesterolemic group compared to age-matched normocholesterolemic group, meanwhile triglyceride levels (Nchol: 0.71±0.04 mmol/L and Hchol: 0.55±0.01 mmol/L, respectively) were significantly lower in the hypercholesterolemic group. HDL level showed no significant difference between groups (Fig.34.).

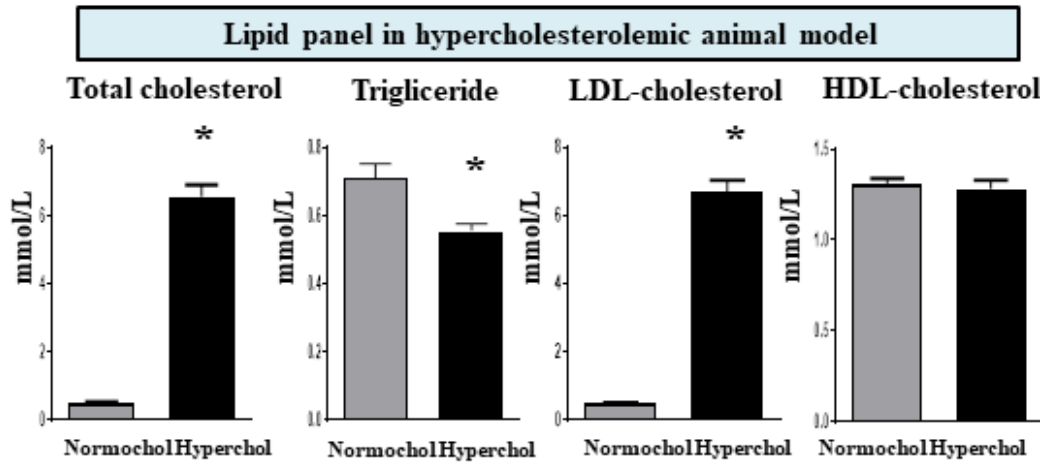

Figure 34. Serum lipid panel of normocholesterolemic and hypercholesterolemic groups. After 12 weeks of diet, animals had a baseline blood sampling to validate the development of metabolic disease. LDL: low-density lipoprotein, HDL: high-density lipoprotein, IDL: intermediate-density lipoprotein. Student's t-test, n=60, *p<0.05.

Since high-fat diet could induces changes in carbohydrate metabolism, therefore blood glucose level was also measured after 12 hours fasting period. Hypercholesterolemic animals had significantly higher blood glucose levels (4.90±0.23 mmol/L) as compared to normocholesterolemic individuals (3.67±0.27 mmol/L) (Fig. 35.).

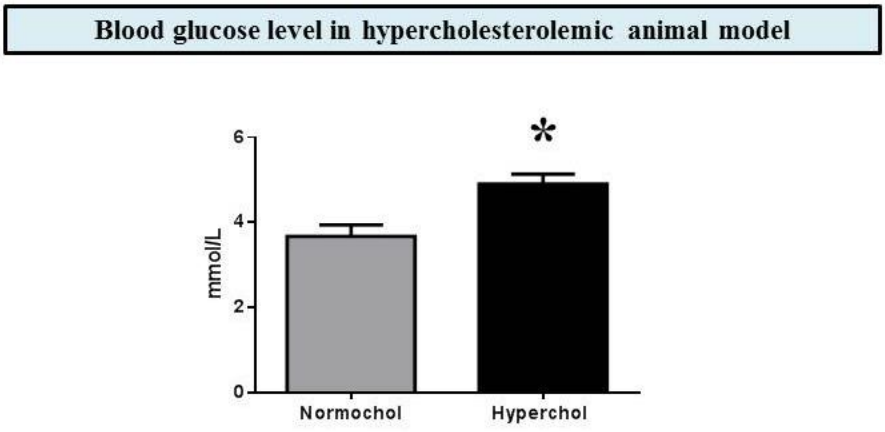

Figure 35. Blood glucose level of age-matched normocholesterolemic and hypercholesterolemic groups. After 12 weeks of diet, animals had a baseline blood sampling to validate the development of metabolic disease. Student's t-test, n=60, *p<0.05.

4.6.2. All-cause mortality

In the second *in vivo* experimental set up, hypercholesterolemia did not affect all-cause mortality, which was similar among experimental groups and mortality rate was low (Fig. 36.). In most cases, mortality occurred before the administration of MMP inhibitors during surgical preparation of the animals or in the early ischemic period.

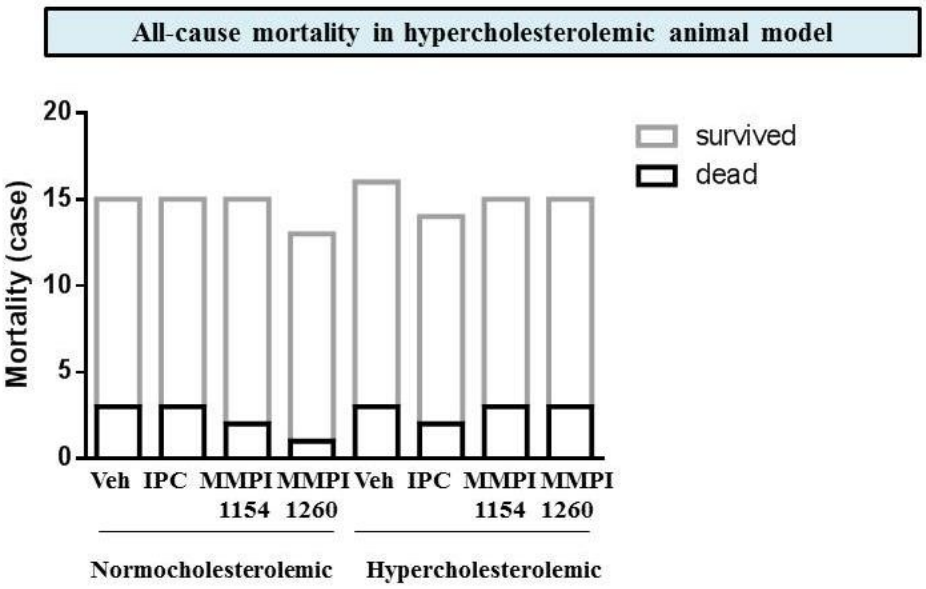

Figure 36. All-cause mortality of age-matched normocholesterolemic and hypercholesterolemic comorbid model. Khi-square test, n=14-16, *p<0.05, no significant difference between groups.

4.6.3. Infarct size limiting effect

In the age-matched normocholesterolemic group, ischemic preconditioning significantly reduced infarct size (26.23±6.16 %) as compared to the vehicle-treated ischemic group (55.58±3.41 %) and both MMP inhibitor molecules provided cardioprotection (MMPI-1154: 40.61±3.38 % and MMPI-1260: 36.75±4.63 %). Based on the results of the first *in vivo* experimental set up (Figure 30.A,B), the infarct size limiting effect of the compound was reproducible, as expected (Figure 37.A). In the presence of hypercholesterolemia (Figure 37.B), ischemic preconditioning (36.77±6.6 %) and both MMP inhibitors, MMPI-1154 at 1 µmol/kg (44.82±6.1 %) and MMPI-1260 at 3 µmol/kg (44.03±2.4 %) failed to reduce infarct size when compared to the vehicle-treated control group (45.59±4.8 %).

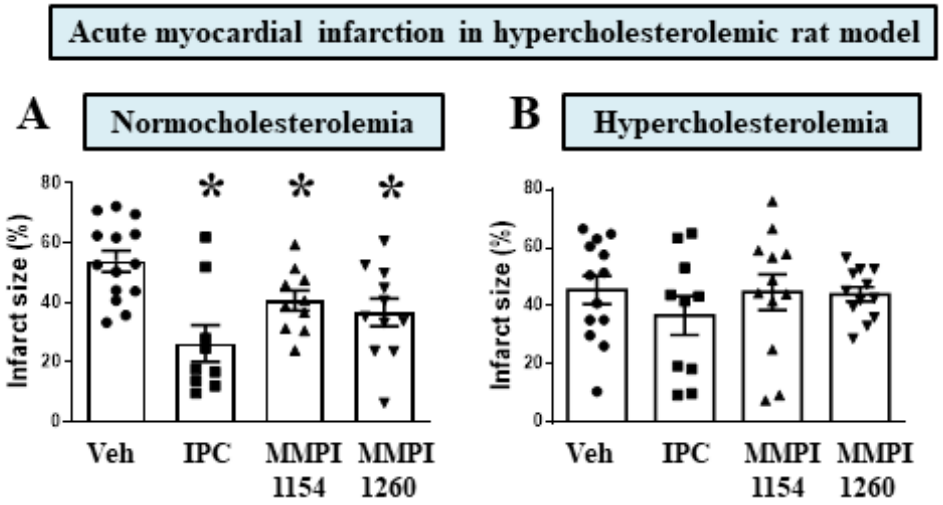

Figure 37. The effects of ischemic preconditioning and MMP inhibitors on infarct size in A) age-matched normocholesterolemic and B) hypercholesterolemic rats subjected to *in vivo* 30 min coronary occlusion followed by 120 min reperfusion. Veh: Dimethyl sulfoxide, IPC: ischemic preconditioning. One-way ANOVA followed by Dunnett's multiple comparisons test, n=9-14, data are expressed as means ± SEM, *p<0.05.

Although it is important to see that hypercholesterolemia on its own were showing smaller infarct size (45.59±4.8 %) when compared to the normocholesterolemic group (55.58±3.41 %) (Fig. 38).

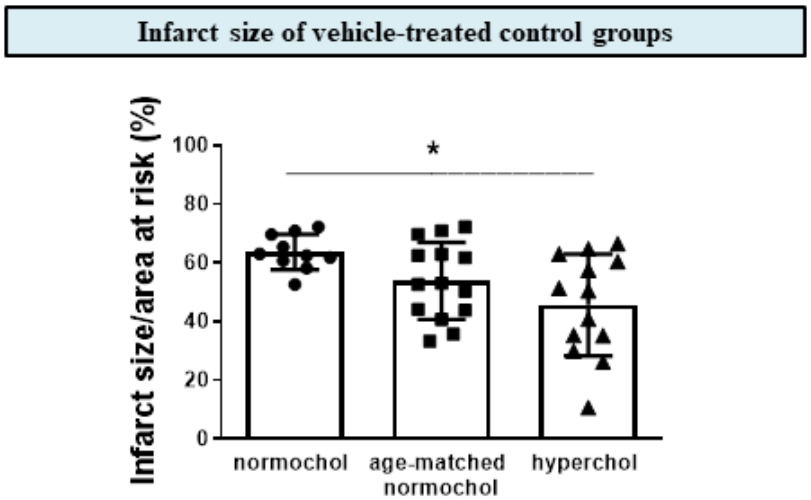

Figure 38. Infarct size/area at risk of vehicle-treated groups. One-way ANOVA, Dunnett's multiple comparison *post hoc* test, n=10-15, *p<0.05.

4.6.4. Microvascular obstruction

Ischemia/reperfusion cause damage not only in cardiomyocytes but also in the coronary circulation, including endothelial cells. Microembolization of debris, impairment of endothelial integrity with subsequently increased permeability and oedema formation, platelet activation and leukocyte adherence and ultimately structural damage to the capillaries with eventual no-reflow. This phenomenon is called microvascular obstruction (MVO), which could be visualized with thioflavine-S staining under UV-linght; see representative photos in Figure 39.A).

The percentage of MVO was significantly reduced in the positive control, ischemic preconditioning both in normocholesterolemic and hypercholesterolemic groups (Nchol: 4.45±0.75 %, Hchol: 5.35±1.68 %) compared to the vehicle-treated ischemic group (Nchol: 9.67±1.47 %, Hchol: 12.57±2.70 %). The tested MMP inhibitor molecules showed similar amount of MVO as vehicle-treated ischemic group (Nchol MMPI-1154: 7.49±1.56 % and MMPI-1260: 9.19±2.23 %; Hchol MMPI-1154: 11.09±2.34 % and MMPI-1260: 13.45±2.07 %), see Figure 39.B and C.

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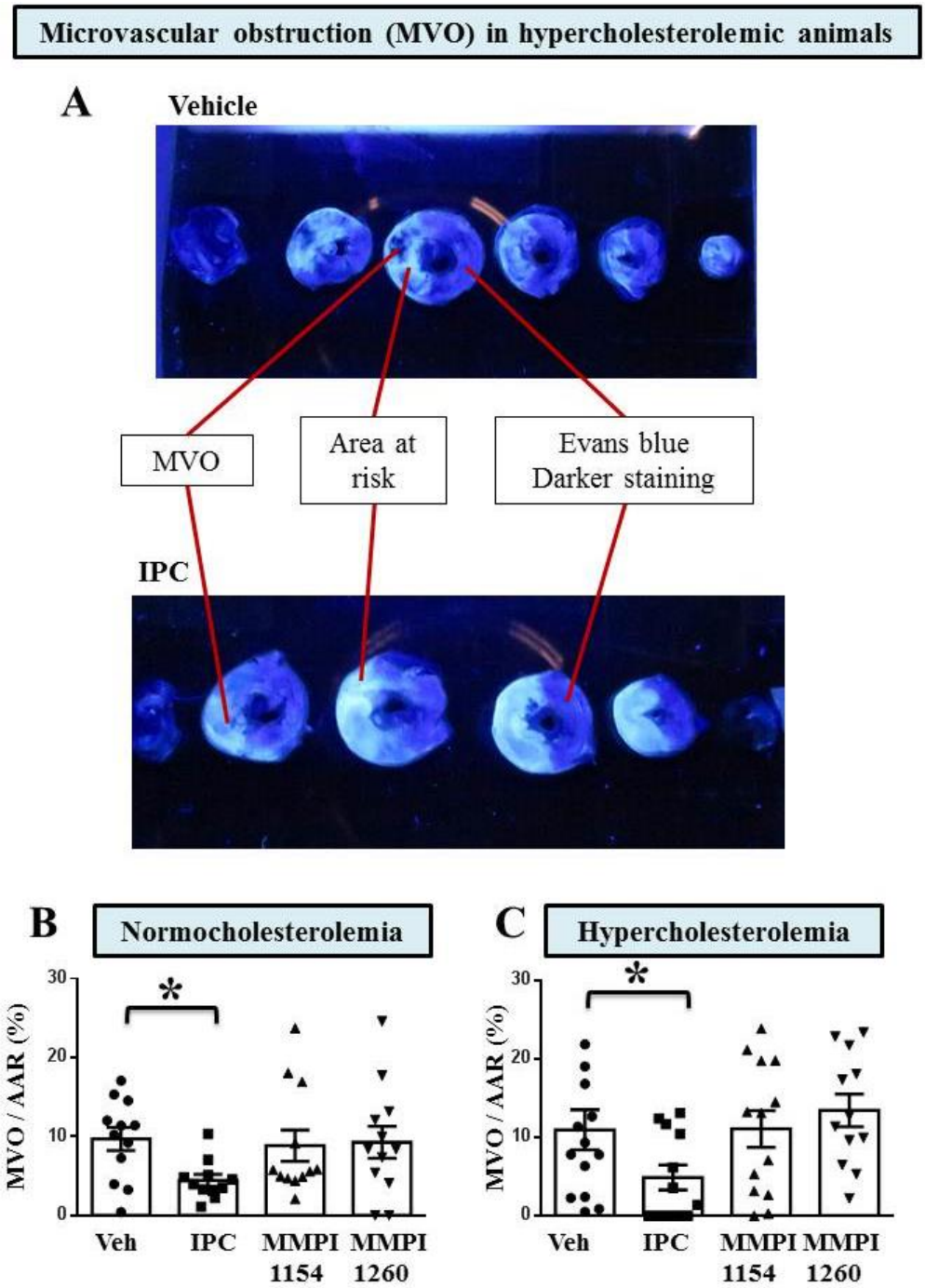

Figure 39. Microvascular obstruction (MVO) in rat heart after myocardial ischemia/reperfusion injury. A) Representative image of MVO in Vehicle-treated group and IPC group. The effects of IPC and MMP inhibitors on MVO in B) normocholesterolemic and C) hypercholesterolemic rats subjected to *in vivo* 30 min coronary occlusion followed by 120 min Veh: Dimethyl sulfoxide, IPC: ischemic preconditioning. One-way ANOVA followed by Fisher LSD post hoc test, n=14-16, data are expressed as means ± SEM, *p< 0.05.

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4.6.5. Incidence of Severe Arrhythmias

Ventricular arrhythmias, namely ventricular tachycardias (VT) and fibrillations (VF) were monitored during the ischemic period in comorbid model as well. Similarly to normocholesterolemic model, there were no significant differences among the groups (Fig. 40.). In ischemic preconditioning no serious arrhythmias were occurring.

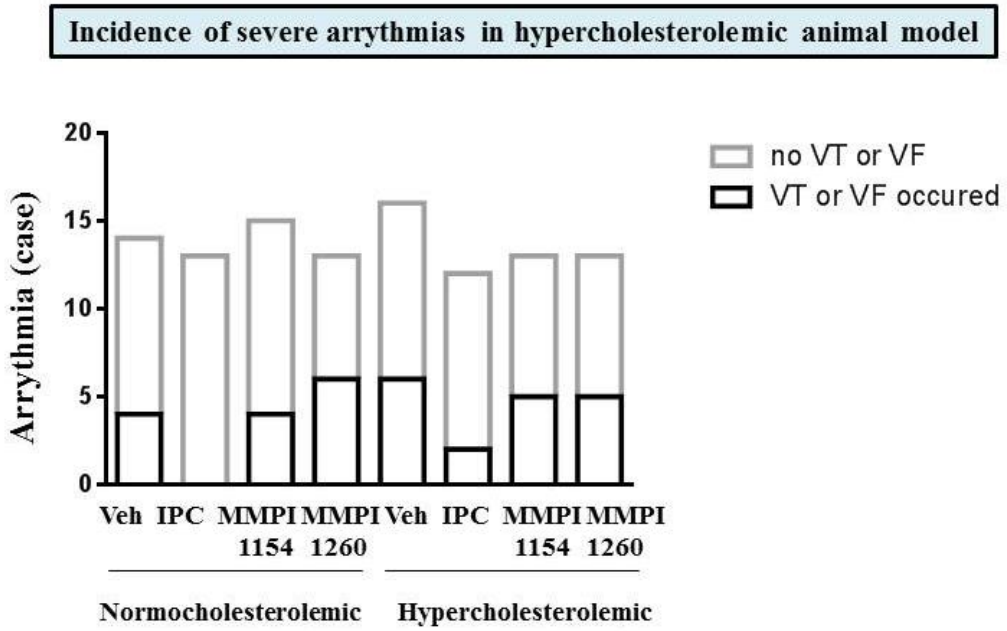

Figure 40. Incidence of severe arrhythmias in hypercholesterolemic animals with positive control (IPC: ischemic preconditioning) and vehicle. Khi-square test, n=14-16, *p<0.05, no significant difference between groups.

4.6.6. Haemodynamics

Haemodynamic data was recorded 6 times throughout all the experiments, at the beginning as baseline (BL), at the time of compound administration during ischemia (I25), at the end of ischemia (I29), 10th minute of reperfusion (R10), 60th of minute of reperfusion (R60) and at the end of reperfusion (R120). In all of those instances, we could see no significant differences in mean arterial blood pressure or heart rate between tested compounds and control groups either in normocholesterolemic (Fig. 41.) or hypercholesterolemic (Fig. 42.). Due to coronary occlusion the mean arterial blood pressure dropped in the beginning of the ischemia. The altered function adjusted to the injury at the end of the ischemic period with gradual depletion in the reperfusion phase.

The heart rate was quite constans, there was no significant difference during the time-course of measurement among experimental groups.

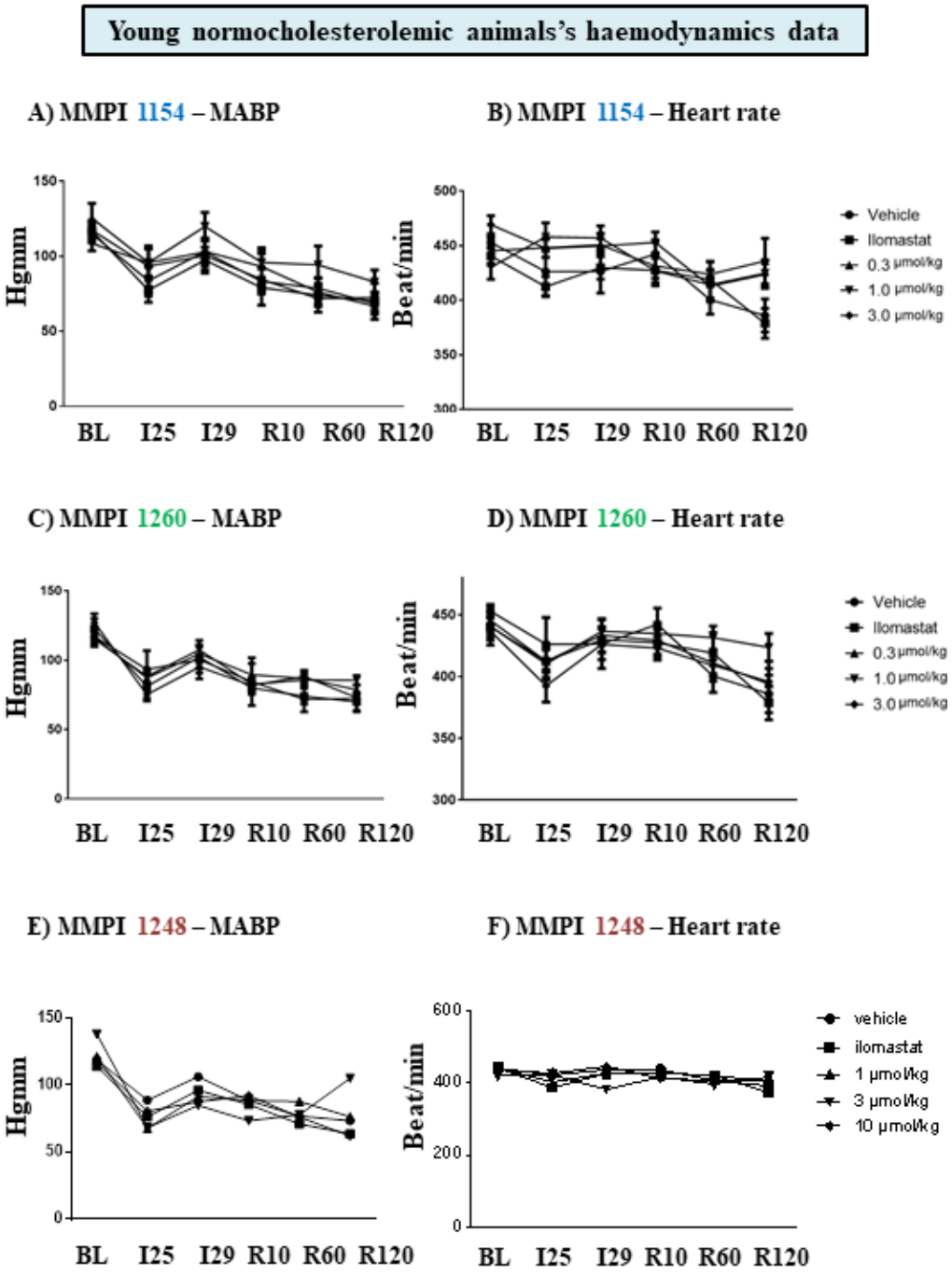

Figure 41. Mean arterial blood pressure (MABP) and Heart Rate for MMPI-1154, -1260 and -1248 test groups with Ilomastat and vehicle. BL: baseline, I: ischemia, R: reperfusion. Two-way ANOVA, n=11-15, *p<0.05, no significant difference.

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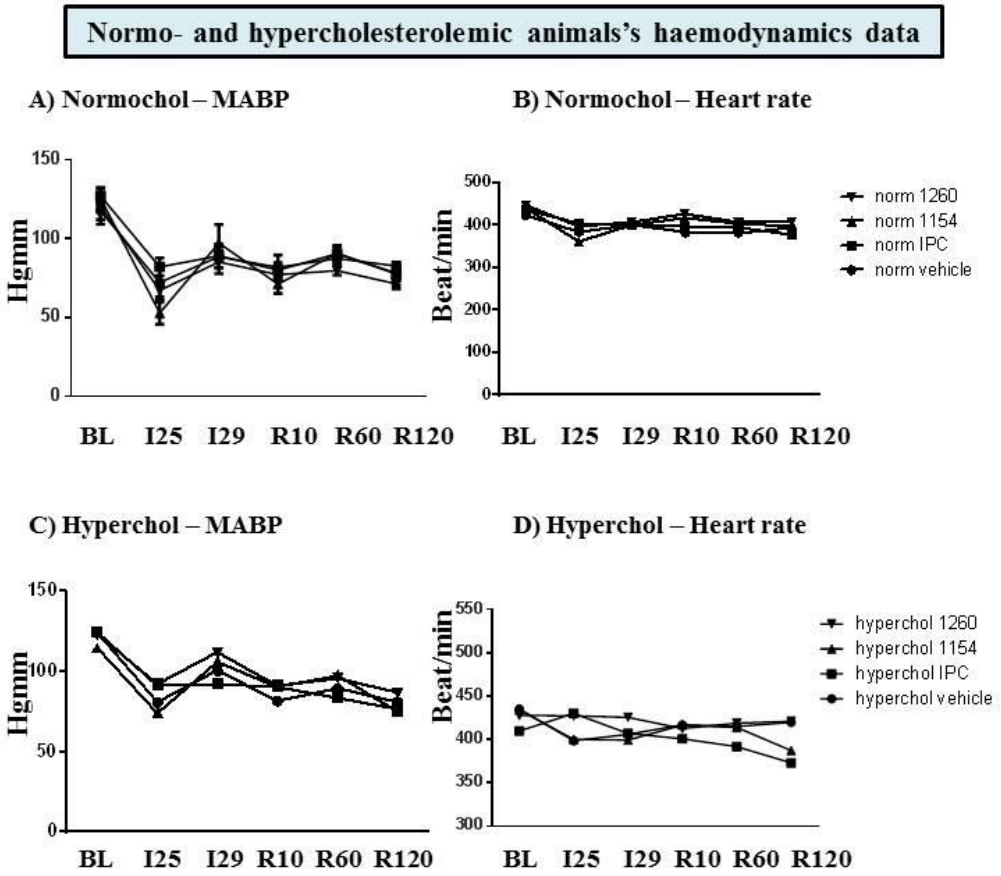

Figure 42. Mean arterial blood pressure (MABP) and Heart Rate for MMPI-1154 and MMPI-1260 test groups with ischemic preconditioning (IPC) and vehicle. BL: baseline, I: ischemia, R: reperfusion. Two-way ANOVA, n=11-15, *p<0.05, no significant difference.

4.6.7. Matrix metalloproteinase activities

MMP activity was measured by gelatin zymography from plasma samples. Due to the proteolytic activation of MMPs there are several active forms that could be measured. The different forms were separated by gel electrophoresis (Fig.13.).

In order to investigate baseline MMP-2 and MMP-9 activity, these enzymes were tested in plasma samples of age-matched normocholesterolemic and hypercholesterolemic animals via gelatin zymography. The baseline 72 kDa MMP-2 and 86 kDa MMP-9 activity were not different in hypercholesterolemic animals in comparison to age-matched normocholesterolemic animals (Fig. 43.).

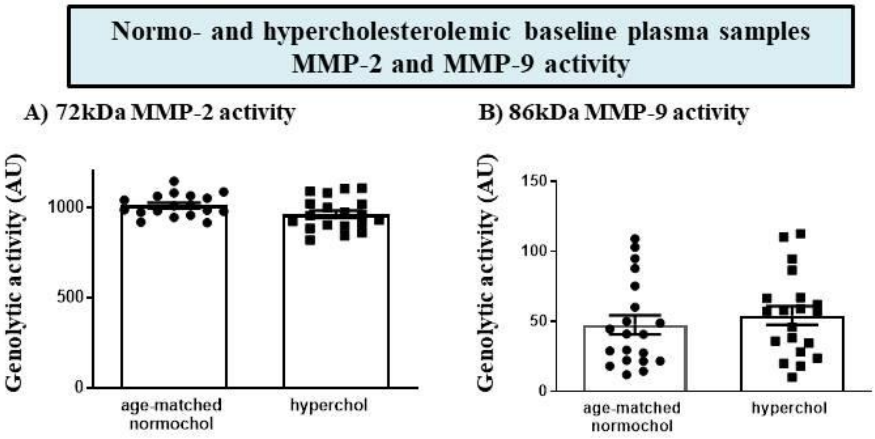

Figure 43. Gelatinolytic activity of (A) MMP-2 and (B) MMP-9. AU: arbitrary units.

After the ischemic insult 72 kDa MMP-2 was not changed in any group (Fig. 44.). MMP-9 activity differed between IPC and MMPI-1154 in normocholesterolemic animals at the late reperfusion (Fig. 45.A,B), and in the hypercholesterolemic animals the significant difference showed between IPC and MMPI-1260 (Fig. 45.C,D).

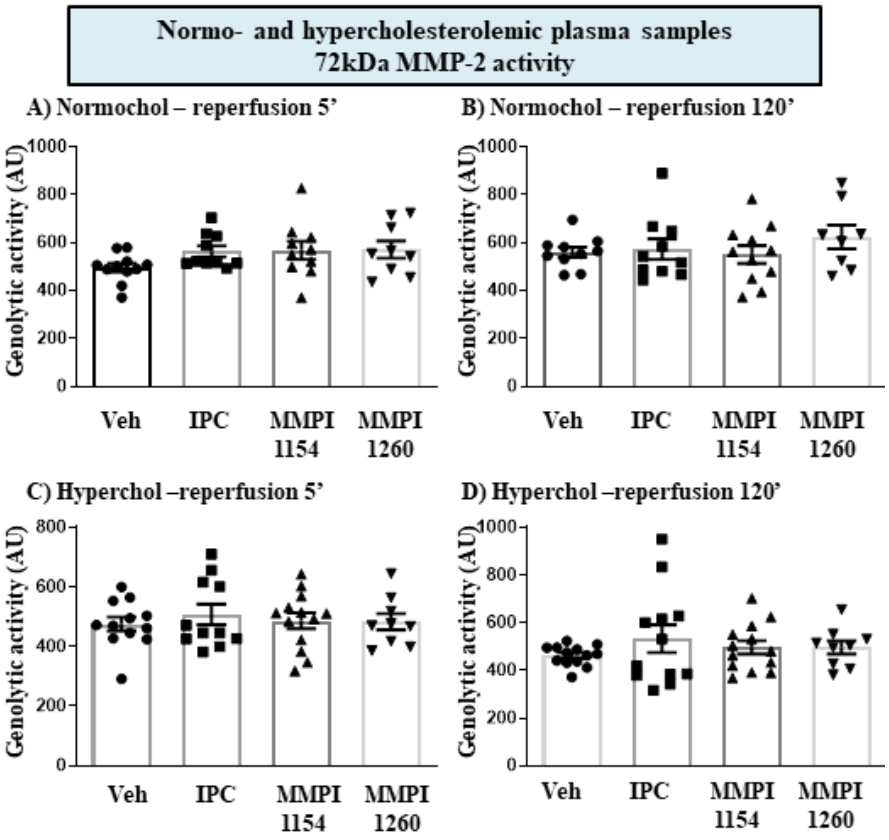

Figure 44. Gelatinolytic activity of 72 kDa MMP-2 from plasma samples. AU: arbitrary units.

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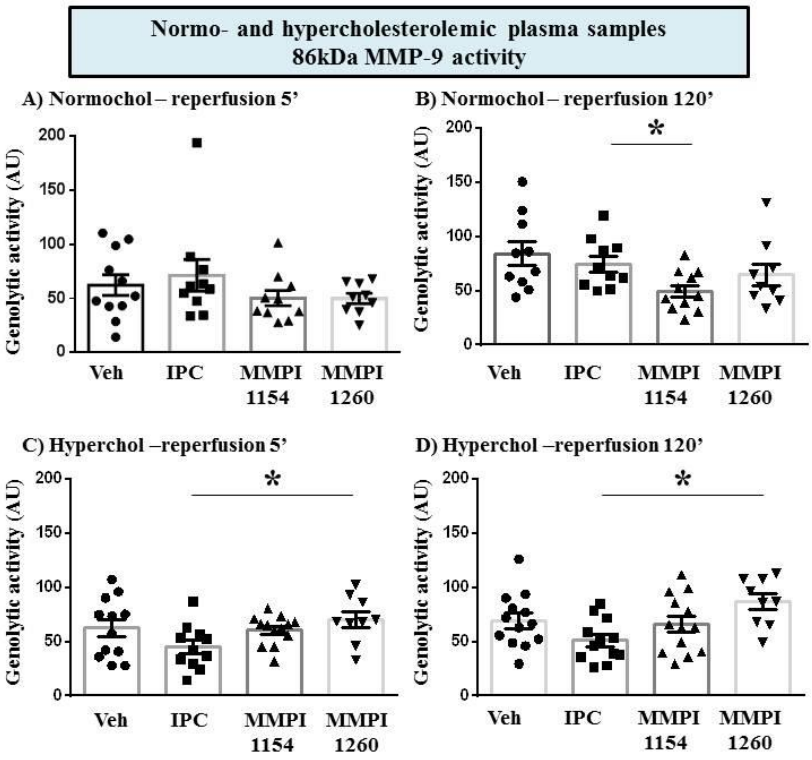

Figure 45. Gelatinolytic activity of 86 kDa MMP-9 from plasma samples. AU: arbitrary units.

5. Discussion

5.1. Main findings

In this thesis the cardioprotective effects of potentially cardioprotective compounds were evaluated. We used relevant, well-established and reproducible *in vitro* (Gorbe et al. 2010; Bencsik et al. 2018; Gaspar et al. 2016) and *in vivo* (Bencsik et al. 2014) animal experimental models for discovering protective compounds against acute myocardial infarction. In cardiac myocyte cell culture, the SI/R injury caused significant cell death, making the system suitable for testing new potentially cardioprotective compound.

GPC was cardioprotective at 100µM after 3 hours pretreatment based on its ability to reduce oxidative stress-related injury (Tuboly et al. 2019). Decorin showed protective effect at 3nM after 20 hours pretreatment. The exact mechanism of action has not been mapped, however, we found that, unlike its sister molecule, biglycan (Gaspar et al. 2016), probably does not act through the NO-cGMP-PKG pathway or the TLR-4 receptor mediated response (Gaspar et al. 2020). MMP inhibitor molecules were used in normocholesterolemic and hypercholesterolemic animals in clinically relevant timing and doses. Cardioprotective effect was shown of novel MMP inhibitors administered before reperfusion at a dose of 1µmol/kg MMPI-1154 and 3µmol/kg MMPI-1260 in an *in vivo* rat model against AMI. However, in the presence of hypercholesterolemia, their infarct size-limiting effect was not seen (Gomori et al. 2020).

Effective cardioprotective drug for treatment of ischemic heart disease is still an unmet clinical need since several promising drug candidates failed to protect the ischemic myocardium in large clinical studies. Among several cardioprotective drug targets, the formation of ROS and the participation of MMP-2 in the development of reperfusion injury following an extended myocardial ischemia have been clearly demonstrated in the early 2000s. After global ischemia in ex vivo isolated, perfused rat hearts, acute release of MMP-2 during reperfusion contributed to cardiac mechanical dysfunction and it was improved by MMP inhibitors such as doxycycline (10 to 100 mmol/L) and o-phenanthroline (3 to 100 mmol/L) (Cheung et al. 2000). In 2015 a clinical trial (Cerisano et al. 2015) was conducted, in which doxycycline marginally reduced infarct size ($p=0.052$) and significantly attenuated myocardial remodeling.

During myocardial ischemia/reperfusion injury Schulz and colleagues have shown that MMP-2 is activated and thereby degrades myocardial contractile proteins such as myosin light chain (Gao et al. 2014), titin (Ali et al. 2010) and troponins (Wang et al. 2002), SERCA2a (Roczkowsky et al. 2020) or junctophilin-2 (Chan et al. 2019) (see for review (Schulz 2007; Hughes and Schulz 2014)). Simultaneously with these discoveries, the therapeutic potential has arisen to inhibit MMP-2 activity to prevent the degradation of the abovementioned intracellular myocardial proteins.

5.2. Ischemia/reperfusion models for testing cardioprotective agents

To investigate the cardioprotective effect of different compounds we used *in vitro* and *in vivo* models. The levels of complexity in animal models are the followings: in silico computer based simulations, *in vitro* biochemical assays and cell culture experiments, ex vivo isolated organ based experiments and finally *in vivo* animal models.

In vitro biochemical assays could be used to determine of the interaction of two compounds, in our case we used zymography to measure IC_{50} value of MMP inhibitors and MMP recombinant enzyme (Bencsik et al. 2018). The next level is *in vitro* cell culture, where it is not only on the level of molecules interacting, but we could investigate phenomenons at a cellular level, which is the smallest unit of living creatures. In case of cardioprotective effect, we were focusing on primary cardiac myocytes cell cultures, which were either coming from neonatal rats or a more difficult to culture, adult ones. Primary cell cultures are better models, than immortalised cell line, because they have the true cardiac features, especially the adult cardiac myocytes. On the other hand cell lines are commercially available, like H9c2 (Kung et al. 2021), and working with them does not require the sacrafice 1-3 days old pups of rats or adult animals.

For *in vitro* cell culture models, our simulated ischemia/reperfusion injury contained a combination of hypoxic solution and 4 hours of hypoxia. Using hypoxic solution combined with hypoxic environment is a better, more efficient way than using only hypoxic chamber, Liu et al used 36 hours to induce apoptosis in primary cultured human cardiac myocytes from left ventricule tissue (Liu et al. 2020). Instead of hypoxic solution some groups are using H_2O_2 to induce hypoxia/reoxygenation–induced injury. Shan et al applied short-term pretreatment (10 minutes) with H_2O_2 at concentrations

ranging from 100 to 200 μmol/L of NRCMs and found a protection of cardiomyocytes against hypoxia/reoxygenation–induced injury, whereas prolonged treatment of H_2O_2 (24 hours) triggered cell death even at a concentration as low as 50 μmol/L (Shan et al. 2020). Yang et al used NRCMs which were subjected to simulated I/R injury in a 2 hours of ischemia and 6 hours of reperfusion set up (Yang et al. 2019).

After cellular level, the next in line is organ level. Therefore ex vivo isolated heart experiments could be designed for, using a Langendorff retrograde perfusion system or a Neely working heart perfusion system (Giricz et al. 2006). From the cardioprotective compounds that was evaluated in this thesis, only MMPI-1154 was tested on ex vivo level (Bencsik et al. 2018).

The most complex level of animals experiments is the in vivo models, where above molecular, cellular and organ level, we could investigate a novel drug in a systemic way. Our *in vivo* AMI protocols were developed for many years. The 30 min coronary occlusion followed by 120 min reperfusion considered a standard and reliable model, where ischemic group showed similar infarct size, 66.1±4.6% (Bencsik et al. 2014); 59.8±4.5% (Kiss et al. 2016) to what we had in normocholesterolemic animals (63.7±1.9%). Similar surgical procedure were done by Finnish researchers, but in their case the time of reperfusion varied from 24 hours to 12 weeks (Palojoki et al. 2001). They were looking for signs of myocardial injury (eosinophilia, karyolysis, and leukocyte infiltration) or collagen scars and analyzed them by examination of Van Gieson-stained transverse LV sections. This method also based on planimetry, although their infarct size results varied between small (4–30%), moderate (31–49%), or large (>50%). Shan et al used 45 minutes of ischemia followed by 24 hours of reperfusion and they determined the myocardial injury by infarct size measurement (27.3±2.0% %) and LDH release (Shan et al. 2020).

There is an effort called the EU-CARDIOPROTECTION COST Action (CA16225) by leading experts in experimental and clinical cardiologist and researchers from pan-European research network to standardize experimental cardiology and infarct size measurement methods and to improve the translation of novel experimental cardioprotective therapies into the clinical setting for the benefit of patient, http://www.cardioprotection.eu/ .

5.3. Drug testing platform – finding or developing novel candidates

Despite several decades of investigation to develop cardioprotective agents against AMI, there is still an utmost need for new cardioprotective drug candidates. Therefore, in this thesis the aim was to test novel compounds in preclinical models and show the way of drug development through experimental, potentially cardioprotective agents. MMP inhibitor development became a novel potential strategy of cardiovascular drug development as well as GPC treatment with its antioxidant capacity.

GPC is known for its neuroprotective effect and it is also hepatoprotective against ischemia/reperfusion injury (Strifler et al. 2016), therefore investigating its cardiac effects seemed reasonable.

Protective effect of decorin on acute kidney ischaemia-reperfusion injury in rats were published by Alan et al (Alan et al. 2011). Sprague Dawley rats suffered from acute kidney ischaemia-reperfusion injury then decorin was administered intraperitoneally at the dose of 0.1 mg/kg for 9 days after reperfusion. Other, similar structure proteoglycan, biglycan were shown to be cardiocytoprotective (Gaspar et al. 2016) against SI/R injury. Based on these previous studies we found decorin worthy of testing in primary neonatal and adult cardiac myocytes against SI/R injury.

The first types of MMP inhibitors were the hydroxamic acid-based molecules, which were binding to the catalytic zinc ion of MMP's active centrum resulting in poor selectivity among MMP isoforms (Jacobsen et al. 2010). Non-selective MMP inhibitors were tested as potential cardioprotective compounds in several preclinical studies, but the problem with these prototype matrix metalloproteinase inhibitors, e.g. marimastat, were its musculoskeletal syndrome triggering effect (Hutchinson et al. 1998). In another case coronary flow and heart rate were improved by *o*-phenanthroline (100 μM) in isolated rat hearts subjected to I/R injury (Baghirova et al. 2016).

Nevertheless, a more specific and moderate MMP-2 inhibitor with limited side effects are needed (Hughes and Schulz 2014; Cathcart and Cao 2015). According to Jacobsen et al., the way of achieving highly specific MMP inhibitors could lay in the targeting the different substrate pockets of individual MMPs (Jacobsen et al. 2010). During the development of novel MMP inhibitor candidates we have found that the MMP-inhibiting effects of imidazole and thiazole carboxylic acid-based compounds were better than the conventional hydroxamic acid type derivatives (Bencsik et al.

2018). Their cardio-cytoprotective effect was investigated on isolated neonatal cardiac myocytes subjected to simulated I/R injury, and in case of MMPI-1154, on ex vivo isolated rat hearts as well (Bencsik et al. 2018)

5.4. Drug testing platform – choosing the right dosage

Finding potential cardioprotective compounds is only the first step. The second step is to find the correct dosage. For the different candidates we used different strategies.

1) For GPC we performed an extended literature search, as follows. Some studies examining GPC dosages up to 1000 mg (approx. 4 mM) *per se* and others describe a dosage within a nutritional supplement range that was at a dose of 150 mg (approx. 600 µM) (Hoffman et al. 2010). There is a huge variation in the literature regarding acute versus long-term administration. In a human study on high-dosages of GPC examined the effects of a 6 days treatment on muscle strength (Bellar, LeBlanc, and Campbell 2015), which showed limited effectiveness. According to the authors, there is a considerable issue with the current research on GPC because of the large variety in terms of dosage. The majority of studies used dosages of around 600 mg (approx. 2.4 mM) in an acute fashion, although the chronic dosing may vary heavily based on the health condition of the human subjects and could reach a daily dosage of 1000 mg for 28 days (Barbagallo Sangiorgi et al. 1994).

There is broad, reliable evidence for GPC tolerance in laboratory animals. Acute oral, as well as parenteral toxicity of GPC, has been shown to be very low in several animal species including mice, rats and dogs (LD_{50}>1000 mg/kg i.v./i.p. in rodents; LD_{50}>500 mg/kg i.m. and LD_{50}>2000 mg/kg p.o. in dogs). Additionally, sub-chronic and chronic oral toxicity of GPC is considered to be very low. A 4-week administration of 100 and 300 mg/kg GPC in rats did not alter behaviour nor produce any signs of general toxicity. A 26-week administration of 300 mg/kg GPC did not produce any toxic effects in rats and it showed only mildly reduced activity in dogs. A high dose of 1000 mg/kg induced reduced activity in rats (Brownawell, Carmines, and Montesano 2011). In addition, GPC is considered not to be genotoxic both *in vitro* and *in vivo:* it exerts no mutagenicity in as high a dose as 3000 µg/mL of concentration in yeasts, and at 10.000 µg/plate concentration in bacterial colonies (Brownawell, Carmines, and Montesano 2011). With respect to its toxicity, it should be pointed out that while GPC

toxicity was tested mostly *in vivo* following oral administration (Brownawell, Carmines, and Montesano 2011), here we have tested the direct *in vitro* effects of GPC on neonatal rat cardiomyocyte cultures. Since GPC is hydrolyzed in the gut mucosa (Abbiati et al. 1993), *in vivo* (after oral administration) and *in vitro* effects of GPC may differ. Nevertheless, cardiac toxicity and cardiac effects of orally administered GPC have not yet been reported. Orally administered GPC showed several beneficial, non-cardiac effects such as neuroprotective effects in different types of brain damage (Tomassoni et al. 2006; Ricci et al. 1992; Ciriaco et al. 1992) and mental disorders (Sigala et al. 1992), as well as in acute stroke or transient ischemic attacks (Barbagallo Sangiorgi et al. 1994). It has also been shown that GPC can rapidly cross the blood-brain-barrier (Lee et al. 2017). Applied GPC doses in these studies were relatively high. It is important in future *in vivo* experiments to examine the dose–response relationship between GPC and risk organs which are different from the target one, with a special emphasis on the possible side-effects.

After all, our chosen concentration range of GPC (1-100 µM) was based on papers describing the free choline concentrations in plasma (Veenema et al. 2008; Nurk et al. 2013) and on pilot experiments.

2) For decorin treatment we based the dosage of the treatment on our previous data, from the experiments of biglycan treatment, which has very similar chemical properties to decorin (Gaspar et al. 2016). The same doses of inhibitors were used as in our previous studies (Gaspar et al. 2016; Gorbe et al. 2010) where they did not affect cell viability after SI/R on their own, while in the present study they affected cell viability.

3) The chosen concentration of 3 selected MMPIs, imidazole (MMPI-1154) and thiazole (MMPI-1248 and -1260) type MMP-2 inhibitors, were based on previous IC_{50} values (IC_{50} for MMP-2 MMPI-1154: 2.5 µM, MMPI-1260: 2.6 µM and MMPI-1248: 9 µM) performed in our laboratory (Bencsik et al. 2018). MMPI-1154 at 1 µmol/kg and MMPI-1260 at 3 µmol/kg showed cardioprotection by significantly lowering the infarct size compared to the vehicle-treated group.

Although, we assessed *in vitro* data on our novel compound's IC_{50} data, I checked the literature for similar experimental set ups. Only a few studies have been performed in *in vivo* rat model of acute myocardial infarction to investigate the

cardioprotective effect of MMP inhibitors. One group was using a 48 hours daily pretreatment of doxycycline in rats prior to 30 min of coronary occlusion followed by 2 days of reperfusion. Doxycycline pretreatment significantly reduced the infarct size compared to the untreated group (Griffin et al. 2005). In another study, rats were administered vehicle or minocycline, a semi-synthetic tetracycline type MMP inhibitor via intraperitoneal injection at 25 mg/kg every 12 hours for 48 hours before and after 45 mins of LAD occlusion. After 48 hours of reperfusion, infarct size was significantly reduced by minocycline treatment (Romero-Perez et al. 2008). In our previous study, a non-selective MMP-inhibitor, ilomastat was tested in an *in vivo* rat model of AMI. AMI was induced by 30 min LAD occlusion followed by 120 min reperfusion. When administered 5 min prior to ischemia, ilomastat at 0.75 and 1.5 μmol/kg doses decreased infarct size significantly as compared to the vehicle-treated group. Furthermore, a higher dose (6.0 μmol/kg) of ilomastat was able to reduce infarct size significantly when administered 5 min before the onset of reperfusion (Bencsik et al. 2014). The latter finding was confirmed by another research group in an *in vivo* mouse model of AMI (Bell et al. 2013). Controversially, in our present study, ilomastat (6.0 μmol/kg administered 5 mins before reperfusion) failed to reduce infarct size *in vivo*, but our novel MMP inhibitors were cardioprotective. ARP-100, a biphenylsulfonamide-type MMP inhibitor is the most selective small molecule MMP inhibitor for MMP-2 over other MMP inhibitors available on the market nowadays (IC_{50} for MMP-2= 12 nM, MMP-9= 200 nM, and MMP-3= 4500 nM) (Roczkowsky et al. 2020). ARP-100 shows selectivity towards MMP-2 and MMP-9 (Rossello et al. 2004). A recent study on isolated rat hearts showed a cardioprotective effect of ARP-100 (10 mM) against myocardial stunning after I/R injury (Chan et al. 2019).

5.5. Drug testing platform – testing of cardioprotective candidates in *in vitro* cell cultures and normoxic conditions

After finding novel drug candidates and their optimal concentration,finding a safe and beneficial therapeutic window for their application is crucial. To confirm their biosafety, first, the normoxic experiments were carried out in neonatal rat cardiac myocytes. To date, no effects on cardiac tissue or cells of GPC treatment are described. Neither the acute, 15 min nor the short-term, 3-hour exposure to GPC affected the cell

viability of NRCMs. Nevertheless, significant, acute effects of GPC at 80-100 µM concentration were noted as regards mitochondrial respiration which was considered as a part of some physiological intra-mitochondrial ROS-producing mechanism that was in line with the short-term ROS-reduction at 100 µM treatment. We proposed that in response to elevated mitochondrial respiration, the cells harbor compensatory increases in other ROS-scavenging mechanisms. Regarding 24 h experiments, long-term exposure to each GPC concentration in normoxic conditions resulted in extensive cellular death of the cardiac myocytes alongside with significantly increased overall ROS level at each applied concentration. This was the first demonstration of the potential cardiotoxicity of GPC.

The viability of cells was not influenced by any of the applied GPC concentrations in any of the experimental setups, except for the 24-hour treatment at which time point profound superoxide and overall ROS accumulation were also detected. Other *in vitro* studies indicated the emerging oxidative stress of 24-hour GPC administration applied on astrocytes (Grasso et al. 2014) and excessive neuronal cell death was described in another scenario in response to exogenous, chronic GPC (de Pablo et al. 2013). It was also reported that *in vitro,* long-term exposure to muscarinic agonists significantly reduced the expression of a series of proteins belonging to the cytoskeletal structure and led to a complete change in the cellular shape. This possibly made the cells more prone to be disturbed by inflammatory factors (Stamatiou et al. 2014). In our case, this latter was likely due to the increasing amount of ROS. This was fully supported by our data. Indeed, after 24 hours of GPC treatment, a significant increase in both the superoxide and overall ROS formation was observed at 1 and 100 µM, and superoxide level was detected to be significantly higher at 80 µM. In line with these results, cellular viability was significantly reduced at 1, 80 and 100 µM concentrations, respectively.

In case of exogenously administered decorin the viability was improved after 20 hours of treatment and the proliferation rate of NRCMs was triggered in normoxic condition. Viability assay based on measurement of intracellular enzyme activity, meanwhile proliferation assay based on cell division, how much tagged uracil (BrdU) is able to incorporate in, 1nM concentration increased while at 100nM decreased cell proliferation of NRCMs.

Therefore, the positive effect of 1 nM decorin seen in the viability assay after SI/R can be due to the proliferative effect of this concentration of decorin. However, decorin at other concentrations did not affect proliferation, therefore, the results of viability assay of these concentrations of decorin have not been influenced by proliferation. The ability of decorin to differentially regulate the cell proliferation is well known for many years (Yamaguchi and Ruoslahti 1988), and the anti-proliferative effects of decorin are considered to be involved in its anti-cancer effects (Jarvinen and Prince 2015; Yang et al. 2017).

In the *in vivo* experimental set up with the MMPI candidates the tolerability testing stood for the normoxic conditions.

5.6. Drug testing platform – testing of cardioprotective candidates in *in vitro* cell cultures and simulated ischemia/reperfusion injury

SI/R challenge was performed to ascertain some protective potential of GPC and decorin. In case of MMP inhibitors the cardio-cytoprotective effect had already been confirmed by Bencsik et al (Bencsik et al. 2018). A 3-hour treatment with 80 µM concentration of GPC had a cytoprotective effect as indicated by the cell viability of cells challenged with SI/R. No significant changes were recorded regarding the pro-oxidant parameters although a strong propensity for an increase in the total ROS level but not the superoxide quantity was considered at 80 and 100 µM GPC concentration. With decorin treatment we have shown exerted cardiocytoprotective effect in both isolated neonatal and adult rat cardiomyocytes exposed to SI/R.

Previous studies have shown that GPC is able to prevent oxidative stress and decrease radical production in different I/R models, e.g. liver or mesenteric tissue (Strifler et al. 2016; Hartmann et al. 2014; Tokes et al. 2015). It has also been demonstrated that dietary supplementation with a mixture of GPC and other four compounds reduced the production of ROS in mice brain (Suchy, Chan, and Shea 2009). At this point in time, no myocardial protection effects were investigated related to GPC. Therefore, the same concentrations of GPC treatment as used in normoxic conditions were applied in a clinically relevant I/R model of cardiac myocytes. After SI/R protocol, viability assay was performed and showed that 80 µM GPC treatment resulted in a significant improvement of cell viability after SI/R. This observation suggested that GPC in 80 uM concentration was cytoprotective against I/R, likely via

facilitating some as-of-yet un-described, fine-tuning mechanism in ROS-mediated cell signaling/cellular death pathways. Although 80µM GPC was cardiocytoprotective, there was no nice dose-effect curve from measured parameters, therefore this results could be an artefact, rather than solid pharmacological response.

Decorin treatment, similarly, as findings of our previous study documenting a dose-dependent protective effect of another matrix proteoglycan – biglycan (Gaspar et al. 2016), the cardioprotective effect of decorin on NRCMs cell viability exposed to SI/R was dose-dependent. The protective effect of decorin on ARCMs in the present study has been shown to be dose-independent, but it should be pointed out that for ARCMs, only those concentration of decorin were used which exerted protective effects in NRCMs. Taken together, small extracellular matrix proteoglycans including biglycan and decorin may represent a powerful tool for cardioprotection. Biglycan and decorin have been shown to differentially regulate signaling in the fetal membranes suggesting their different biological activities (Wu et al. 2014). There are also differences in enzymatic degradation of these two proteoglycans by matrix metalloproteinases (MMPs) (Monfort et al. 2006; Tufvesson et al. 2002) which points to different regulation of these two proteoglycans and may also potentially influence their biological activities.

5.7. Drug testing platform – *in vivo* animal model of acute myocardial infarction

Potential cardioprotective effect of novel MMP inhibitors, MMPI-1154, -1260, and -1248 inhibitors were tested *in vivo* in a rat model of AMI. Rats were subjected to myocardial ischemia/reperfusion injury at the 25th min of coronary occlusion (5 min before the onset of reperfusion) as a single bolus dose. The timing and the way of administration of the drugs are clinically relevant. In this study, MMPI-1154 and -1260 showed significant reduction in infarct size *in vivo*.

5.8. Drug testing platform – *in vivo* animal model of acute myocardial infarction combined with comorbidities

According to the latest guidelines on preclinical study designing of novel cardioprotective therapies, attention must be paid to the importance of clinically relevant animal models of different risk factors and co-morbidities and their

medications to gather more knowledge on individual responses to cardioprotective therapies. Investigating novel drug candidates in comorbid animal models allows better planning of clinical studies for cardioprotection in different patient populations (Schulz et al. 2020). Hypercholesterolemia is a common comorbidity associated with cardiovascular diseases (Ferdinandy et al. 2014), which severely interfere with endogenous cardioprotective conditioning procedures (Andreadou et al. 2017). Hyperlipidemia interferes with pharmacological interventions on cardioprotective signaling pathways (Andreadou et al. 2020). Therefore, investigating the role of MMP inhibition in acute myocardial infarction in the presence of hypercholesterolemia has a significant importance. We have previously shown, that MMP-2 activity correlated positively with total ($r = 0.55$; $p < 0.05$) and LDL cholesterol ($r = 0.45$; $p < 0.05$) in coronary artery disease patients (Bencsik et al. 2015). In this thesis, hypercholesterolemia was induced by 12 weeks high fat diet (2 % cholesterol + 0.25 % cholic acid). The impact of ageing on I/R injury and cardioprotection can also depend on factors unrelated to comorbidities. Therefore we established an age-match control for the 12 weeks diet. The aged heart displays some features that reduce its functional and adaptive capacity for adequate response to conditions of increased demand, such as stress or damage. At the cellular level, ageing could change the cardiomyocyte cytoarchitecture and metabolic or biochemical pathways (Ruiz-Meana et al. 2020).

We tested the cardioprotective doses of MMP-1154 and -1260 in hypercholesterolemic rats, which have previously reduced infarct size in young normocholesterolemic animals. MMPI-1154 (1 µmol/kg) and MMPI-1260 (3 µmol/kg) administered i.v. 5 minutes before reperfusion showed significant cardioprotection reproducibly in normocholesterolemic age-matched animals but not in hypercholesterolemic ones.

Interestingly, hypercholesterolemia could disguise the protective effect of these novel MMP inhibitors, since it was demonstrated that the altered metabolism due to hypercholesterolemia leads to a better tolerance of ischemia/reperfusion injury and thus to a smaller infarct size (Inserte et al. 2019). In this work, the infarct size data derived from hypercholesterolemic rats were significantly lower than that of in normocholesterolemic animals (see Fig 36), which difference could be feasible to abolish significant infarct size-limiting effect of abovementioned novel MMP inhibitors. In light of these factors influencing infarct size data, MMPIs cardioprotective potential

cannot be excluded, although we could not show a significant infarct size-lowering effect of our novel MMP inhibitors in hypercholesterolemic conditions. Since we tested them in only single doses, testing further doses of MMPI-1154 and -1260 may show their cardioprotective capacity even in the presence of hypercholesterolemia. Furthermore, our novel MMP inhibitors may possess late beneficial effects on cardiac function in post-MI heart failure models.

5.9. Possible molecular mechanisms behind cardioprotection

GPC is previously reported to increase inositol phosphate (IP) formation and to promote PKC translocation in the brain (Aleppo et al. 1994). However (to date), no similar observations were obtained in cardiac cells. In fact, it is well-known that cardiac dysfunction with marked changes in the membrane inositol phosphate-Protein Kinase C (IP-PKC)-mediated signaling and subsequent Ca^{2+}-handling abnormalities in cardiac myocytes arise primarily from oxidative stress as well as reduced antioxidant defenses (Tappia, Asemu, and Rodriguez-Leyva 2010). It is thus possible that long-term exposure to a modulator of the IP-PKC cascade led to a critical accumulation of ROS with inescapable cell death. The possible explanation of the long-term cytotoxic effect might also be due to the feature of GPC as being a muscarinic acetylcholine receptor (mAChR) agonist (Muccioli et al. 1996). Several mAChR subtypes may be potentially linked to the ROS production through multiple effector cascades, i.e. by elevating intracellular calcium levels, activating PKC, or by increasing the formation of arachidonic acid through phospholipase A2 activation (Naarala et al. 1997). More recently, long-term stimulation of ACh production by cigarette smoke extract for 4 days was reported to enhance the traditional PI3/PKC/PEBP1/Raf-ERK1/2 pathway activation as well as the release of pro-inflammatory and oxidative mediators in 16HBE cell line *in vitro* (Albano et al. 2018). The existence of different subtypes of mAChRs on rat neonatal cardiomyocytes is well-supported (Yang et al. 1993). Physiological ROS-formation and the redox signaling of cardiac cells through superoxide play an important role to maintain the normal function (Zhang and Shah 2014).

In case of decorin treatment ROS production measurement were not performed, since there is no direct link between them. We focused on the pathways that are involved in the cardioprotective effect of biglycan. The effect of decorin was not

influenced by the NO-synthase inhibitor, L-NAME, the PKG inhibitor, KT-5823, or the inhibitor of TLR-4, TAK-242, respectively.

We suggests that the molecular mechanisms involved in the cardioprotective effect of decorin are different from those of biglycan. While cardiocytoprotective action of biglycan includes activation of TLR-4 and its downstream effectors (Gaspar et al. 2016), the protective effect of decorin documented here seems to be TLR-4-independent. This might be explained by possibly different cellular effects of decorin and biglycan via TLR signaling found e.g. in tumor cells, where opposite effects of these proteoglycans on tumor growth via TLR have been shown (Neill, Schaefer, and Iozzo 2012; Schaefer and Iozzo 2012). In addition, the protective effect of biglycan has been shown to involve enhanced production of NO, while the effect of decorin in the present study was not affected by NOS inhibition suggesting a NO-independent action of decorin in preventing the SI/R-induced cell death of NRCMs. Finally, inhibition of PKG in the current study had also no impact on decorin action in NRCMs exposed to SI/R suggesting these effects to be independent of NO-cGMP-PKG signaling. Gaspar et al showed that pharmacological blockade of Toll-like receptor 4 (TLR-4) signaling and its downstream signaling contributors (IRAK1/4, ERK, JNK and p38 MAP kinases) abolished the cytoprotective effect of exogenously administered recombinant biglycan core protein against SI/R injury (Gaspar et al. 2016). In another study, decorin secreted by human adult renal stem cells through the TLR-2 receptor induce renal tubular cell regeneration (Sallustio et al. 2017).

These can be explained by structural differences (Lord and Whitelock 2013) as well as by differences in their biological actions which have been documented in some studies (Wu et al. 2014). Besides cardiac myocytes, other myocardial cell types, like cardiac fibroblasts, may also contribute to cardioprotection. Expression of proteoglycans by fibroblasts is influenced by TNF-α and TGF-β (Mauviel et al. 1995). Recently it was documented that certain amount of decorin is produced in the scar tissue, the border zone close to the infarction, as well as in remote region of pig hearts after myocardial infarction. In that study, biglycan and decorin levels were higher in the border zone and are suggested to support the stabilization of collagen fibrillogenesis (Nagaraju et al. 2017).

Recent studies have demonstrated that post-infarction gene therapy with adenoviral vector expressing decorin mitigates cardiac remodeling and dysfunction (Li et al. 2009) suggesting promising therapeutic potential of exogenous decorin for the treatment of acute myocardial infarction. Diabetic male Wistar rats received recombinant adeno-associated viral decorin attenuated diabetic cardiomyopathy with improved LV function compared with control animals, these protective effects were associated with TGF-β pathway (ERK1/2 and smad-2) and NF-κB pathway, which could be due to the decreased activation level of IGF-IR, increased expression of PKC-α and Hsp70 (Chen et al. 2020). Gubbiotti et al showed in decorin knock out (Dcn-/-) and wild type mice that decorin has a new role as a nutrient sensor that modulates cardiac autophagy and metabolism (Gubbiotti et al. 2015), and exogenous decorin treatment (10 mg/kg core protein) restores fasting-induced autophagy in Dcn−/− hearts and can also salvage cardiac function after 25 hours of fasting (Gubbiotti et al. 2018). Decorin affects rate of apoptosis in different experimental models. Decorin induced apoptosis via activation of caspase-3 in A431 tumor engraftment cells (Seidler et al. 2006). In another model using skin fibroblasts decorin treatment resulted a significant increase in the expression of apoptotic markers, histone-1, caspase-1, caspase-8, and p53 in superficial fibroblasts when compared with deep dermal fibroblasts (Honardoust et al. 2012). Overexpression of decorin inhibited mesangial cells proliferation by inducing apoptosis and cell growth arrest *in vitro* and it also downregulates expression of TGF-β (Wu et al. 2008). Therefore we evaluated the apoptotic effect of decorin in simulated ischemic conditions by measuring caspase-3 activity and by performing TUNEL assay in NRCMs. In the present study, however, decorin treatment had no significant effect on apoptosis in neonatal cardiac myocytes.

P-Akt/Akt is considered as pro-survival protein which is included in the downstream signaling pathway e.g. reperfusion injury salvage kinase (RISK) pathway (Rossello and Yellon 2018), therefore Akt kinase has a cardioprotective role against ischemia/reperfusion injury. We evaluated the activity of Akt kinase by measuring the p-Akt/Akt ratio (Akt is activated by its phosphorylation) by western blot to see if an Akt-dependent pathway is potentially involved in the mechanism of decorin action.

Suzuki et al investigated the effect of decorin in myogenic cells. Decorin activated Akt downstream of IGF-IR and enhanced the differentiation of C2C12 myoblast cells

(Suzuki et al. 2013). In endothelial cells decorin inhibited anti-autophagic signaling via suppression of Akt/mTOR/p70S6K activity with the concurrent activation of pro-autophagic AMPK-mediated signaling cascades (Goyal et al. 2014). Activation of autophagy *in vitro* using mouse embryonic NIH-3T3 fibroblasts induced decorin expression via mTOR pathway, while biglycan expression showed no changes (Gubbiotti et al. 2015). Overexpression of decorin ameliorated diabetic cardiomyopathy and promoted angiogenesis through the IGF1R-Akt-VEGF signaling pathway in endothelial cells *in vivo* and *in vitro* (Lai et al. 2017). To our best knowledge, there are no available data of the direct effect of decorin on Akt signaling in cardiac myocytes. Our results point to a decreased phosphorylation/activation of Akt after simulated ischemia, which is not in line with majority of data regarding to cardioprotection against ischemia/reperfusion injury. Therefore, decorin probably protects cardiac cells activating different downstream signaling pathways.

We performed RNA sequencing analysis to monitor direct effect of decorin in isolated cardiac myocytes. This is the first demonstration that zinc finger, MYND-type containing 19/Zmynd19 and eukaryotic translation initiation factor 4E nuclear import factor 1(eIF4E) are significantly upregulated due to decorin action. eIF4E is best known for its function in the initiation of protein synthesis on capped mRNAs in the cytoplasm and involved in the nuclear export of specific mRNAs (Goodfellow and Roberts 2008). It has been shown, that eIF4E plays an important role in the nucleocytoplasmic export of human iNOS mRNA in colon carcinoma cell line (Bollmann et al. 2013). Culjkovic et al showed, that eIF4E associates and promotes the nuclear export of cyclin D1 in the nucleus and be involved in regulation of cell proliferation (Culjkovic et al. 2005). In the present study, we showed that decorin treatment in 1 nM concentration enhanced the proliferation of cardiac cell culture. This finding also in line with our findings using GO analysis, which presented overrepresentation mitotic cell cycle (GO:0000278) genes. GO analysis resulted overrepresentation genes in response to oxidative stress (GO:0006979) as well, which phenomenon has been studied in traumatic brain injury model, where decorin protected neuronal cells reducing level of oxidative stress (Ozay et al. 2017). To the best of our knowledge there is no direct link between Zmynd19 and oxidatice stress, pro-death or cytoprotection.

MMP inhibitors mechanism of action could be based on the limited cleavage of its intracellular target proteins. MMP-2 has a wide repertoire of both intracellular and extracellular substrate that they are capable of proteolyzing, like myosin light chain (Gao et al. 2014), titin (Ali et al. 2010) and troponins (Wang et al. 2002), SERCA2a (Roczkowsky et al. 2020) or junctophilin-2 (Chan et al. 2019) (see for review (Schulz 2007; Hughes and Schulz 2014)), therefore the inhibiton of protease enzyme could protect the sarcomere proteins. Troponin I, a regulator of actin–myosin interaction, is also a target of MMP-2. The presence of TnI and its fragments in the circulation is well-known and recognized as a biomarker for cardiac injury. In isolated rat hearts subjected to I/R injury, cardiac troponin I levels are decreased and MMP inhibitors (doxycycline (100 μmol/L), *o*-phenanthroline (100 μmol/L)) prevented troponin I loss and decreased the severity of contractile dysfunction after I/R injury (Wang et al. 2002).

5.10. Limitations

Limitations and challenges of preclinical models. *In vitro* studies are the very first line in biological testing new compounds. We can assess data of the direct effect on living cells and to first-time we investigated the effects of a broad concentration range of GPC on cardiac cells. Nevertheless, *in vivo* experiments would certainly supply further information regarding the functional cardiac effects of GPC. The mechanisms of action of GPC on cardiac cells is poorly characterised, it should be studied with multiple omics technologies in any depth.

Limitations of the decorin study: Although we clearly showed the cardiocytoprotective effect of decorin in both neonatal and adult cardiomyocytes, it did not reveal the mechanism of its action because none of the used inhibitors affected decorin-induced cardiocytoprotection. Although NOS, TLR-4 and PKG inhibitors did not affect decorin induced cytoprotection in the present study, the variability of response of cardiac myocytes to NOS, TLR-4, PKG inhibitors in different studies may cause an uncertainty as to whether the selected pathways may contribute to the cardiocytoprotective effects of decorin in the present study. Indeed, other studies also showed diverse effects of these inhibitors in cardioprotection. For example PKG inhibitor KT-5823 in the same dose (1 μM) blocked the cardioprotective effect of ischemic postconditioning (IPostC) in some studies (Inserte et al. 2013; Inserte et al.

2014) while had no effect on cardioprotective effect of IPostC in other studies (Sun et al. 2013; Tong et al. 2014) in isolated perfused heart. Also, opposite effects of TLR-4 inhibitor TAK-242 have been documented in different types of cardiomyocyte injury: it has been shown to dramatically block the high glucose-induced cytotoxicity leading to an increase in cell viability in H9c2 cells (Liang et al. 2017), on the other hand it attenuated biglycan-induced cardiocytoprotection and had no effect when applied alone in SI/R model in neonatal rat cardiomyocytes (Gaspar et al. 2016). It has been shown that NOS inhibitor L-NAME at the dose of 30 μM exerted both cardioprotective (Woolfson et al. 1995) as well as no effects (Weselcouch et al. 1995) in isolated hearts exposed to I/R. It is well known that the different doses of NOS inhibitors can induce either cardioprotection or block cardioprotective pathways, see for reviews: (Andreadou et al. 2015; Ferdinandy and Schulz 2003). Unfortunately, there is no study showing the dose response curve for TAK-242 and KT-5823 in I/R injury. This limitation reflects the fact that multiple pathways may be individually and sequentially activated in cardiomyocytes due to I/R (Pavo, Lukovic, Zlabinger, Lorant, et al. 2017; Pavo, Lukovic, Zlabinger, Zimba, et al. 2017; Perrino et al. 2017) which may cause the variability of cardiomyocyte responses to I/R as well as to NOS, TLR-4, PKG inhibitors used in individual experiments. Indeed, a recent study by Heusch's group (Kleinbongard et al. 2018) showed that reproducibility of studies of cell signaling of cardioprotection performed even in the same laboratory can be variable.

In vivo studies have other limitations and challenges. Finding a reliable and reproducible positive control is challenging. It is now clearly demonstrated that ischemic postconditioning may also lack cardioprotective effects both in patients as well as in preclinical animal models (see for Review: (Botker, Lassen, and Jespersen 2018)). In a recent study, we have highlighted the presence of a phenomenon, which is accompanied with ischemic postconditioning and led to diverse effects distinguishing responders and non-responders to postconditioning stimuli in terms of infarct size reduction. This phenomenon is supposed at least partially being based on individual polymorphism of down-stream signaling pathways of postconditioning stimuli including jun dimerization protein type 2 (JDP2) and activator protein-1 (AP-1) (Schreckenberg et al. 2020). Therefore, in our study, we may suppose that the ratio of animals non-

responding to the cardioprotective signals evoked by ilomastat was higher than previously published, which might lead to the loss of significant infarct size reduction.

In the experiments with young normocholesterolemic animals, we used ilomastat as positive control to decrease myocardial infarct size after I/R injury. However, in spite of previous studies, which have demonstrated the infarct size-limiting ability of ilomastat in rodent models of AMI (Bell et al. 2013; Bencsik et al. 2014), this time it was unable to achieve cardioprotection in terms of infarct size reduction as compared to the vehicle control. Therefore, due to the inefficacy of ilomastat to reduce infarct size in young normocholesterolemic rats, we changed the positive control to IPC in the hypercholesterolemic model despite its clinical relevance and applicability is limited. We also provided a detailed discussion above about the potential reasons of the absence of the cardioprotective effect of ilomastat with this administration algorithm.

Another limitation of drug testing experiments are the lack of examination of pharmacokinetic or full ADMETox properties of the inhibitor compounds.

5.11. Clinical translation

Kleinbongard et al. reviewed the fact that translation of cardioprotection from robust experimental evidence to beneficial clinical outcome for patients has been largely disappointing (Kleinbongard et al. 2020). In clinical practice, infarct size following ST-segment elevation myocardial infarction (STEMI) varies widely depending on the location of the coronary occlusion, ischaemia duration, collateral blood flow, and spontaneous recanalization but in animal models all these factors are standardized. In animal models the end-point measurement is often the infarct size. However in humans, especially at low infarct sizes, the translation of infarct size reduction into improved clinical outcome by any cardioprotective therapy is challenging.

Different cardiovascular risk factors, like ageing, sex, obesity, and smoking are known to modify the efficacy of cardioprotective strategies in experimental settings (Ferdinandy et al. 2014). A major limitation of studies in human patients is that the assessment of interaction between risk factors and the efficacy of cardioprotective strategies relies on surrogate markers of cardioprotection and retrospective or post hoc analyses (Kleinbongard et al. 2020).

Finding a good cardioprotective compound becomes even more complex when we realize that the cardioprotection could not be achieved by a single molecule,

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therefore we should use combination of therapeutics. Nevertheless basic research is important and a step-by-step approach with validated and relevant animal models combined with the correct doses and timing of novel potential cardioprotective compound are essential for new discoveries and for the improvement of therapeutics options of cardiovascular diseases.

6. Conclusions

In conclusion, we have shown for the first time that choline donor L-alpha-GPC (which is widely used as a food supplement and generally considered as safe for human use) had ambiguous effects on cardiac cells. It may be beneficial in short-term administration to maintain the physiological balance of ROS-production under normoxic, healthy conditions and could be also protective in I/R conditions, but could, in fact, be cytotoxic if it surrounded the cells for long enough. Besides the duration of the treatment, the correct dosage can also be a crucial factor, as a fine-tuning effect seemed to occur in a small, but dietary-relevant concentration range. Thus (despite many limitations of this *in vitro* study), our results indicate the need for a comprehensive cardiac safety testing of GPC.

The small leucine-rich proteoglycan, decorin exerts cardiocytoprotective effects against SI/R, suggesting a therapeutic potential of exogenously administered decorin for the treatment of acute myocardial infarction. The molecular mechanism of its action still remains to be uncovered; however, it seems to be independent of NO-cGMP-PKG and TLR-4 signaling.

MMPI-1154 and MMPI-1260 shown to be cardiocytoprotective *in vitro* and ex vivo and have been further proved here to be cardioprotective *in vivo* when administered before the onset of reperfusion, which is a clinically relevant therapeutics approach in a rat model of AMI. Although, the presence of hypercholesterolemia abolished the cardioprotective effects of MMPI-1154 and -1260 in single doses that shown cardiprotection *in vivo*, whether it is due to a shift in the dose-response relationship of these compounds remained unknown. Further development of these promising cardioprotective MMPIs to be continued in different dose ranges in hypercholesterolemia and other comorbidities.

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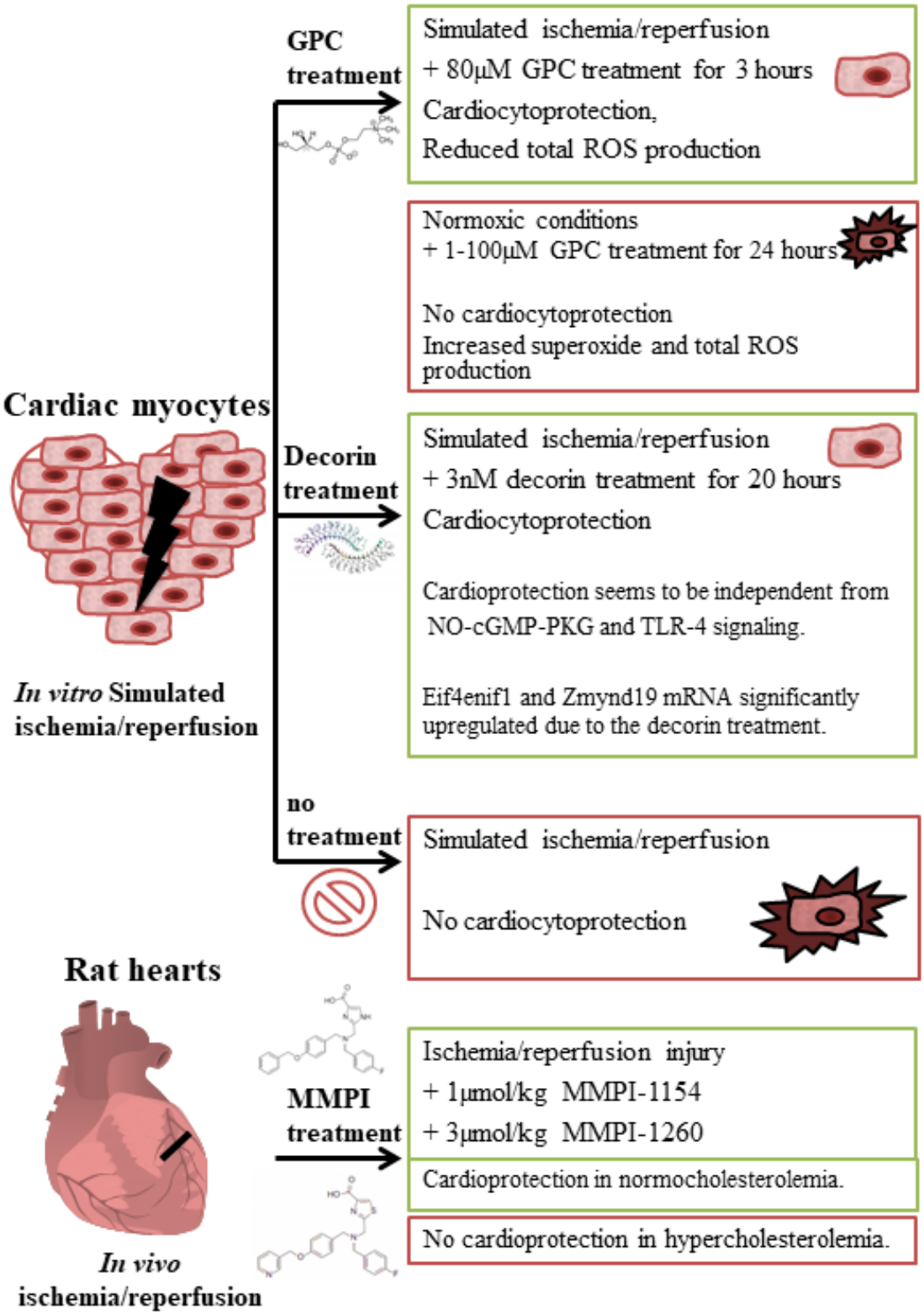

Figure 46. Visual summary of tested compound's cardioprotective effects.

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7. Summary

In my thesis I presented relevant *in vitro* and *in vivo* animal experimental models for discovering protective compounds against acute myocardial infarction. In myocardial cell culture, the simulated ischemia / reperfusion injury caused significant cell death, making the system suitable for testing new potentially cardioprotective compound. GPC was able to protect myocardial cells from oxidative stress-related reperfusion injury at 80µM after 3 hours pretreatment, while decorin at 3nM for 20 hours pretreatment. In the case of GPC, the reduced reactive oxygen species were behind the positive effect, but chronic treatments have detrimental effect. In case of decorin the exact mechanism of action has not been mapped, however, we found that, unlike its sister molecule, biglycan, decorin seems not act through the NO-cGMP-PKG pathway or the TLR-4 receptor mediated response. Eif4enif1 and Zmynd19 mRNA significantly upregulated due to the decorin treatment.In addition, the protective effect of matrix metalloproteinase inhibitors has been demonstrated *in vivo* in a rat model of acute myocardial infarction. Matrix metalloproteinases are able to degrade a number of intracellular and extracellular proteins, therefore the novel inhibitors could protect the sarcomere proteins responsible for myocardial cell contractility. The use of the new inhibitory molecules can be used in normocholesterolemic animals at safe, clinically relevant timing and doses, however, further research is needed in the presence of comorbidities. We show the dose-dependent cardioprotective effect of novel MMP inhibitors MMPI-1154 at 1µmol/kg and MMPI-1260 at 3µmol/kg in an *in vivo* rat model of AMI when administered before reperfusion. However, in the presence of hypercholesterolemia, their infarct size-limiting effect was not seen in a single dose that showed cardioprotective effects in normal rats. This is the first demonstration that MMPI-1154 and MMPI-1260 are cardioprotective *in vivo* against myocardial infarction. Whether hypercholesterolemia inhibits their cardioprotective effect or may only shift the dose–response relationship of these compounds remains unknown.

Finding cardioprotective molecules are complex because the duration of the treatment and the correct dosage are both crucial factors. We were aiming to test 5 different molecules, from which we have found 4 to be cardioprotective (Fig. 46.).

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8. Összefoglalás

Tézisemben releváns *in vitro* és *in vivo* állatkísérleti modelleket mutattam be az akut miokardiális infarktus elleni védő vegyületek felderítésére. A szívizom sejttenyészetben a szimulált ischaemia / reperfúziós károsodás jelentős sejthalált okozott, alkalmassá téve a rendszert új potenciálisan kardioprotektív vegyületek tesztelésére. A GPC 3 órás előkezelés után tudta megvédeni a szívizomsejteket az oxidatív stressz okozta reperfúziós sérüléstől 80 µM-os dózisnál, míg a dekorin 3 nM-on 20 órás előkezelés után. A GPC esetében a lecsökkent reaktív oxigén gyökök álltak a pozitív hatás hátterében, azonban a krónikus GPC kezelés hatására átbillen a sejtek reaktív oxygen gyök termelése és sejtelhalás következik be. Dekorin esetén a pontos hatásmechanizmus nem került feltérképezésre, azonban azt tapasztaltuk, hogy testvérmolekulájától, a biglikántól eltérően valószínűleg nem az NO-cGMP-PKG útvonalon vagy a TLR-4 receptor által közvetített válaszon keresztül hat. Decorin hatására az Eif4enif1 és Zmynd19 mRNS expressziója nőtt meg szignifikáns mértékben.

Ezenkívül a mátrix metalloproteináz inhibitorok védőhatását *in vivo* igazoltuk akut miokardiális infarktus patkány modelljében. Ez az első bizonyíték arra, hogy az MMPI-1154 és az MMPI-1260 *in vivo* kardioprotektív hatású a szívinfarktus ellen. A mátrix metalloproteinázok képesek lebontani számos intracelluláris és extracelluláris fehérjét, ezért az új inhibitorok megvédhetik a szívizomsejtek kontraktilitásáért felelős szarcomer fehérjéit. Az MMPI-1154 dózisfüggő kardioprotektív hatását 1 µmol/kg és MMPI-1260 3 µmol / kg dózisban igazoltuk akut miokardiális infarktus *in vivo* patkány modelljében, reperfúzió előtti intravénás adagolással. Az új gátló molekulák alkalmazása normokoleszterinémiás állatokban biztonságos, klinikailag releváns időzítéssel és dózisokkal alkalmazható, azonban társbetegségek jelenlétében további kutatásokra van szükség. Teszteltük a normokoleszterinémiás állatokban védőhatást mutató molekulákat hiperkoleszterinémiában is, azonban az infarktuscsökkentő hatás elveszett.

A kardioprotektív molekulák megtalálása bonyolult, mivel a kezelés időtartama és a helyes dózis egyaránt döntő tényező. A klinikai transzlálhatóság végett törekszünk klinikailag releváns beadási időpontot és módot találni. Tézisemben 5 különböző molekula tesztelése volt célül kitűzve, amelyekből négyet kardioprotektívnek bizonyult.

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10. List of own publications

DISSERTATION-RELATED PUBLICATIONS:

L-Alpha-glycerylphosphorylcholine can be cytoprotective or cytotoxic in neonatal rat cardiac myocytes: a double-edged sword phenomenon.
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