SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

INVESTIGATIONS FOR THE MECHANISM OF ACTION OF NOVEL POSITIVE INOTROPIC AGENTS

by

Nagy László MD

Supervisor: Zoltán Papp MD, PhD, DSc

UNIVERSITY OF DEBRECEN

Kálmán Laki Doctoral School
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By László Nagy MD

Supervisor: Prof. Zoltán Papp MD, PhD, DSc
Kálmán Laki Doctoral School, University of Debrecen

Head of the **Examination Committee**:	Prof. György Balla MD, PhD, DSc, member of HAS
Members of the Examination Committee:	György Kerekes MD, PhD István Szokodi MD, PhD

The Examination takes place at the Szontagh Library, Department of Pediatrics, , Faculty of Medicine, University of Debrecen, 14th of November, 2017, 11:00 AM.

Head of the **Defense Committee**:	Prof. György Balla MD, PhD, DSc, member of HAS
Reviewers:	Endre Zima MD, PhD István Lekli DP, PhD
Members of the Defense Committee: György Kerekes MD, PhD	
	István Szokodi MD, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
14th of November, 2017, 13:00 PM.

1. Introduction

1.1. Definition, epidemiology and classification of heart failure

Heart failure (HF) can be defined as an abnormality of cardiac structure or function leading to failure of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues. HF is defined, clinically, as a syndrome in which patients have typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) resulting from an abnormality of cardiac structure or function. HF is the most common disease in the human population, approximately 0.5–2% of the adult population in developed countries has HF, with the prevalence rising to ≥10% among persons 70 years of age or older. In Europe, nearly 10 million people suffer from HF with an incidence of about 1–5/1000. Once hospitalized for HF, the mortality rates at 30 days, 1 year, and 5 years were as high as 10%, 22%, and 50%, overrating the mortality rates of cancer.

Based on the recommendation of the European Society of Cardiology (ESC), the main terminology used to describe HF is historical and is based on measurement of LV ejection fraction (EF). When the signs and symptoms of decompensation are associated with an EF >50%, the diagnosis of HF with preserved EF (HFpEF) is satisfied. The diagnosis of HF with reduced EF is established, when the characteristics signs are associated with an EF <50%.

Patients with an EF in the range 40 – 50% therefore represent a “grey area”, and most probably have primarily mild systolic dysfunction. This category is termed as HF with moderately reduced EF (HFmREF).

1.2. Pathogenesis of heart failure

HF is a complex, progressive clinical-pathological entity which is initiated by various etiological factors (ischaemic and non-ischaemic triggers) given rise to compensatory mechanisms occurring in the surviving myocytes and extracellular matrix. Neurohumoral activation (renin – angiotensin – aldosterone system, sympathetic nervous system and natriuretic factors) may counterbalance the decreased cardiac performance as short-time effects, but leading to pathological remodeling of the left ventricle (LV) with dilatation and further impaired contractility. Two mechanisms are thought to account for this progression. The first is occurrence of further events leading to additional myocyte death (e.g. recurrent myocardial infarction). The other is the systemic responses induced by the decline in systolic function, particularly due to neurohumoral activation by triggering pathological signaling pathways resulting in further cardiomyocyte necrosis, apoptosis and extracellular matrix fibrosis.

1.3. Acute heart failure

Acute heart failure (AHF) relates to the rapid decline in cardiac pump function requiring urgent medical care. AHF is also associated with high mortality rates: the estimated risk of death was reported around 3-4% in the hospital and approximately 10% after discharge within 60-90 days. AHF

may arise as a *de novo* entity in a previously asymptomatic patient, as an acute exacerbation of previously diagnosed chronic HF or as a terminal stage of chronic HF. Briefly, the pathophysiology of AHF relies on a complex interaction between the weakened cardiac performance and increased systemic vascular resistance (SVR). A novel paradigm suggests that episodes of AHF can be classified as either an acute vascular or cardiac failure. Decline in the cardiac performance by diverse pathological processes (e.g. myocardial ischaemia, arrhythmia) results in a forward and backward failure, manifesting as low peripheral perfusion with renal impairment and fluid accumulation with severe pulmonary congestion. The vascular pathway is related to the increased SVR and arterial stiffness leading to elevations in the LV end-diastolic pressure (LVEDP). Hence, increased LV filling pressure contributes to pulmonary congestion with concomitant signs of AHF. Although one or another pathway dominates in certain clinical cases, the combination of the above pathologic pathways results in the initiation of AHF by promoting a vicious circle.

1.4. Pharmacological treatment of AHF

Current pharmacological therapies should respect the distinct clinical and pathophysiological entities of the AHF syndromes. Accordingly, in those patients with hypotension (RR <90 mmHg), hypoperfusion or shock, intravenous inotropic support should be considered to maintain the peripheral perfusion via increasing the cardiac output (CO) and the blood pressure (BP), while intravenous diuretics and vasodilators are recommended in patients with pulmonary and/or systemic venous congestion, as well as with signs of elevated filling pressures and vascular volume redistribution (RR >90 mmHg).

Majority of the positive inotropic agents were shown to evoke beneficial hemodynamic effects but failed to improve the prognoses of HF, indeed the long-term morbidity and mortality rates were impaired after those inotropic intervention. Positive inotropic agents may improve the contractility of the myocardium via three different mechanism of action:

1.) calcium (Ca^{2+}) mobilizers acting by increasing the amplitude of the intracellular Ca^{2+} transient via interfering with the adrenergic signaling pathways,

2.) modulation of the Ca^{2+} homeostasis of the sarcoplasmic reticle can augment Ca^{2+} release from the SR during systole and Ca^{2+} uptake during diastole,

3.) sarcomere targeted agents activate directly the myofilaments.

1.5. Mechanism of action and clinical implications for traditional inotropic agents

Myocardial contractility depends partially on the intracellular Ca^{2+} concentration ($[Ca^{2+}]$). Traditional positive inotropic agents load the cardiomyocytes with Ca^{2+} to improve cardiac contractility, termed thereby as Ca^{2+} mobilizers. β-adrenergic agonists are the most frequently used drugs for the treatment of AHF Dobutamine and adrenalin, acting as β-adrenergic agonists increase the intracellular Ca^{2+} level

via activating this signaling pathway leading to protein kinase A (PKA) activation. Phosphodiestherase III (PDE III) inhibitors (e.g. milrinone, enoximone) enhance the contractility in cardiac myocytes and relaxation in the vascular smooth muscle cells by reducing the rate of cyclic adenosine monophosphate (cAMP) break-down. Despite of the increased myocardial contractility, this inotropic intervention can be complicated by deleterious side effects limiting its applicability in long-term therapies for patients with AHF syndrome. This is because cardiomyocyte Ca^{2+}-loading is associated with enhanced myocardial oxygen (O_2) consumption, increased heart rate (HR) and greater risk of arrhythmias contributing to the higher morbidity and mortality rates.

1.6. Sarcomere targeted agents for the treatment of AHF

Based on the deleterious side effects of the traditional inotropes, novel inotropic agents with different mechanism of action were required for the treatment of AHF. Theoretically, clinical use of sarcomere targeted drugs would avoid the disadvantages of Ca^{2+} mobilizers in the therapy of AHF syndrome. This is because pharmacological modification of the cardiac sarcomere is not expected to interfere with the intracellular Ca^{2+} homeostasis at all or not to the same degree. Several different terms and concepts have been associated with cardiac sarcomere targeted agents, i.e. 1.) Ca^{2+} sensitization of contractile filaments, 2.) direct cardiac myosin activation and 3.) drugs with added myofilamental effects (e.g. SR-33805 and HNO donors). Of note, sarcomere activating inotropes may exert pleiotropic cardiovascular effects, as direct activation of the cardiac sarcomere may be further supported by added non-myofilamental mechanisms (i.e. PDE III inhibition), evoking potentially additional hemodynamic and metabolic advantages or perhaps deleterious side effects. Minority of the sarcomere targeted agents possess only pure direct myofilamental effects, which makes them possible to evoke a clear cardiac selective mechanism of action.

1.6.1. Potential usefulness of Ca^{2+} sensitizer drugs in AHF syndrome

Ca^{2+} sensitization refers to increased contractile force production at a given [Ca^{2+}]. In fact force augmentation can be achieved via different molecular mechanisms leading to various modifications in the [Ca^{2+}] - contractile force relationship. Considering the interaction between Ca^{2+} and the cardiac troponin C (cTnC) as a reference point within the contractile activation process, inotropic interventions can be classified as those with (a) upstream mechanisms (increasing the amplitude of the intracellular Ca^{2+} transient), (b) central mechanisms (promoting the interaction between Ca^{2+} and cTnC; e.g. levosimendan) or (c) downstream mechanisms (directly modulating the actin – myosin interactions; e.g. by EMD-53998). The latter two actions are conventionally lumped together as Ca^{2+}-sensitizing positive inotropy.

Levosimendan interacts specifically with the hydrophobic region of cTnC close to its D/E linker domain on the N-terminal region, where the consequence of levosimendan binding is the stabilization of the open conformation of cTnC – Ca^{2+} complex strengthening its binding to cTnI. In

other words, levosimendan increases the affinity of cTnC – Ca^{2+} complex for the cTnI and thus promoting a Ca^{2+} sensitizer effect via a disinhibition mechanism. Diastolic function is not impaired by levosimendan treatment, as drug binding to the N-terminal region of cTnC is highly Ca^{2+} dependent with a subsequent release from the binding site at diastolic Ca^{2+} levels. Levosimendan was reported to be highly selective enzyme inhibitors for the PDE III and especially at higher plasma concentration for the PDE IV isozymes *in vitro.* Preclinical data suggested that levosimendan can exert a positive inotropic effect via a Ca^{2+} sensitizing mechanism without modifications in the intracellular [Ca^{2+}]. Levosimendan was also characterized as a potent cardio-protective drug, which is attributable directly to the vasodilation by opening adenosine triphosphate-dependent K^+- channels (K_{ATP}) in the vascular smooth muscle and indirectly to the activation of similar K_{ATP} channels in the mitochondria. Despite of the positive messages of previous preclinical trials, novel clinical studies failed to confirm the superiority of levosimendan to the traditional inotropic agents. The initial optimism driven by the improvement of short and mid-tem mortality in early clinical trials (LIDO and RUSSLAN) was tempered by the less favorable outcomes of recent studies (SURVIVE and REVIVE). Nevertheless, more recent meta-analyses have reported that administration of levosimendan is associated with a significant reduction of mortality in critically-ill patients and in those of undergoing cardiac surgery.

The chemical substitution of the structure of levosimendan resulted in the development of a novel levosimendan related chemical entity, ORM-3819, exerting its hypothesized positive inotropic effect via a cTnC-dependent Ca^{2+} sensitizing mechanism. ORM-3819 may have a different pattern of side effects than that of levosimendan evoked, which would represent a new therapeutic possibility for the treatment of AHF when levosimendan is contraindicated or well-tolerated. Common side effects of levosimendan are hypotension and headache due to its vasodilating properties occurring more frequently in case of application with high loading doses. Atrial fibrillation, hypokalaemia and tachycardia are considered as less common adverse events. This study is therefore a complete pharmacological characterization of ORM-3819 regarding its Ca^{2+} sensitizing mechanism, cTnC-binding capacity and other pleiotropic effects.

1.6.2. Cardiac myosin activation represents a novel therapeutic approach for AHF

Myosin activation represents potentially another mechanism of sarcomere targeting by increasing the ATPase rate of the cardiac myosin heads. Omecamtiv mecarbil (OM) previously referred as CK-1827452 exerts a positive inotropy via its selective binding to the S1 domain of the cardiac myosin where the relay helix and converter domain converge at the base of the force producing lever arm. The mechanism of action evokes a conformational change in the nucleotide binding domain of the cardiac myosin head contributing to the allosteric activation of its mechanical and enzymatic properties. Consistent with the allosteric modulation in the nucleotide binding domain of the cardiac myosin, omecamtiv mecarbil accelerates the inorganic phosphate release from the myosin heads, which is the rate limiting step of the acto-myosin cycle. In other words, OM increases the ATPase rate of the

cardiac myosin, accelerating thereby the transition rate from the weakly to the strongly actin-bound conformation. This kind of mechanism of action also suggests that myosin activation may result in an increase of the available force-producing myosin heads in the sarcomere, indicating "more hands pulling on the rope". Since application of OM is associated with increased myocardial contractility without any changes in the Ca^{2+} homeostasis, myosin activation might be regarded as a potential downstream Ca^{2+} sensitizing positive inotropy.

In preclinical trials, OM significantly increased the contractility of isolated rat cardiomyocytes without any changes in the Ca^{2+} homeostasis measured by the fluorescent Ca^{2+} indicator. Furthermore, myosin activation resulted in an increase not only in the magnitude but also in the duration of contraction.

In an *in vivo* dog model with pacing induced systolic HF after myocardial infarction or chronic pressure overload, OM infusion was shown to enhance the LV systolic performance without increasing the myocardial O_2 consumption. Moreover, OM failed to affect the diastolic performance of the chambers.

As the preclinical data demonstrated beneficial cardiovascular effects of myosin activation, OM was tested in clinical studies in order to determine the dose-dependent augmentation of the cardiac function, as well as the maximum tolerated doses and plasma concentration of the drug. In healthy human volunteers and patients with NYHA III-IV HF, OM was reported to enhance the systolic functions of the LV. Cardiac myosin activation was not accompanied by impairments in the diastolic functions, although the dose-limiting toxic effect was myocardial ischaemia due to the detailed prolongation of the systolic ejection time. Furthermore, OM was shown to evoke beneficial hemodynamic effects after oral administration, as well. Most recently, a randomized, controlled phase IIb trial (ATOMIC-AHF) was undertaken to evaluate the safety and efficiency of OM in those of hospitalized with AHF. The ATOMIC-AHF revealed that myosin activation did not meet the primary end-point of the study, as no significant effect on dyspnea was demonstrated. Nevertheless, administration of OM proved to be clinically safe and the results also suggested a tendency towards reduction of worsening HF. In the COSMIC-HF study, OM was administered for chronic HF patients and was confirmed effective similarly to that of previous studies. Despite the growing preclinical and clinical evidence for OM, there is less data for OM related to its long effects on the morbidity and mortality rates of HF.

1.7. Potential usefulness of sarcomere targeted agents in other diseases

Acute or chronic HF is also associated with the decreased contractility of the diaphragm. Similar findings were reported after long term mechanical ventilation, in patients with chronic obstructive pulmonary disease (COPD) and in neuromuscular diseases. Those reports have shown that decreased contractility is attributable to its decreased Ca^{2+} sensitivity of the contractile machinery. Previous studies preclinical studies have revealed that levosimendan and EMD-57033 improved the

contractility of the failing diaphragm through their Ca^{2+} sensitizing mechanisms. Clinical data also confirmed that levosimendan increased the contractility of the respiratory muscles in patients with COPD or after long term mechanical ventilation. Myosin activation also represents a potential therapeutic approach for the treatment of respiratory insufficiency, because the slow-skeletal muscle fibres co-express the same MHC isoform (MHC-β) than that of in the heart, resulting in an OM-evoked Ca^{2+} sensitization.

2. Aims

This study is a complete pharmacological characterization of the myosin activator OM, and the levosimendan-related novel chemical entity, ORM-3819. Our goal was to elucidate the potential mechanisms underlying the inotropic effects of the above agents.

Therefore, we aimed:

1. To demonstrate and quantify the effects of OM on the mechanical (ΔpCa_{50}, n_{Hill}, F_{max}, F_{active}, $F_{passive}$) and kinetic (k_{tr}, $t_{1/2akt}$, t_{relax}) contractile parameters of isolated and permeabilized cardiomyocytes.
2. To report on the tissue selectivity of OM by repeating the above investigations on skeletal muscle preparations of the rat diaphragm
3. To compare the OM-evoked mechanical and kinetic changes between skeletal muscle fibres with slow and fast intrinsic kinetics.
4. To demonstrate the hypothesized Ca^{2+} sensitizing effect of ORM.3819
5. To identify another pleiotropic mechanisms of action of ORM-3819 underlying its potential positive inotropic effect.

3. Material and methods

3.1. Chemicals and muscle specimens

OM, previously referred to as CK-1827452, was purchased from AdooQ BioScience (Irvine, CA, USA), ORM-3819 was provided by Orion Pharma. Stock solutions with a final OM concentration of 10 mM were prepared in dimethyl sulfoxide (DMSO) as solvent and stored at 4 ^{o}C. Appropriate volumes of the concentrated stock solutions were dissolved in activating and relaxing solutions to obtain test solutions containing OM in the concentration range between 3 nM and 10 µM. The final concentration of DMSO never exceeded 0.1%. DMSO itself did not modify the contractility of the myocyte-sized preparations.

Investigations were carried out on male 8-15-week-old Wistar-Kyoto rats and guinea pigs, weighing 250-500 g (Toxi-Coop Toxicological Research Center, Dunakeszi, Hungary; n = 20). The animals were fed a standard chow and drank tap water *ad libitum* and were housed in groups of two to three per polycarbonate cages in a room controlled thermostatically at 20 ± 2 °C with a target relative humidity of 55 ± 15%. The animals were anaesthetized with an intraperitoneal injection of sodium

pentobarbital (Release, Garbsen, Germany; 150 mg kg^{-1}). The heart and the diaphragm were quickly excised and the LV was dissected in cold isolating solution ($MgCl_2$: 1 mM, KCl: 100 mM, EGTA: 2 mM, adenosine triphosphate (ATP): 4 mM, imidazole: 10 mM; pH 7.0). Diaphragm and LV tissue samples were then stored at -80 ^oC. All procedures employed in this work conformed strictly to Directive 2010/63/EU of the European Parliament and were approved by the Ethical Committee of the University of Debrecen (Ethical Statement number: 1/2013/DE MÁB).

3.2. In vitro isometric contractile force measurements

Contractile force measurements were performed on single, mechanically isolated cardiomyocytes and diaphragm muscle fibres, as described previously. Briefly, deep-frozen (-80 ^oC) LV and diaphragm tissue samples were mechanically disrupted in isolating solution using a tissue homogenizer at 4 C, and thereafter subjected to chemical permeabilization with 0.5% Triton X-100 detergent for 5 min. The Triton X-100 eliminated all membrane structures, allowing characterization of the mechanical properties of single myocytes under standardized conditions with the avoidance of potential disturbing factors present *in vivo*. Isolated and permeabilized single cardiomyocytes or skeletal muscle fibres being kept in Ca^{2+}-free relaxing solution were attached with silicone adhesive (DAP 100% all-purpose silicone sealant; Baltimore, MD, USA) to two stainless insect needles, connected to a very sensitive force transducer (SensoNor, Horten, Norway) and an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). After adjustment of the sarcomere length to 2.3 µm, the contractile machinery was activated by transferring the preparation from the relaxing to the activating solution (the same components apart from containing Ca^{2+}-EGTA instead of EGTA). The compositions of the activating and relaxing solutions were calculated by using a previously reported approximation. [Ca^{2+}] were indicated as pCa values calculated by -lg[Ca^{2+}]. The pCa of the relaxing solution was 9.0, and that of the activating solution was pCa 4.75.

The Ca^{2+}-induced contractions of the preparations were recorded with a custom-built LABVIEW Data Acquisition (DAQ) platform and analysed with LabVIEW analyzing software (Myo; National Instruments, Austin, TX, USA) after transfer from a droplet of relaxing to a droplet of activating solution. When the Ca^{2+}-activated force production reached its maximum level (F_{max}), a quick release – restretch manoeuvre (20 ms) was applied in the activating solution. As a result of this intervention, force first dropped to zero and then started to re-develop allowing the determination of the total force (F_{total}). The Ca^{2+}-independent passive force level ($F_{passive}$) was approximated by shortening of the preparations to 80% of the original lengths in relaxing solution for 8 sec. The active isometric force (F_{active}) level was calculated as the difference between F_{total} and $F_{passive}$. Force values were normalized for the cross-sectional area determined from the width and height of the myocytes, approximating their dimensions with elliptic geometry. Values indicating the force production of the myocyte-sized preparations at a given [Ca^{2+}] (pCa 5.4 – 9.0) normalized to F_{max} were fitted to a modified Hill equation in Origin 6.0 (Microcal Software, Northampton, MA. USA) or GraphPad

Prism 5.02 (GraphPad Software, Inc., La Jolla, CA, USA): The pCa value for the half-maximum contraction indicated by Ca_{50} or pCa_{50} defines the Ca^{2+} sensitivity of force generation of the contractile machinery, while the steepness of the Ca^{2+} sensitivity curve describing the myofilamental co-operativity was also calculated and expressed as a coefficient (n_{Hill}).

In addition to the mechanical parameters of the myocyte-sized preparations, the kinetic parameters of the contractions were also assessed. The rate of isometric force production was determined by fitting the phase of isometric force generation following the Ca^{2+}-activation until the release – -restretch manoeuvre to a single exponential by means of LabView analysing software. The force re-development after the release – restretch manoeuvre was also fitted to a single exponential function in order to evaluate the rate constant of force re-development ($k_{tr,max}$). The half-time of activation ($t_{1/2}$ of activation) was determined by estimating the duration for half-maximum contraction after the preparations were placed in activating solution. The relaxation time (t_{relax}) was evaluated from the time point of the preparation being placed in relaxing solution until the baseline force level was reached.

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) followed by Dunnet's two-tailed test and paired or unpaired Student *t* test. Values are given as means ± S.E.M. Statistical significance was accepted when $P < 0.05$. Statistical analyses were carried out with GraphPad Prism software.

3.3 Analysis of the MHC isoform composition of the rat LV and diaphragm.

The MHC isoform composition of the rat diaphragm and LV was analysed by using sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. Deep-frozen muscle samples were homogenized and then boiled in sample buffer for 2 min. The adjusted final protein concentration was 0.25 mg ml^{-1}. Separate upper and lower buffers were used. The running conditions were 70 V (constant voltage) for 24 h at 4 C. The gels were stained with Coomassie Blue.

4. Results

4.1. Pharmacological characterization of the positive inotropic effect of OM

4.1.1. The effects of OM on the mechanical contractile properties of the isolated rat cardiomyocytes.

The possibility of a Ca^{2+} sensitizing effect of OM was investigated in permeabilized LV myocyte-sized preparations. Isolated and permeabilized cardiomyocytes were firstly treated with OM-free test solutions containing saturating (pCa 4.75) and submaximal (pCa 6.1) [Ca^{2+}] to determine the Ca^{2+}-activated maximum and submaximal contractile force production. The submaximal [Ca^{2+}] was chosen to achieve a contractile force of 10-15% related to the F_{max}. Then, increasing concentrations of OM were administered at the submaximal [Ca^{2+}] to determine the F_{active} and $F_{passive}$ of the cardiomyocytes.

The OM-evoked increase in F_{active} at pCa 6.1 was significant in the OM concentration range between 0.1 µM and 3 µM. The concentration – response relationship of OM was bell-shaped, with

the highest levels of Ca^{2+} sensitization between concentrations of 0.3 µM and 1 µM (F_{active} in OM-free medium = 2.74 ± 0.40 kN m^{-2} *vs.* 8.66 ± 1.32 kN m^{-2} and 8.71 ± 1.29 kN m^{-2} at 0.3 and 1 µM OM, respectively; P <0.001, n = 8 individual preparations), while no Ca^{2+}-sensitizing effect of OM was apparently exerted at 10 µM OM. The concentration – response relationship was fitted to a sigmoid function in the OM concentration range between 3 nM and 1 µM resulting in an EC_{50} of 0.08 ± 0.01 µM.

ΔpCa_{50} was assessed based on the pCa – force relationship after exposing the myocyte-sized preparations to test solutions (pCa 4.75 – pCa 9.0) containing 0.1 µM and 1 µM concentration of OM. 0.1 µM and 1 µM OM increased the Ca^{2+} sensitivity of force production suggested by a leftward shift on the pCa – force relationship. The force augmentation was pronounced in the presence of 1 µM OM at low – intermediate [Ca^{2+}] levels (*i.e.* between pCa 9.0 and pCa 6.0) referring to Ca^{2+} sensitization at diastolic Ca^{2+} levels. However, decreases in F_{max} together with force reductions at high – intermediate Ca^{2+} concentrations were also observed, when illustrating isometric force production in absolute terms. When the leftward shifts of the pCa – force relationships evoked by 0.1 µM and 1 µM OM are illustrated by their respective mean pCa_{50} values, OM increased the Ca^{2+} sensitivity of force production (pCa_{50} in the OM-free medium = 5.86 ± 0.02 *vs.* 5.97 ± 0.02 and 6.20 ± 0.03 at 0.1 and 1 µM OM; P < 0.05 and P <0.001 *vs.* the control, respectively; n = 8 individual preparations). 0.1 and 1 µM OM decreased the n_{Hill} values of the pCa – force relationships (n_{Hill} in OM-free medium = 2.56 ± 0.07 *vs.* 1.66 ± 0.06 and 0.96 ± 0.05 at 0.1 and 1 µM OM; P <0.001 in both groups *vs.* the control, respectively).

Thereafter, we investigated the OM-dependent decrease of F_{max} and the Ca^{2+} sensitization at diastolic Ca^{2+} levels in the presence of wide OM concentration range (10 nM - 10 µM). OM decreased F_{max} in a monotonous fashion, the decreases proving significant at 1 µM OM concentration or higher (F_{max} in OM-free medium = 14.75 ± 0.70 kN m^{-2} and at 1 µM OM concentration = 9.42 ± 0.97 kN m^{-2}, P <0.001 *vs.* the control; n = 8).

Ca^{2+} sensitization at diastolic Ca^{2+} levels was determined in relaxing solutions (pCa 9.0). OM increased the contractile force production between concentrations of 0.3 µM and 1 µM (F_{active} in OM-free medium ~ 0 kN m^{-2} *vs.* 1.77 ± 0.18 kN m^{-2} at 0.3 and 1 µM OM, respectively; P < 0.01; n = 6) similarly to that of observed at submaximal [Ca^{2+}].

OM increased $F_{passive}$ at 0.3 µM and higher ($F_{passive}$ in OM-free medium = 1.2 ± 0.12 kN m^{-2} and at 0.3 µM OM = 2.29 ± 0.31 kN m^{-2}; P < 0.001 *vs.* the control; EC_{50} = 0.31 ± 0.1 µM; n = 8).

4.1.2. Effects of OM on the kinetics of activation–relaxation cycles in isolated rat cardiomyocytes

To obtain a more detailed picture as concerns how OM affects the kinetics of Ca^{2+}-dependent activations and relaxations, contractile force measurements were carried out at saturating (pCa 4.75) and submaximal (pCa 6.0) [Ca^{2+}] in a wide range of OM concentrations (between 3 nM and 10 µM).

At pCa 4.75, OM decreased k_{tr} at 10 nM and higher concentration (ktr in OM-free medium: 5.70 ± 0.33 s^{-1} and 4.44 ± 0.37 s^{-1} at 10 nM OM; P <0.01 vs. the control; EC_{50}: 0.05 ± 0.01 μM; n = 8). Interestingly, OM-dependent changes of k_{tr} were biphasic at pCa 6.0, as k_{tr} increased in the presence of 0.1 μM OM from the drug-free control of 1.31 ± 0.08 s^{-1} to 1.57 ± 0.08 s^{-1} (P < 0.05 vs. the control), albeit k_{tr} decreased in the presence of 0.3 μM and higher OM concentrations (to 1.00 ± 0.11 s^{-1} at 0.3 μM OM; P <0.01 vs. the control; n = 8).

OM treatment resulted in the prolongation of duration of contractile force production. 1 μM and higher concentrations of OM increased significantly the $t_{1/2}$ of activation at pCa 4.75 ($t_{1/2}$ in OM-free conditions: 0.77 ± 0.12 s and 4.02 ± 0.56 s at 1 μM OM, P <0.001 vs. the control) and at pCa 6.0 ($t_{1/2}$ in OM-free conditions: 2.55 ± 0.19 s and 4.23 ± 0.53 s at 1 μM OM, P <0.05 vs. the control; n = 8).

Similar kinetic features were observed on the relaxation of the preparations. The relaxation was slowed following Ca^{2+}- removal from the preparations at 1 μM and higher OM concentrations at pCa 4.75 (t_{relax} in the OM-free medium: 1.19 ± 0.10 s and 3.75 ± 0.31 s at 1 μM OM; P <0.001 vs. control) and at pCa 6.0 (t_{relax} in the OM-free medium: 0.61 ± 0.06 s and 4.36 ± 0.77 s at 1 μM OM; P <0.001 vs. control; n = 8).

4.2. The effect of OM in isolated muscle fibres of rat diaphragm

A complicating factor in diaphragm muscle preparations is the composition of muscle fibres with different MHC isoforms. A mixture of abundant proteins with distinct molecular weights close to those of MHC was visualized during the SDS-PAGE of the rat diaphragm, reflecting skeletal muscle fibres of types I(β), IIx, IIb and IIa. Importantly, MHC isoform type I co-migrated with the α+β isoforms of the LV samples, indicating similar molecular weights and hence suggesting similar structures for these MHC isoforms. Although we could not extend the mechanical measurements on isolated muscle fibres to biochemical MHC isoform subtyping, the $k_{tr,max}$ parameters (measured at pCa 4.75) allowed the differentiation between muscle fibres with slow ($k_{tr,max}$ < 2 s^{-1}) and fast kinetics ($k_{tr,max}$ = 2-7 s^{-1}), most probably reflecting isoform compositions of type I(β) *vs.* type II fibres, respectively in the investigated preparations.

4.2.1. OM-dependent changes in the mechanical contractile parameters of isolated muscle fibres of rat diaphragm with slow intrinsic kinetics

The effect of OM the mechanical contractile parameters of isolated skeletal muscle fibres with slow endogenous kinetics ($k_{tr,max}$ = 1.61 ± 0.22 s^{-1}) was assessed similarly to that of investigated in cardiomyocytes. OM increased the F_{active} at pCa 6.0 significantly in the concentration range between 0.1 μM and 3 μM. The OM-dependent Ca^{2+} sensitization was characterized by an apparent EC_{50} of 0.48 ± 0.07 μM (n = 14 isolated myofibers).

The Ca^{2+}-sensitizing effect of OM was also demonstrated by comparing pCa – force relationships in diaphragm muscle preparations with low $k_{tr,max}$ (1.50 ± 0.10 s^{-1}) following exposure to 1 µM OM. OM increased the Ca^{2+} sensitivity of force production in the isolated rat diaphragm muscle fibres (pCa_{50} in OM-free controls = 5.61 ± 0.02 and at 1 µM OM = 5.85 ± 0.03; P <0.001; n = 9). This OM concentration exerted less effect on the force production at either the highest or the lowest applied [Ca^{2+}] than those in the isolated cardiomyocytes.

Although 1 µM OM had no effect on F_{max} previously, we investigated its hypothesized effects in the presence of wide concentration range (10 nM - 10 µM). 3 µM and higher OM decreased F_{max} significantly in muscle fibres with low $k_{tr,max}$ (F_{max} in OM-free medium = 21.37 ± 3.16 kN m^{-2} and at 3 µM OM = 13.26 ± 1.78 kN m^{-2}; P <0.001; *vs.* the control; n = 6).

$F_{passive}$ of the slow skeletal fibre preparations were significantly increased at 1 µM and higher OM concentrations ($F_{passive}$ in OM-free medium = 1.2 ± 0.15 kN m^{-2} and at 1 µM OM = 1.75 ± 0.42 kN m^{-2}; P <0.01 *vs.*the control; EC_{50} = 0.79 ± 0.16 µM; n = 6).

4.2.2. The effect of OM on the kinetic contractile parameters of the isolated muscle fibres with slow intrinsic kinetics

OM decreased $k_{tr,}$ both at pCa 4.75 and 6.0, however OM was more potent at pCa 4.75 (EC_{50} = 0.13 ± 0.09 µM) than that of at pCa 6.0 (EC_{50} = 0.29 ± 0.04 µM)

The kinetic features of the OM effects were also reflected by increased $t_{1/2}$ of activation and t_{relax} in diaphragm myofibres with low $k_{tr,max}$. OM significantly increased the $t_{1/2}$ of activation at 1 µM and higher concentration either at pCa 4.75 ($t_{1/2}$ of activation in OM-free medium: 5.99 ± 0.98 s and at 1 µM OM: 13.98 ± 1.46 s, P <0.001 *vs.* the control) or at pCa 6.0 ($t_{1/2}$ of activation in OM-free medium: 6.96 ± 0.86 s and at 1 µM OM: 10.50 ± 0.93 s, P <0.001 *vs.* the control). 0.3 µM and higher OM slowed the relaxation at pCa 4.75 (t_{relax} in OM-free medium: 3.15 ± 0.68 s and at 0.3 µM OM: 5.49 ± 0.81 s; P <0.05 *vs.* the control), while proving significant at 1 µM OM and higher at pCa 6.0 (t_{relax} in OM-free medium: 0.86 ± 0.09 s and at 1 µM OM: 7.26 ± 0.83 s; P <0.01 *vs.* the control; n = 11-14)

4.2.3. Mechanical and kinetic effect of OM for the isolated muscle fibres with fast intrinsic kinetics

OM exhibited a significant Ca^{2+}-sensitizing effect at 1 µM concentration and higher at pCa 5.8 (F_{active} in OM-free medium: 4.59 ± 0.99 kN m^{-2} and 6.97 ± 1.30 kN m^{-2} at 1 µM OM; P <0.05 *vs*. the control) in fast skeletal muscle preparations ($k_{tr;max}$: 4.40 ± 0.29 s^{-1}). OM-dependent changes in the F_{active} were less when compared to that of evoked in cardiomyocytes and slow skeletal muscle preparations. F_{max} of the preparations was not affected by OM.

OM treatment did not increase the $F_{passive}$ of the myocyte-sized preparations either at pCa 4.75 or at pCa 5.8.

OM decreased the k_{tr} at 1 μM concentration and higher at pCa 4.75 (EC_{50}: 1.23 ± 0.28 μM), however the decrease of the k_{tr} was abolished at pCa 5.8.

OM effect was associated with faster Ca^{2+}-contractions at pCa 5.8 ($t_{1/2}$ of activation in OM-free medium: 8.73 ± 0.70 s and at 1 μM OM: 4.92 ± 0.97 s, P <0.05 *vs*. the control), whereas the relaxation of the preparations was not affected following Ca^{2+} removal. Investigations were performed at 6 - 6 isolated and permeabilized myofibers.

4.3. Ca^{2+}-sensitizing effects of ORM-3819 in isolated and permeabilized cardiomyocytes

After characterizing the pharmacological effect of OM, we have focused on the hypothesized positive inotropic effect of the novel levosimendan-related chemical entity, ORM-3819. Isolated and permeabilized cardiomyocytes from guinea pig LV were treated at a submaximal [Ca^{2+}] (pCa 5.8) with increasing concentrations of ORM-3819 while registering the F_{active} and $F_{passive}$ of the myocyte-sized preparations. The ORM-3819-evoked increase in F_{active} was significant at 3 μM and higher OM concentration. (F_{active} in ORM-3819-free medium: 3.38 ± 0.32 kN/m^2 kN m^{-2} *vs*. 4.77 ± 0.4 kN m^{-2} at 3 μM ORM-3819, respectively; P <0.05; n = 10) and its Ca^{2+}-sensitizing effect was characterized by a half-maximal concentration (EC_{50}) of 2.88 ± 0.14 μM.

To obtain more details on the Ca^{2+}-sensitizing effect of ORM-3819, isometric force production was also assessed at maximal (pCa 4.75) and submaximal [Ca^{2+}] (i.e. between pCa 5.4 – pCa 7.0) in the absence and in the presence ORM-3819, respectively. 10 μM, 30 μM and 100 μM ORM-3819 exerted comparable Ca^{2+}-sensitizing effects, and therefore 10 μM test concentration of ORM-3819 was used to determine the drug evoked changes on the pCa – isometric force relations. Accordingly, ORM-3819 treatment resulted in a leftward shift on the pCa – isometric force relationship, when compared to that of untreated cardiomyocytes (n = 10 – 10 cardiomyocytes). ORM-3819 did affect the isometric force production neither at the maximal (i.e. pCa 4.75) nor at the lowest (i.e. pCa 7.0) applied [Ca^{2+}]. The [Ca^{2+}] required to evoke half of the maximal force production (i.e pCa_{50}) reflected the change in Ca^{2+} sensitivity of force production: i.e. it was pCa 5.69 ± 0.01 in the absence and pCa 5.81 ± 0.01 in the presence of 10 μM ORM-3819 (ΔpCa: 0.12 ± 0.01; P <0.001; n = 10). ORM-3819 did not affect the Ca^{2+}-independent $F_{passive}$ production at any applied concentration.

Thereafter, the ORM-3819-dependent changes in the activation-relaxation cycles were investigated similarly to that of carried out to assess the OM-evoked kinetic effects. Cardiomyocytes were exposed to increasing concentrations of ORM-3819 in a wide concentration range (0.3 μM – 10 μM), while assessing the drug-induced changes of the k_{tr}, $t_{1/2}$ of activation and t_{relax}. Previously, 10 μM had no effect on F_{max} and $F_{passive}$, therefore investigations were not repeated at pCa 4.75. ORM-3819 increased the k_{tr} at submaximal [Ca^{2+}] (pCa 5.8) at 10 μM and higher concentrations (k_{tr} in ORM-3819-free medium: 0.30 ± 0.05 s^{-1} and 0.49 ± 0.04 s^{-1} at 10 μM ORM-3819, P <0.05; EC_{50}: 5.34 ± 0.45 μM; n = 10). ORM-3819 seemed to accelerate the contraction of the myocyte-sized preparation,

but the evoked changes failed to prove significance. ORM-3819 had no effect on the relaxation of the preparations.

5. Discussion

This study is a pharmacological characterization of the positive inotropic effect of the myosin activator OM and the levosimendan-related ORM-3819 regarding those of mechanical and kinetic effects on the contractility of the myocyte-sized preparations.OM was demonstrated to increase the Ca^{2+} sensitivity, and $F_{passive}$, slowed the kinetics the activation – relaxation cycles of the rat cardiomyocytes while increasing force production at very low [Ca^{2+}] comparable to that of diastolic levels. OM-evoked changes in the mechanical and kinetic contractile properties were less in skeletal muscle fibres with slow intrinsic kinetics and even lesser in those with fast intrinsic kinetics than that of exerted in cardiomyocytes. ORM-3819 evoked a cTnC-dependent Ca^{2+} sensitizing effect with totally different kinetics when compared to that of OM exerted.

This study is the first quantitation of the Ca^{2+} sensitizing effect of the novel myosin activator OM in isolated and skinned cardiomyocytes. Previous studies reported on the OM-evoked changes in the Ca^{2+} sensitivity of myosin ATPase activity, although similar relationship for the OM-evoked changes in the Ca^{2+}-dependence of the contractile force production was previously not demonstrated. Hereby, 0.1 µM and 1 µM OM increased the Ca^{2+} sensitivity of force production suggested by a leftward shift on the pCa – force relationship. Considering the interaction between Ca^{2+} and the cTnC as a reference point within the contractile activation process, Ca^{2+} sensitization can be classified as those with central and downstream mechanism of action. This suggests that myosin activation can be regarded actually as a downstream Ca^{2+}-sensitizing mechanism interfering with the actin – myosin interaction. Ca^{2+} sensitizers may affect myocardial force production at a wide [Ca^{2+}] range. Nevertheless, it is hard to estimate, how much is the physiological systolic and diastolic intracellular [Ca^{2+}] because those of the continuous fluctuation by Ca^{2+} channels and pumps of the cell membrane and SR. Hence, the systolic [Ca^{2+}] may be approximated as 1 µM concentration (pCa 6.0), which decreases to 10 nM during diastole. Ca^{2+} sensitizers acting on the central pathway of positive inotropy (i.e. levosimendan) may increase force production especially at systolic Ca^{2+} levels, while those of affecting the cross-bridge cycling of the myofilaments may alter force production at systolic and diastolic [Ca^{2+}], as well. EMD-53998, a known downstream Ca^{2+} sensitizer binding to the myosin motor at the base of the lever arm located close to the proposed binding site of OM, increased the contractility of the myocyte-sized preparations at pCa 9.0 and pCa 4.75. OM also exerted robust Ca^{2+} sensitizing effects through a wide range of [Ca^{2+}], but both the extent and the direction of the evoked changes (force enhancement *vs.* force mitigation) depended on the applied OM concentration. 0.1 µM OM increased the isometric force production mainly at systolic [Ca^{2+}], while at 1 µM OM, contractile force development was also observed at very low [Ca^{2+}] (pCa 7.0 – 9.0), as suggested by the upward shift in the pCa – force relationships. Furthermore, the Ca^{2+} sensitizing effect of OM was absent at

pCa 4.75, suggesting that the available number of strongly-bound cross-bridges are maximal under this condition. Moreover, F_{max} rather tended to decline at high OM concentrations due to the excessive cross-bridge activation consistently with the abolished Ca^{2+} sensitization at submaximum [Ca^{2+}] in response of high OM concentrations. Our present data corroborate a similar relationship for the OM-evoked changes in the Ca^{2+}-dependence of the myosin ATPase activity suggested by previous studies. Accordingly, Malik at al. found increased ATPase rate at [Ca^{2+}] lesser than that of pCa 7.0 related to the isometric force increase at diastolic Ca^{2+} levels, resulting potentially in diastolic dysfunction. Additionally, OM was also demonstrated to inhibit the actin – myosin complex dissociation rate following P_i release while our results reported on increased $F_{passive}$ and relaxation time of the cardiomyocytes. The increased Ca^{2+} sensitivity of force production has been observed in advanced stages of HF. Moreover, Ca^{2+} sensitization at diastolic [Ca^{2+}] may be complicated by an already elevated cytosolic [Ca^{2+}] during diastole in HF. All of the above components may complicate ventricular relaxation during OM treatment especially at high doses. Accordingly, increases in the time constant of isovolumic relaxation (τ) and the rate of the LV pressure decrease (dP/dt_{min}) reflected impaired diastolic performance after intravenous OM administration in rats with volume overload HF. Despite of the *in vitro* mechanical and kinetic effects, OM treatment did not compromise the diastolic functions in healthy volunteers or in patients with systolic HF at plasma concentrations similar to those applied in this study (400 – 600 mg ml^{-1}/0.5 – 3 μM). It must therefore be acknowledged that extrapolation from *in vitro* data to *in vivo* conditions can be complicated by the distinct interactions of OM with the actin – myosin system in permeabilized preparations and in the structurally intact myocardium. Through the use of an isolated single-cell experimental arrangement, the contractile machinery was directly exposed to OM, in contrast with the structurally intact fibres, and hence resulting in a potentially greater extent of myosin activation at the applied concentration range than that achieved *in vivo*. Furthermore, the intracellular concentration of OM remained unknown, even complicated by its binding to the plasma proteins (82%).

Previous studies suggested that the increased ATPase rate is the major determinant for cardiotonic effect of OM. Nevertheless, more recent data showed decreased rate of the steady-state ATPase activity in response to OM, while myosin activation was only associated with increased ATP hydrolysis and P_i release rates. Acceleration of the P_i release rate is in contrast to the slowed cross-bridge cycling manifesting in increased duration of contraction, which is mainly attributable to OM-evoked decrease in the rate of the actin – myosin cycling. Anyhow, it is widely accepted that the increased P_i release and unchanged ADP dissociation by OM results in increased available number of cross-bridges during cardiac contractions without affecting the intracellular Ca^{2+} homeostasis. Recruiting of strongly-bound cross-bridges by OM may delay the inactivation of the thin filaments as Ca^{2+} level fall and hence resulting in their sustained activation paralleled by increased force generation. Increased number of strongly-attached cross-bridges may therefore lead to the prolonged activation of the contractile machinery. Hence, our kinetic data of increased $t_{1/2}$ of activation and

decreased k_{tr} corroborate the slowed *in vitro* motility of the myosin filaments and decreased contraction and relaxation velocity of the cardiomyocytes, potentially manifesting in increased systolic ejection time. Additionally, sustained thin filament activation may delay the kinetics of the force decay. Furthermore, OM decreased the ATP-initiated dissociation rate of acto-myosin. Together these changes may impair the relaxation of the myocardium, further supported by our results of increased t_{relax}. Hence, OM treatment may result in stronger, slower and prolonged cardiac contractions consistently with echocardiographic results. These results may indicate that in therapeutic concentration range, OM does not necessarily act as a myosin ATPase activator but rather is an allosteric modulator on the myosin motor.

Our study firstly demonstrated that OM increased the contractility of the isolated and permeabilized skeletal muscle fibres of the rat diaphragm. A complicating factor in diaphragm muscle preparations is the composition of muscle fibres with different slow [types I (β)] and fast (type IIa, IIx and IIb) MHC isoforms. Although we could not extend the mechanical measurements on isolated muscle fibres to biochemical MHC isoform subtyping, the $k_{tr,max}$ parameters allowed the differentiation between muscle fibres with slow ($k_{tr,max}$ < 2 s^{-1}) and fast kinetics ($k_{tr,max}$ = 2-7 s^{-1}), most probably reflecting isoform compositions of type I (β) *vs.* type II fibres, respectively in the investigated preparations. In the present study, OM exerted its Ca^{2+} sensitizing effect in the slow skeletal muscle fibres of the rat diaphragm because of co-expressing the same MHC isoform (MHC-β) than that of in the heart. Our present data additionally demonstrated that OM exerted its effect on the Ca^{2+} sensitivity of force production and on the kinetic characteristics of the slow diaphragm preparations at a higher concentration range than in isolated cardiomyocytes due to their different thin filament composition. Moreover, our data probably suggest that OM may influence the contractility of the muscle fibres with fast-skeletal MHCs, although its Ca^{2+} sensitizing potency was even lower in isolated muscle fibres with high $k_{tr,max}$ than in those developing low $k_{tr,max}$, with less effects on $F_{passive}$ production and the kinetics of activation – relaxation cycling. HF is also associated with the decreased contractility of the diaphragm attributable to its decreased Ca^{2+} sensitivity. Further, mechanical ventilation is accompanied by the impaired contractile performance of the respiratory muscles. Previous studies have revealed that levosimendan and EMD-57033 improved the contractility of the failing diaphragm through their Ca^{2+} sensitizing mechanisms. In the present study, OM exerted its Ca^{2+} sensitizing effect in the skeletal muscle fibres of the rat diaphragm. As reported on the ATOMIC-HF trial, the treatment of HF patients with OM may decrease the incidence of dyspnoea. Our present study suggests that this might be due in part to the enhanced contractility of the diaphragm resulting particularly from Ca^{2+} sensitization.

Our study also characterized the mechanism action of ORM-3819 related to its hypothesized positive inotropic effect. Due to structural similarities to that of levosimendan, ORM-3819 was also suggested to evoke a cTnC-dependent Ca^{2+} sensitization. NMR results clearly confirmed that similarly to that of levosimendan, ORM-3819 binds to the N-terminal domain of cTnC, however the binding

site of ORM-3819 to cTnC could not be located. After demonstrating the binding of ORM-3819 to the cTnC, the hypothesized Ca^{2+} sensitizing effect was investigated on isolated and permeabilized cardiomyocytes derived from guinea pigs LV in response to ORM-3819 application. Although the magnitude of the Ca^{2+} sensitizing potency of ORM-3819 was comparable to that of levosimendan (ΔpCa_{50}: ~ 0.1 pCa unit for levosimendan *vs.* ~ 0.11 pCa unit for ORM-3819), ORM-3819 exerted its Ca^{2+} sensitizing effect at higher drug concentrations than that of levosimendan (ECa_{50}: ~ 9 nM for levosimendan *vs.* ~ 3 µM for ORM-3819) Therefore, it is to be acknowledged, that although ORM-3819 may sensitize the contractile protein machinery for the activating Ca^{2+} via binding to cTnC, the higher EC_{50} for the Ca^{2+} sensitizing effect points to a relatively small affinity of ORM-3819 for cTnC-binding, when compared to that for levosimendan. Similar differences of concentration-dependence was observed, when the *in vitro* and *ex vivo* positive inotropic effect was compared. The concentration-dependence for the Ca^{2+}-sensitizing effect of ORM-3819 in isolated cardiomyocytes clearly did not overlap that obtained in Langendorff-perfused working heart preparations, where the concentration – response relationship for the positive inotropic effect of ORM-3819 was positioned leftward from that of Ca^{2+} sensitization. Therefore, our collaborating partners showed that ORM-3819 application progressively decreased the activity both of the PDE III and PDE IV isozymes. Of note, ORM-3819 appeared to be a more potent inhibitor of PDE III when compared to that of PDE IV. Structure-to-activity investigations revealed that the pyridazinone ring, also present in ORM- 3819 is a critical determinant for PDE III inhibition with the nitrogen preferably unsubstituted, which interacts directly with the catalytic domain of the PDE III isozyme. These data suggest, that ORM-3819 may possess a dual mechanism of action, at low ORM-3819 concentrations PDE III inhibition could be dominantly involved in its positive inotropic effect, while at higher ORM-3819 concentrations Ca^{2+} sensitization could also contribute. This dual mechanism of action of ORM-3819 is in contrast to that for levosimendan, which acts mainly on TnC-dependent Ca^{2+} sensitization to exert its positive inotropic effect in its therapeutic concentration range. It is to be pointed out that the PDE inhibitory effect of ORM-3819 was highly selective for PDE III. This is not the case with most other known PDE III inhibitors (i.e. enoximone and milrinone), where the selectivity ratio for PDE III vs. PDE IV inhibition is significantly lower than that for ORM-3819. The above factors may contribute to avoid the deleterious side effects of the Ca^{2+} mobilizers in case of ORM-3819 administration. Indeed, the pleiotropic effects of ORM-3819 and other less pure Ca^{2+}-sensitizing agents (i.e. levosimendan) represent a fascinating approach for the treatment of HF while avoiding the potential drawbacks of pure Ca^{2+}-sensitizers and PDE inhibitors. As potential limitations of our study, the *in vivo* PDE III inhibitory effect of ORM-3819 had to be addressed based on *in vitro* measurements in response to ORM-3819 application without determining the changes of the intracellular cAMP and phosphorylation level. Furthermore, concerning the anti-stunning effect of levosimendan, the potential effect of ORM-3819 on the opening of ATP-dependent potassium channels was not investigated in this study.

6. Summary

While Ca^{2+}-mobilizer positive inotropic agents are nowadays less favored in acute heart failure, direct activation of the cardiac sarcomere became an attractive strategy for the treatment of acute decompensation. The Ca^{2+}-sensitizer levosimendan is now widely accepted to give inotropic support for the failing heart and cardiac myosin activation by omecamtiv mecarbil (OM) is also increasingly considered as a future therapeutic agent in heart failure.

Our aim was to assess the mechanical and kinetic effects of OM and the levosimendan-related ORM-3819 on the contractile function of isolated and permeabilized cardiomyocytes. In addition, we also aimed at the clarification of the tissue selectivity of OM during contractile force measurements in skeletal muscle myofiber preparations from the diaphragm. At a given submaximal [Ca^{2+}] OM increased the Ca^{2+}-sensitivity of force production in cardiomyocytes in the presence of low and intermediate OM concentrations, but high OM concentrations did not augment force production. OM dependent Ca^{2+}-sensitization was also observed at very low [Ca^{2+}] levels mimicking diastolic conditions, and it also increased $F_{passive}$. OM decreased the rate of the actin-myosin cross-bridge cycle. Due to the above mechanic and kinetic effects, OM treatment led to strong, slow and long-lasting Ca^{2+}-contractures. OM evoked similar effects in skeletal muscle fibers with slow intrinsic kinetics, nevertheless, OM possessed lower affinity for skeletal muscle fibers than for cardiomyocytes. In fast skeletal muscle preparations, OM-evoked mechanical changes were observed only at high OM concentrations. The levosimendan-related ORM-3819 also increased the Ca^{2+}-sensitivity of force production of the cardiomyocytes, however with a different mechanism of action than OM. ORM-3819-evoked Ca^{2+}-sensitization was accompanied by strong Ca^{2+}-contractures due to increased rates of cross-bridge cycling. ORM-3819 failed to evoke Ca^{2+}-sensitization at very low [Ca^{2+}] and it did not increase $F_{passive.}$

Our data demonstrated OM as a Ca^{2+}-sensitizing agent with a downstream mechanism of action in both cardiomyocytes and diaphragmatic muscle fibers. We propose that OM should be administered with care in patients with heart failure because of its potential adverse effects on myocardial relaxation. ORM-3819 was characterized as a cTnC-dependent Ca^{2+}-sensitizer, thereby acting centrally in the contractile process. The positive inotropic effect of ORM-3819 is complemented by its selective inhibition of PDE III isoenzymes.

7. *In extenso* publications of the author

Registry number: DEENK/177/2017.PL
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Candidate: László Nagy
Neptun ID: HJJZL4
Doctoral School: Kálmán Laki Doctoral School
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List of publications related to the dissertation

1. Nagy, L., Pollesello, P., Haikala, H., Végh, Á., Sorsa, T., Levijoki, J., Szilágyi, S., Édes, I., Tóth, A., Papp, Z., Papp, G. J.: ORM-3819 promotes cardiac contractility through Ca2+ sensitization in combination with selective PDE III inhibition, a novel approach to inotropy.
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   IF: 2.73 (2015)
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   J. Cardiovasc. Pharmacol. 64 (3), 199-208, 2014.
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List of other publications

4. Nagy, L., Gödény, I., Nánási, P. P., Leskó, Á., Balogh, L., Bánhegyi, V., Bódi, B., Csípő, T., Csongrádi, A., Fülöp, G. Á., Kovács, Á., Lódi, M., Papp, Z.: A szív pozitív inotróp támogatása a miozin-aktivátor hatású omecamtiv-mecarbil segítségével.
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

5. Kovács, Á., Papp, Z., Nagy, L.: Causes and pathophysiology of heart failure with preserved ejection fraction.
   Heart Fail. Clin. 10 (3), 389-398, 2014.
   DOI: http://dx.doi.org/10.1016/j.hfc.2014.04.002
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   Exp. Clin. Cardiol. 20 (1), 2026-2035, 2014.

Total IF of journals (all publications): 11,968
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