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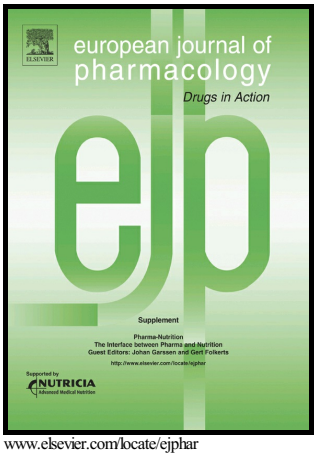

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ORM-3819 promotes cardiac contractility through Ca^{2+} sensitization in combination with selective PDE III inhibition, a novel approach to inotropy

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ABSTRACT

This study is the first pharmacological characterization of the novel chemical entity, ORM-3819 (L-6-{4-[N'-(4-Hydroxi-3-methoxy-2-nitro-benzylidene)-hydrazino]-phenyl}-5-methyl-4,5-dihydro-2H-pyridazin-3-one), focusing primarily on its cardiotonic effects. ORM-3819 binding to cardiac Troponin C (cTnC) was confirmed by nuclear magnetic resonance spectroscopy, and a selective inhibition of the phosphodiesterase III (PDE III) isozyme (IC_{50} = 3.88 ± 0.3 nM) was revealed during *in vitro* enzyme assays. The Ca^{2+}-sensitizing effect of ORM-3819 was demonstrated *in vitro* in permeabilized myocyte-sized preparations from left ventricles (LV) of guinea pig hearts (ΔpCa_{50} = 0.12 ± 0.01; EC_{50} = 2.88 ± 0.14 µM). ORM-3819 increased the maximal rate of LV pressure development ($+dP/dt_{max}$) (EC_{50} = 8.9 ± 1.7 nM) and LV systolic pressure (EC_{50} = 7.63 ± 1.74 nM) in Langendorff-perfused guinea pig hearts. Intravenous administration of ORM-3819 increased LV $+dP/dt_{max}$ (EC_{50} = 0.13 ± 0.05 µM/kg) and improved the rate of LV pressure decrease ($-dP/dt_{max}$); (EC_{50} = 0.03 ± 0.02 µM/kg) in healthy guinea pigs. In an *in vivo* dog model of myocardial stunning, ORM-3819 restored the depressed LV $+dP/dt_{max}$ and improved % segmental shortening (%SS) in the ischaemic area (to 18.8 ± 3), which was reduced after the ischaemia-reperfusion insult (from 24.1 ± 2.1 to 11.0 ± 2.4). Our data demonstrate ORM-3819 as a potent positive inotropic agent exerting its cardiotonic effect by a cTnC-dependent Ca^{2+}-sensitizing mechanism in combination with the selective inhibition of the PDE III isozyme. This dual mechanism of action results in the concentration-dependent augmentation of the contractile performance under control conditions and in the postischaemic failing myocardium.

Keywords: ORM-3819; levosimendan; Ca^{2+} sensitization; phosphodiesterase III inhibition; positive inotropic agent; myocardial stunning

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Abbrevation's list

Ca^{2+}: calcium ion

$[Ca^{2+}]$: calcium ion concentration

cTnC: cardiac troponin C

$cTnC_{cs}$: recombinant chicken cTnC with mutation of cysteine-35 to serine

DMSO: dimethyl-sulfoxide

DTT: dithiothreitol

F_{active}: active force

$F_{passive}$: passive force

F_{max}: maximum force level

F_{total}: total force level

HSQC: heteronuclear single-quantum coherence

LAD: anterior descending branch of the left coronary artery

LCX: circumflex branch of the left coronary artery

LV: left ventricle

n_{Hill}: Hill coefficient

NOE: nuclear Overhauser-effect

NOESY: nuclear Overhauser-effect spectroscopy

NMR: nuclear magnetic resonance

pCa: -log of calcium ion concentration

pCa_{50}: -log of calcium ion concentration at half-maximal isometric force production

PDE: phosphodiesterase

$+dP/dt_{max}$: peak of the first derivative of the LV pressure development

$-dP/dt_{min}$: maximal rate of LV pressure decrease

%SS: percent segment shortening

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1. INTRODUCTION

Different pharmacological approaches of positive inotropy have been considered for compensation of the weakened cardiac contractions in heart failure. Traditional inotropes mobilize intracellular calcium ions (Ca^{2+}) in cardiomyocytes to improve the contractility of the left ventricle (LV) while increasing the myocardial oxygen consumption, heart rate and the risk for arrhythmias (Endoh, 2001). LV performance can be also supported by Ca^{2+} sensitizers, designed to tune the Ca^{2+}-dependence of force production via targeting directly the contractile protein machinery, theoretically without direct interference with the Ca^{2+} homeostasis (Nagy et al., 2014).

The interaction between Ca^{2+} and cardiac troponin C (cTnC) plays a central role in the myocardial contractile regulation. Contraction is induced by a conformational change of cTnC triggered by its Ca^{2+} binding, allowing myosin to interact with actin filaments, leading ultimately to force production and cardiomyocyte shortening (Gomes et al., 2002). Drug molecules promoting cTnC function may improve the contractility of the failing heart (Endoh, 2001). Levosimendan, the (-)-enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile was discovered during Ca^{2+}-dependent affinity chromatography in the presence of cTnC (Haikala et al., 1995). Subsequent investigations revealed its binding to a hydrophobic patch formed in the regulatory domain of cTnC following a Ca^{2+}-induced conformational change (Ovaska and Taskinen, 1991; Pollesello et al., 1994). Levosimendan was shown to interact with the Ca^{2+}-bound cTnC close to its D/E linker domain of the N-terminal region (Sorsa et al., 2001; Robertson et al., 2010), thereby stabilizing the open conformation of the Ca^{2+} – cTnC complex.

Based on structure to activity relationships of known Ca^{2+} sensitizers, novel inotropes possessing selective Ca^{2+}-dependent binding to cTnC and fast pharmacokinetics *in vivo* are constantly looked for (Lindert et al., 2015). In line, ORM-3819 (L-6-{4-[N'-(4-Hydroxi-3-

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methoxy-2-nitro-benzylidene)-hydrazino]-phenyl}-5-methyl-4,5-dihydro-2H-pyridazin-3-one) was designed as a new chemical entity following *in silico* drug discovery developments (Fig. 1A).

Until now not a single Ca^{2+}-sensitizing agent reached the clinical arena as a "pure" Ca^{2+} sensitizer and additional cardiovascular effects through independent drug targets have been pinpointed. For example, levosimendan and its active metabolite, OR-1896 (the (-)-enantiomer of N-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]acetamide) (Fig. 1B and 1C) exhibit vasodilator and cardio-protective effects due to opening ATP-dependent potassium channels in vascular smooth muscle cells and in cardiomyocyte mitochondria (Kopustinskiene et al., 2001; Lepran et al., 2006; Godeny et al., 2013). The vascular effects of levosimendan and OR-1896 can be effectively antagonized by selective potassium channel blockers (Pataricza et al., 2003; Hohn et al., 2004). Moreover, highly selective phosphodiesterase III (PDE III) inhibitory potentials were also reported for the above compounds (Szilagyi et al., 2004).

ORM-3819 could have a different pattern of action in comparison with previous inotropes, deriving from its binding to cTnC and inhibition of PDE isozymes. Hereby we report on the pharmacological characterization of ORM-3819 through a wide range of *in vitro*, *ex vivo* and *in vivo* studies. To these ends, (1) first we confirmed the binding of ORM-3819 to cTnC, then we evaluated its (2) positive inotropic effects in permeabilized myocyte-sized preparations and in working heart models of non-failing and failing hearts and we also highlighted its (3) phosphodiesterase inhibitor effects.

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2. MATERIAL AND METHODS

2.1. Binding of ORM-3819 to cardiac troponin C

2.1.1. Protein samples

Recombinant chicken cTnC with mutation of cysteine-35 to serine ($cTnC_{cs}$) was used throughout this study. In structural and binding studies, the presence of cysteine-84 of cTnC in its reduced state was considered to be critical for ORM-3819 binding (similarly to that for levosimendan). Therefore, mutant cTnCs (Cys-35 to Ser) were employed to prevent disulfide formation between cysteine-35 and cysteine-84 (Sorsa et al., 2001). The expression and purification of the unlabelled, ^{15}N-, and $^{15}N/^{13}C$-labelled $cTnC_{cs}$ were achieved as described elsewhere (Finley et al., 1999). Purified $cTnC_{cs}$ was characterized by high performance liquid chromatography, mass spectometry analysis and sodium dodecylsulfate gel electrophoresis. Lyophilized proteins were solubilised in 20 mM Bis-Tris, 10 mM $CaCl_2$, and 5% of D_2O, while the pH was adjusted to 6.8 at room temperature. 1 mM dithiothreitol (DTT) was also added to the protein solution. All data were collected with fully Ca^{2+}-loaded $cTnC_{cs}$.

2.1.2. Drug samples

ORM-3819 was kindly provided and specifically ^{13}C-labelled for the nuclear Overhauser-effect spectroscopy (NOESY) by Orion Pharma (Espoo, Finland). The stock solutions of ORM-3819 were prepared by dissolving the weighted amounts of powder in deutereted dimethyl-sulfoxide (DMSO). Drug concentrations between 10 mM to 20 mM in stock solutions were used, while protected from light and stored at room temperature. Small amounts of DMSO neither affected the $cTnC_{CS}$ spectra nor the drug binding to $cTnC_{CS}$ (Li et al., 2000).

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2.1.3. Nuclear magnetic resonance (NMR) spectroscopy

All NMR experiments were acquired by Varian Inova 500, 600 or 800 MHz spectrometers at the temperature from 5 °C to 40 °C. In order to determine the binding site of the drug molecule to $cTnC_{cs}$, the following NMR spectra were recorded: one-dimensional proton spectra (1D ^{1}H spectra), 2D ^{1}H-^{13}C-, or ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC), and 2D ^{13}C-edited ^{1}H-^{1}H NOESY of the selectively labelled ORM-3819, as detailed elsewhere (Sorsa et al., 2001). Briefly, 1D ^{1}H spectra were collected to monitor the stability of ORM-3819 under various experimental conditions. The interaction of ORM-3819 with $cTnC_{CS}$ was initially followed by ^{1}H-^{15}N HSQC of cTnC, recorded at 800 MHz using 256 time increments (ni) and 16 transients (nt) and spectral widths of 11000 Hz in proton dimension and 2200 Hz in nitrogen dimension in the presence and absence of ORM-3819. Then, the temperature behaviour of the $cTnC_{CS}$/ORM-3819 complex was evaluated by 1D ^{1}H-^{13}C HSQC of specifically ^{13}C-labelled ORM-3819 and unlabelled $cTnC_{CS}$. In order to localize the exact binding sites of ORM-3819 in $cTnC_{CS}$, 2D ^{13}C-edited ^{1}H-^{1}H NOESY spectra of the $cTnC_{CS}$/selectively labelled ORM-3819 complex (ni = 256, nt = 48, spectral widths for both dimensions 10000 Hz, 800 MHz) were acquired for the drug and protein – drug samples. The ^{1}H chemical shifts were referenced to the water signal (4.62 ppm), and the ^{13}C- and ^{15}N-chemical shifts were referenced indirectly relative to 3-trimethylsilyl-propionate sodium salt.

2.2. Inhibition of phosphodiesterase (PDE) isozymes

High purity of PDE III and PDE IV isozymes were isolated from human platelets and promonocytic cell line of patients with myeloid leukaemia (U-937), respectively. The procedures of isozyme isolation and the determination of PDE activity were reported elsewhere (Weishaar et al., 1986; Torphy et al., 1992). In brief, the supernatant fraction of the tissue homogenate was added to a diethylaminoethanol-sepharose column and then eluted with a linear sodium acetate gradient buffer. Collected fractions with peak PDE activities

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were analysed for cAMP PDE activity. Purified PDE isozymes were incubated at 30 ^{o}C for 30 min in a reaction mixture containing [^{3}H]-cAMP (0.1 µM) and cAMP (0.1 µM) in the presence or in the absence of ORM-3819. The amount of [^{3}H]-5'-AMP regarded as a degradation product was determined by using liquid scintillation detection. Milrinone (IC_{50} = 0.247 µM for PDE III) and rolipram (IC_{50} = 0.11 µM for PDE IV) were used as reference compounds to verify the specificity of the PDE assay. Inhibitory assays were performed in duplicates.

2.3. Determination of the cardiotonic effect of ORM-3819

2.3.1. Chemicals

Concentrated stock solutions of ORM-3819 (10 mM) were prepared in DMSO and then stored at 4 ^{o}C for *in vitro* and *ex vivo* investigations. All test solutions containing ORM-3819 were freshly prepared on each experimental day. The final DMSO concentration of the experimental solutions never exceeded 0.1% in working heart studies and 1% in single-cell force measurements. DMSO itself did not affect the contractility of the preparations. ORM-3819 was dissolved in isotonic saline vehicle (0.9% NaCl + NaOH *q.s.*) for that of *in vivo* studies. DMSO and the other chemicals were obtained from Sigma (St. Louis, USA) and Merck (Darmstadt, Germany).

2.3.2. Animals

Investigations were performed on 8 – 15-week-old male guinea pigs, weighing 250 – 500 g and mongrel dogs of either sex with a mean body weight of 25.2 ± 0.9 kg (ordered from Toxi-Coop Toxicological Research Centre Zrt., Dunakeszi, Hungary; Mollegaard Breeding Center Ltd., Denmark and Charles River, Germany). Animals were fed a standard chow and drank tap water *ad libitum*. Animals were housed in an animal room (temperature: 10 – 20 °C; humidity: 40 – 70%; lightening: 12 h per day, 2 – 3 animals per pen/cages). Anaesthesia is detailed in the corresponding subsections of the *in vitro*, *ex vivo* and *in vivo* investigations. All

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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).

2.3.3. In vitro isometric force measurements in isolated and skinned myocyte-sized preparations

Contractile force measurements were carried out in mechanically isolated and chemically permeabilized LV cardiomyocytes of guinea pigs, as described elsewhere (Papp et al., 2004). Guinea pigs were anesthetized with an intraperitoneal injection of sodium pentobarbital (Release, Garbsen, Germany; 150 mg/kg). Hearts were quickly excised and the LVs were dissected in cold isolating solution ($MgCl_2$: 1 mM, KCl: 100 mM, EGTA: 2 mM, ATP: 4 mM, imidazole: 10 mM; pH 7.0) and then stored in -80 ^{o}C. Deep-frozen myocardial tissue samples were defrosted, mechanically disrupted and then permeabilized by incubating for 5 min in cold isolating solution supplemented with 0.5% Triton X-100. Isolated and permeabilized cardiomyocytes were then attached with silicone adhesive (DAP 100% all-purpose silicone adhesive sealant, Baltimore, USA) between two stainless insect needle, connected to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada) and a sensitive force transducer (SensoNor, Horten, Norway). After adjustment of the sarcomere length to 2.3 µm, the contractile machinery was activated by transferring the cardiomyocyte from a relaxing to activating solution. The compositions of the activating and relaxing solutions were calculated as detailed elsewhere (Fabiato and Fabiato., 1979). Relaxing solutions contained N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (100 mM), KCl (37.11 mM), $MgCl_2$ (6.41 mM), ATP (6.94 mM), creatine-phosphate (15 mM), pH 7.2. Additionally, activating solutions contained Ca^{2+}-EGTA (7 mM). The Ca^{2+} concentrations ($[Ca^{2+}]$) expressed in pCa units were estimated by -lg $[Ca^{2+}]$. The pCa of the activating and relaxing solutions was 4.75 and 9.0, respectively. Protease inhibitors were added to all

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solutions: phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (40 μM), E-64 (10 μM). Once the isometric force reached the maximum, a rapid release-restretch manoeuvre (30 ms) was applied to determine the baseline of the force generation and hence the Ca^{2+}-activated total force (F_{total}). Fitting of the force re-development phase to a single exponential following the release-restretch manoeuvre allowed the characterization of the kinetics of the actin-myosin cross-bridges (rate constant of force redevelopment in the presence of saturating Ca^{2+} concentration). Ca^{2+}-independent passive force ($F_{passive}$) of the cardiomyocytes was then measured by shortening to 80% of the original preparation length at pCa 9.0 for 8 sec. The active force (F_{active}) was calculated as a difference of the F_{total} and $F_{passive}$. Maximal activation at pCa 4.75 was used to determine the maximal Ca^{2+}-activated isometric force (F_{max}), while activations with intermediate [Ca^{2+}] (pCa 5.4 – 7.0) yielded the pCa – isometric force relationship. Isometric forces at submaximal [Ca^{2+}] normalized to F_{max} were plotted and then fitted to a modified Hill-equation (Origin 6.0, Microcal Software, Northampton, MA. USA). The midpoint of the pCa – isometric force relationship defines the Ca^{2+} sensitivity of the contractile machinery (pCa_{50}), while the steepness of the relationship indicates the co-operativity of the myofilaments (n_{Hill}). Experimental procedures were carried out at 15 ^{o}C to maintain the stability of the myocyte-sized preparations.

2.3.4. Langendorff-perfused hearts

ORM-3819-evoked changes in the LV contractility were approximated *ex vivo* by using Langendorff-perfused guinea pigs hearts, as detailed elsewhere (Szilagyi et al., 2004). Briefly, hearts of the guinea pigs, anesthetized with an intraperitoneal injection of sodium pentobarbital (Release, Garbsen, Germany; 150 mg/kg) were quickly excised, rinsed in oxygenated perfusion buffer at room temperature and cannulated through the aorta. Modified Tyrode-solution used as perfusion buffer containing NaCl (135 mM), $MgCl_2$ (1.0 mM), KCl (5.0 mM), $CaCl_2$ (2.0 mM), $NaHCO_3$ (15.0 mM), Na_2HPO_4 (1.0 mM), glucose (10.0 mM), pH

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7.3–7.4 (37 C^o) was saturated with carbogen (95% O_2 and 5% CO_2). Every experiment was carried out in the presence of constant coronary flow (10.6 ml/min) and constant heart rate (280 beats/min). Hence, aortic cannulated and retrograde perfused hearts were suspended in a temperature-controlled moist chamber of the Langendorff apparatus (Hugo Sachs Elektronik, Germany). After a short period of stabilization (10 min) the left atrium was cannulated through the pulmonary vein inserting a latex balloon (size 4) connected to a stainless steel cannula and hence to a pressure transducer to determine the isovolumetric LV pressure. The volume of the balloon was adjusted to develop a LV diastolic pressure of approximately 5 mmHg. The pressure signal was then amplified by a two-channel bridge amplifier (Type 301, Hugo Sachs Elektronik; Germany) and collected by using the Acquisition v3.1 program (Orion Pharma). The cardiac contractility was indexed by determining the magnitude (LV systolic pressure) and the positive peak of the first derivative of the LV pressure development ($+dP/dt_{max}$) (Mason et al., 1971). The hearts were then allowed to stabilize for 30 – 50 min before the first data sampling. This approach allowed the characterization the baseline of the cardiac contractility regarded as drug free control. Thereafter, ORM-3819 solved in DMSO was added to the buffer in the concentration range between 3 nM – 3 µM to determine the drug induced changes in the contractile properties of the LV.

2.3.5. In vivo characterization of the left ventricular contractility

The *in vivo* inotropic effect of ORM-3819 was studied in anaesthetized healthy guinea pigs and mongrel dogs following the induction of myocardial stunning.

Guinea-pigs were anesthetized with isoflurane (Forene, Abbott Laboratories, North Chicago, IL, USA) and placed on a temperature-controlled pad. Millar catheter pressure transducer was inserted into the LV to determine the cardiac contractility indicated by the $+dP/dt_{max}$. Additionally, the relaxation of the LV was evaluated by measuring the maximal rate of the LV pressure decrease ($-dP/dt_{max}$). After a stabilization period, incremental

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intravenous doses of ORM-3819 (0.01 – 3.0 µmol/kg) were injected into the brachial vein. Every dose was injected in 30 sec, while the amount of injected fluid was 1 ml/kg. For each dose, the maximum effect (one time point) after the bolus injection was used for evaluation.

In mongrel dogs, myocardial stunning was induced by the occlusion, then reperfusion of the anterior descending branch of the left coronary artery (LAD) in the presence of the critical constriction of the circumflex branch (LCX) of the same artery. Dogs were anaesthetized with sodium-pentobarbitone (Nembutal, Serva, 30 mg/kg intravenously), and then artificially ventilated with room air using a variable stroke Harvard ventilator, sufficient to maintain blood gases and pH within the normal range (pH: 7.4 ± 0.03; pO_2: 87 ± 3.3; pCO_2: 25 ± 2.2). Arterial blood gases were regularly checked with a blood gas analyzer (OP-216, Radelkis, Hungary). Polyethylene catheter was introduced into the LV via the left carotid artery to measure LV pressure changes in the absence and presence of ORM-3819. The $+dP/dt_{max}$ was measured by means of an electronic differentiator, implicated in the System-6 apparatus (Triton Technology, San Diego, USA). Segmental shortening was determined to assess the regional myocardial contractile performance (Theroux et al., 1974; Szigeti et al., 2004). Two pairs of piezoelectric crystals were implanted at the level of subendocardium, approximately 10 mm apart, in the minor axis of the heart to follow the regional myocardial function by sonomicrometry (Triton Technology, San Diego, USA). The crystals were inserted into the inner third of the myocardium through small stab wounds, perpendicular to the long axis of the ventricle. One pair or crystals were located in the area supplied by the LAD and the other in the area supplied by the LCX. Subendocardial segment lengths and the LV pressure were recorded simultaneously and analyzed as follows: (a) segment length at end-diastole, which was identified as the point just before the onset of the $-dP/dt_{max}$; (b) segment length at the end-systole defined at the nadir of $+dP/dt_{max}$ (as the maximum systolic excursion occurring at peak $+dP/dt_{max}$). The extent of segment shortening was calculated as a

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difference in millimetres between the segment length at end-diastole and end-systole and expressed as a percentage change (%SS) from the end-diastolic dimensions. After the surgery, the animals were allowed to stabilize for 60 min before the commencement of the study according to the protocol. Myocardial stunning was induced by a 40 min occlusion and then reperfusion of LAD in the presence of the critical constriction of the LCX. After this, both arterial bed sides were reperfused. ORM-3819 (1.5 μg/kg/min in six dogs) as well as the solvent (0.5 ml per dog/min in seven dogs) were administered intravenously over an 80 min period, starting the infusion 30 min after the commencement of the reperfusion.

2.3.6. Statistical analysis

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) followed by Dunnet's two-tailed test and the paired or unpaired Student's *t* test. Values are given as means ± S.E.M.. Statistical significance was accepted at $P < 0.05$. Statistical analyses were carried out with GraphPad Prism 5.02 software.

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3. RESULTS

3.1. Binding of ORM-3819 to cTnC

ORM-3819 was solved in a buffer solution at pH 6.8 suitable to a protein binding test (0.1 mM ORM-3819 in 20 mM Bis-Tris, 10 mM $CaCl_2$, 1 mM DTT, pH 6.8) and preliminary analysis demonstrated that the samples remained stable over several days (not shown). Nevertheless, the solubility of ORM-3819 under our experimental conditions was limited. Thus, drug concentrations used in protein binding studies were kept around 0.1 mM.

The interaction of ORM-3819 with $cTnC_{CS}$ was initially followed by ^{1}H-^{15}N HSQC assays. No resonance doublings were observed in the ORM-3819 titrations, but only chemical shift changes were detected, from the ^{1}H-^{15}N HSQC titration experiments it is clear that ORM-3819 interacts with cTnC (Fig. 2A). The interaction of ORM-3819 with $cTnC_{CS}$ involved fast kinetics on the NMR timescale with a residence time of < 0.001 sec resulting in shift changes of up to 80 Hz without any major line broadening.

The temperature dependence of $cTnC_{CS}$ – ORM-3819 complex was followed by 1D ^{1}H-^{13}C HSQC of specifically ^{13}C-labelled ORM-3819 and unlabelled $cTnC_{CS.}$ In the presence of $cTnC_{CS}$, the ^{1}H resonances of ORM-3819 became broader at lower temperatures, and eventually broadened below detection limit, which is an indication of direct interaction of the drug with $cTnC_{CS}$. If $cTnC_{CS}$ was not present, these signals remain sharp at all temperatures (Fig. 2B).

In order to localize the exact binding sites of ORM-3819 in $cTnC_{CS}$, 2D ^{13}C-edited ^{1}H-^{1}H NOESY spectra of the $cTnC_{CS}$ – ORM-3819 complex were recorded at various temperatures (10 – 40 °C). However, no specific NOE correlations between ORM-3819 and $cTnC_{CS}$ could be observed despite long acquisition times. In fact, due to the low solubility of ORM-3819, the spectral quality remained fairly modest and only intra-molecular correlations of ORM-3819 were observed (data not shown).

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3.2. PDE inhibition: the effect of ORM-3819 on the activity of PDE III and PDE IV

The PDE inhibitory effect of ORM-3819 was tested on purified PDE III and PDE IV isozymes (two experimental runs) (Fig. 3). The activity of purified PDE isozymes decreased with increasing concentrations of ORM-3819 (1 nM – 10 μM). However, ORM-3819 appeared to be a more potent inhibitor of PDE III than that of PDE IV. Half-maximal inhibition (IC_{50}) for PDE III was achieved at a drug concentration of 3.88 ± 0.3 nM, while the IC_{50} for the PDE IV inhibition was 4.95 ± 0.02 μM. Apparently, ORM-3819 did not inhibit completely PDE IV even at its 100 μM concentration. The selectivity factor of ORM-3819 for the PDE inhibition was ~ 12000.

3.3. Cardiotonic effects of ORM-3819

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

The Ca^{2+}-sensitizing effect of ORM-3819 was demonstrated in isolated and permeabilized cardiomyocytes from guinea pig LV. The averages of F_{max} and $F_{passive}$ under drug-free conditions, normalized to the cross-sectional area of the preparations were 19.07 ± 1.6 kN/m^2 and 1.08 ± 0.3 kN/m^2, respectively. These data are comparable to those reported elsewhere (Szilagyi et al., 2005).

The concentration – response relationship of the Ca^{2+} sensitization evoked by ORM-3819 was determined during repeated isometric force measurements at an individual submaximal [Ca^{2+}] (pCa 5.8) in response to increasing ORM-3819 (10 nM – 100 μM) concentrations (Fig. 4A). Of note, the water solubility of ORM-3819 might have limited reaching 100 μM concentrations, however, stable and strong biological effects were recorded hereby, suggesting that the highest concentration achieved was well above 30 μM. We, therefore, refer to the 100 μM concentration also in this study. Isometric force production expressed in absolute terms (kN/m^2) was augmented in isolated cardiomyocytes significantly at 3 μM ORM-3819 concentration and higher (F_{active} at drug free medium: 3.38 ± 0.32 kN/m^2

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vs. 4.77 ± 0.40 kN/m^2; P < 0.05), (Fig. 4A). The concentration – response relationship was characterized by a half-maximal concentration (EC_{50}) of 2.88 ± 0.14 μM and a maximal increase in the force production of ~ 101% (n = 10; 5 different animals; Fig. 4B). These data were fully comparable to those recorded at a lower submaximal [Ca^{2+}] (pCa 6.0) with a half-maximal Ca^{2+}-sensitizing effect of 2.91 ± 0.09 μM (n = 10; data not shown). ORM-3819 did not affect the Ca^{2+}-independent $F_{passive}$ production at any applied ORM-3819 concentrations.

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. As illustrated by the concentration – response relationship, 10 μM, 30 μM and 100 μM ORM-3819 exerted comparable Ca^{2+}-sensitizing effects, and therefore10 μM test concentration of ORM-3819 was used to determine the drug evoked changes on the pCa – isometric force relations. ORM-3819 exposures resulted in a leftward shift on the pCa – isometric force relationship, when compared to that of untreated cardiomyocytes (n = 10; 5 different animals, Fig. 4C). ORM-3819 did not affect the isometric force production either at the maximal (i.e. pCa 4.75) or 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 ORM-3819 evoked change in Ca^{2+}-sensitivity of force production (pCa_{50} was 5.69 ± 0.01 in the absence and 5.80 ± 0.01 in the presence of 10 μM ORM-3819; ΔpCa_{50} = 0.11 ± 0.01; P < 0.01; n = 10; 4 different animals, Fig.4D).

3.3.2. Positive inotropic effect of ORM-3819 in Langendorff-perfused hearts

The inotropic effect of ORM-3819 was further investigated *ex vivo* in isolated Langendorff-perfused guinea pig hearts (n = 5) in a concentration range between 3 nM – 3 μM. LV $+dP/dt_{max}$ was regarded here as an indicator of LV contractile function. ORM-3819 application increased $+dP/dt_{max}$ in a concentration-dependent manner (Fig. 5A), proving significant at the drug concentration of 10 nM and higher. The maximal ORM-3819 induced

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increase of $+dP/dt_{max}$ was ~ 140% relative to the baseline, while the EC_{50} value of the drug effect was 8.9 ± 1.7 nM. Improved cardiac contractility was also reflected by LV systolic pressure, reaching significance at 10 nM ORM-3819 concentration and higher (Fig. 5B.).Of note, the LV systolic pressure appeared with a similar concentration-dependence for ORM-3819 (maximal increase, relative to baseline ~ 80%, EC_{50} = 7.63 ± 1.74 nM) when compared to that of changes in the $+dP/dt_{max}$.

3.3.3. In vivo inotropic effects of ORM-3819 in anaesthetized guinea pigs and dogs

The inotropic effects of ORM-3819 were investigated *in vivo* first in anaesthetized control guinea pigs (n = 6) after intravenous infusions with increasing concentrations of ORM-3819 (10 nM/kg – 3 μM/kg). ORM-3819 improved the contractility of the non-failing myocardium indicated by increased $+dP/dt_{max}$, proving significant at the application of 0.1 μM/kg and higher with a corresponding EC_{50} of 0.13 ± 0.01 μM/kg (Fig. 6A.). In addition to the above changes of the $+dP/dt_{max}$, ORM-3819 treatment resulted in decreased $-dP/dt_{max}$ (EC_{50} = 31 ± 13 nM/kg) suggesting improved relaxation of the LV (Fig 6B).

The cardiotonic effect of ORM-3819 was further tested after intravenous administration in mongrel dogs following the induction of myocardial stunning, using LAD ligation and then release in the presence of simultaneous LCX constriction (n = 6). Basically, myocardial stunning was characterized by reduced global and regional contractile function of the LV being apparent even in the presence of restored myocardial perfusion. The depressed cardiac performance was also present after reperfusion in the animals infused with solvent instead of ORM-3819. When ORM-3819 was administered (1.5 μg/kg/min) intravenously for 80 min after the commencement of the reperfusion, the global myocardial contractile function was significantly improved. $+dP/dt_{max}$, which was reduced by 33% following the ischaemia-reperfusion insult was increased again recovering close to the normal level by the end of the infusion period (Fig. 7A.), whereas the control solvent was without effect. In line with the

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above findings, %SS reduced after the ischaemia-reperfusion insult from 24.1 ± 2.1 to 11.0 ± 2.4 in the ischemic area (supplied by the LAD) which was markedly improved following ORM-3819 administration (to 18.8 ± 3) (Fig. 7B).

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4. DISCUSSION

This study was carried out to characterize the pharmacological mechanisms underlying the positive inotropic effects of ORM-3819. The NMR spectroscopy revealed the binding of ORM-3819 to cTnC, resulting in a Ca^{2+} sensitizing effect in isolated and permeabilized cardiomyocytes *in vitro.* ORM-3819 was also reported to evoke a positive inotropic effect in Langendorff-perfused hearts *ex vivo* and in anaesthetized guinea pigs and mongrel dogs with myocardial stunning *in vivo*. The Ca^{2+}-sensitization by ORM-3819 was paralleled with a highly selective PDE III inhibitory action.

Levosimendan, an inodilator with structural similarities to ORM-3819 exerts its positive inotropic effects primarily through a Ca^{2+}-sensitizing mechanism (Papp et al., 2012). Previous NMR spectroscopy studies revealed that the Ca^{2+}-sensitizing effect is attributable to the specific interaction between levosimendan and the Ca^{2+}-saturated regulatory domain located in the N-terminal region of TnC (Levijoki et al., 2000; Sorsa et al., 2001; Sorsa et al., 2004). In this study, binding of the novel molecule, ORM-3819 to the Ca^{2+}-saturated recombinant cTnC was demonstrated similarly to that described previously for levosimendan (Sorsa et al., 2001). Our NMR results clearly showed, that ORM-3819 interferes with cTnC, manifesting in small chemical shift increases as a function of the amino acid sequence determined by ^{1}H-^{15}N HSQC. Nevertheless, no resonance doublings were observed in the ORM-3819 titrations in contrast to that of levosimendan binding (Sorsa et al., 2001), which can be potentially attributable to the differences either in the affinity of the two drugs or the time scale of the exchange reaction. Further, 1D ^{1}H-^{13}C HSQC measurements also supported the binding of the ORM-3819 to the cTnC by the broadening of the ^{1}H resonances of ORM-3819 in the presence of $cTnC_{CS}$. Nonetheless, the binding site of ORM-3819 to cTnC could not be located by direct NOE distances between the drug and cTnC, which was mainly attributable to the low solubility of the drug under protein NMR conditions. Although the

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magnitude of the TnC-dependent Ca^{2+} sensitization of ORM-3819 was comparable to that of levosimendan, ORM-3819 exerted its Ca^{2+}-sensitizing effect at higher drug concentrations when compared to above drug (EC_{50}: ~ 9 nM for levosimendan *vs.* ~ 3 µM for ORM-3819) (Lancaster and Cook, 1997; Szilagyi et al., 2005). Therefore, it is to be acknowledged, that although ORM-3819 may sensitize the contractile protein machinery for the activating Ca^{2+} via binding to TnC, 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. These data may be potentially in line with the results of the NMR spectroscopy, as well.

The concentration-dependence for the Ca^{2+}-sensitizing effect of ORM-3819 in isolated cardiomyocytes clearly did not overlap with 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. In addition to Ca^{2+} sensitization, inhibition of the PDE isozymes represents an alternative mechanism to improve the cardiac performance in heart failure (Schmitz et al., 1992). It is achieved by the cAMP-dependent increase in the protein kinase A (PKA) activity which, in turn, promotes Ca^{2+} entry through sarcolemmal L-type Ca^{2+} channels, triggering Ca^{2+}-induced Ca^{2+} release from the intracellular Ca^{2+} store, sarcoplasmic reticulum. PKA also increases contractile strength by promoting SERCA2b mediated Ca^{2+} uptake into the sarcoplasmic reticulum through the phosphorylation of its regulatory protein phospholamban (Ferrier et al., 1998; Bangash et al., 2012). In this study, ORM-3819 appeared to be a highly selective PDE III inhibitor (IC_{50} ~ 4 nM) while exerting its PDE IV inhibitor effect only at high concentrations (IC_{50} ~ 5 µM). Based on *in vitro* assays, we postulate a direct molecular interaction between ORM-3819 and PDE III or 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

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of the PDE III isozyme (Allcock et al., 2011). Previously, several studies were related to the inotropic effect of many PDE inhibitors. In isolated guinea pig hearts, the pure PDE III inhibitor, milrinone exerted significant positive inotropic effect *ex vivo,* although the magnitude of the evoked changes was lower when compared to that of ORM-3819 in our study (Abe et al., 1996; Kaheinen et al., 2004; Zausig et al., 2006). Similarly, the PDE III/IV inhibitor enoximone was also demonstrated to evoke a positive inotropic effect in isolated guinea pig hearts, however to a lesser extent when compared to levosimendan and than that observed in this study for ORM-3819 (Szilagyi et al., 2005; Zausig et al., 2006). These data suggest, that ORM-3819 may possess with 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. Since ORM-3819 is still under early development, neither its pharmacokinetic distribution nor its final free plasma concentration reached in therapy is known. For the above reasons, both mechanisms are at the moment potentially effective. 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 (Papp et al., 2012). 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 (Szilagyi et al., 2005). The above factors may explain the missing arryhthmogenic potential of ORM-3819 in dogs undergoing ischaemia and reperfusion in contrast to other PDE III inhibitors (Packer et al., 1991). Indeed, the pleiotropic effects of ORM-3819 and other less pure Ca^{2+}-sensitizating agents (i.e. levosimendan) represent a fascinating approach for the treatment of heart failure while

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avoiding the potential drawbacks of pure Ca^{2+}-sensitizers and PDE inhibitors (Pollesello et al., 2015).

Myocardial stunning is a post-ischemic reversible dysfunction of the systolic and diastolic performance of the heart, emerging from the ischaemia – reperfusion injury (Camici et al., 2008). ORM-3819 was tested and proved to be beneficial in a dog model with myocardial stunning evoked by LAD ligation in the presence of LCX constriction, promoting the recovery of the depressed global and local myocardial contractile function from the ischaemia – reperfusion injury. Since a decrease in the Ca^{2+} sensitivity of the contractile machinery is believed to contribute to the reversible contractile dysfunction after the ischaemia – reperfusion injury (Hofmann et al., 1993), it is suggested that improvement of the mechanical properties of the stunned myocardium may result from the favourable Ca^{2+}-sensitizing property of ORM-3819 (Soei et al., 1994; Sonntag et al., 2004). Furthermore, PDE III inhibitors were also reported to improve the contractile performance of the stunned myocardium (Sidi et al., 2007; Shibata et al., 2013). Hence, the dual mechanism of action of ORM-3819, involving Ca^{2+} sensitization supported by the selective inhibition of PDE III may contribute to the functional recovery following myocardial stunning.

Of note, changes in intracellular cAMP and protein kinase A dependent protein phosphorylation levels upon ORM-3819 administrations have not been addressed here, and these can be regarded as potential limitations of our investigations. Furthermore, with regards to 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. Other potential cardiovascular effects, such as central or peripheral vasodilatation have still to be studied for a full characterization of the pharmacological effects of this molecule. Nevertheless, it seemed that ORM-3819 infusion did not decrease blood pressure in our dog model with myocardial stunning. Therefore, in contrast to levosimendan, ORM-3819 might be used in

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decompensated heart failure complicated with hypotension, although the vascular effects of ORM-3819 should be further elucidated experimentally (McMurray et al., 2012).

5. CONCLUSION

Overall, this study characterized the novel chemical entity, ORM-3819 as a potent inotropic agent exerting its cardiotonic effect by using a Ca^{2+}-sensitizing mechanism further supported by the highly selective inhibition of the PDE III isozyme. This dual mechanism of action results in the concentration-dependent augmentation of the contractile performance of the failing and non-failing myocardium. Based on this recent study, TnC still appears to be fascinating for the selection of novel inotropic drug targets, although a safe inotrope needs to possess with pleiotropic effects in addition to the Ca^{2+} sensitization to compensate for the potential drawbacks of pure Ca^{2+} sensitizers and PDE inhibitors.

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CONFLICT OF INTEREST

Piero Pollesello, Jouko Levijoki, Tia Sorsa and Heimo Haikala†, employed by Orion Pharma, have been involved in the development of ORM-3819 and levosimendan. Other authors have nothing to disclose.

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9. Figure legends

Fig. 1. The chemical structure of the novel levosimendan-related chemical entity, ORM-3819 (the (-)-enantiomer of 6-{4-[N'-(4-Hydroxi-3-methoxy-2-nitro-benzylidene)-hydrazino]-phenyl}-5-methyl-4,5-dihydro-2H-pyridazin-3-one) (A); levosimendan, the (-)-enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono}propanedinitrile) (B) and its active metabolite, OR-1896 (the (-)-enantiomer of N-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide) (C).

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Fig. 2. The interaction of ORM-3819 with $cTnC_{CS}$ was initially followed by ^{1}H-^{15}N HSQC. ORM-3819 binding to Ca^{2+}-saturated cTnC gave rise to small chemical shift changes presented as a function of amino acid sequence (A). ^{1}H-^{13}C HSQC of specifically ^{13}C-labelled ORM-3819 and unlabelled $cTnC_{CS}$ illustrated the broadening of the ^{1}H-^{13}C signals of ORM-3819 implying direct interaction of the drug with cTnC. ORM-3819 signal positions are indicated by arrows (B).

Fig. 3. The inhibitory potentials of ORM-3819 on PDE III (indicated by solid black lines and circles) and PDE IV (indicated by grey lines and circles) isozymes were determined via measuring their activities in response to increasing concentration of ORM-3819 (0.1 nM – 100 μM). Means of two experimental run depicted in a function of the applied ORM-3819 concentration were fitted to a sigmoid function to evaluate the concentration – response relationship of ORM-3819 and determine the drug concentration at that of half-maximal effect (IC_{50}). Nevertheless, ORM-3819 did not reach the complete PDE IV inhibition potentially due to its scarce solubility. PDE inhibition by milrinone (PDE III) and rolipram (PDE IV) are included as reference controls.

Fig. 4. The Ca^{2+}-sensitizing effect of ORM-3819 in isolated myocyte-sized preparations of guinea pigs LV. Original force records (A) illustrated, that ORM-3819 promoted the isometric

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force generation at a submaximal [Ca^{2+}] in response to that of increasing drug concentrations. Cardiomyocyte transfers between relaxing (pCa 9.0) and activating solutions (pCa 4.75 or pCa 5.8) were accompanied by brief vertical artefacts. Drug concentrations are depicted under the individual force records, while dotted line illustrates the baseline of the isometric force production. Length and [Ca^{2+}] changes are schematically given above the first activation – relaxation cycle. Means of isometric force increases (relative to that of drug free at pCa 5.8) were fitted to a concentration – response equation to determine the ORM-3819 concentration at its half-maximum effect (EC_{50}) (B). The Ca^{2+}-sensitizing effect of ORM-3819 was quantified by evaluating pCa – isometric force relationships between pCa 7.0 and pCa 4.75 in the absence (black solid lines and circles) and presence of 10 µM ORM-3819 (dark dotted lines and open circles). Means of individual isometric force values expressed in normalized terms were fitted to a modified Hill-equation. A leftward shift on the pCa – isometric force relationships indicates the Ca^{2+} sensitization (C). The Ca^{2+}-sensitizing effect of ORM-3819 was illustrated by the means of the midpoints of the pCa – isometric force relationships (pCa_{50}) in the absence and presence of 10 µM ORM-3819 (D). Symbols illustrate means ± S.E.M, and asterisks (*) depict significant differences *vs.* the ORM-3819-free control ($P < 0.05$) in all graphs (n = 10 – 10 cardiomyocytes from 4 – 5 different animals).

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Fig. 5. The concentration-dependent cardiotonic effect of ORM-3819 was investigated in Langendorff-perfused guinea pig hearts. Values were expressed in absolute terms (mmHg) in the absence (control) and presence of ORM-3819, respectively. The positive inotropic effect of ORM-3819 was determined by assessing the drug-induced changes in the derivative of the positive peak of LV pressure development ($+dP/dt_{max}$) (A) and LV systolic pressure (B).

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Symbols illustrate means ± S.E.M., and asterisks (*) depict significant differences *vs.* the ORM-3819-free control ($P < 0.05$) in all graphs (n = 5 isolated heart preparations).

Fig. 6. *In vivo* investigations for the cardiotonic effect of ORM-3819 were carried out in anaesthetized guinea pigs after intravenous administrations of ORM-3819. The positive inotropic effect of ORM-3819 was indicated by determining the changes in rate of the LV systolic pressure development ($+dP/dt_{max}$) (A). Values were expressed in absolute terms (mmHg) in the absence (control) and presence of ORM-3819. The effect of ORM-3819 on the relaxation of the LV was characterized via determining the maximal rate of the LV systolic pressure decrease ($-dP/dt_{max}$) (B). Symbols illustrate means ± S.E.M., and asterisks (*) depict significant differences *vs*. the ORM-3819-free control ($P<0.05$) in all graphs (n = 6 animals).

Fig. 7. *In vivo* cardiotonic effect of ORM-3819 was also investigated in mongrel dogs with myocardial stunning, induced by a 40-min occlusion and then reperfusion of the anterior descending branch of the left coronary artery (LAD) in the presence of the critical constriction of the circumflex branch (LCX) of the same artery. Both ORM-3819 (solid, dark circles) and the solvent (empty circles) were administered intravenously over an 80 min period, starting the infusion 30 min after the commencement of the reperfusion. Myocardial stunning was characterized by reduced global and regional contractile function of the LV attributable to the transient ischaemia – reperfusion insult, even after restoring the myocardial perfusion. Infusion of ORM-3819 resulted in improved contractile performance of the myocardium indicated by indices of the myocardial contractility, $+dP/dt_{max}$ (A) and % segmental shortening (B) in the presence of ORM-3819 (solid circles) and solvent (empty circles). The experimental protocol is depicted above the graphs (n = 6 – 7 animals for each group). Symbols illustrate means ± S.E.M., and asterisks (*) depict significant differences between

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the data obtained after the application of ORM-3819 *vs.* the respective ORM-3819-free values of reperfusion ($P < 0.05$) in all graphs.

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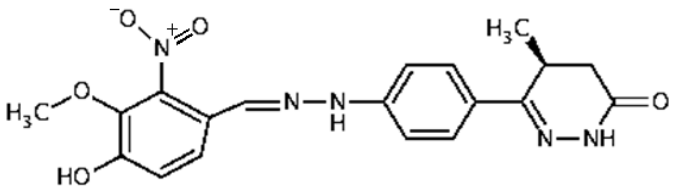
Figure

Figure 1.

A



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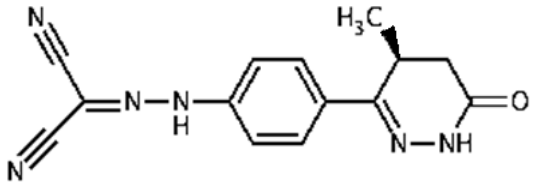

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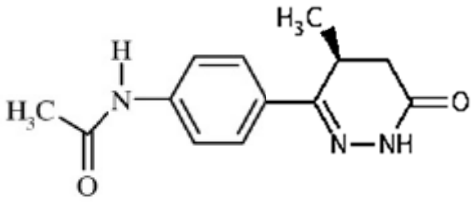

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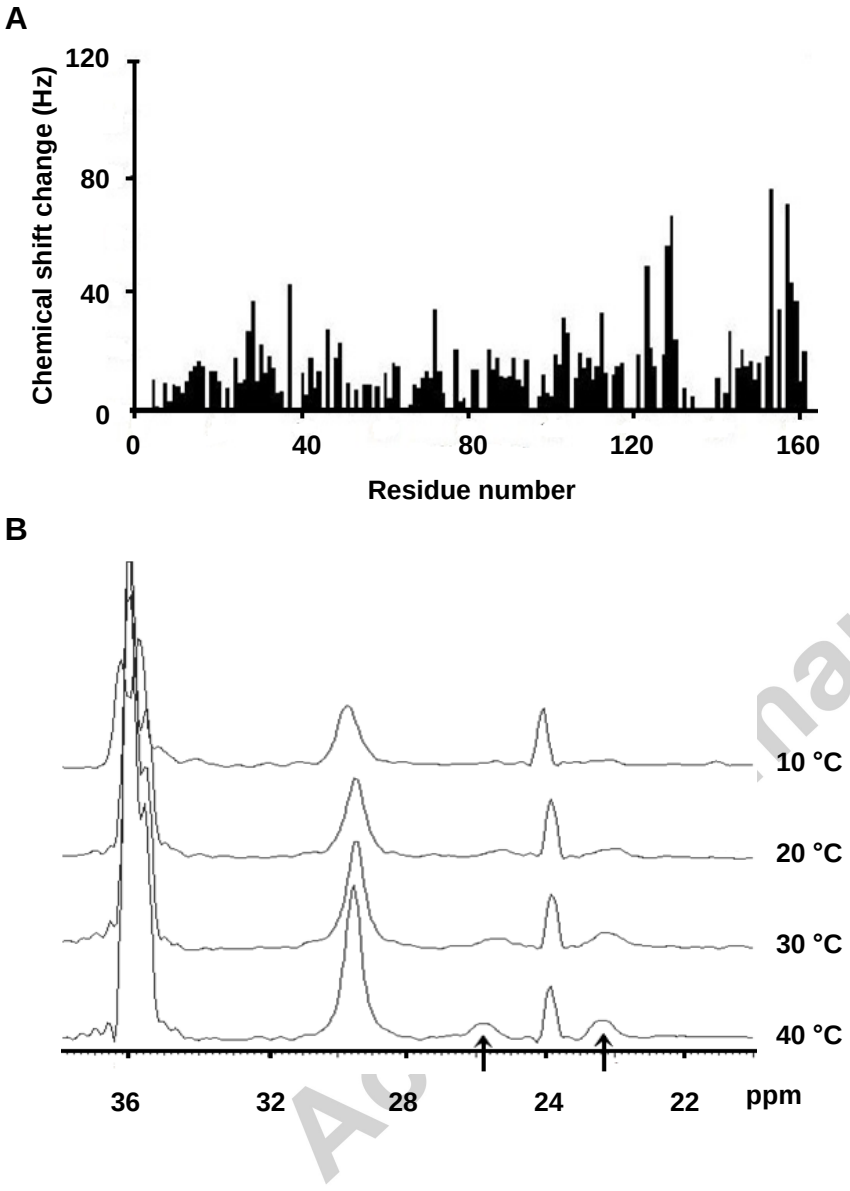

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Figure

Figure 3

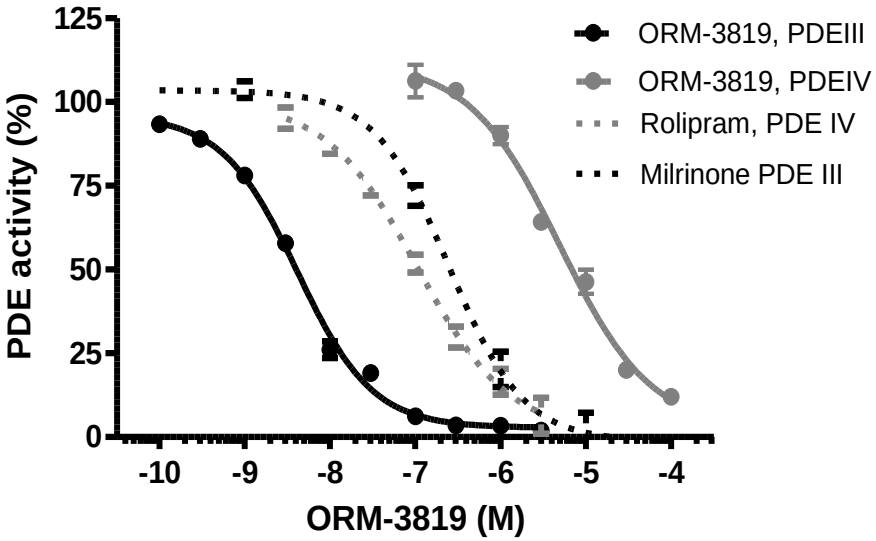

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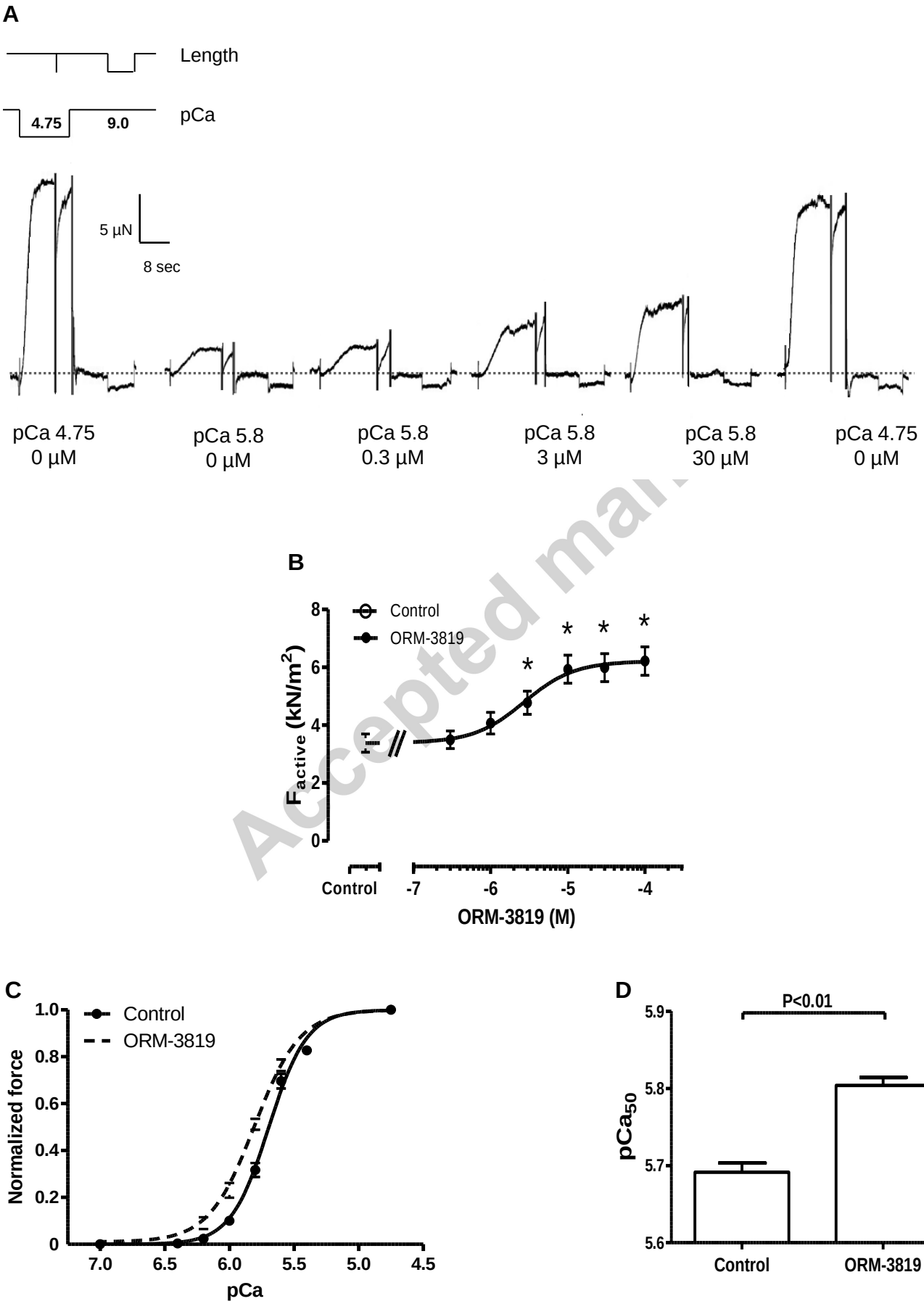
Figure 4.



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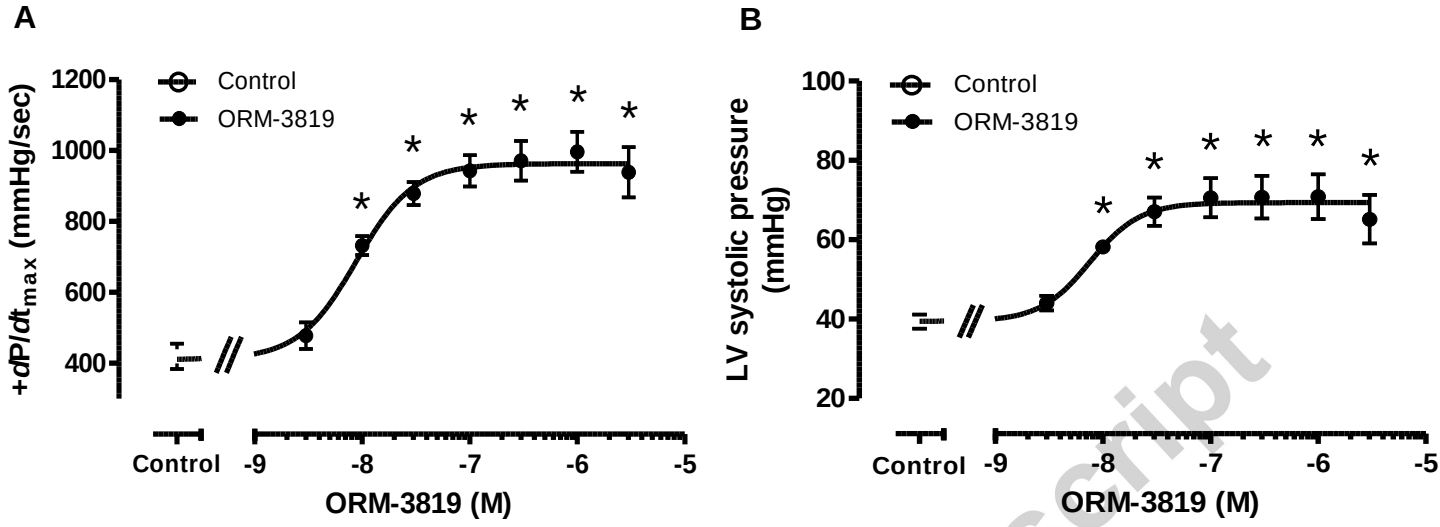

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Figure

Figure 6.

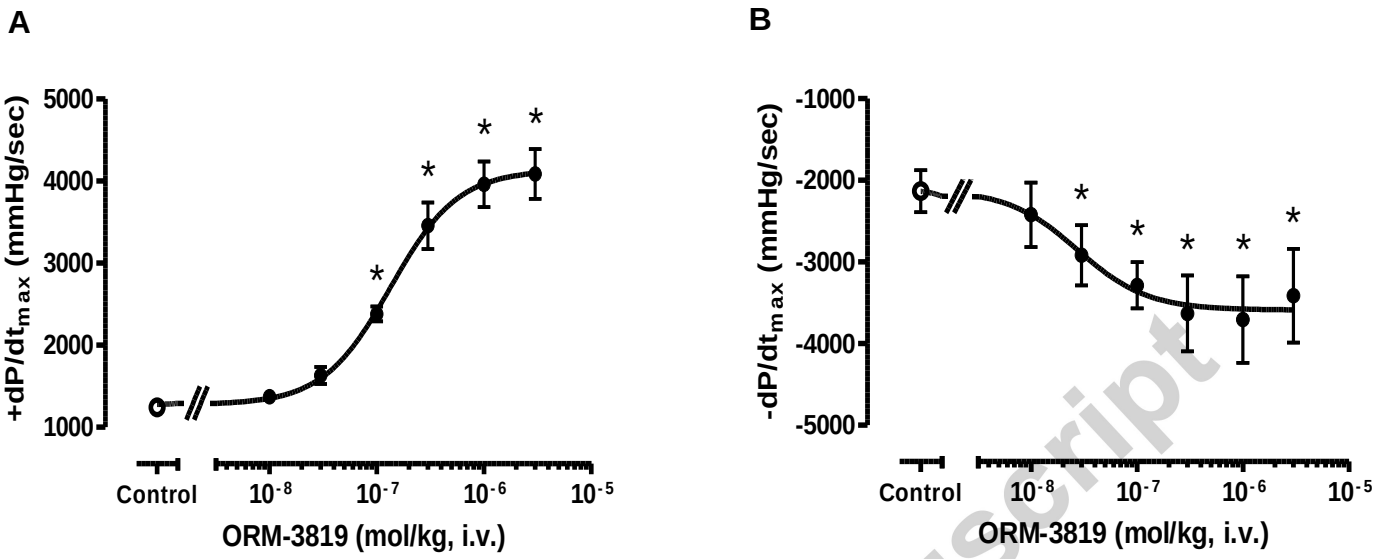

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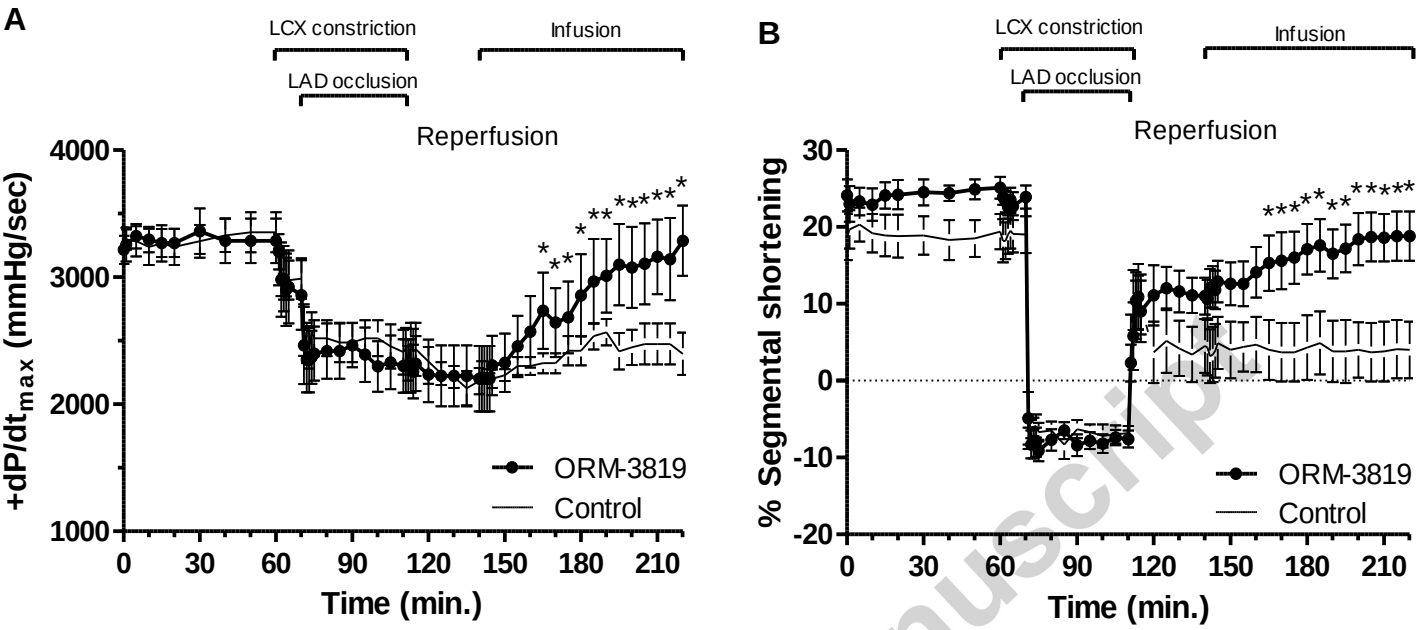
Figure
Figure 7.



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