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RESEARCH PAPER

Combined subthreshold dose inhibition of myosin light chain phosphorylation and MMP-2 activity provides cardioprotection from ischaemic/reperfusion injury in isolated rat heart

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BACKGROUND AND PURPOSE

Phosphorylation and degradation of myosin light chain 1 (MLC1) during myocardial ischaemia/reperfusion (I/R) injury is a well-established phenomenon. It has been established that MMP-2 is involved in MLC1 degradation and that this degradation is increased when MLC1 is phosphorylated. We hypothesized that simultaneous inhibition of MLC1 phosphorylation and MMP-2 activity will protect hearts from I/R injury. As phosphorylation of MLC1 and MMP-2 activity is important for normal heart function, we used a cocktail consisting combination of low (subthreshold for any protective effect alone) doses of MLC kinase, MMP-2 inhibitors and subthreshold dose of an MLC phosphatase activator.

EXPERIMENTAL APPROACH

Isolated rat hearts were subjected to 20 min of global, no-flow ischaemia and 30 min reperfusion in the absence and presence of inhibitors of MLC1 phosphorylation and degradation.

KEY RESULTS

The recovery of cardiac function was improved in a concentration-dependent manner by the MLC kinase inhibitor, ML-7 (1–5 μM), the MLC phosphatase activator, Y-27632 (0.05–1 μM) or the MMP inhibitor, doxycycline (Doxy, 1–30 μM). Co-administration of subthreshold doses of ML-7 (1 μM) and Y-27632 (0.05 μM) showed a potential synergistic effect in protecting cardiac contractility and MLC1 levels in I/R hearts. Further combination with a subthreshold concentration of Doxy (1 μM) showed additional protection that resulted in full recovery to control levels.

CONCLUSIONS AND IMPLICATIONS

The results of this study exemplify a novel low-dose multidrug approach to pharmacological prevention of reperfusion injury that will enable a reduction of unwanted side effects and/or cytotoxicity associated with currently available MMP-2 and kinase inhibiting drugs.

Abbreviations

AMPK, AMP-activated PK; CF, coronary flow; Doxy, doxycycline; I/R, ischaemia/reperfusion; LVDP, left ventricular developed pressure; MI, myocardial infarction; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ROS, reactive oxygen species; RPP, rate pressure product

Introduction

The present goal of pharmacological prevention and therapy in the course of heart disease, including myocardial infarction (MI), is to improve the oxygen supply/demand ratio of the heart (Gross and Gross, 2007; Rosamond *et al.*, 2008; Lloyd-Jones *et al.*, 2009). As no current pharmacological agent alone is capable of preventing ischaemia/reperfusion (I/R) injury, new directions in pharmacological protection and treatment of the injured MI heart are needed.

Although the molecular basis for I/R-induced myocardial injury is yet to be fully understood, recent studies suggest that myofilament protein degradation by proteolytic enzymes in response to increased reactive oxygen species (ROS) is likely to significantly contribute to cardiac contractile dysfunction. ROS have been shown to contribute to I/R injury (Yasmin *et al.*, 1997) through the activation of MMP-2 (Okamoto *et al.*, 1997; 2001; Gu *et al.*, 2002; Viappiani *et al.*, 2009) and the post-translational modification of cardiac contractile proteins, namely myosin light chains (MLCs; Kanski *et al.*, 2005a,b; Doroszko *et al.*, 2009; Polewicz *et al.*, 2011). We have previously shown that MMP-2 contributes to cardiac contractile dysfunction following I/R (Cheung *et al.*, 2000; Wang *et al.*, 2002; Sawicki *et al.*, 2005; Fert-Bober *et al.*, 2008b) and that this is most likely through the degradation of cardiac contractile proteins (Wang *et al.*, 2002; Sawicki *et al.*, 2005; Ali *et al.*, 2010). Moreover, we have shown that I/R-induced post-translational modification of MLCs lead to their increased degradation by MMP-2 (Doroszko *et al.*, 2010; Polewicz *et al.*, 2011; Cadete *et al.*, 2012).

The role of PK signalling pathways in the pathogenesis of cardiovascular diseases is well accepted (Force *et al.*, 2004). Aside from its recognized role in signal transduction, phosphorylation has been associated with the proteolytic degradation of MLC1 in isolated cardiomyocytes (Arrell *et al.*, 2001) and I/R hearts (Cadete *et al.*, 2012). Moreover, the inhibition of MLC1 phosphorylation or of MMP-2 activity results in a protection against I/R-induced cardiac contractile dysfunction (Cadete *et al.*, 2010; 2012; Lin *et al.*, 2012). These observations lead us to hypothesize that the simultaneous pharmacological reduction of post-translational phosphorylation of contractile proteins together with the pharmacological inhibition of MMP-2 activity may protect the heart from I/R injury through additive or synergistic drug effects. Furthermore, as MMPs, kinases and post-translational modifications also play important physiological functions, we propose that by combining multiple approaches, lower dosing can be used that may allow control of pathological overactivation/activity without completely disrupting physiological roles. To that end, we show that complete protection of cardiac contractile function from I/R injury can be achieved by preventing MLC1 phosphorylation and degradation simultaneously, using a combination of low concentrations (subthreshold) of MLC kinase and MMP-2 inhibitors with a low dose of an MLC phosphatase activator.

Material and methods

This investigation conforms to the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Heart preparations

Male Sprague-Dawley rats (300–350 g) were anaesthetized with sodium pentobarbital (40 $mg \cdot kg^{-1}$, i.p.). The hearts were rapidly excised and briefly rinsed by immersion in ice-cold Krebs–Henseleit buffer. Spontaneously beating hearts were placed in a water-jacketed chamber (EMKA Technologies, Paris, France) and maintained at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mmHg with modified Krebs–Henseleit buffer at 37°C containing (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11) and EDTA (0.5), and gassed continuously with 95% O_2/5% CO_2 (pH 7.4).

A water-filled latex balloon connected to a pressure transducer was inserted through an incision in the left atrium into the left ventricle through the mitral valve. The volume was adjusted to achieve an end diastolic pressure of 10 mmHg at the beginning of the perfusion and left untouched throughout perfusion. Coronary flow (CF), heart rate, and left ventricular diastolic and systolic pressures were monitored using an EMKA recording system with IOX2 software (EMKA Technologies). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate pressure product (RPP) was calculated as the product of heart rate and LVDP. Left ventricular mechanical function is determined for each experiment by expressing the RPP measured at the end of perfusion (75 min) as a percentage of the RPP measured after 15 min of aerobic perfusion (stabilization period/baseline). The recovery of RPP and CF was used to calculate the degree of protection provided by the different pharmacological approaches. Diastolic pressure changes between groups, at the end of the perfusion protocol, were used as an indicator of ischaemic injury and/or protection when drugs were used. Stock solutions (140×) of various reagents were infused into the heart via a side-port proximal to the aortic cannula at the rate 140 times lower than CF, usually 0.1 $mL \cdot min^{-1}$ with a Gilson mini pump Minipuls 3 (Gilson, Middleton, WI, USA).

I/R protocol

The scheme of the experimental protocol is shown in Figure 1. Briefly, control hearts (aerobic control, $n = 12$) were perfused aerobically for 75 min. Ischaemic hearts (I/R, $n = 9$), after 25 min at aerobic perfusion, were subjected to 20 min global no-flow ischaemia by closing the aortic inflow line, followed by 30 min of aerobic reperfusion. In three separate groups of I/R hearts ($n = 6$ each) either ML-7 [1–5 μM (Sigma, St Louis, MO, USA)], MLC kinase (MLCK) inhibitor, Y-27632 [0.05–1 μM (Sigma)], an activator of MLC phosphatase (MLCP) or doxycycline [Doxy, 1–30 μM (Sigma, Taufkirchen, Germany)], an inhibitor of MMP-2, were infused 10 min before onset of ischaemia and for the first 10 min of reperfusion. To study the possible synergistic/additive effects of these drugs, different combinations of subthreshold concentrations of ML-7 (1 μM), Y-27632 (0.05 μM) and Doxy (1 μM) were

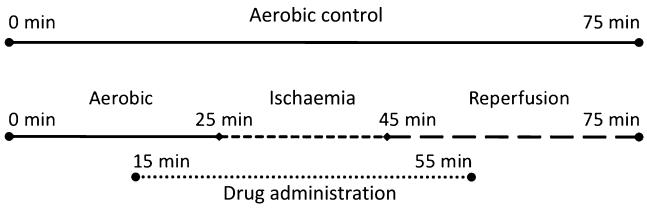

Figure 1

Schematic representation of the perfusion protocols used. Control hearts (aerobic control) were perfused aerobically for 75 min. In I/R protocols, after 25 min of aerobic perfusion (aerobic) hearts were subjected to 20 min of global no-flow ischaemia followed by 30 min of reperfusion. Infusion of the drugs started 10 min before the onset of ischaemia and was maintained during the first 10 min of reperfusion.

infused to the I/R hearts. Water was used as vehicle for Y-27632 and Doxy, while ML-7 was first solubilized in ethanol [10 mM stock solution in 50% (v/v) ethanol] and subsequently diluted in water. The maximal concentration of ethanol infused during the heart was less than or equal to 0.025% (v/v). Ethanol was 0.025% when used as a vehicle for ML-7 at 5 μM concentration. When ML-7 was infused to the heart at 1 μM or in the mixture with the other compounds, it was 0.005%. At the end of perfusion the hearts were freeze clamped in liquid nitrogen and used for biochemical studies.

Preparation of heart protein extracts

Frozen heart tissue powder was homogenized on ice in 150 mM NaCl, 50 mmol·L^{-1} Tris-HCl (pH 7.4) containing protease inhibitor cocktail (Sigma) and 0.1% Triton X-100. Homogenates were centrifuged at 10 000× *g* at 4°C for 10 min, and the supernatant was collected and stored at –80°C until use. Protein samples for two-dimensional gel electrophoresis (2-DE) were prepared by mixing frozen (–80°C), powdered heart tissue (40–60 mg wet weight) with 200 μL rehydration buffer [8 mol·L^{-1} urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 10 mmol·L^{-1} DTT, 0.2% Bio-Lytes 3/10 (Bio-Rad, Hercules, CA, USA)] at room temperature. Samples were sonicated twice for 5 s and centrifuged for 10 min at 10 000× *g* to remove any insoluble particles.

2-DE

Protein samples (0.4 mg) were applied to 11 cm immobilized linear pH gradient (5–8) strips (IPG, Bio-Rad, Hercules, CA, USA), with rehydration for 16–18 h at 20°C. For isoelectrofocusing, the Bio-Rad Protean IEF cell was used with the following conditions at 20°C with fast voltage ramping: step 1: 15 min with end voltage at 250 V; step 2: 150 min with end voltage at 8000 V; step 3: 35 000 V-hours (approximately 260 min). After isoelectrofocusing, the strips were equilibrated according to the manufacturer's instructions. The second dimension of 2-DE was then carried out with Criterion pre-cast gels (8–16%; Bio-Rad). After separation, proteins were detected with Coomassie Briliant Blue R250 (Bio-Rad).

MS

MLC1 protein spots were manually excised from the 2-DE gel. These spots were then processed using a MassPrep Station (Waters, Milford, MA, USA) using the methods supplied by the manufacturer. The excised gel fragment containing the protein spot was first destained in 200 μL of 50% acetonitrile with 50 mM ammonium bicarbonate at 37°C for 30 min. Next, the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 μL of a mixture of formic acid, water, and isopropanol (1:3:2, vol : vol). The resulting solution was then subjected to trypsin digestion and mass spectrometry analysis. For electrospray, quadruple time-of-flight (Q-TOF) analysis, 1 μL of the solution was used. LC/MS was performed on a CapLC HPLC unit (Waters) coupled with Q-TOF-2 MS (Waters). A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from MS (MS/MS) analysis were used to search against the NCBInr and SwissProt databases with Mammalia specified. We used the Mascot (http://www.matrixscience.com) search engine to search the protein database.

Immunoblot analysis

MLC1, LDH and tubulin content in heart extracts were determined by immunoblotting. Twenty micrograms of protein from each heart extract were analysed by SDS-PAGE, using 12% gels. After electrophoresis, protein was transferred to a PVDF membrane (Bio-Rad). MLC1, tubulin and LDH were identified using mouse monoclonal anti-MLC1, rabbit polyclonal anti-tubulin and goat polyclonal anti-LDH (Abcam, Cambridge, MA, USA).

Co-localization of myocardial MLC1 with myocardial MLCK or MLCP was analysed by separating protein complexes in non-reducing and non-denaturing conditions on native mini-PROTEAN TGX pre-cast gels (Bio-Rad), followed by dual-immunoblot detection. MLC1 was detected with mouse monoclonal anti-MLC1 antibody (Abcam) using goat anti-mouse IgG tagged with Alexa fluor 488 (Invitrogen, Eugene, OR, USA) as secondary antibody. MLCK and MLCP were detected with rabbit monoclonal antibody (Abcam) and rabbit polyclonal antibody (Affinity BioReagents, Golden, CO, USA), respectively, with goat anti rabbit IgG tagged with Alexa fluor 647 (Invitrogen) as a secondary antibody.

Fluorescence from immunoblot analysis was detected with VersaDoc 5000 (Bio-Rad) and band densities were measured with Quantity One software (Bio-Rad).

Statistical analysis

ANOVA or Kruskal–Wallis tests were used in functional studies (followed by Tukey's *post hoc* test) and an unpaired *t*-test was used in immunoblot analysis. Data are expressed as mean ± SEM. A $P < 0.05$ was considered statistically significant.

Results

Co-localization of MLC1 with MLCK and MLCP in I/R hearts

Co-localization of MLC1 with MLC kinase (MLCK) and MLC phosphatase (MLCP) was evaluated by separating protein complexes from heart homogenates under non–reducing and non-denaturing conditions (see methods), followed by dual-immunoblotting (Figure 2). Under these conditions two dis-

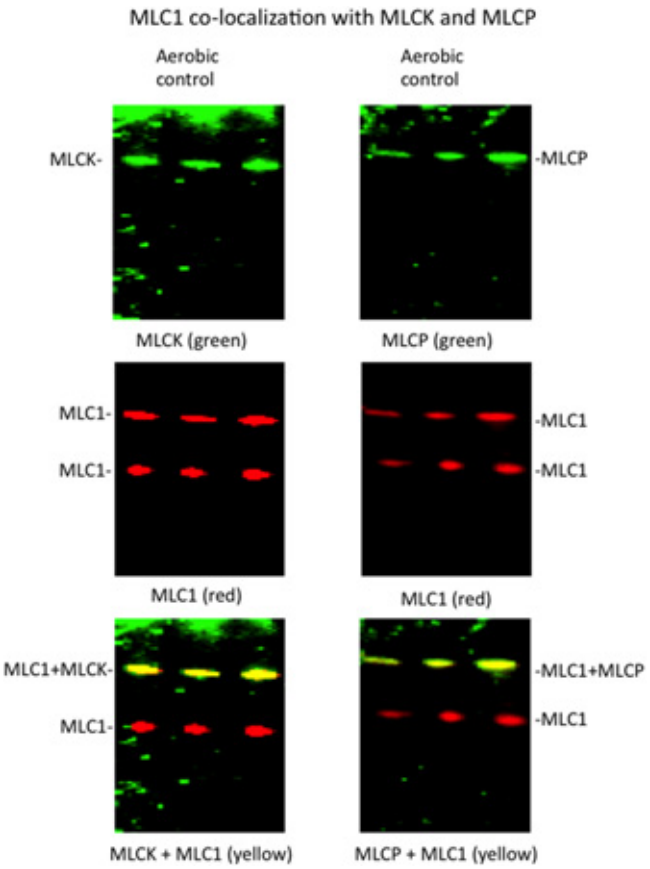

Figure 2

Co-localization of MLC1 with MLCK and MLCP in rat hearts. Co-localization of MLC1 with MLCK or MLCP was determined by using double immunoblotting under non-reducing and non-denaturing conditions to preserve protein–protein interactions. Co-localization is determined by overlap of the dual immunoblots and represented by a change in colour (yellow) when both proteins co-localize.

tinct bands for MLC1 (red colour) were observed (middle panel), while for MLCK or MLCP only one band (green colour) was seen (top panel). Overlap of both blots revealed co-localization (yellow colour) of either MLCK or MLCP with MLC1 (bottom panel).

Inhibition of MLC1 phosphorylation improves mechanical function of I/R hearts

Mechanical heart function, shown as recovery of RPP (Figure 3A and B) and CF (Table 1), was significantly decreased in hearts subjected to ischaemia followed by reperfusion, in comparison with hearts perfused aerobically for 75 min. This was accompanied by a significant increase in end-diastolic pressure (Table 1).

Pharmacological inhibition of MLCK by ML-7 resulted in increased contractile recovery during reperfusion in a concentration-dependent manner (Figure 3A). ML-7 (5 μM) resulted in an almost full recovery of mechanical function and CF of I/R heart whereas 1 μM was subthreshold for effect on heart contractility (Figure 3A), CF and diastolic pressure

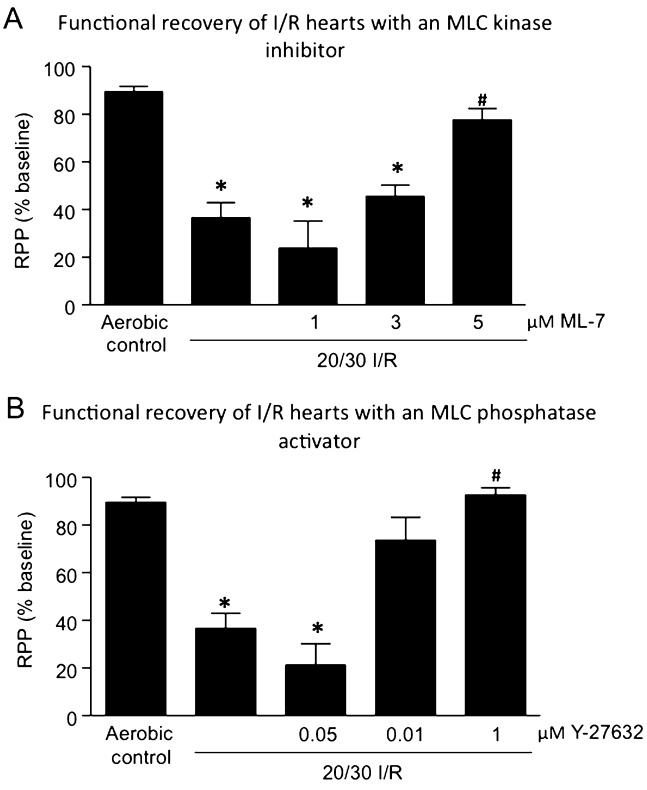

Figure 3

Cardioprotective effect of inhibition of MLC1 phosphorylation with ML-7 an inhibitor of MLCK (A) and with Y-27632 activator of MLCP (B). Isolated rat hearts were subjected to 20 min of ischaemia followed by 30 min of reperfusion in the presence or absence of ML-7 or Y-27632. Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. $^{*}P < 0.05$ versus aerobic control; $^{\#}P < 0.05$ versus I/R; $n = 6$–9 per group.

(Table 1). Infusion of ethanol at 0.025% concentration to the heart showed no effect on neither aerobically perfused hearts (controls) nor hearts subjected to I/R (data not shown).

In order to assess whether the activation of MLCP (leading to the reduction of MLC1 phosphorylation) could have a protective effect on the mechanical function in I/R hearts, we used Y-27632, an activator of MLCP through inhibition of the Rho-kinase pathway. Y-27632 protected cardiac contractility in a concentration-dependent manner (Figure 3B). Y-27632 (1 μM) resulted in the full recovery of cardiac mechanical function, whereas 0.05 μM represents a subthreshold concentration that did not improve mechanical function of I/R hearts (Figure 3B). Similarly, CF was decreased in I/R hearts and 1 μM Y-27632 protected it, whereas 0.05 μM Y-27632 did not (Table 1). End-diastolic pressure was unaltered by 0.05 μM Y-27632. Administration of 1 μM Y-27632 reduced en-diastolic pressure, in comparison with I/R, but not to aerobic levels (Table 1).

Role of kinase inhibition and phosphatase activation in I/R heart functional recovery

Potential synergistic or additive effects of ML-7 and Y-27632 were determined by using subthreshold concentrations of

Table 1

Average coronary flow and end-diastolic pressures at the end of perfusion in the isolated perfused rat hearts

	Type of perfusion							
	Aerobic		I/R (20/30)					
Compound	–	–	ML-7		Y-27632		Doxy	
Concentration (μM)	–	–	1	5	0.05	1	1	30
n	9	9	7	9	7	6	9	9
Coronary flow (mL·min^{-1})	17.30	6.267*	4.029*	15.44$^{\#}$	6.671*	14.83$^{\#}$	6.111*	15.18$^{\#}$
SEM	± 0.5548	± 1.452	± 1.587	± 0.8577	± 2.149	± 0.5578	± 1.404	± 1.033
End-diastolic pressure (mmHg)	12.11	41.67*	28.44*	11.00$^{\#}$	28.78	21.44*,$^{\#}$	33.33*	22.33*,$^{\#}$
SEM	± 0.5386	± 6.205	± 4.451	± 0.7638	± 5.449	± 1.634	± 3.329	± 2.421
Compound	Y-27632 + ML-7		Y-27632 + Doxy		ML-7 + Doxy		Y-27632 + ML-7 + Doxy	
Concentration (μM)	0.05 + 1		0.05 + 1		1 + 1		0.05 + 1 + 1	
N	9		9		9		9	
Coronary flow (mL·min^{-1})	9.667*		10.67*		10.99*		15.69$^{\#}$	
SEM	± 1.936		± 1.785		± 1.787		± 0.3638	
End-diastolic pressure (mmHg)	18.89*,$^{\#}$		35.33*		31.11*		15.89$^{\#}$	
SEM	± 1.720		± 6.526		± 4.931		± 2.150	

*$P < 0.05$ versus aerobic; $^{\#}P < 0.05$ versus I/R.

both drugs and evaluated in terms of their protection of mechanical function and preservation of MLC1 levels in hearts subjected to I/R (Figure 4). Co-administration of Y-27632 (0.05 μM) and ML-7 (1 μM) at subthreshold doses, that have no protective effect when administered alone, resulted in approximately 60% recovery of cardiac function during reperfusion (Figure 4A). Similarly, an increase of CF (not significant, however) was observed when both drugs were co-administrated at subthreshold concentrations (Table 1). This was accompanied by a decrease in end-diastolic pressure (Table 1). Furthermore, co-administration of these two drugs at subthreshold concentrations protected MLC1 from degradation in I/R hearts (Figure 4B).

Effect of an MMP-2 inhibitor and inhibitors of MLC1 phosphorylation on recovery of mechanical function in I/R hearts

Because Doxy is known for its inhibition of MMPs (Cheung *et al.*, 2000; Fert-Bober *et al.*, 2008b) and improving cardiac functional recovery following I/R in a concentration-dependent manner (Figure 5A), we used this drug in our studies as an inhibitor of MMP-2. A subthreshold concentration of Doxy (1 μM) did not protect cardiac contractility, whereas 30 μM showed more than 70% protection of function as well as abolishment of the degradation of MLC1 in I/R hearts (Figure 5A and B). Doxy produced a similar result on CF (Table 1). End-diastolic pressure was reduced, in comparison with I/R, only with 1 μM of Doxy (Table 1).

Administration of a mixture containing subthreshold concentrations of Doxy (1 μM) and ML-7 (1 μM) showed a protective effect on cardiac contractility of approximately 60% (Figure 6A). Similarly, administration of a mixture of subthreshold concentrations of Doxy (1 μM) and Y-26732 (0.05 μM) also showed protection of cardiac contractility (~50%, Figure 6B), but failed to protect diastolic function (Table 1). Although not significant, CF showed an increasing trend for both combinations (Table 1). When used separately, subthreshold concentrations of these inhibitors did not protect MLC1 from degradation during I/R (Figure 6C).

Although complete recovery of cardiac contractility was never obtained using subthreshold doses of any combination of two pharmacological agents, combination of all three (Doxy, ML-7 and Y-27632) at subthreshold concentrations did (Figure 7A). The protection of contractile function by combining subthreshold concentrations of these three drugs was also associated with a complete preservation of MLC1 protein levels (Figure 7B). Furthermore, complete protection (more than 90% restoration of flow) of CF and diastolic function was observed when the three drugs were co-administrated at subthreshold concentrations (Table 1).

In addition to improvement of contractility and CF, LDH (marker of heart injury; twofold increased in I/R hearts) was normalized to pre-ischaemic levels by co-administration of subthreshold concentrations of Doxy, ML-7 and Y-27632 (Figure 7C).

Effect of co-administration of subthreshold concentrations of phosphorylation inhibitors on MLC1 phosphorylation

The MLC1 from I/R hearts treated with ML-7, an inhibitor of MLCK, or Y-27632, an activator of MLCP, was analysed first by 2-DE (protein separation) and next MS for detection of phosphorylation. As shown in Table 2, ML-7 (5 μM) inhibited phosphorylation of T69, T164, S184 and Y190. A similar

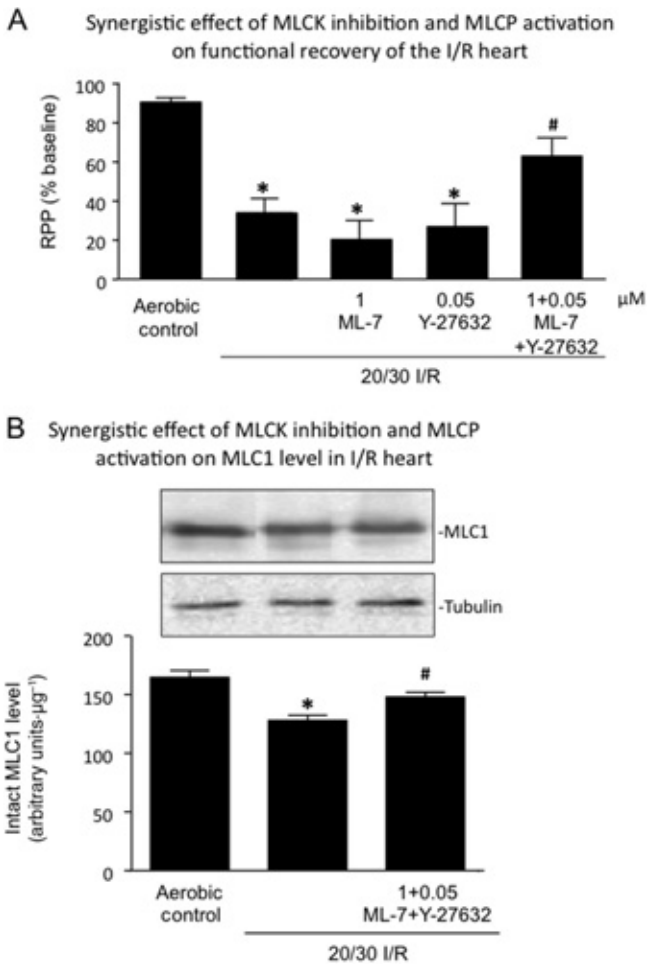

Figure 4

Effect of action between two different inhibitors of MLC1 phosphorylation on contractile function recovery (A) and MLC1 protein levels (B). Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. *$P < 0.05$ versus aerobic control; $^{\#}P < 0.05$ versus I/R; $n = 6$–9 per group in functional study, $n = 3$ per group in immunoblot analysis.

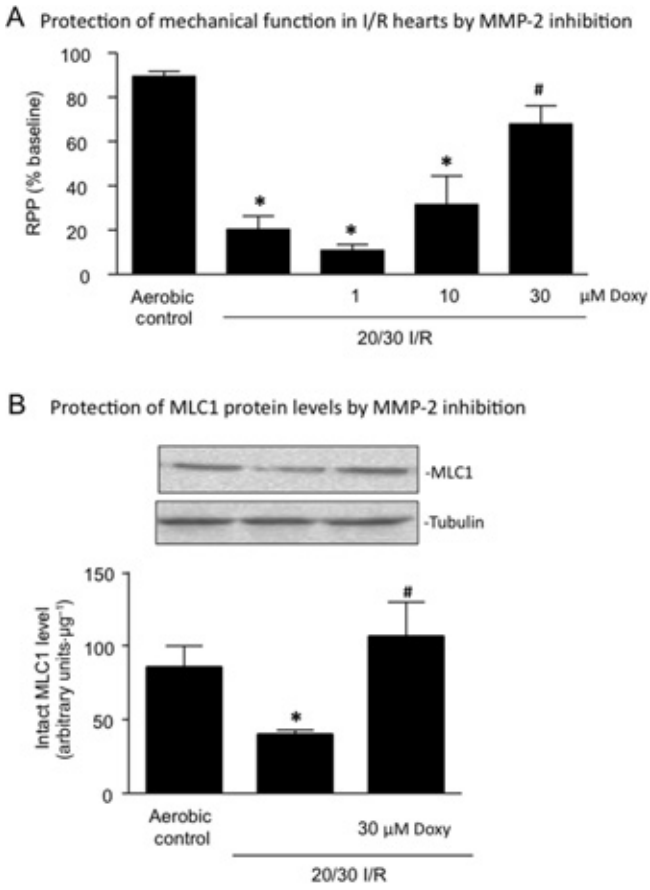

Figure 5

Cardioprotection by inhibition of MMP-2 with Doxy. (A) Concentration–response histogram of Doxy on contractile function recovery. (B) Protection of MLC1 protein levels by the MMP-2 inhibitor Doxy. Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. *$P < 0.05$ versus aerobic control; $^{\#}P < 0.05$ versus I/R; $n = 6$–9 per group for functional study, $n = 3$ per group in immunoblot analysis.

phosphorylation pattern was observed in the MLC1 from I/R hearts treated with Y-27632 (1 μM). Additionally, Y-27632 inhibited phosphorylation of T77/Y78, whereas ML-7 did not (Table 2). Co-administration of subthreshold concentrations of ML-7 and Y-27632 (1 and 0.05 μM, respectively) inhibited phosphorylation of MLC1 similarly to the pattern visible for full protective doses of ML-7 and Y-27632 alone (Table 2).

Discussion

The development of new therapeutic strategies aimed at preventing and treating I/R injury are needed. Here we show a new and promising therapeutic strategy for the prevention of I/R injury. The use of low (subthreshold) concentrations of drugs that prevent both phosphorylation and degradation of MLC1 showed a complete cardioprotective effect in I/R injury in an isolated rat heart model. As these drugs are used at low subthreshold concentrations, toxicity and drug–drug interactions should also be reduced. Furthermore, as complete antagonism is likely not achieved, non-pathological processes may be partially intact for improved functional outcomes. Hence, this cocktail approach can prove to be a valid strategy for prevention and protection of the heart against I/R injury.

It has been previously shown that MLC1 is phosphorylated in response to oxidative stress (Arrell *et al.*, 2001; 2006; Cadete *et al.*, 2012). We have recently shown that, in a model of I/R injury, MLC1 phosphorylation is MLCK dependent and results in increased degradation of MLC1 by MMP-2 (Cadete *et al.*, 2012). The loss of MLC1 by the myocardium is associated with a deterioration of cardiac contractile function in I/R injury, and its presence in the serum has been validated as a good marker for predicting the prognosis of acute MI patients (Yamada *et al.*, 1998). Consistent with this, our model of I/R

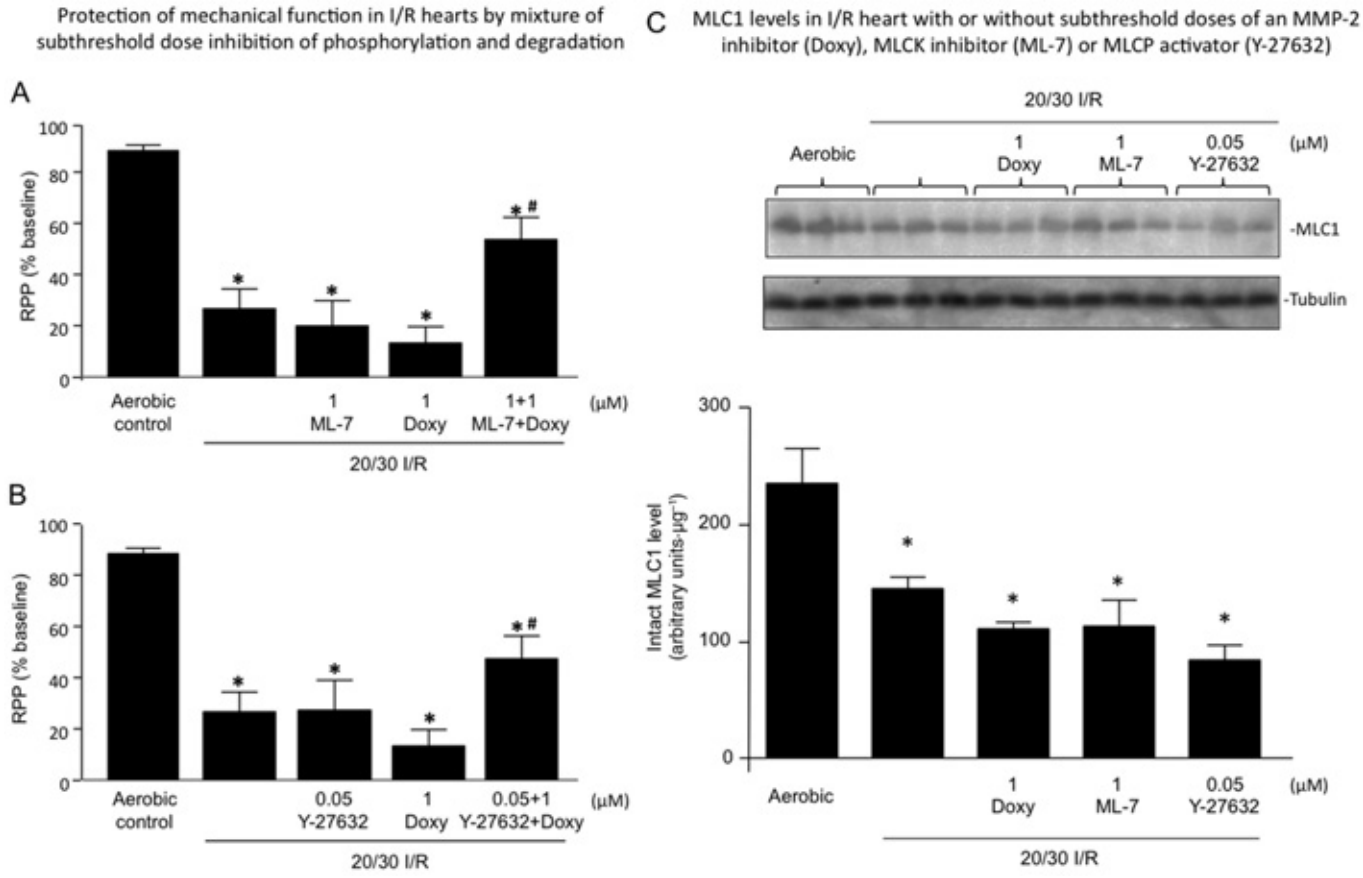

Figure 6

Effect of multidrug administration on recovery of the mechanical function of I/R hearts and MLC1 protein levels. (A) Synergystic/additive effect between inhibition of MLCK and MMP-2 with subthreshold concentrations of ML-7 and Doxy on the protection of contractile function recovery. (B) Synergystic/additive effect between stimulation of MLCP and inhibition of MMP-2 with subthreshold concentrations of Y-27632 and Doxy on the protection of contractile function recovery. (C) MLC1 protein levels measured by immunoblotting in hearts subjected to I/R in the presence or absence of subthreshold concentrations of ML-7, Y-27632 and Doxy. Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. *$P < 0.05$ versus aerobic control; $^{\#}P < 0.05$ versus I/R; $n = 6–9$ per group, $n = 3$ per group in immunoblot analysis.

Table 2

Phosphorylation of MLC1 from rat hearts subjected to I/R and perfused in the presence of high doses of ML-7 or Y-27635 or with a mixture of low doses of ML-7 and Y-27632

Identified phosphorylation in myocardial MLC1				
	I/R heart (20/30)			
Aerobic heart		**+ML-7 (5 μM)**	**+Y-27632 (1 μM)**	**+ML-7 (1 μM) + Y-27632 (0.05 μM)**
T69	T69	n/d	n/d	n/d
n/d	T77 or Y78	T77 or Y78	n/d	T77 or Y78
T132 orT134 or T135	T132 orT134 or T135	T132 orT134 or T135	T132 orT134 or T135	T132 orT134 or T135
n/d	T164	n/d	n/d	n/d
n/d	S184	n/d	n/d	n/d
n/d	Y190	n/d	n/d	n/d

T, threonine; Y, tyrosine; S, serine; n/d, not detected.

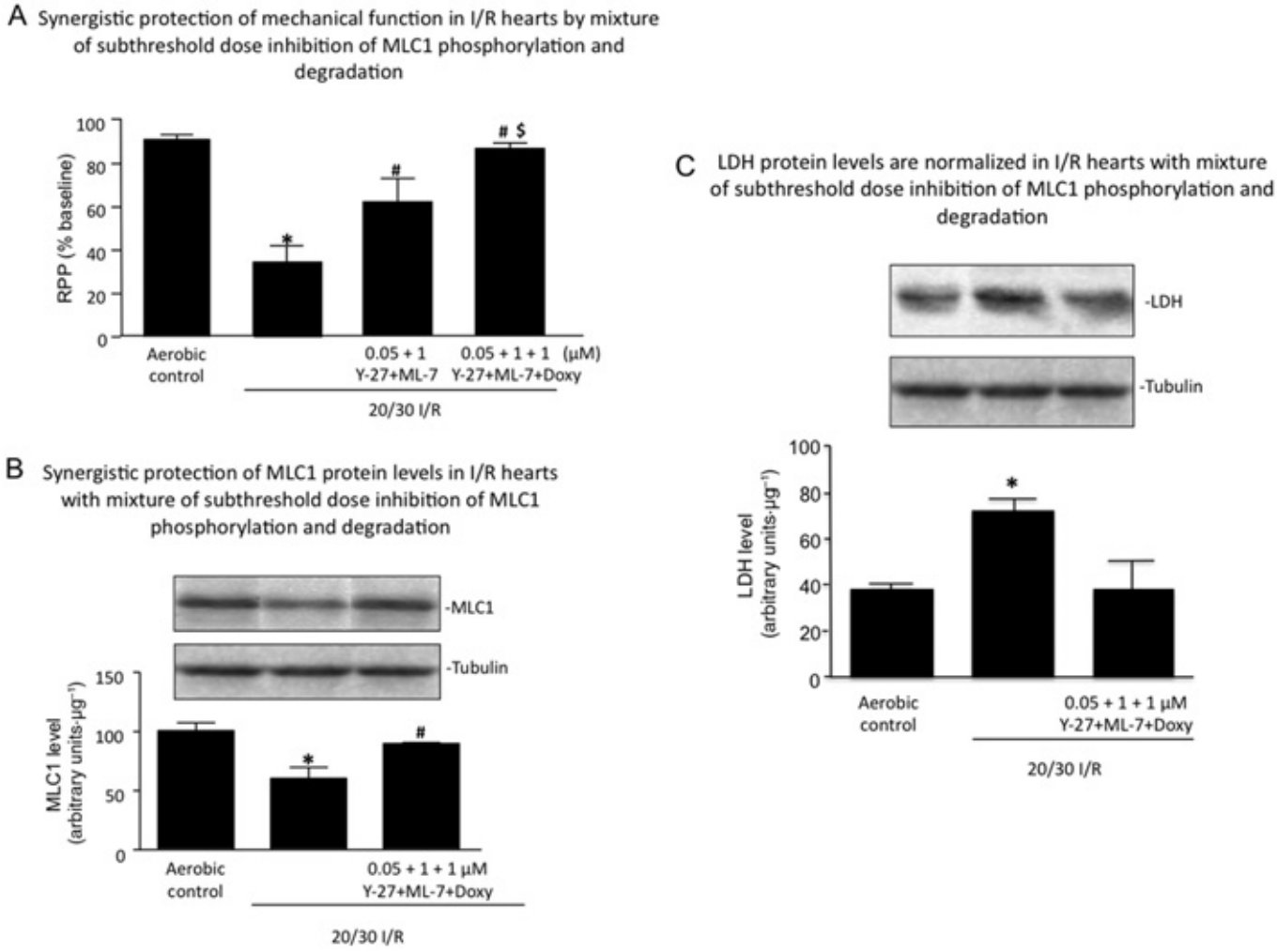

Figure 7

Effect of action between two different inhibitors of MLC1 phosphorylation and an inhibitor of MMP-2 activity on contractile function recovery (A) and MLC1 protein levels (B). (A) Comparison of synergistic/additive effects of inhibition of MLC1 phosphorylation (with subthreshold concentrations of ML-7 and Y-27632) and inhibition of MLC1 phosphorylation together with MMP-2 activity (with additional subthreshold concentration Doxy) on the protection of contractile function recovery. (B) Synergystic/additive effect between inhibition of MLC1 phosphorylation (with ML-7 and Y-27632) and MMP-2 activity with subthreshold concentrations of ML-7, Y-27632 and Doxy on the protection of MLC1 protein levels ($n = 3$). Percent recovery was calculated based on RPP during the first 25 min of the perfusion protocol. $^{*}P < 0.05$ versus aerobic control; $^{\#}P < 0.05$ versus I/R; $n = 6$–9 per group, $n = 3$ per group in immunoblot analysis.

injury has consistently shown loss of MLC1 from myocardium, which was detected in the effluent (Sawicki *et al.*, 2005; Cadete *et al.*, 2012). These observations are consistent with previous reports in which MLC1 has been validated as a serum marker of myocardial injury (Ravkilde *et al.*, 1994; Omura *et al.*, 1995). Additionally, we have shown that creatine kinase (CK) is increased (200-fold) in our I/R injury model and can be rescued with MMP-2 inhibition (Fert-Bober *et al.*, 2008b). Although detectable, samples must be concentrated hundreds of times before analysis making this impractical as a routine measure. On the other hand, LDH is readily measureable as a marker of tissue injury and routinely performed showing near complete normalization with the cocktail treatment. In our model, we show that LDH protein levels are significantly increased with I/R. Although these results might appear counter-intuitive (one would expect LDH to be decreased with I/R because higher levels are detected in the plasma or perfusates) they are in line with many reports using this perfusion model (Fert-Bober *et al.*, 2008a,b; Cadete *et al.*, 2010). LDH levels are increased both in the tissue and in the perfusates of hearts subjected to I/R. The increase in LDH levels in the myocradial tissue might reflect a metabolic adaptation in response to I/R as previously suggested (Cadete *et al.*, 2010).

As phosphorylation of MLC1 is involved in triggering MMP–2-mediated degradation (Cadete *et al.*, 2012), inhibition of MLC1 phosphorylation is a viable target for protection of the heart. The phosphorylation status of any protein

can be affected by targeting the corresponding kinase or phosphatase. With respect to MLC1, MLCK has been shown to phosphorylate MLC1 both *in vitro* and *ex vivo* (Arrell *et al.*, 2001; Cadete *et al.*, 2012), although its primary described target is MLC2 (Sweeney and Stull, 1986; Mizuno *et al.*, 2008; Stull *et al.*, 2011). MLC phosphatase (MLCP) is the enzyme responsible for counteracting the actions of MLCK in terms of MLC2 phosphorylation status (Cole and Welsh, 2011). Here we show that in heart protein extracts, both MLCK and MLCP co-localize with MLC1. Moreover, selective inhibition of MLCK with ML-7 or stimulation of MLCP with the Rho-kinase inhibitor Y-27632 can result in almost full recovery of cardiac contractile function, with a concomitant reduction in MLC1 degradation. It is then plausible to speculate that, in the heart, MLCK and MLCP are responsible for the regulation of MLC1 phosphorylation status under both physiological and pathological states. Indeed, our previous study (Cadete *et al.*, 2012) has shown that in aerobically perfused hearts, MLC1 is phosphorylated by MLCK at position T69, suggesting a role for MLC1 phosphorylation by MLCK in the regulation of MLC1 function. This previous study has also reported a phosphorylation site within MLC1 that can be attributed to either PKC or the AMP-activated PK (AMPK). Interestingly, the measurement of the phosphorylation status of MLC1 in I/R hearts revealed four additional phosphorylation sites that were abolished when hearts were perfused in the presence of the MLCK inhibitor ML-7 (Cadete *et al.*, 2012). These data strongly support our pharmacological approach of using low (subthreshold) concentrations of ML-7 to target the pathological fraction of MLCK activity triggered by I/R.

In our concentration–response studies, we identified the subthreshold concentration for ML-7 being 1 μM and for Y-27632 being 0.05 μM. Used individually, these doses show no protection of cardiac contractile function. Although no changes in total MLC1 protein levels were observed in hearts subjected to I/R in the presence of subthreshold concentrations of any of the drugs given alone, we believe that at these concentrations we are targeting the pathological component of MLC1 phosphorylation. In support of this, when both drugs are administered together to hearts undergoing an I/R protocol, a significant recovery of cardiac contractile function is observed. Additionally, MS data showed that co-administration of the mixture of ML-7 and Y-27632 at subthreshold concentrations inhibited phosphorylation of MLC1 similarly to the inhibition achieved by full doses of ML-7 and Y-27632.

Post-translational modification of MLC1 has previously been shown to increase affinity for, and degradation by, MMP-2 in I/R injured hearts (Sawicki *et al.*, 2005; Doroszko *et al.*, 2009; Polewicz *et al.*, 2011; Cadete *et al.*, 2012). Inhibition of MLC1 phosphorylation with subthreshold concentrations of ML-7 and Y-27632 showed an improvement in contractile function during reperfusion, but did not confer full protection. This was likely due to the fact that the subsequent step of MLC1 degradation by MMP-2 was not inhibited. MMP-2 activity can be inhibited using the broad sprectrum MMP inhibitor, Doxy (Moir *et al.*, 2011; Nagareddy *et al.*, 2012). Doxy, a member of the tetracycline family, has shown promising functions through its capability to inhibit MMPs in coronary artery disease (Bench *et al.*, 2011) and other cardiac ailments resulting from I/R and oxidative stress (Chow *et al.*, 2007). Full protective concentrations (30 μM) of Doxy confers ~70% recovery of contractile function following I/R. When combining a subthreshold dose of 1 μM Doxy together with either ML-7 or Y-27632 (inhibitors of MLC1 phosphorylation) at subthreshold doses, Doxy appears to act in concert to confer an ~50% protection of cardiac function. When the three drugs were present at subthreshold concentrations, a full recovery of cardiac contractile function is observed with concomitant preservation of MLC1 levels.

Given the profound cardioprotective effect this drug cocktail provides when given prior to injury and with reperfusion, simulating a potential prophylatic treatment approach, the obvious question becomes whether or not this cocktail would have any effect if administered solely after the ischaemic period, as would be the case with acute MI patients arriving at hospital emergency. Our previous studies addressing the effect of ischaemic duration on the myocardial proteome, showed that many proteins, including MLC1, are degraded within the ischaemic period (Fert-Bober *et al.*, 2008a). Thus, inhibition of these processes during reperfusion is likely to yield little effect. Future studies will address this question, but this cocktail appears best suited for use as a prophylactic treatment regimen.

This is the first report showing that targeting molecular mechanisms involved in the development of I/R injury and contractile dysfunction with a cocktail of multiple drugs at subthreshold concentrations, is a viable therapeutic approach to prevent I/R injury. Given the use of reduced (subthreshold) drug doses, it is expected that drug–drug interactions, side effects and disruption of physiological processes will be minimized.

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Conflicts of interest

None.

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