

Original Article

Inhibition of MLCK attenuates pulmonary artery hypertension

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Abstract: Objectives: To investigate the therapeutic effect of myosin light chain kinase (MLCK) inhibition on pulmonary artery hypertension (PAH). Methods: Primary rat pulmonary artery small muscle cells (PASMC) were cultured *in vitro*. The effect of ML-7 on myosin light chain (MLC) kinase (MLCK) expression, MLC phosphorylation, and PASMC growth and apoptosis *in vitro* were analyzed by Western blot, MTT and ³H-TdR incorporation assays, and flow cytometry. The therapeutic effects of MLCK inhibition by ML-7 on PAH were evaluated in a rat pulmonary hypertension model. Results: Inhibition of MLCK by inhibitor ML-7 significantly enhanced the apoptosis of PASMC and suppressed ET1-induced PASMC growth *in vitro*. Delivery of ML-7 markedly reduced pulmonary right ventricular hypertrophy index, the width of inferior vena cava, and mean pulmonary arterial pressure and mean right ventricular pressure in the rat PAH model. Conclusion: Inhibition of MLCK effectively reduces PASMC proliferation, pulmonary arterial remodeling and pulmonary hypertension.

Keywords: Myosin light chain kinase, pulmonary smooth muscle cell, apoptosis, pulmonary artery hypertension

Introduction

Pulmonary artery hypertension (PAH) is a devastating hemodynamic disease, characterized by a persistent elevation of pulmonary arterial remodeling and pulmonary arterial pressure [1]. Patients with PAH often suffer from right ventricular hypertrophy and right heart failure, and eventually die if left untreated [2]. Over the last decade, distinct classes of drugs including prostacyclins, endothelin receptor antagonists (ERAs), and phosphodiesterase-5 (PDE-5) inhibitors/soluble guanylate cyclase stimulators have been developed for the treatment of PAH with improvements in clinical outcomes [2-4], however, the disease still remains incurable.

Pulmonary artery smooth muscle cells (PASMC), a major component of the vascular media, play crucial roles in the pathogenesis of PAH through pulmonary vascular remodeling and vasoconstriction [5]. Excessive proliferation of PASMC induced by growth factors PDGF, VEGF, TGF- β , and mitogens such as endothelin-1 (ET1) results in the narrowing or occlusion of pulmonary arteries [6, 7]. Thus, enhancement of PASMC apoptosis and suppression of PASMC

proliferation can reverse pulmonary vascular remodeling and attenuate PAH [8].

Myosin light chain (MLC) kinase (MLCK), a ubiquitous Ca²⁺/calmodium (CaM)-activated kinase, is highly expressed in smooth muscle cells [9, 10], and required for smooth muscle contraction [11-13], which regulates blood pressure and blood flow distribution [14]. Here, we demonstrate that inhibition of MLCK activity by a MLCK specific pharmacological inhibitor ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1 H-hexahydro-1, 4-diazepine hydrochloride [15], enhances PASMC apoptosis *in vitro* and reduced pulmonary pressure in a preclinical rat PAH model.

Materials and methods

Cell culture

Primary rat PASMC were isolated as previously described [16], and cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, USA) and penicillin/streptomycin antibiotics at 37°C in a 5% CO₂ incubator. The cells were monitored under a microscopy (Kodak 4400CF, PELS Co. USA),

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and verified by immunohistochemical staining with anti- α actin antibody after 3-5 passages. For proliferation, PASMOC were cultured in DMEM with 1% FBS for 24 hours, then treated with ET1 (10^{-8} mol/L) or ET1 plus MLCK specific inhibitor ML-7 (20 μ mol/L), or DMSO control for 72 hours before subjected to Western blot and other assays.

Western blot

PASMOC were harvested 72 hours after treatments with ET1 (Bachem, USA), ET1 and ML-7 (Calbiochem, Germany), or DMSO, and washed with cold PBS. Total proteins extracted from the cells using a RIPA cell lysis buffer (Wolsen, China) were separated on 12% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membranes were treated with mouse anti-rat MLCK, anti-MLC, anti-pMLC or mouse anti- β -actin antibodies (1:1000 dilution) (Sigma, USA). The membranes were then reacted with the HRP-conjugated secondary antibodies before subjected to enhanced chemiluminescent (ECL) detection on an ECL machine (Pierce, USA). The blots were scanned and the band density was measured on the Quantity One imaging software. The level of phosphorylation of MLC was calculated as $pMLC/(pMLC+MLC) \times 100\%$.

Cell proliferation assay

PASMOC were seeded into wells of a 96-well plate. PASMOC growth was assessed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as described [17]. The cells were then treated with MTT solution (15 μ l/well) for 4 hours at 37°C. After removal of culture supernatant, the cell pellets were dissolved with 200 μ l of Dimethyl sulfoxide (DMSO). Optical density (OD) at 570 nm was read on a FLUOstar OPTIMA machine (BMG Labtech). Experiments were performed in triplicate. Alternatively, $^3\text{H-TdR}$ (Purchased from Shanghai Atomic Energy Institute, Shanghai, China) was used to measure DNA synthesis on a liquid scintillation counter for measuring the number of pulses per minute (cpm). The cpm values are representatives of cell division and growth of PASMOC as previously described [16].

Cell apoptotic assay

PASMOC (10^6 cells/well) were harvested from culture in 12-well plates as described [16].

Apoptotic and viable cells fractions were assessed with Annexin-V staining (Invitrogen, USA) following the manufacturer's instruction and analyzed on a flow cytometer (Becton, USA). The apoptotic cells were gated on Annexin-V positive population. Alternatively, the cells were harvested and washed in PBS, followed by fixation with ice-cold ethanol overnight. The cells were then washed in PBS and incubated in 1 ml staining solution (20 μ g/ml propidium iodide (PI) and 10 U/ml RNaseA) for 15 min at room temperature, the cell cycle distributions were assayed by fluorescence-activated cell sorting (FACS) using a flow cytometer as previously described [18].

Rat pulmonary hypertension model

Male rats (4-5 weeks) were purchased from the Experimental Animal Facility in the Third Military University in China, and used to establish a pulmonary hypertension model through high pulmonary flow by the shunt between abdominal aorta and the inferior vena cava. A sham operation served as control. Rats with pulmonary artery hypertension were by intraperitoneally injected with MLCK inhibitor ML-7 (3 mg/kg/day) twice a day for 8 weeks, or without treatment ($n=10$ per group). Eight weeks after shunt, mean right ventricular pressure (MRVP), mean pulmonary arterial pressure (MPAP), and the right ventricular hypertrophy (RVH) index and the width of inferior vena cava (IVC) were measured by right cardiac catheterization procedure. After the measurement of MPAP, MRVP, RVH index and IVC width, the rats were sacrificed, and the total body weight and the right ventricular weight each rat were measured. The ratios of right ventricular/body weight (RV/BW) per group were calculated.

Statistical analysis

All data were presented as mean \pm SD (standard deviation), and analyzed by One-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Inhibition of MLCK abrogates the ET1-augmented phosphorylation of MLC in PASMOC

MLCK regulates smooth muscle contraction by phosphorylation of MLC. ET1 as a potent vasoconstrictor can promote phosphorylation of

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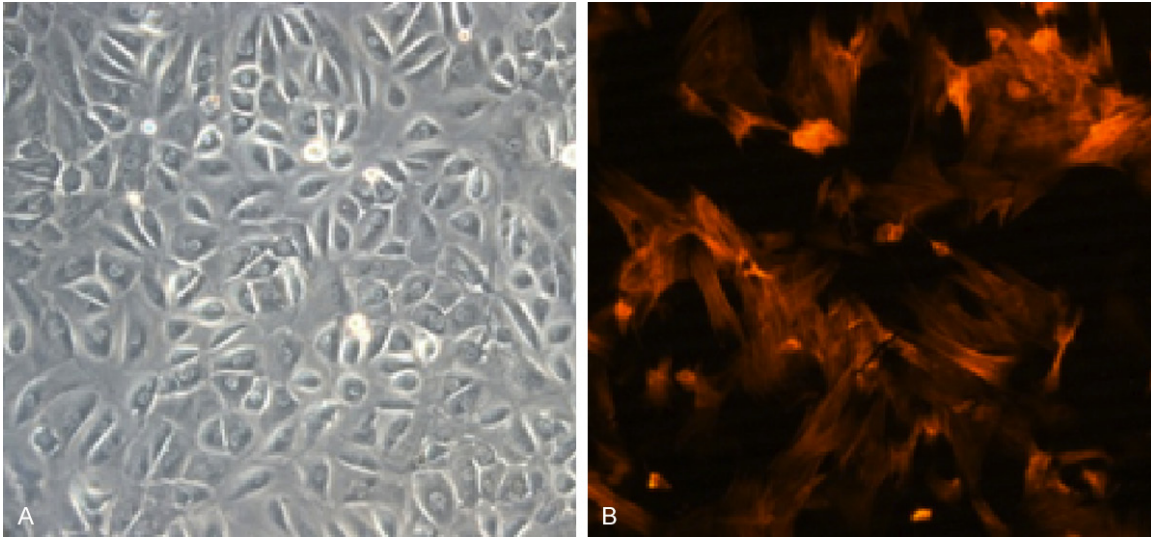


Figure 1. Morphology of PASMC. A. Primary PASMC was grown in DMEM medium and photographed under a microscope ($\times 20$). B. PASMC was stained with PE-conjugated anti- α -actin antibodies ($\times 200$).

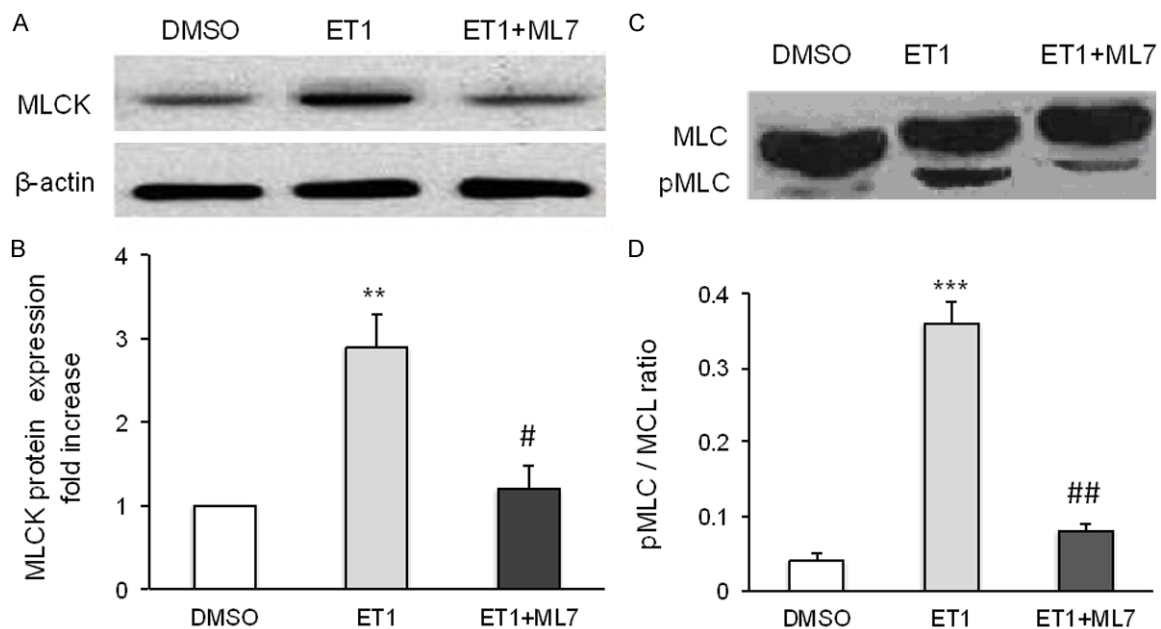


Figure 2. Inhibitory effect of ML-7 on MLCK expression and phosphorylation of MLC in rat PASMCs. A. Protein lysates isolated from PASMC treated with ET1, ET1 plus ML-7 (ML7), or DMSO control were subjected to Western blot analysis with anti-rat MLCK antibody. B. MLCK protein expression level relative to DMSO control treatment was presented. C. Phosphorylation of MLC protein in rat PASMCs were analyzed by Western blot with anti-rat MLC or pMLC antibodies. D. MLC phosphorylation levels compared with total MLC protein were calculated and presented. Data were presented as mean \pm SD. ** $P < 0.01$, *** $P < 0.001$, comparison between ET1 treatment and DMSO control; # $P < 0.05$, ## $P < 0.01$, comparison between ET1 and ET1 plus ML-7 treatments.

MLC and enhance vascular remodeling [19]. To test that MLCK inhibition with a specific inhibitor could reduce ET1-enhanced phosphorylation of MLC in PASMC, we grew primary PASMC from rat and grew it in DMEM medium. PASMC cells (Figure 1A) were stained with PE-conju-

gated anti- α -actin antibodies. Immunohistochemical analysis demonstrated that the cells were α -actin positive (red color) (Figure 1B). Western blot showed that ET1 treatment robustly increased MLCK protein expression in PASMC; two fold higher than DMSO control

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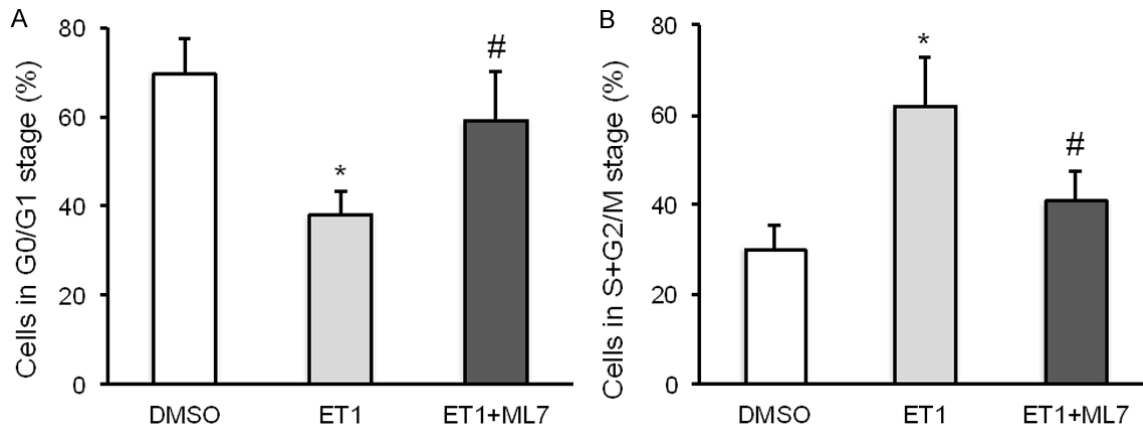


Figure 3. Effect of ML-7 on PASMC cell cycle. PASMC were treated with ET1, ET1 plus ML-7, or DMSO control for 72 hours, and subjected to cell cycle analysis by FACS. A. Percentage of PASMC in the G0/G1 stage. B. Percentage of PASMC in the S+G2/M stage. * $P < 0.05$ comparison between ET1 treatment and DMSO control; # $P < 0.05$ comparison between ET1 and ET1 plus ML-7 treatments.

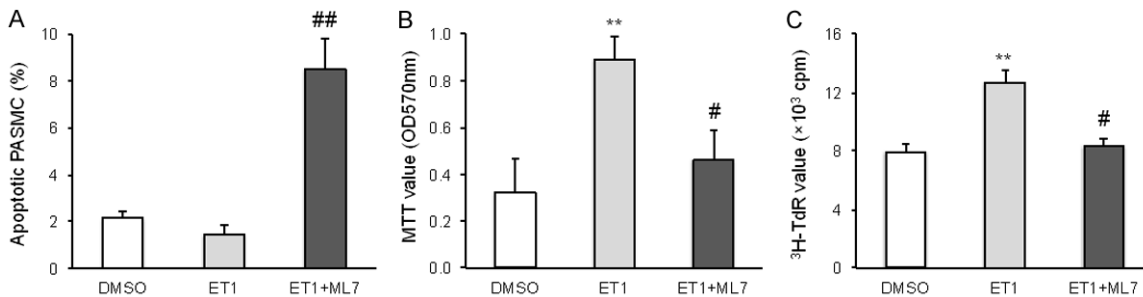


Figure 4. ML-7 treatment-induced apoptosis in PASMC. (A) PASMC treated with ET1, ET1 plus ML-7 or DMSO control was subjected to FACS analysis with an apoptotic kit. Percentages of apoptotic PASMC in the treatments were calculated and presented. (B, C) The growth of PASMC in ET1, ET1 plus ML-7, or DMSO treatments was analyzed by MTT (B) and ³H-TdR (C) assays. Data were presented as Mean ± SEM. ** $P < 0.01$ comparison between ET1 treatment and DMSO control; # $P < 0.05$, ## $P < 0.01$ comparison between ET1 and ET1 plus ML-7 treatments.

(Figure 2A and 2B). While, MLCK inhibitor treatment significantly reduced the kinase protein expression in ET1-stimulated PASMC, similar to the expression level in DMSO-treated PASMC (Figure 2A and 2B). Analysis of MLC phosphorylation in PASMC showed that ET1 stimulation enhanced the phosphorylation of MLC (44%) in comparison with DMSO control (9.6%) (Figure 2C and 2D). In contrast, MLCK inhibitor ML-7 treatment significantly suppressed ET1-augmented MLC phosphorylation in PASMC (20.6%) (Figure 2C and 2D).

MLCK inhibition promotes apoptosis of PASMC

To examine the effect of MLCK inhibition on the cell cycle of PASMC, we performed FACS analyses on the treated PASMC cells. FACS analysis demonstrated that ET1 markedly reduced the PASMC population on G0/G1 stage (Figure 3A)

and increased the portion of S+G2/M cells (DNA synthesis and mitosis stage) (Figure 3B). In contrast, ML-7 treatment abrogated ET1-induced DNA synthesis and PASMC division when co-culture the cells with the MLCK inhibitor and ET1 (Figure 3B). To test whether MLCK inhibition could induce the apoptosis of PASMC, we stained PASMC with Annexin-V after treatment with ET1, ET1 plus ML-7, or DMSO. Flow cytometry assay showed that ML-7 treatment markedly increased apoptosis of PASMC even in comparison with DMSO or ET1 treatment (Figure 4A). Consequently, ML-7 treatment suppressed ET1-induced proliferation and growth of PASMC (Figure 4B and 4C).

MLCK inhibition reduced rat pulmonary vascular remodeling in vivo

RVH index, RV/BW ratio and IVC width are widely used as typical parameters for pulmonary

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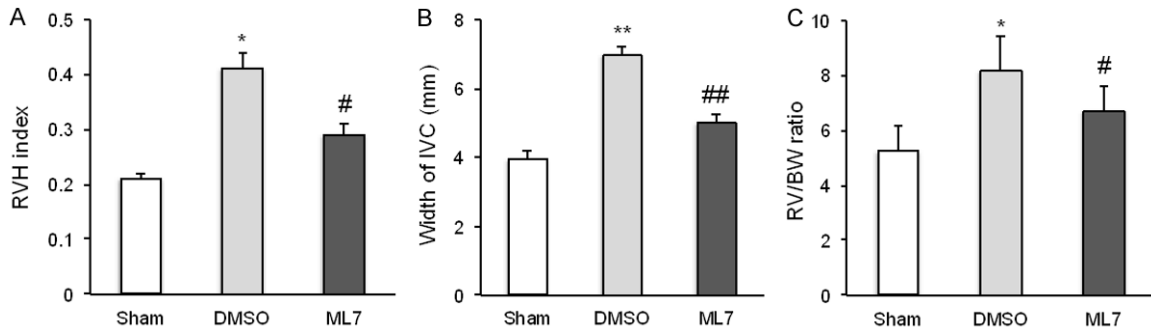


Figure 5. Effect of MLCK inhibition on pulmonary vascular remodeling in rat pulmonary hypertension model. Rat pulmonary hypertension was induced by high pulmonary flow after shunting between abdominal aorta and the inferior vena cava. The rats were then intraperitoneally treated with ML-7 or DMSO. A sham operation without shunting served as control. (A) RVH index and (B) IVC width were measured by right cardiac catheterization procedure 8 weeks after shunt. (C) The RV/BW ratio was also calculated after the rats were sacrificed. Each group had 10 rats. * $P < 0.05$, ** $P < 0.01$ comparison between ET1 treatment and DMSO control; # $P < 0.05$, ## $P < 0.01$ comparison between ET1 and ET1 plus ML-7 treatments.

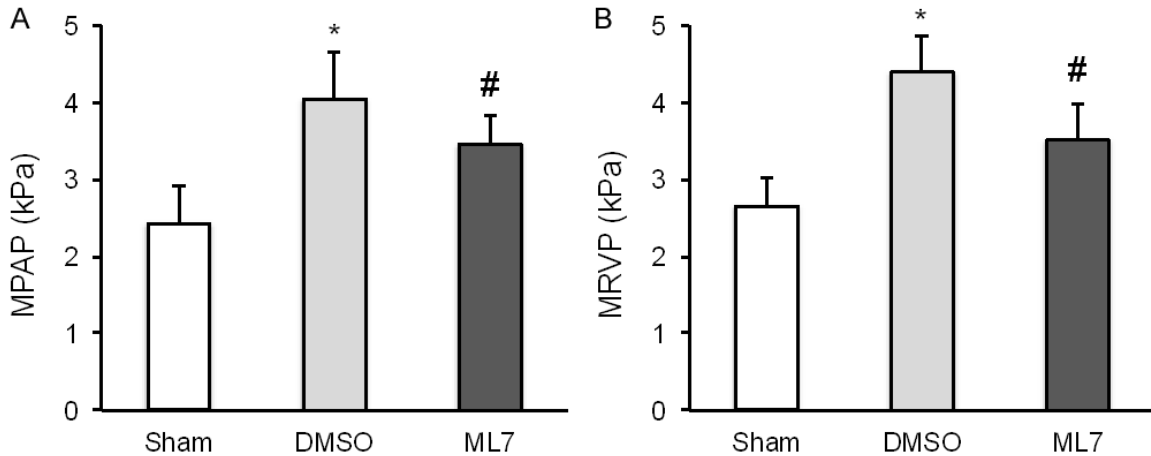


Figure 6. Effect of MLCK inhibition on rat pulmonary hypertension *in vivo*. Rat under pulmonary hypertension induction were treated with ML-7 or DMSO. Sham surgery served as control. (A) Mean pulmonary arterial pressure (MPAP) and (B) mean right ventricular pressure (MRVP) in the three groups of rats were quantified by right cardiac catheterization procedure 8 weeks after shunt. Each group had 10 rats. * $P < 0.05$ comparison between ET1 treatment and DMSO control; # $P < 0.05$ comparison between ET1 and ET1 plus ML-7 treatments.

vascular remodeling. To test whether blocking MLCK activity could attenuate pulmonary vascular modulation *in vivo*, we established a rat PAH model through high pulmonary flow and treated the rats with MLCK specific inhibitor ML-7 for 8 weeks. Examination of RVH index and the width of inferior vena cava demonstrated that shunting between abdominal aorta and the inferior vena cava (DMSO group) significantly increased RVH index (Figure 5A), IVC width (Figure 5B) and RV/BW ratio (Figure 5C) in comparison with sham group (Figure 5A-C). Conversely, administration of ML-7 into shunted rats markedly reduced the three parameters

in comparison with the group without ML-7 treatment (Figure 5A-C).

MLCK inhibition reduced rat pulmonary hypertension in vivo

Pulmonary hypertension is characterized with an increase of MPAP and MRVP [20]. To check whether ML-7 treatment could reduce pulmonary hypertension, we performed right cardiac catheterization procedure on the rats treated with ML-7 or DMSO, or with sham operation to evaluate both MPAP and MRVP. Measurement demonstrated that rats under shunt had signifi-

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cant higher MPAP (**Figure 6A**) and MRVP (**Figure 6B**) in comparison with sham group (**Figure 6A** and **6B**). After ML-7 treatment, rats with shunt had significantly lower MPAP (3.45 ± 0.44 vs. 4.04 ± 0.61 , $P < 0.05$) and MRVP (3.51 ± 0.47 vs. 4.19 ± 0.67 , $P < 0.05$) compared with DMSO-treated rats (**Figure 6A** and **6B**).

Discussion

In this study, we demonstrate that inhibition of MLCK by ML-7 inhibitor induces the apoptosis of PASMC and suppresses its growth; delivery of ML-7 reduces rat vascular remodeling and consequent pulmonary hypertension. To our knowledge, it is the first report that inhibition of MLCK could attenuate pulmonary hypertension.

Dysfunctional extensive proliferation of PASMC is the major cause of pulmonary arterial remodeling that results in arterial medial layer hyperplasia and the occlusion of the distal pulmonary arteries, increasing both pulmonary arterial pressure and vascular resistance [5]. One approach for reversing the proliferation of PASMC is to induce its apoptosis. It has been shown that promoting the apoptosis of PASMC could reverse the remodeling of the pulmonary arteries [21-25]. In this study, we demonstrated that inhibiting MLCK activity by its specific inhibitor ML-7 reduced the apoptosis of PASMC, which suggests a potential role of MLCK in the pathogenesis of pulmonary arterial remodeling. Indeed, ML-7 treatment markedly suppressed the proliferation of PASMC triggered by ET1 stimulation. Phosphorylation of MLC by MLCK allows ATP-dependent mechanochemical interaction between actin and myosin, and plays a critical role in the regulation of PASMC contractile activity and relaxation [26]. Our result that inhibition of MLCK by ML-7 abrogated the phosphorylation of MLC further informs the potential function of MLCK in the pathogenic process of vascular remodeling in PAH.

Preclinical PAH animal model has been established to evaluate the therapeutic effect of drugs targeting vascular remodeling in PAH [27-30]. It has been showed that targeting vascular remodeling prevented pulmonary arterial occlusion and reduced pulmonary arterial pressure *in vivo* [31-34]. We utilize a rat pulmonary hypertension model that is induced by high pulmonary flow with the shunt between abdominal

aorta and the inferior vena cava to examine the therapeutic effect of MLCK inhibition on pulmonary hypertension. Consistent with our *in vitro* finding, we demonstrated that ML-7 treatment not only reduced the right ventricular hypertrophy index and the inferior vena cava width, but also rat pulmonary arterial pressure and the right ventricular pressure in the rat PAH model. Our results strongly suggest that inhibition of MLCK by ML-7 could attenuate rat pulmonary artery hypertension. PAH-targeted drugs including prostacyclin and endothelin receptor blockers can oppose abnormal vasoconstriction [35], but could not impact the root causes of pulmonary vascular remodeling, since pulmonary vascular lesions remaining in the explanted lung tissue samples obtained from patients [36]. Our study on the effect of a MLCK inhibitor in rat PAH suggests that the inhibition of MLCK activity could offer a potential new approach to treat PAH.

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Disclosure of conflict of interest

None.

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