

# Larixyl acetate, a TRPC6 inhibitor, attenuates pressure overload-induced heart failure in mice

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**Abstract.** Heart failure is a primary cause of global mortality. In the present study, whether larixyl acetate, an inhibitor of transient receptor potential cation channel subfamily C member 6, attenuates pressure overload-induced heart failure in mice was investigated. To test this hypothesis, a transverse aortic constriction (TAC) animal model and an angiotensin II (Ang II)-treated H9c2 cell model were used. Cardiac and cellular structure, function and the expression levels of hypertrophy, endoplasmic reticulum (ER) stress, apoptosis, autophagy and pmTOR/mTOR related mRNAs or proteins were assessed to explore the underlying molecular mechanisms. The results indicated that treatment with TAC or Ang II leads to significant hypertrophy and dysfunction of the heart or H9c2 cells, accompanied by an increase in ER stress, apoptosis and activation of the mTOR signaling pathway, and a decrease in autophagy. The administration of larixyl acetate attenuated these impairments, which can be reversed by inhibiting autophagy through the activation of the mTOR signaling pathway. These findings suggested that larixyl acetate can effectively protect against pressure overload-induced heart

failure by enhancing autophagy and limiting ER stress and apoptosis through inhibition of the mTOR pathway.

## Introduction

Heart failure, which is characterized by increased intracardiac pressures or decreased cardiac output, is a life-threatening condition and is recognized as a global health issue (1). In the early stages of cardiovascular diseases like hypertension and aortic valve stenosis, cardiac hypertrophy often occurs as a response to increased pressure overload (2). This hypertrophy initially boosts cardiac work efficiency. However, if it persists under adverse conditions, it can lead to excessive cell enlargement, cardiomyocyte death and impaired contraction (3-5). These changes will ultimately result in irreversible heart failure (6). Despite significant progress in understanding pressure overload-induced heart failure, its incidence is still increasing (7,8). Currently, there are no effective approved pharmacological treatments for this condition (9). Therefore, it is important to further explore the mechanisms behind pressure overload-induced heart failure, which will help identify new and promising therapeutic targets.

The endoplasmic reticulum (ER) is a vital cellular organelle with multiple functions, including protein synthesis, folding and translocation, as well as the uptake and storage of cellular calcium and the production of cellular lipids (10). There is mounting evidence that pressure overload increases the protein synthesis and folding load of the ER, resulting in the accumulation of misfolded and unfolded proteins, which in turn disturbs ER homeostasis (11). These disturbances trigger ER stress, also known as the unfolded protein response, which aims to eliminate accumulated unfolded proteins and maintain ER homeostasis (12,13). However, prolonged or severe ER stress from continued pressure overload can escalate and lead to increased levels of ER chaperones, like GRP78, and activation of the pPERK/PERK-ATF4-CHOP signaling pathway (12). These changes can result in myocardial apoptosis and potentially fatal heart failure (14,15).

Autophagy is a cellular process that helps maintain intracellular homeostasis by removing damaged proteins and organelles and recycling intracellular components through

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lysosomal degradation (16-18). Autophagy also promotes protein synthesis and energy production (19). Numerous studies have demonstrated the role of autophagy in protecting against heart failure induced by pressure overload (20-22). The mammalian target of rapamycin (mTOR), a well-established negative regulator of autophagy, has been increasingly recognized for its role in cardiac health. Evidence suggests that inhibiting the mTOR signaling pathway can enhance autophagy in cardiomyocytes. This enhancement in turn may reduce ER stress-induced apoptosis in cardiomyocytes, offering potential protection against heart failure (23-25).

Transient receptor potential cation (TRPC) channels, comprising seven subfamilies (TRPC1-7), play a crucial role in regulating the functions of cardiomyocytes, smooth muscle cells and endothelial cells (26,27). Among these, TRPC6 is particularly significant in the pathophysiology of cardiac hypertrophy and heart failure (28,29). Larixyl acetate, extracted from larch balsam, has been identified as a specific inhibitor of TRPC6. It has been reported that larixyl acetate can effectively block the  $\text{Ca}^{2+}$  entry and ionic current following the activation of TRPC6 channels (30). Notably, this compound demonstrates 12- and 5-fold selectivity over the closely related TRPC3 and TRPC7 channels, respectively (30). Previous studies have shown that larixyl acetate can prevent traumatic brain injury-induced systemic endothelial dysfunction (31), ozone or lipopolysaccharide-induced airway or lung injury (32,33) and neuropathic pain resulting from spared nerve injury (34) through TRPC6-dependent mechanisms. These findings underscore the potential of larixyl acetate as a therapeutic TRPC6 inhibitor in various diseases. However, its effects on cardiac hypertrophy and heart failure remain unexplored.

Thus, the present study employed both *in vivo* and *in vitro* experiments, along with pharmacological interventions, to investigate this issue. While the calcineurin A/NFAT pathway has been extensively studied (28,35), the present research aimed to uncover other potential new mechanisms. It is widely recognized that inhibiting the mTOR signaling pathway can alleviate heart failure by promoting autophagy (36). Furthermore, recent research has demonstrated that the inhibition of TRPC6 can reduce mTOR phosphorylation (37). Therefore, it is reasonable to hypothesize that larixyl acetate, as a TRPC6 inhibitor, might protect against pressure overload-induced heart failure. Mechanistically, the protective role of larixyl acetate may involve promoting autophagy through the inhibition of the mTOR signaling pathway, which could subsequently reduce ER stress-induced cardiomyocyte apoptosis.

## Materials and methods

**Reagents.** Angiotensin II (Ang II) was purchased from MilliporeSigma (Merck KGaA), and larixyl acetate and MHY1485 were purchased from MedChemExpress.

**Animals and treatments.** A total of 70 male C57BL/6 mice (age, 8-10 weeks; weight, 23-25 g) were purchased from GemPharmatech Co. Ltd. All animal procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (38). All the experimental procedures were approved by The Ethical

Review Board of Drum Tower Hospital of Nanjing University Medical School (Nanjing, China; approval no. 2022AE01017). The mice were housed in a controlled environment, with a temperature of 23°C ( $\pm 2^\circ\text{C}$ ), a relative humidity of 50-60%, and under a 12-h light/dark cycles. Food and water were provided *ad libitum*. Throughout the course of the experiment, eight mice reached the predetermined humane endpoints and were subsequently euthanized. The remaining mice were euthanized upon completion of the experiment, and their heart tissues were harvested for further experimental analysis. The method of euthanasia was rapid decapitation following anesthesia induced with 5% isoflurane.

In the present study, specific humane endpoints were established to ensure ethical treatment of the animals. These endpoints were designed to minimize suffering and distress. They included: i) Clinical signs: Animals showing severe signs of heart failure, such as labored breathing, inability to remain upright or decreased activity levels; ii) weight loss: A weight loss more than 20% of the baseline body weight of the animal; iii) failure to eat or drink: Animals with a complete loss of appetite for 24 h or poor appetite ( $<50\%$  of the normal amount) for 3 days; and iv) disease progression: Animals exhibiting rapid progression of disease symptoms without any signs of recovery. These endpoints were determined in consultation with veterinary staff and were in compliance with the guidelines provided by The Experimental Animal Ethics Committee of Drum Tower Hospital of Nanjing University Medical School. Throughout the present study, animals were monitored daily for these indicators, and any decisions for euthanasia were made with the utmost consideration for the welfare of the animals. Throughout the course of the experiment, eight mice reached the predetermined humane endpoints and were subsequently euthanized. The remaining mice were euthanized upon completion of the experiment, and their heart tissues were harvested for further experimental analysis.

### *Pressure overload model, echocardiography and treatment.*

Transverse aortic constriction (TAC) or sham surgery was performed according to previous studies with mild modifications (39,40). Briefly, mice were anesthetized using 5% isoflurane for induction, followed by maintenance with 1.5% isoflurane. The aortic arch was exposed through a median incision at the upper sternal segment under a stereomicroscope. A 6-0 silk ligature was made between the right brachiocephalic and left common carotid artery around a 27-gauge, and then the wire was removed to generate a defined constriction of the aorta. As for the sham group, an identical operation was performed except for the ligature of the aorta. Doppler echocardiography was applied on mice after TAC to assess the pressure gradient across the constriction. A pressure gradient  $>45$  mmHg was considered a successful surgery. A total of four weeks after TAC, echocardiography was performed and analyzed in a blinded manner to evaluate the cardiac function using a Small Animal Ultrasound Imaging System (VEVO2100, FUJIFILM VisualSonics, Inc.). The measurements were taken in M-mode with a 30 MHz linear ultrasonic transducer.

Previous studies have reported that the intraperitoneal (i.p.) injection of larixyl acetate at a dosage of 5 mg/kg/day for 1 week can safely and effectively inhibit TRPC6 (31,34). In the present study, the same dosage of larixyl acetate was selected,

with 5 mg/kg i.p. administered daily after surgery to investigate its protective effects against pressure overload-induced heart failure using a 4-week TAC animal model. It has been reported that i.p. administration of 10 mg/kg MHY1485, an mTOR activator, is safe and effective for activating the mTOR signaling pathway when administered daily for 28 days (41). Therefore, this dosage was selected to investigate the impact of the mTOR signaling pathway on the efficacy of larixyl acetate.

**Group and tissue harvest.** A total mortality rate of 18.18% (8/44) was observed in the TAC model. To investigate the effects of larixyl acetate on pressure overload-induced heart failure, the number of mice in each experimental group was adjusted as necessary. The goal was to ensure that 10 mice/group successfully completed the 4-week TAC procedure, providing robust data for analysis and sufficient tissue samples for subsequent experiments. The final composition of each group was as follows: i) Sham + Vehicle (n=10); ii) Sham + Larixyl (n=10); iii) TAC + Vehicle (n=13; with 3 mice deceased); and iv) TAC + Larixyl (n=12; with 2 mice deceased). Additionally, all spare mice were euthanized at the conclusion of the experiment. For the subsequent experiments, the mice were further divided in each group. The hearts of 5 mice underwent apical perfusion with ice saline and paraformaldehyde, followed by fixation, dehydration, OCT embedding and preparation for tissue staining. In the remaining 5 mice, after blood removal, the ventricles were divided for western blotting analysis and reverse transcription quantitative polymerase chain reaction (RT-qPCR).

In experiments testing the interaction of larixyl acetate with the mTOR activator, MHY1485, it was aimed to have 8 mice/group complete the 4-week TAC procedure. The groups were: i) TAC + Larixyl (n=9; with 1 mouse deceased); and ii) TAC + Larixyl + MHY (n=10; with 2 mice deceased). Only one additional spare mouse was euthanized post-experiment.

**Hematoxylin-eosin staining.** The heart tissues embedded in OCT were stored at -80°C. Frozen heart sections (10 µm) were prepared in a cryostat at -20°C. The sections were then recovered to room temperature before staining and then rinsed 3x5 min with ddH<sub>2</sub>O, incubated for 2 min with hematoxylin staining solution (Beijing Solarbio Science & Technology Co., Ltd.; H8070) followed by 1% hydrochloric acid alcohol for a few sec, and then rinsed with ddH<sub>2</sub>O. After that, the sections were soaked in eosin staining solution (Beijing Solarbio Science & Technology Co., Ltd.; G1100) for 1 min and then rinsed with ddH<sub>2</sub>O, and then dehydrated with gradient alcohol, and transparentized with dimethyl benzene, and sealed the sections with sealing cement. All incubations were carried out at room temperature. The images were captured under an Olympus BX43 light microscope (Olympus Corporation).

**Sirius red staining.** The heart tissues embedded in OCT were stored at -80°C. Frozen heart sections (10 µm) were prepared in a cryostat at -20°C. The sections were then recovered to room temperature before staining and then rinsed 3x5 min with ddH<sub>2</sub>O, and the staining was performed according to the manual of the modified Sirius Red Stain Kit (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. G1472) at room temperature. Briefly, heart frozen sections were rinsed 3x5 min with ddH<sub>2</sub>O and then incubated with iron hematoxylin

staining solution for 2 min and rinsed with tap water for 5 min. Next, sections were incubated with sirius red staining solution for 30 min, rinsed with running water and then dehydrated with gradient alcohol and transparentized with dimethyl benzene. Finally, the sections were sealed with sealing cement. The images were captured by Olympus BX43 light microscope (Olympus Corporation). The collagen volume fraction of the left ventricular (LV) was analyzed with Fiji software (Version 1.54f; NIH) by using the interest grayscale threshold analysis and was calculated as sirius red staining area divided by total area. A total of three sections/heart and five mice/group were sampled. The images were captured and analyzed by two individuals blinded to the experimental group. Average data were used to represent the data for each mouse.

**TUNEL staining.** The heart tissues embedded in OCT were stored at -80°C. Frozen heart sections (10 µm) were prepared in a cryostat at -20°C. The sections were then recovered to room temperature before staining and then rinsed 3x5 min with ddH<sub>2</sub>O, and the staining was performed with a One-step TUNEL cell apoptosis detection kit (Beyotime Institute of Biotechnology; cat. no. C1090) according to the manufacturer's instructions. Briefly, heart frozen sections were rinsed 2x10 min with phosphate buffer saline (PBS), then permeabilized by 0.3% Triton X-100 in PBS for 5 min at room temperature, and again rinsed 2x10 min with PBS. Next, sections were incubated with TUNEL solution at 37°C for 60 min, rinsed 3x10 min with PBS and then sealed with anti-fluorescence quenching seal liquid. The images were captured by the Olympus FV3000 confocal microscope (Olympus Corporation) with 550 nm excitation light and analyzed with Fiji software (Version 1.54f; NIH). A total of three random fields from the LV-free wall/section, three sections/mouse and five mice/group were sampled. The images were captured and analyzed blindly. Average data were used to represent the data for each mouse.

**Cell culture.** The rat cardiomyocyte line, H9c2 was cultured in DMEM (cat. no. 319-015; Wisent, Inc.) supplemented with 10% fetal bovine serum (cat. no. 085-150; Wisent, Inc.) and 1% penicillin/streptomycin (cat. no. 15140-122; Gibco) at 37°C. Previous studies have demonstrated that Ang II is capable of activating TRPC6 and the mTOR signaling pathway, leading to cardiomyocyte hypertrophy, ER stress and apoptosis (23,28,42-44). Therefore, Ang II (100 nM) was administered to H9c2 cells for 72 h at 37°C to establish a cell model and to confirm whether larixyl acetate could provide protective effects by upregulating autophagy via inhibiting the mTOR signaling pathway and reducing ER stress. Groups were incubated with larixyl acetate (5 µM) with or without MHY1485 (10 µM) at 37°C to block TRPC6 only or to activate mTOR signaling simultaneously, 30 min before Ang II administration. Cellular size, and the expression of cardiac hypertrophy-related genes, ER stress markers, autophagy-related proteins and apoptosis-related proteins were then assessed. The specified dosage for administering larixyl acetate (30,45) and MHY1485 (46) were determined based on previous research.

**Wheat germ agglutinin (WGA) staining.** WGA staining was used to measure the cardiomyocyte crosssectional area. Briefly,

Table I. Primers for qPCR.

Genes	Forward sequence (5'-3')	Reverse sequence (5'-3')
Mouse <i>Anp</i>	GCTTCCAGGCCATATTGGAG	GGGGGCATGACCTCATCTT
Mouse <i>Bnp</i>	GAGGTCACTCCTATCCTCTGG	GCCATTTCCTCCGACTTTTCTC
Mouse <i>Myh7</i>	CAACCTGTCCAAGTTCGCCA	TACTCCTCATTGAGGCCCTTG
Mouse <i>Col1a1</i>	TGCTAACGTGGTTCGTGACCGT	ACATCTTGAGGTCGCGGCATGT
Mouse <i>Col3a1</i>	ACGTAAGCACTGGTGGACAG	CCGGCTGGAAAGAAGTCTGA
Mouse <i>Gapdh</i>	ATGTGTCCGTCGTGGATCTG	AGTTGGGATAGGGCCTCTCTT
Rat <i>Anp</i>	AAAGCAAAGTGAAGGCTCTGCTCG	TTCGGTACCGGAAGCTGTTGCA
Rat <i>Bnp</i>	TGCCCCAGATGATTCTGCTC	TGTAGGGCCTTGCTCCTTTG
Rat <i>Myh7</i>	AGTTCGGGCGAGTCAAAGATG	CAGGTTGTCTTGTTCGCCT
Rat <i>Gapdh</i>	ACTCTACCCACGGCAAGTTC	TGGGTTTCCCGTTGATGACC

heart tissues embedded in OCT were stored at  $-80^{\circ}\text{C}$ . Frozen heart sections ( $10\ \mu\text{m}$ ) were prepared in a cryostat at  $-20^{\circ}\text{C}$ . The sections were then recovered to room temperature before staining and the sections were then rinsed for 3x5 min with PBS. Next, sections were incubated with 1  $\mu\text{g}/\text{ml}$  WGA (cat. no. W6748; Invitrogen; Thermo Fisher Scientific, Inc.) at  $37^{\circ}\text{C}$  for 10 min, rinsed 3x5 min with PBS and then sealed with anti-fluorescence quenching seal liquid. The images were taken by an Olympus FV3000 confocal microscope (Olympus Corporation) with 488 nm excitation light and analyzed with Fiji software (Version 1.54f; NIH). A total of three random fields from the LV-free wall/section, three sections/mouse and five mice/group were sampled. The images were captured and analyzed blindly. Average data was used to represent the data for each mouse.

**Phalloidin staining.** Phalloidin staining was used to measure the cellular area of H9c2 cells. Briefly, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, rinsed 3x5 min with PBS and further permeabilized by 0.3% Triton X-100 in PBS for 5 min. Next, cells were rinsed 3x5 min with PBS and then incubated with 0.005 unit/ $\mu\text{l}$  phalloidin (A12381, Invitrogen; Thermo Fisher Scientific, Inc.) for 60 min at room temperature, rinsed 3x5 min with PBS and then sealed with anti-fluorescence quenching seal liquid. The images were taken by an Olympus FV3000 confocal microscope (Olympus Corporation) with 594 nm excitation light and analyzed with Fiji software (Version 1.54f; NIH). A total of five random fields from each coverslip and five coverslips/group were sampled. The images were captured and analyzed in a blind manner. Average data were used to represent the data for each coverslip.

**Western blotting.** Proteins in LV tissues or H9c2 cells were extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) containing proteinase and phosphatase inhibitors. BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) was used. A total of 20  $\mu\text{g}$  protein samples were separated by SDS/PAGE on 4-20% gels and then transferred to PVDF membranes. The membranes were blocked in 5% defatted milk for 1 h at room temperature and then incubated with primary antibodies including TRPC6

(1:500; cat. no. 18236-1AP; Proteintech Group, Inc.), GRP78 (1:1,000; cat. no. sc-13539; Santa Cruz Biotechnology, Inc.), pPERK (1:500; cat. no. sc-32577; Santa Cruz Biotechnology, Inc.), PERK (1:1,000; cat. no. sc-377400; Santa Cruz Biotechnology, Inc.), ATF4 (1:1,000; cat. no. sc-390063; Santa Cruz Biotechnology, Inc.), CHOP (1:1,000; cat. no. sc-7351; Santa Cruz Biotechnology, Inc.), pmTOR (1:500; cat. no. 2971; CST Biological Reagents Co., Ltd.), mTOR (1:500; cat. no. 2972; CST Biological Reagents Co., Ltd.), P62 (1:500; cat. no. ab155686; Abcam), LC3B (1:1,000; cat. no. ab51520; Abcam), Bcl-2 (1:500; cat. no. 3498; CST Biological Reagents Co., Ltd.), Bax (1:1,000; cat. no. ab32503; Abcam), cleaved caspase-3 (1:100; cat. no. 9664; CST Biological Reagents Co., Ltd.) and GAPDH (1:2,000; cat. no. ab8245; Abcam) at  $4^{\circ}\text{C}$  overnight. The membranes were rinsed 3x5 min with TBST (0.1% Tween; cat. no. ST825; Beyotime Institute of Biotechnology) and then incubated with goat anti-mouse (1:2,000; cat. no. A0216), goat anti-rat (1:2,000; cat. no. A0192), or goat anti-rabbit (1:2,000; cat. no. A0208) HRP-conjugated secondary antibodies (all from Beyotime Institute of Biotechnology) according to primary antibodies at room temperature for 1 h, rinsed 3x5 min with TBST and detected by enhanced chemiluminescence solutions (cat. no. KGC4602; Nanjing KeyGen Biotech Co., Ltd.). The protein expression level was quantified using Fiji software (Version 1.54f; NIH) with GAPDH as the loading control.

**RNA isolation and RT-qPCR.** Total mRNA in LV tissues or H9c2 cells was extracted using Trizol reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1  $\mu\text{g}$  total RNA was used to reverse transcribe into cDNA using HiScript III RT SuperMix for qPCR (cat. no. R323-01; Vazyme Biotech Co., Ltd.). The obtained cDNA was mixed with gene-specific primers (Table I) and ChamQ Universal SYBR qPCR Master Mix (cat. no. Q711-02; Vazyme Biotech Co., Ltd.) was used for RT-qPCR in light cycler 480 instruments (Roche Diagnostics). The thermocycling conditions were as follows: Initial denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 10 sec and annealing/extension at  $60^{\circ}\text{C}$  for 30 sec. The cycle time values were standardized to GAPDH of the same sample and  $2^{-\Delta\Delta\text{Cq}}$  was used to represent relative quantity (47).



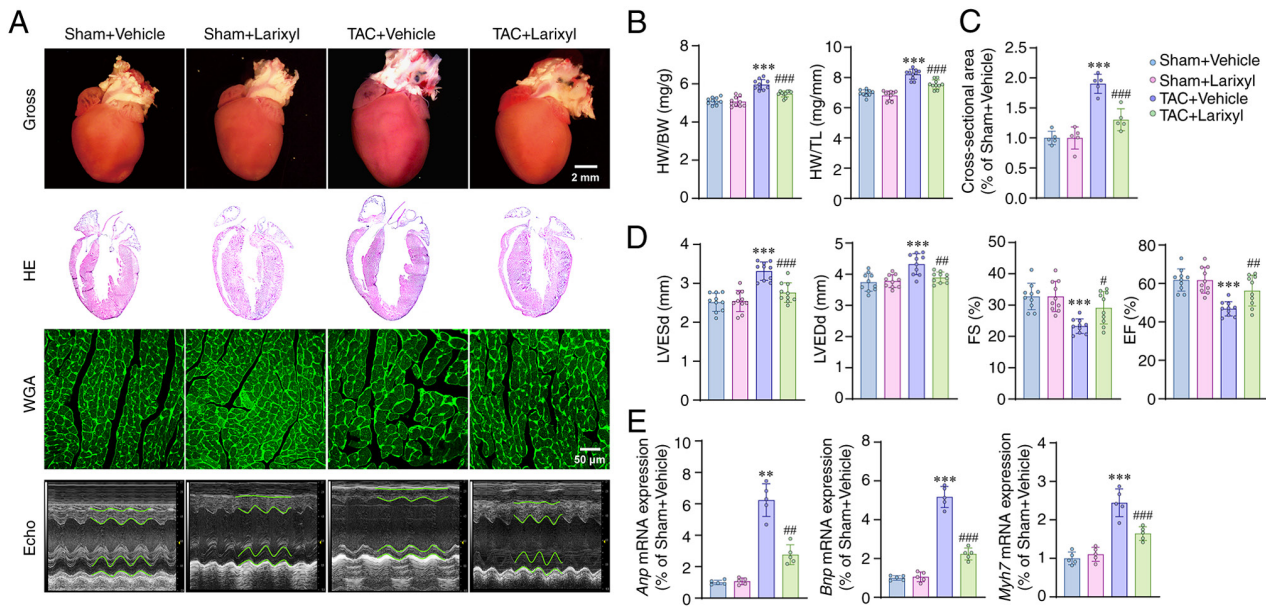


Figure 1. Larixyl acetate attenuates pressure overload-induced heart failure. (A) Representative images of gross morphology of hearts, HE staining, WGA staining and transthoracic echocardiography of the hearts in the Sham + Vehicle, Sham + Larixyl, TAC + Vehicle and TAC + Larixyl groups at 4 weeks after TAC (n=10/group). (B) HW/BW, HW/TL ratios in the Sham + Vehicle, Sham + Larixyl, TAC + Vehicle and TAC + Larixyl groups at 4 weeks after TAC (n=10/group). (C and D) Quantitative results of the cross-sectional area of cardiomyocyte (n=5/group), LVESd, LVEDd, FS and EF of the hearts (n=10/group) from indicated groups. (E) Relative mRNA levels of hypertrophy marker genes, *Anp*, *Bnp* and *Myh7* in the hearts from the indicated groups (n=5/group). Data are presented as the mean  $\pm$  SD. For *Anp* mRNA expression, the homogeneity of variance test showed significant differences, therefore, these data were analyzed using Welch's ANOVA. For the rest of the datasets, where the homogeneity of variance test did not reveal significant differences, one-way ANOVA was employed for analysis. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. the Sham + Vehicle group; \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the TAC + Vehicle group. HE, hematoxylin-eosin; WGA, wheat germ agglutinin; TAC, transverse aortic constriction; HW, heart weigh; BW, body weight; TL, tibia length; LVESd, left ventricular end-systolic diameter; LVEDd, left ventricular end-diastolic diameter; FS, fractional shortening; EF, ejection fraction; *Anp*, atrial natriuretic peptide; *Bnp*, B-type natriuretic peptide; *Myh7*,  $\beta$ -myosin heavy chain.

**Statistical analysis.** All data were presented as means  $\pm$  SD. An unpaired t-test was utilized to evaluate the statistical significance between two groups. For multiple comparisons, one-way ANOVA followed by Tukey's or Welch ANOVA followed by Dunnett's post hoc test was employed, contingent on the results of the homogeneity test of variance. The analysis of data and the creation of figures were carried out with GraphPad Prism 9.0 (Dotmatics).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Larixyl acetate attenuates pressure overload-induced heart failure.** Compared with the Sham + Vehicle group, the TAC + Vehicle group exhibited significant increases in heart weight (HW)/body weight (BW;  $P < 0.001$ ;  $t = 12.45$ ,  $df = 36$ ), HW/tibia length (TL;  $P < 0.001$ ;  $t = 13.50$ ,  $df = 36$ ; Fig. 1B), LV end-systolic diameter (LVESd;  $P < 0.001$ ;  $t = 7.11$ ,  $df = 36$ ) and LV end-diastolic diameter (LVEDd;  $P < 0.001$ ;  $t = 10.16$ ,  $df = 36$ ) (Fig. 1D). Whereas the fractional shortening (FS;  $P < 0.001$ ;  $t = 6.99$ ,  $df = 36$ ) and ejection fraction (EF) were significantly decreased ( $P < 0.001$ ;  $t = 7.57$ ,  $df = 36$ ; Fig. 1D). In addition, TAC induced upregulation of cardiac hypertrophy-related genes, such as atrial natriuretic peptide (*Anp*;  $P = 0.0016$ ;  $t = 11.14$ ,  $df = 4.141$ ), B-type natriuretic peptide (*Bnp*;  $P < 0.001$ ;  $t = 27.13$ ,  $df = 16$ ) and  $\beta$ -myosin heavy chain (*Mh7*;  $P < 0.001$ ;  $t = 13.77$ ,  $df = 16$ ; Fig. 1E). However, treatment with larixyl acetate for 4 weeks reversed the above alterations in the TAC + Larixyl group compared with the TAC + Vehicle group.

The expression of TRPC6 among groups was also detected and the results showed that 4 weeks of TAC induced a significant upregulation of TRPC6 expression when compared with the Sham + Vehicle group ( $P < 0.001$ ;  $t = 9.94$ ,  $df = 8$ ; Fig. S1A). Whereas the administration of larixyl acetate reversed the aforementioned alteration.

**Larixyl acetate ameliorates pressure overload-induced cardiac fibrosis.** Next, the effect of larixyl acetate on cardiac fibrosis, an important pathophysiological mechanism of heart failure, was evaluated. The findings revealed that TAC induced a substantial interstitial ( $P < 0.001$ ;  $t = 17.30$ ,  $df = 16$ ) and perivascular ( $P < 0.001$ ;  $t = 11.63$ ,  $df = 16$ ) fibrosis of the heart, as observed through sirius red staining when compared with the Sham + Vehicle group (Fig. 2A and B). Additionally, a significant upregulation of fibrosis-related genes, such as collagen type I  $\alpha 1$  (*Col1a1*;  $P = 0.0033$ ;  $t = 9.27$ ,  $df = 4.155$ ) and collagen type III  $\alpha 1$  (*Col3a1*;  $P = 0.0025$ ;  $t = 7.88$ ,  $df = 4.596$ ), in the TAC + Vehicle group (Fig. 2C) was observed. The results also demonstrated that the effects of larixyl acetate on fibrosis were consistent with the observations made during cardiac hypertrophy and heart failure. Specifically, the administration of larixyl acetate effectively inhibited the development of cardiac fibrosis after TAC.

**Larixyl acetate decreases pressure overload-induced cardiomyocyte apoptosis.** TUNEL staining revealed a significant increase in apoptotic cells in the TAC + Vehicle group compared with the Sham + Vehicle group ( $P < 0.001$ ;  $t = 8.18$ ,

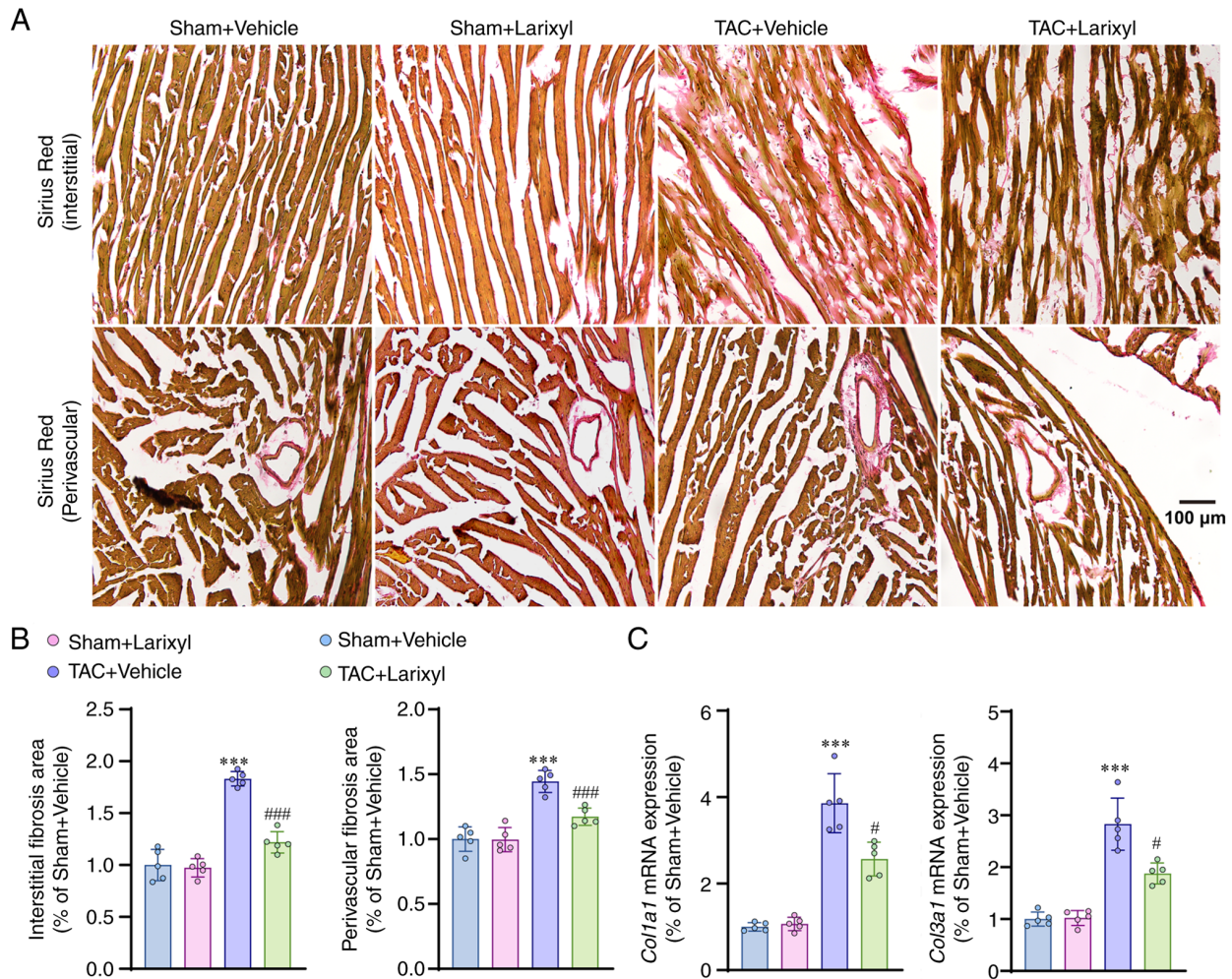


Figure 2. Larixyl acetate ameliorates pressure overload-induced cardiac fibrosis. (A) Representative images of sirius red staining in the hearts of mice from the sham + vehicle, Sham + Larixyl, TAC + Vehicle and TAC + Larixyl groups at 4 weeks after TAC. (B) Quantitative results of interstitial and perivascular fibrosis area of the heart from the indicated groups (n=5/group). (C) Relative mRNA levels of fibrosis marker genes, *Col1a1* and *Col3a1* in the heart from the indicated groups (n=5/group). Data are presented as the mean  $\pm$  SD. For (B) the homogeneity of variance test indicated no significant differences, hence, data analysis was conducted using one-way ANOVA. For (C) significant differences were observed in the homogeneity of variance test and Welch's ANOVA was used for data analysis. \*\*\* $P < 0.001$  vs. the Sham + Vehicle group; # $P < 0.05$  and ### $P < 0.001$  vs. the TAC + Vehicle group. TAC, transverse aortic constriction; *Col1a1*, collagen type I  $\alpha 1$ ; *Col3a1*, collagen type III  $\alpha 1$ .

df=16; Fig. 3A). Western blotting analysis demonstrated that TAC downregulated the anti-apoptotic Bcl2/Bax pathway ( $P = 0.0048$ ;  $t = 5.70$ , df=16) and upregulated the pro-apoptotic cleaved caspase-3 pathway ( $P < 0.001$ ;  $t = 13.89$ , df=16; Fig. 3B). These results indicated that TAC induced cardiomyocyte apoptosis by disrupting the balance between anti- and pro-apoptotic pathways. Treatment with larixyl acetate via i.p. injection for 4 weeks effectively reversed these impairments.

*Larixyl acetate inhibits ER stress and the mTOR signaling pathway and promotes autophagy.* To elucidate the mechanisms underlying the protective effect of larixyl acetate against cardiomyocyte apoptosis and heart failure, the involvement of ER stress, the mTOR signaling pathway and autophagy was investigated. Firstly, it was confirmed that 4 weeks of TAC significantly upregulated the expression of ER stress-related proteins, such as GRP78 ( $P < 0.001$ ;  $t = 7.30$ , df=16), pPERK/PERP ( $P < 0.001$ ;  $t = 11.29$ , df=16), ATF4 ( $P < 0.001$ ;  $t = 12.49$ , df=16) and CHOP ( $P < 0.001$ ;  $t = 12.39$ , df=16), which are closely related to promoting cardiomyocyte

apoptosis, in the TAC + Vehicle group compared with the Sham + Vehicle group (Fig. 4A). Secondly, it was found that TAC increased the level of pmTOR/mTOR ( $P = 0.001$ ;  $t = 9.61$ , df=16) in the TAC + Vehicle group, indicating the activation of the mTOR signaling pathway (Fig. 4B). Thirdly, whether mTOR activation was accompanied by inhibition of autophagy was investigated by assessing the autophagy markers P62 and the ratio of light chain 3B II to light chain 3B I (LC3B II/I). The results showed that the expression level of P62 ( $P = 0.0047$ ;  $t = 5.71$ , df=16) was increased, while LC3B II/I ( $P < 0.001$ ;  $t = 8.40$ , df=16) was decreased in the TAC + Vehicle group compared with the Sham + Vehicle group (Fig. 4B), suggesting that autophagy was inhibited. Treatment with larixyl acetate effectively reversed these alterations.

*Larixyl acetate alleviates Ang II-induced H9c2 cell hypertrophy, ER stress and apoptosis while promoting autophagy; mTOR activation reverses these alterations, except for cellular hypertrophy.* Compared with the Con + Vehicle group, the Ang II + Vehicle group cells showed



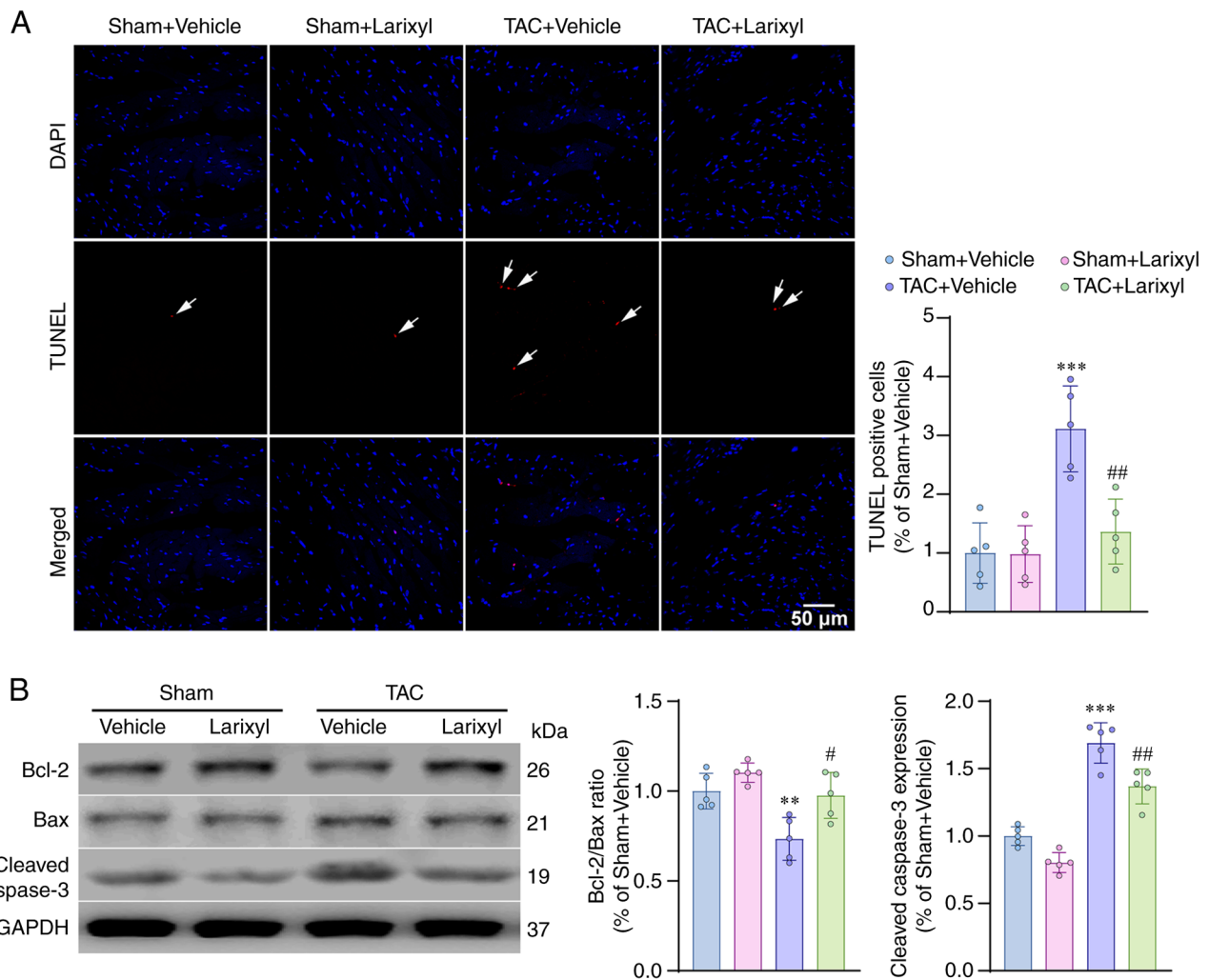


Figure 3. Larixyl acetate decreases pressure overload-induced cardiomyocyte apoptosis. (A) Representative images of TUNEL staining (left) in the hearts of mice from the Sham + Vehicle, Sham + Larixyl, TAC + Vehicle and TAC + Larixyl groups at 4 weeks after TAC. Quantitative results of TUNEL<sup>+</sup> cells (right) from indicated groups (n=5/group). (B) Relative protein levels of apoptosis markers Bcl-2, Bax and cleaved caspase-3 in the heart from the indicated groups (n=5/group). Data are presented as the mean  $\pm$  SD. The homogeneity of variance test applied to the data revealed no significant differences, therefore, one-way ANOVA was used for the analysis. \*\*P<0.01 and \*\*\*P<0.001 vs. the Sham + Vehicle group; #P<0.05 and ##P<0.01 vs. the TAC + Vehicle group. TAC, transverse aortic constriction.

significant hypertrophy, as evidenced by an enlarged cellular area (P<0.001; t=17.91, df=16), upregulation of *Anp* (P<0.001; t=13.58, df=16), *Bnp* (P<0.001; t=14.28, df=16) and *Myh7* (P<0.001; t=16.75, df=16) genes (Fig. 5A). Additionally, Ang II significantly increased the expression levels of GRP78 (P<0.001; t=10.36, df=16), pPERK/PERK (P<0.001; t=13.08, df=16), ATF4 (P<0.001; t=13.31, df=16), CHOP (P<0.001; t=7.71, df=16), pmTOR/mTOR (P=0.0056; t=5.84, df=5.569), P62 (P=0.0033; t=5.97, df=16) and cleaved caspase-3 (P<0.001; t=7.80, df=16), while decreasing the ratio of LC3B-II/I (P<0.001; t=8.87, df=16) and Bcl-2/Bax (P<0.001; t=10.14, df=16; Fig. 5B-D). These results suggested that Ang II increases ER stress, mTOR signaling pathway and apoptosis, while inhibiting autophagy. Preincubation with larixyl acetate alleviated the above impairments, whereas the co-incubation with MHY1485 abolished the effects of larixyl acetate, except for cellular hypertrophy. These findings indicated that larixyl acetate can enhance autophagy and reduce ER stress and apoptosis by inhibiting the mTOR signal pathway, thereby playing a protective role in cardiac dysfunction.

Additionally, the expression of TRPC6 across different groups was assessed. The results showed that Ang II significantly upregulated TRPC6 expression compared with the Con + Vehicle group (P<0.001; t=9.41, df=8; Fig. S1B). The administration of larixyl acetate, with or without MHY1485, reversed this alteration. This finding aligns with the *in vitro* experiments, demonstrating that not only mechanical but also chemical activation of TRPC6 can further enhance its expression. Since MHY1485 did not have an additional influence, it suggests that the mTOR pathway is not involved in this feedback loop.

**Activation of the mTOR signal abrogates the protective effects of larixyl acetate *in vitro*.** Based on the evidence presented, it was hypothesized that mTOR activation may influence the protective effects of larixyl acetate in TAC-induced heart failure. Accordingly, larixyl acetate was injected i.p. at a dose of 5 mg/kg, once/day, with or without the mTOR activator MHY1485 at 10 mg/kg, also once/day. This treatment was continued for 4 weeks following TAC to test the hypothesis. At the end of the TAC model, heart function was evaluated

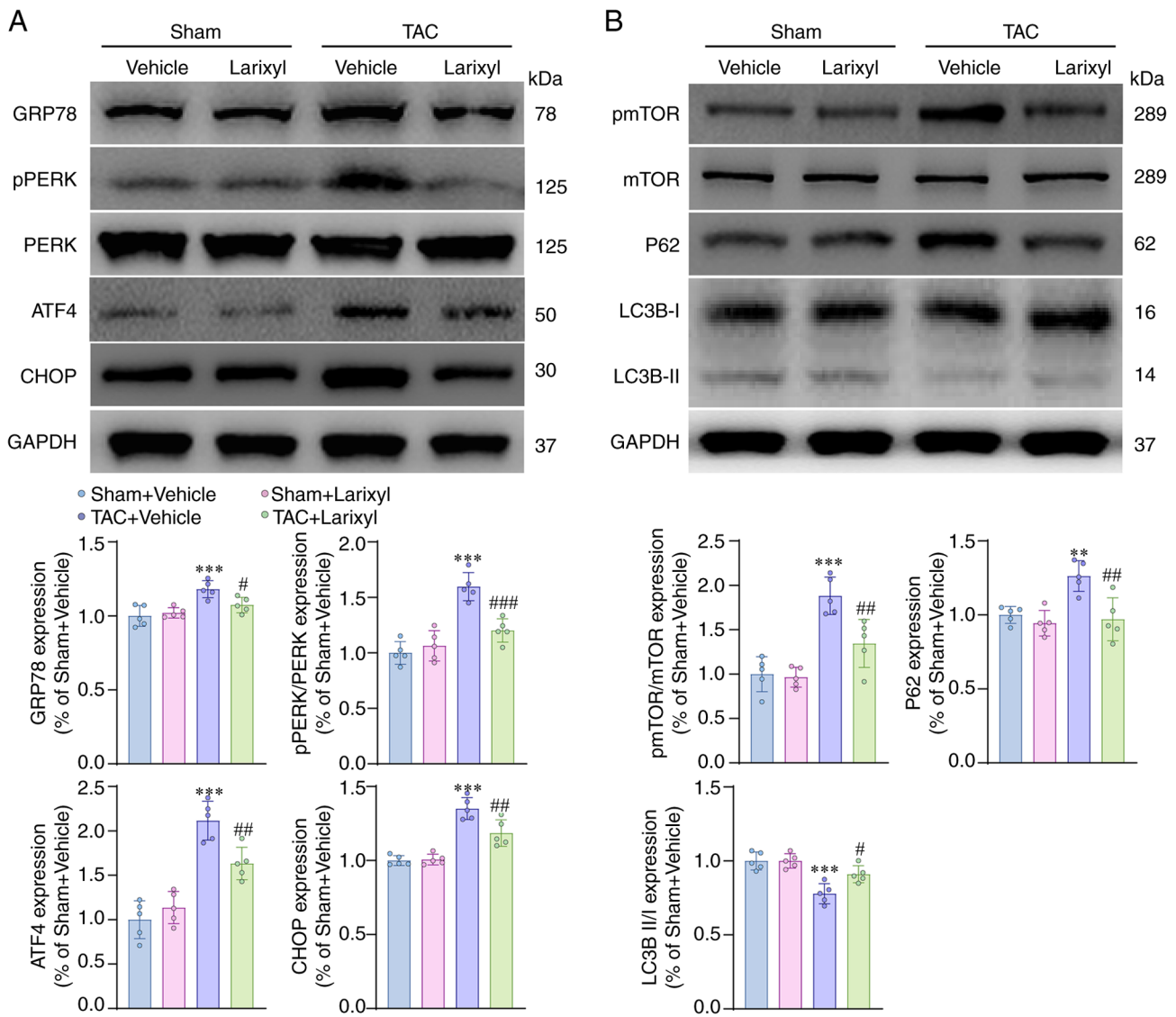


Figure 4. Larixyl acetate inhibits ER stress and mTOR signal pathway and promotes autophagy. (A) Relative protein levels of ER stress markers, GRP78, PERK, ATF4 and CHOP in the heart from the Sham + Vehicle, Sham + Larixyl, TAC + Vehicle and TAC + Larixyl groups at 4 weeks after TAC (n=5/group). (B) Relative protein levels of pmTOR/mTOR and autophagy markers, P62 and LC3BII/I in the heart from indicated groups (n=5/group). The data are presented as the mean  $\pm$  SD. The homogeneity of variance test for the data did not reveal significant differences, therefore, the data were analyzed using one-way ANOVA. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the Sham + Vehicle group; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. the TAC + Vehicle group. ER, endoplasmic reticulum; mTOR, mammalian target of rapamycin; TAC, transverse aortic constriction.

using transthoracic echocardiography. The results showed that LVESd ( $P < 0.001$ ;  $t = 5.15$ ,  $df = 14$ ) and LVEDd ( $P = 0.0236$ ;  $t = 2.54$ ,  $df = 14$ ) were significantly increased, whereas FS ( $P < 0.001$ ;  $t = 4.27$ ,  $df = 14$ ) and EF ( $P < 0.001$ ;  $t = 4.40$ ,  $df = 14$ ) were significantly decreased in the TAC + Larixyl + MHY group when compared with the TAC + Larixyl group (Fig. 5E). These findings suggested that inhibition of the mTOR signal pathway is essential for the protective effects of larixyl acetate in maintaining cardiac function under pressure overload stress.

## Discussion

It is widely recognized that persistent pressure overload resulting from pathological conditions can induce heart failure, which is a leading cause of morbidity and mortality (2,8). To improve the understanding of the pathophysiologic mechanisms underlying heart failure and identify promising

therapeutic targets, it is necessary to conduct further research. The present study has demonstrated that larixyl acetate, a TRPC6 inhibitor, can effectively reverse cardiac dysfunction induced by pressure overload. Notably, it was observed that the protective role of larixyl acetate involves the inhibition of autophagy through decreased phosphorylation of mTOR, which consequently diminishes ER stress and apoptosis in cardiomyocytes. These findings provided valuable insights into the pathogenesis of heart failure and highlight the potential utility of larixyl acetate as a therapeutic intervention (Fig. 6).

The ER is a multifunctional organelle that plays an important role in the folding of secretory and membrane proteins (12,48). In cases of pressure overload, there is an increase in protein synthesis, which can lead to ER stress and subsequent cardiomyocyte apoptosis (49,50). Autophagy is a crucial lysosome-dependent catabolic process that plays a vital role in the degradation and recycling of cytoplasmic

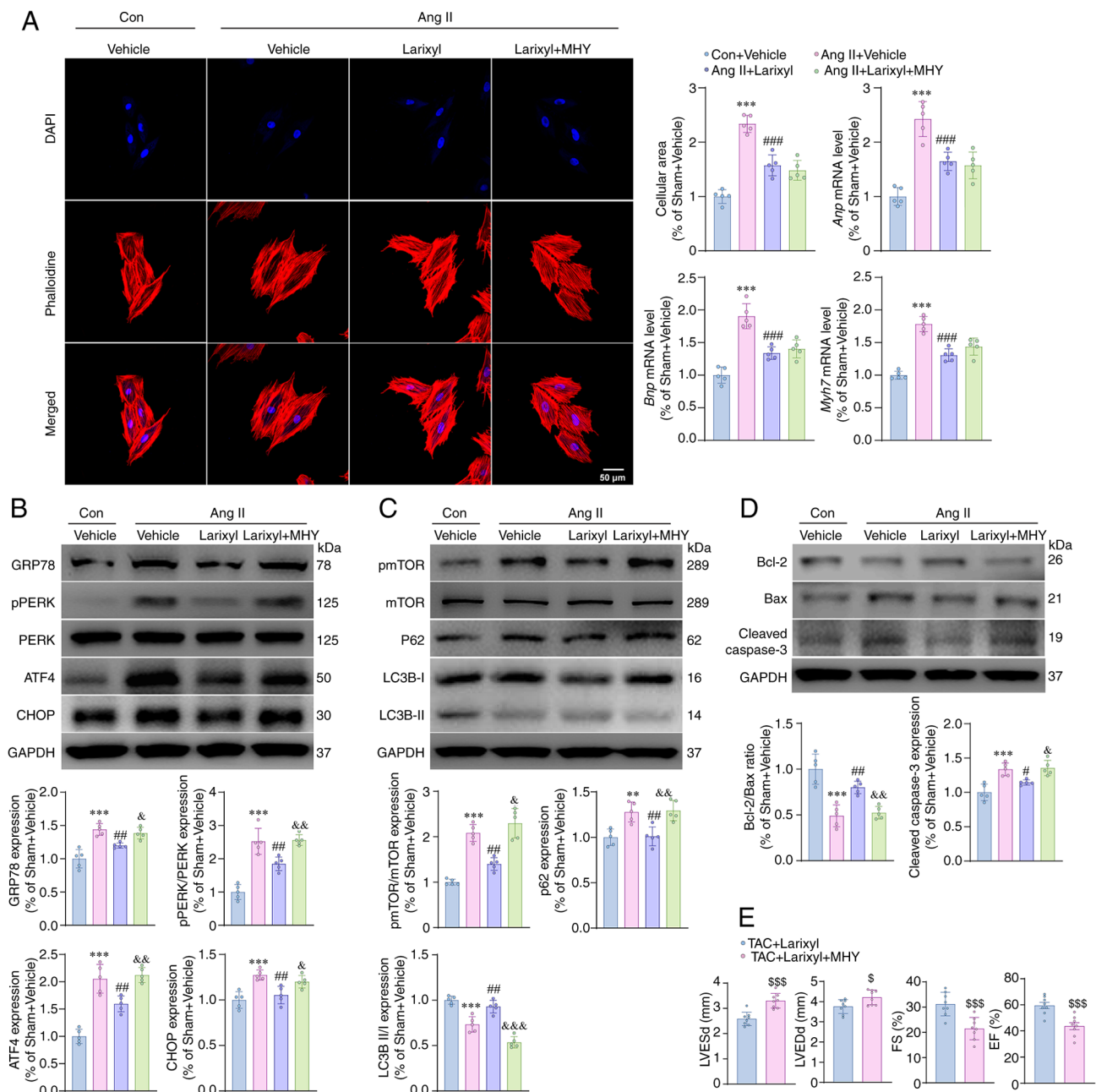


Figure 5. Activation of the mTOR signal reverses the protective effects of larixyl acetate *in vitro* and *in vivo*. (A) Representative images of phalloidine staining (left) of H9c2 cells from the Con + vehicle, Ang II + Vehicle, Ang II + Larixyl and Ang II + Larixyl + MYH groups at 72 h after drugs treatment. Quantitative results (right) of cellular area (n=5/group) and relative mRNA levels of hypertrophy markers of cells (n=5/group) from the indicated groups. (B) Relative protein levels of ER stress markers in cells from the indicated groups (n=5/group). (C) Relative protein levels of pmTOR/mTOR and autophagy markers in the heart from indicated groups (n=5/group). (D) Relative protein levels of apoptosis markers in cells from the indicated groups (n=5/group). (E) EF of the hearts from indicated groups evaluated by transthoracic echocardiograph (n=8/group). Data are presented as the mean  $\pm$  SD. For pmTOR/mTOR expression, the homogeneity of variance test showed significant differences, therefore, the data were analyzed using Welch ANOVA. The homogeneity of variance test for the other data did not reveal significant differences, thus, the data were analyzed using one-way ANOVA followed. \*\*P<0.01 and \*\*\*P<0.001 vs. the Con + Vehicle group; #P<0.05, ##P<0.01 and ###P<0.001 vs. the Ang II + Vehicle group; &P<0.05, &&P<0.01 and &&&P<0.001 vs. the Ang II + Larixyl group; \$P<0.05 and \$\$\$P<0.001 vs. the TAC + Larixyl group. mTOR, mammalian target of rapamycin; Con, control; Ang II, angiotensin II; MYH, MYH1485; ER, endoplasmic reticulum.

components to maintain intracellular homeostasis (51,52). Numerous studies have demonstrated that autophagy can protect against ER stress-induced apoptosis of myocytes by limiting the accumulation of misfolded proteins, thereby preventing the deterioration of cardiac function (23,53). This suggests that autophagy serves as a critical mechanism to maintain the proper functioning of cardiac cells under stressful conditions (54,55). The present study revealed that either

4 weeks of TAC or 72 h of incubation with Ang II significantly upregulated ER stress while downregulating autophagy, which are key factors for the malfunction of cardiomyocytes (25). However, it was found that the administration of larixyl acetate can mitigate this damage by promoting autophagy and limiting ER stress. This highlights the potential therapeutic value of targeting TRPC6 to alleviate cardiac dysfunction associated with ER stress and impaired autophagy.



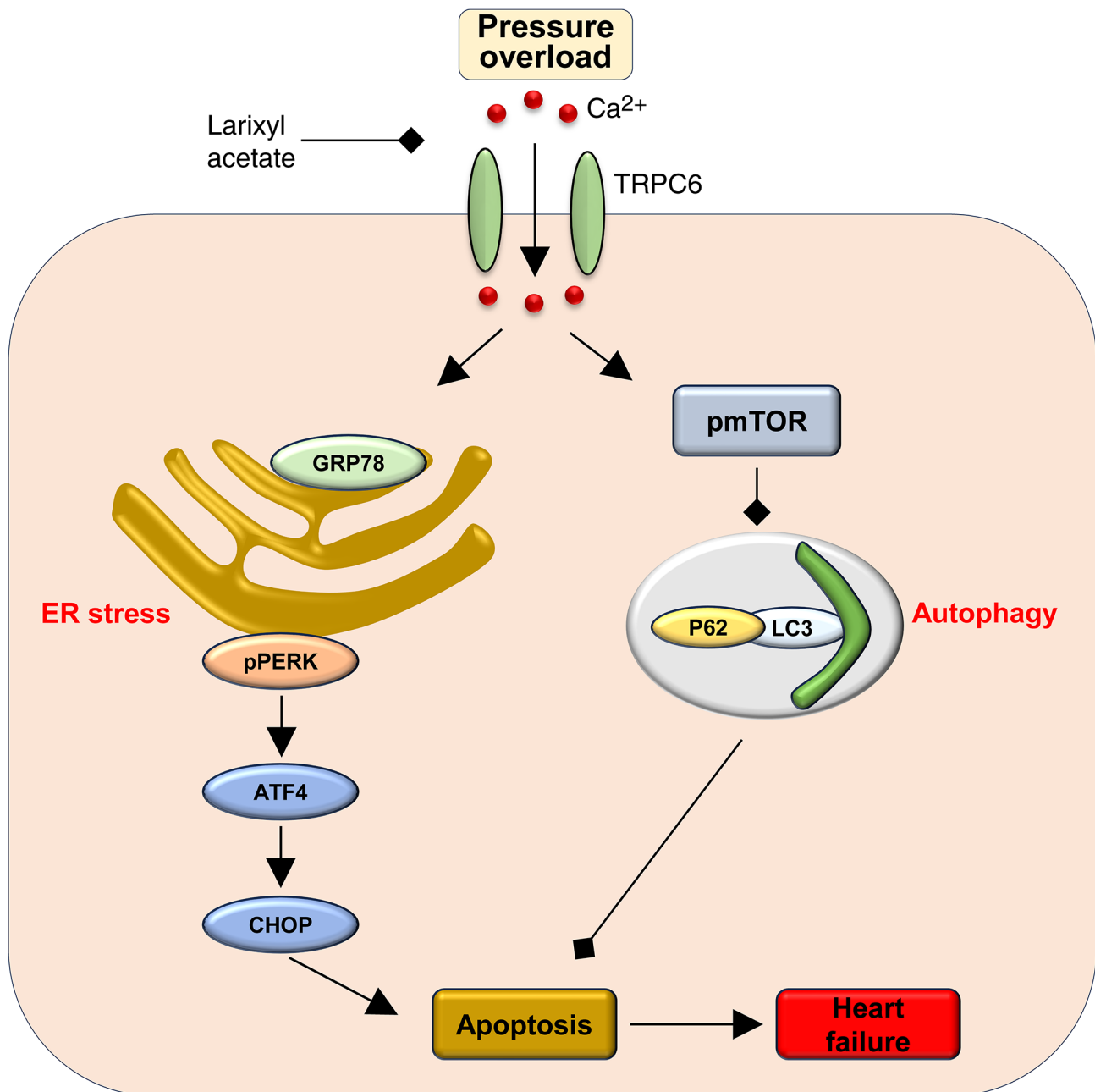


Figure 6. The general overview of the main highlights of this study. Larixyl acetate improves heart failure by reducing ER stress and apoptosis while promoting autophagy through mTOR signaling inhibition.

Previous studies have indicated that pressure overload or Ang II-induced activation of TRPC6 can initiate a positive feedback loop, resulting in increased TRPC6 expression. This activation and upregulation contribute to cardiac hypertrophy through the calcineurin/NFAT signaling pathway (35,56). However, it is unclear whether TRPC6 inhibition can modulate ER stress and autophagy, which are key processes involved in cardiomyocyte function and survival. It has been reported that TRPC1 can regulate ER stress in dopaminergic neurons (57), and TRPC6 has been shown to regulate the mTOR signaling pathway, a well-established mediator of autophagy, in human pulmonary arterial smooth muscle cells (37). Therefore, it is reasonable to hypothesize that TRPC6 may also modulate ER stress and autophagy in the context of cardiac function. Additionally, it has been well

established that pressure overload-induced heart failure is partly driven by excessive cardiomyocyte apoptosis following TAC (46). Furthermore, recent studies have highlighted that downregulation or inhibition of TRPC6 can exert anti-apoptotic effects (47,48). Consequently, it can be hypothesized that larixyl acetate may preserve cardiac function post-TAC, potentially by mitigating cardiomyocyte apoptosis. Consistent with the aforementioned hypothesis, the present study found that blocking TRPC6 by larixyl acetate significantly decreased ER stress and phosphorylation of mTOR, while promoting autophagy and decreased apoptosis.

The aforementioned results have established that TRPC6 inhibition can mitigate ER stress and apoptosis; however, these effects might be attributed mainly to the inhibition of the calcineurin A/NFAT pathway (12). It remains unclear

whether the reduced phosphorylation of the mTOR signaling pathway and the resulting enhancement of autophagy also contribute to the reduction of ER stress and the protection against apoptosis. To investigate this, larixyl acetate was administered to block TRPC6, while concurrently using the mTOR activator MHY1485 to specifically activate the mTOR pathway and suppress autophagy in an Ang II-induced H9C2 cellular hypertrophy model. The findings of the present study revealed a notable phenomenon that although MHY1485 does not affect the anti-hypertrophic effects of larixyl acetate, it still abolished the protective role of larixyl acetate by pronounced significant ER stress and apoptosis through increasing mTOR phosphorylation and inhibiting autophagy.

A reasonable explanation is that administering larixyl acetate, both *in vivo* and *in vitro*, does not completely diminish cardiac hypertrophy. This partial effect is likely because there are multiple mechanisms (2), apart from TRPC6, that mediate pressure overload or Ang II-induced cardiac hypertrophy and heart failure. The results from the present study lend strong support to this hypothesis. Although some hypertrophy persists, the blockade of larixyl acetate of the mTOR signaling pathway simultaneously promotes the protective effects of autophagy. This action effectively counters the adverse effects of residual hypertrophy and prevents myocardial damage. Conversely, the activation of mTOR using MHY1485 can negate this protective effect. This evidence indicated that TRPC6 inhibitors may exert a cardioprotective role through two mechanisms. The first is the traditional inhibition of the calcineurin A/NFAT pathway and the second is via the newly identified mTOR signaling pathway. This dual-mechanism action potentially makes TRPC6 inhibitors more effective than drugs operating through a single mechanism.

In addition to cardiomyocyte apoptosis, myocardial fibrosis represents a key pathological alteration in cardiac hypertrophy and heart failure (58). Studies have shown a strong association between TRPC6 activation and fibrosis. For example, cardiac-specific overexpression of TRPC6 in transgenic mice triggers cardiac fibrosis, hypertrophy and heart failure (35). In cases of renal injury induced by unilateral ureteral obstruction in mice, an increase in *TRPC6* gene expression is observed, leading to significant interstitial fibrosis. This effect is notably reduced in TRPC6-deficient mice (59). Similarly, TRPC6 knockout mice exhibit less lung fibrosis in the bleomycin-treated mice model (60). In the present research, it was also discovered that the administration of larixyl acetate significantly reduces myocardial fibrosis. This anti-fibrotic effect is two-fold, firstly, through the inhibition of cardiomyocyte apoptosis, as aforementioned, and secondly, it may directly inhibit the transdifferentiation of fibroblasts into myofibroblasts. A previous study indicated that overexpression of TRPC6 in fibroblasts markedly facilitates their conversion to myofibroblasts, whereas the absence of TRPC6 prevents TGF- $\beta$ -mediated myofibroblast conversion (61).

In the present study exploring cardiac hypertrophy, heart failure and the effects of larixyl acetate, some limitations were acknowledged and future research directions identified. Firstly, the use of the H9C2 rat cardiomyocyte line, instead of primary cultured cardiomyocytes, may not fully replicate the complex *in vivo* environment or the exact behavior of

primary cardiomyocytes, potentially biasing the conclusions of the present study. Secondly, while larixyl acetate significantly reduced myocardial fibrosis *in vivo*, there was a lack of *in vitro* evidence to confirm its direct impact on fibrosis. Additionally, its high selectivity in inhibiting TRPC6 may not rule out off-target effects. Future research should focus on validating the findings of the present study with primary cultured cardiomyocytes for more accurate insights, exploring the direct effects of larixyl acetate on fibroblasts, and using more specific molecular or genetic interventions to clarify the role of TRPC6 in cardiac fibrosis and hypertrophy.

In summary, the present study demonstrated that larixyl acetate, a TRPC6 inhibitor, plays a significant cardioprotective role against cardiac hypertrophy and heart failure. The mTOR signaling pathway was newly identified as a key component in this cardioprotective effect. This novel mechanism involves the enhancement of autophagy through the decrease in phosphorylation of mTOR, which subsequently reduces ER stress and cardiomyocyte apoptosis. These findings not only enhance understanding of the pathophysiology of heart failure but also underscore the therapeutic potential of larixyl acetate. It offers a dual-mechanism approach that could potentially surpass the efficacy of single-mechanism drugs in heart failure treatment. Additionally, this research provides a new perspective for developing novel anti-heart failure drugs targeting the mTOR signaling pathway.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

MJ and WXL conducted the experiments and contributed to data acquisition; KYZ, RSL and ZGW performed data analysis; MJ and WXL wrote the manuscript; JP, JJY and DJW made significant contributions to the conception and design of the study. JJY and DJW reviewed the manuscript and gave critical suggestions for revision. MJ, WXL, KYZ, ZGW, RSL, JP, JJY and DJW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

All the experimental procedures were approved by The Experimental Animal Ethics Committee of Drum Tower Hospital of Nanjing University Medical School (Nanjing, China; approval no. 2022AE01017).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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