

## Original Article

# Pachyman serves as a protective agent for cardiac hypertrophy via inhibiting the PI3K/Akt/mTOR signaling pathway

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Received September 3, 2017; Accepted May 26, 2018; Epub September 15, 2018; Published September 30, 2018

**Abstract:** Pachyman, a polysaccharide isolated from wild mushrooms, is commonly applied as an anti-inflammatory agent in traditional Chinese medicine. But whether they are effective in the improvement of cardiac hypertrophy has never been evaluated. First, a cellular cardiac hypertrophy model was established using 5  $\mu\text{M}$  AngII. Cell surface was observed after pachyman treatment with or without AngII pre-incubation. Our data showed that H9c2 cell surface area was gradually increased after AngII treatment for 24 h and 48 h. Furthermore, AngII treatment increased the mRNA levels of atrial natriuretic peptide (ANP) and  $\beta$ -myosin heavy chain ( $\beta$ -MHC). In contrast, we found that pachyman gradually decreased cell surface area in a dose dependent manner. Additionally, qPCR analysis indicated that 10  $\mu\text{g}/\mu\text{l}$  pachyman incubation decreased the mRNA levels of ANP and  $\beta$ -MHC. Based on GFP-LC3 transfection assay, pachyman treatment induced prominent autophagic dots in H9c2 cells with or without AngII. Meanwhile, preincubation of 10  $\mu\text{g}/\mu\text{l}$  pachyman significantly increased the ratio between LC3II/LC3I and the expression of Beclin11. Besides, we found that pachyman treatment decreased the phosphorylation levels of PI3K, Akt, mTOR, S6K, S6. Furthermore, we also explored whether pachyman induced cell autophagy was mediated via PI3K/Akt/mTOR signaling pathway. Hence, H9c2 cells were pre-incubated with 10  $\mu\text{M}$  3-Methyladenine (3-MA), a selective class III PI3k inhibitor. We found that pachyman-induced up-regulation of Beclin11 and LC3II/LC3I could be largely reversed by 3-MA pre-incubation. In summary, in vitro study showed that pachyman may serve as a protective agent for cardiac hypertrophy via activating autophagy through the PI3K/Akt/mTOR signaling pathway.

**Keywords:** Pachyman, cardiac hypertrophy, PI3K/Akt/mTOR signaling, autophagy

## Introduction

To maintain the cardiac output, the heart has to undergo morphological changes due to long term pressure overload, thereby resulting in compensated hypertrophy [1, 2]. But sustained cardiac hypertrophy is a major risk factor for lethal cardiovascular events since it can induce arrhythmias, heart failure, and sudden cardiac death [3]. Therefore, it is of great importance for us to explore the underlying mechanisms of cardiac hypertrophy and then develop novel interventions for this disease.

Increasing evidence has indicated the important role of autophagy in the progression of car-

diac hypertrophy [4, 5]. As an evolutionary conserved cellular process, autophagy mainly aims to degrade and recycle protein aggregates and organelles [6]. Autophagy is activated by various stimuli, including hypoxia and ischemia. In multiple cardiovascular diseases, including pressure overload-induced cardiac hypertrophy and heart failure, aberrant activation of autophagy is identified [7, 8]. The phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway is indicated to play an important role in the cardiovascular system through the negative regulation of autophagy in mammal cells [9, 10]. Furthermore, suppression of mTOR prevents cardiac hypertrophy [11]. Based on the molecular basis of

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pathological myocyte hypertrophy, formulating therapeutic strategies to prevent cardiac hypertrophy is necessary.

Pachyman, a polysaccharide isolated from wild mushrooms, is commonly applied as an anti-inflammatory agent in traditional Chinese medicine. But whether they are effective in the improvement of cardiac hypertrophy has never been evaluated. In the current study, we first showed that pachyman treatment improved cardiac hypertrophy via suppressing Akt/mTOR pathway. These data may provide novel prevention and therapy methods for cardiac hypertrophy.

### Materials and methods

#### Cell culture

H9c2 cells were purchased from ATCC and maintained in DMEM with 4.5 g/L glucose supplemented with 10% (v/v) FBS. Cells were cultured to 80% confluence before passage and harvest for experiments. To establish cardiac hypertrophy cell model, cells were seeded in growth media for 24 h and then were exposed to angiotensin II (AngII) alone or in combination with pachyman (Ningbo Dekang Biological Product Co., Ltd.), which were added to the culture media at various concentrations for 48 h.

#### Cell treatment

H9c2 cells were incubated with 5  $\mu$ M of AngII in culture for 12, 24 or 48 h. To evaluate the mechanism by which pachyman affected AngII-induced cellular hypertrophy, cells were incubated with 10  $\mu$ g/ $\mu$ l pachyman. All experiments were performed in triplicate.

#### Assessment of cellular hypertrophy

H9c2 cells were fixed with 4% paraformaldehyde and then stained with 1% crystal violet (Fisher Scientific). After treatment, the cells were incubated with 0.4% Triton X-100 for 90 min and then the cells were incubated with goat serum at room temperature for 60 min. Then, the samples were treated with anti-sarcomeric actin antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. Next, cells were stained with FITC-conjugate goat anti-mouse antibody for 1 h. Images were obtained under a fluorescence microscope (Nikon 80i,

Japan). Cell surface area was determined using Image-Pro Plus Data Analysis Software (Media Cybernetics).

#### Western immunoblotting

Proteins samples were isolated from RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) with a supplementation of a protease and phosphatase inhibitor cocktail (Sigma). Next, the samples were separated by 12% SDS-PAGE, and then electrophoretically transferred onto a PVDF membrane. The membranes were further blocked with 8% milk in PBST (pH 7.5) for 2 h at room temperature and were incubated with the primary antibodies, anti-p-Akt, anti-p-mTOR, anti-p-S6K, anti-LC3 and anti-GAPDH (Cell signaling). Immunodetection was performed using enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. GAPDH was used as the internal control.

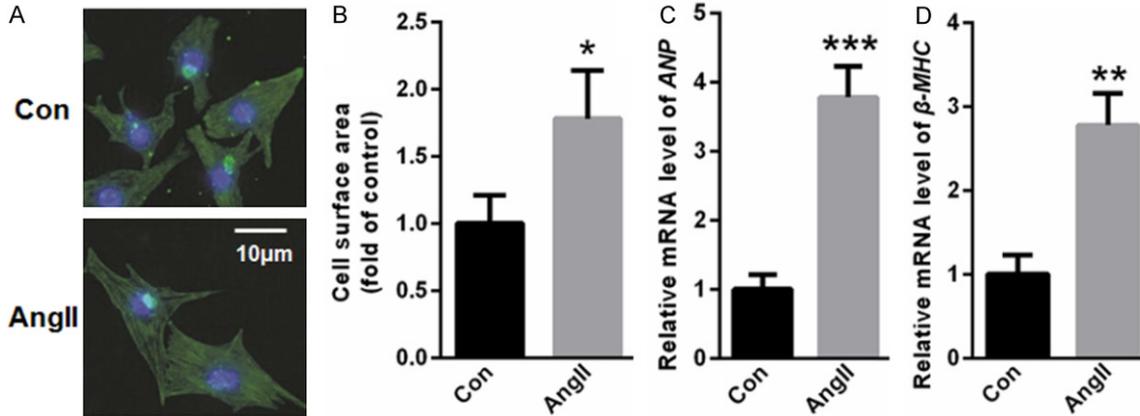
#### Quantitative real-time RT-PCR

RNA was isolated in an RNA TRIZOL (Invitrogen) according to the manufacturer's instructions. Then, the RNA was transcribed into complementary DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems). To quantify the expression of mRNAs, a quantitative real-time PCR assay was carried out with SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCyclerIQ real-time PCR detection system. The primers used in the current study were listed as follows: GAPDH-F: 5'-ATGG GAAGCTGGTCATC AAC-3'; GAPDH-R: 5'-GTGGTTCACACCCATCAC AA-3'; ANP-F: 5'-ATACAGTGC GGTTGTTCCAACA-3'; ANF-R: 5'-AGCCCTCAGTTTGTCTTTTCA-3';  $\beta$ -MH-C-F: 5'-GGAGAAAGAGAAGAGCGAGTTC-3';  $\beta$ -MH-C-R: 5'-GGC ACATCTTCTCCAGTTAG-3'.

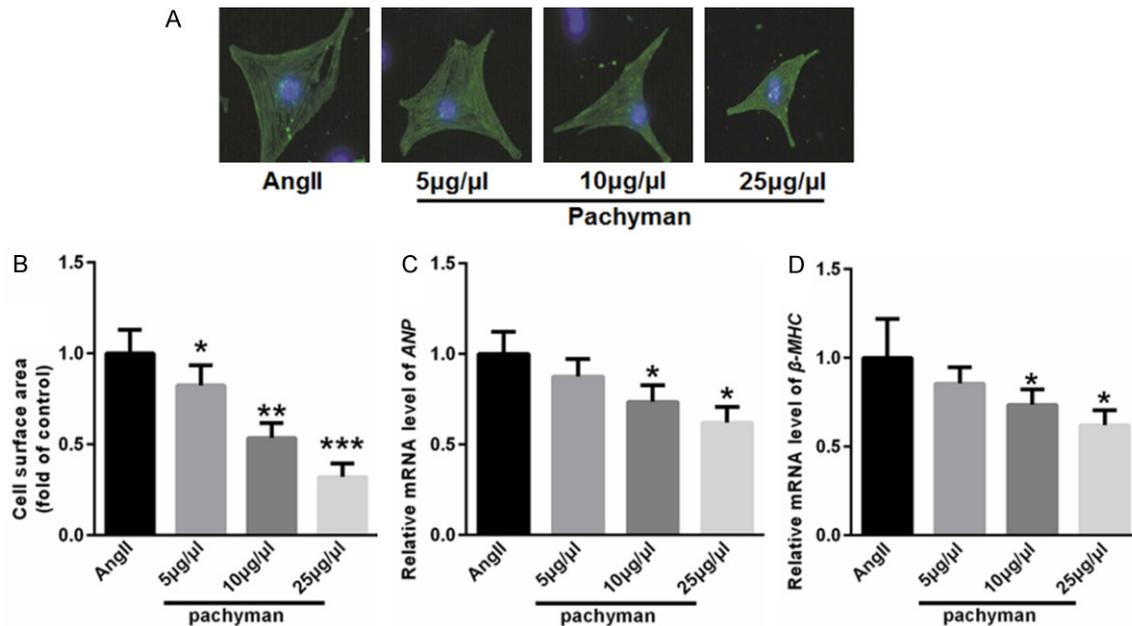
#### Statistical analysis

Data were expressed as mean  $\pm$  SEM. For comparison between 2 groups, 2-tailed Student's t-test was used; for comparison among multiple groups (more than 3 groups), one-way ANOVA with Bonferroni's post hoc test was used. *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software version 22.0 (IBM, Armonk, NY).

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**Figure 1.** Cardiac myocyte hypertrophy model was established using AngII treatment. (A) H9c2 cell surface area was gradually increased after AngII treatment after 24 h and 48 h. (B) Statistical analysis of cell surface area after AngII treatment for 24 h and 48 h. Real time PCR analysis indicated that the mRNA levels of ANP (C) and  $\beta$ -MHC (D) were increased in H9c2 cells after 12, 24 and 48 h of AngII exposure. \* $p < 0.05$ , \*\* $p < 0.01$  vs. control.



**Figure 2.** Pachyman ameliorates AngII-induced myocyte hypertrophy. (A) Preincubation with 5, 10, 25  $\mu\text{g}/\mu\text{l}$  pachyman decreased cell surface area gradually after H9c2 cells exposure to 5  $\mu\text{M}$  of AngII for 48 h. (B) Statistical analysis of cell surface area after H9c2 cells were pre-incubated with 5, 10, 25  $\mu\text{g}/\mu\text{l}$  pachyman. qPCR analysis indicated that 10  $\mu\text{g}/\mu\text{l}$  pachyman incubation decreased the mRNA levels of ANP (C) and  $\beta$ -MHC (D). \* $p < 0.05$ , \*\* $p < 0.01$  vs. control.

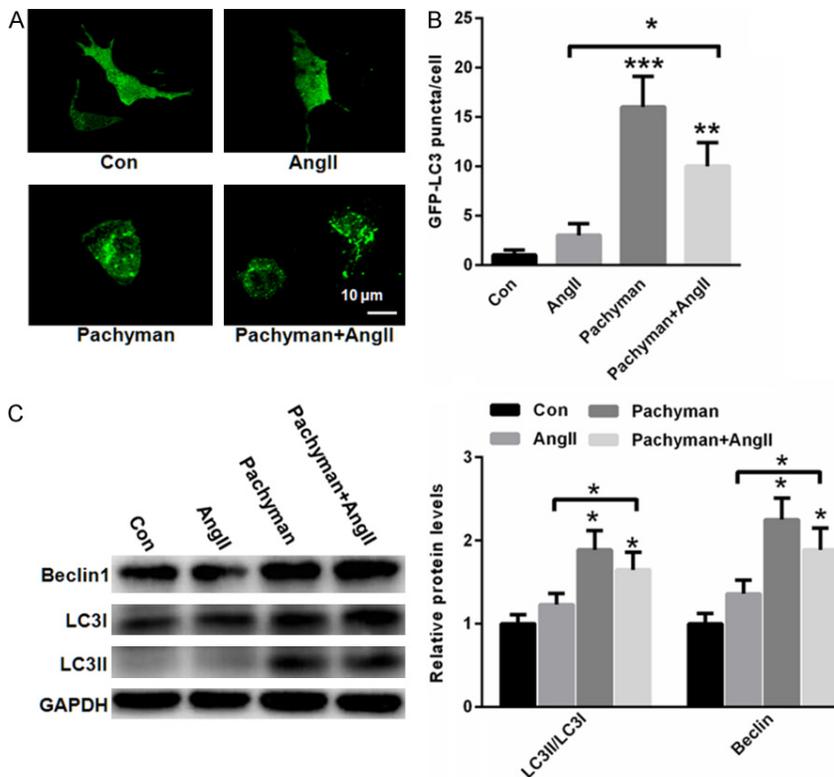
## Results

### *Establishment of cellular myocyte hypertrophy model induced by AngII*

First, we evaluated whether cellular myocyte hypertrophy model was successfully established by AngII treatment. As shown in **Figure 1A**, H9c2 cell surface area was gradually in-

creased after AngII treatment for 48 h. According to the statistics, the average cell surface was enhanced 1.78-fold after 48 h of AngII exposure, respectively (**Figure 1B**). Furthermore, we explored the mRNA levels of atrial natriuretic peptide (ANP) and  $\beta$ -myosin heavy chain ( $\beta$ -MHC), two important molecular markers related to cardiac hypertrophy. Real time PCR analysis indicated that the mRNA levels of ANP and

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**Figure 3.** Pachyman induced cell autophagy in H9c2 cell myocyte hypertrophy model induced by AngII. A. GFP-LC3 transfection assay showed that treatment with 5  $\mu$ M for 48 h did not significantly induced autophagic dots in H9c2 cells. B. Statistical analysis of GFP-LC3 dots. C. Preincubation of 10  $\mu$ g/ $\mu$ l pachyman significantly increased the ratio between LC3II/LC3I and the expression of Beclin1 even in the presence of 5  $\mu$ M AngII. \* $p$ <0.05, \*\* $p$ <0.01 vs. control.

$\beta$ -MHC were increased by 3.78-fold and 2.78~ in H9c2 cells after 48 h of AngII exposure (Figure 1C and 1D).

### *Pachyman ameliorates AngII-induced myocyte hypertrophy*

To evaluate whether pachyman protected H9c2 cells from AngII-induced myocyte hypertrophy, H9c2 cells were pre-incubated with 5, 10, 25  $\mu$ g/ $\mu$ l pachyman after their exposure to 5  $\mu$ M of AngII for 48 h. As shown in Figure 2A and 2B, pre-incubation with 5, 10, 25  $\mu$ g/ $\mu$ l pachyman decreased cell surface area gradually. Furthermore, qPCR analysis indicated that pachyman incubation (5, 10, 25  $\mu$ g/ $\mu$ l) decreased the mRNA levels of ANP and  $\beta$ -MHC by 12.5%, 24.3%, 36.8% and 14.5%, 26.4%, 37.9%, respectively (Figure 2C and 2D). These data indicated that pachyman alleviated AngII-induced myocyte hypertrophy.

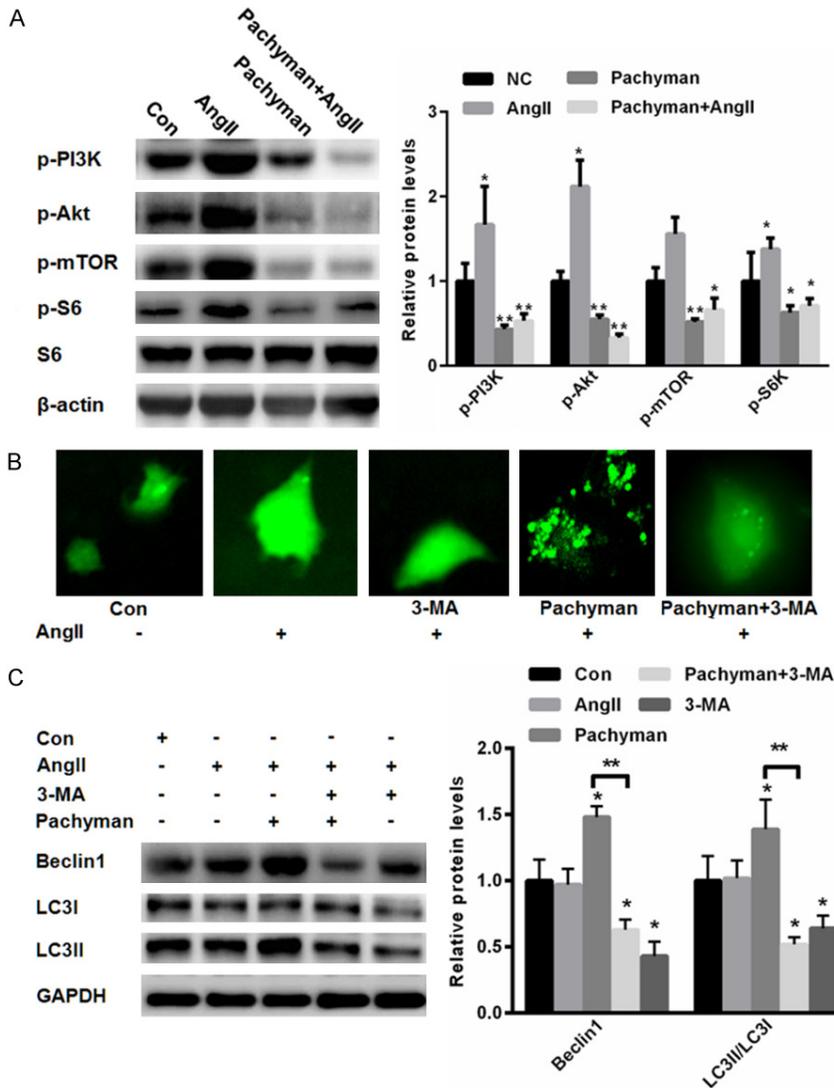
### *Induction of H9c2 cell autophagy by pachyman treatment*

Next, we examined the effects of AngII in H9c2 cell autophagy and the specific role of pachyman in this process. GFP-LC3 transfection assay showed that treatment with 5  $\mu$ M AngII for 48h did not induce obvious autophagic dots in H9c2 cells (Figure 3A and 3B). However, pachyman treatment significantly increased autophagic dots in H9c2 cells with or without AngII (Figure 3A and 3B). Meanwhile, western blot assay was carried out to explore the changes of autophagy markers, including LC3 and Beclin1.

Western blot assay showed that pre-incubation of 10  $\mu$ g/ $\mu$ l pachyman significantly increased the ratio of LC3II/LC3I and the expression of Beclin1 even in the presence of 5  $\mu$ M AngII (Figure 3C). These data suggested that pachyman induced cell autophagy in H9c2 cell myocyte hypertrophy model induced by AngII.

### *Pachyman-induced autophagy was mediated through the PI3K/Akt/mTOR signaling*

To explore the specific molecular mechanism by which pachyman induced H9c2 cell autophagy, H9c2 cells were treated with 10  $\mu$ g/ $\mu$ l pachyman after exposure to 5  $\mu$ M of AngII for 48 h. Western blot analysis showed that 10  $\mu$ g/ $\mu$ l pachyman treatment decreased the phosphorylation levels of PI3K, Akt, mTOR, S6K, and S6 compared with that of AngII incubation alone (Figure 4A). Meanwhile, we suppressed H9c2 cell autophagy by 10  $\mu$ M 3-Methyladenine (3-MA), a selective class III PI3K inhibitor, in the presence of 5  $\mu$ M AngII. Autophagy is regulated



**Figure 4.** Pachyman activates autophagy through the PI3K/Akt/mTOR signaling pathway. A. Pachyman treatment decreased the phosphorylation levels of p-PI3K, p-Akt, p-mTOR, p-S6K, p-S6 in H9c2 cells with or without AngII treatment. B. GFP-LC3 transfection assay showed that pachyman-induced H9c2 cell autophagy could be largely abolished by 3-MA incubation. C. Western blot assay showed that pachyman-induced upregulation of Beclin11 and LC3II/LC3I could be largely reversed by 3-MA preincubation. \*p<0.05, \*\*p<0.01 vs. control.

positively by Class III phosphoinositide 3-kinase (PI3K) [9]. In line with previous finding, GFP-LC3 transfection demonstrated that 3-MA suppressed H9c2 cell autophagy, while 10 μg/μl pachyman-induced H9c2 cell autophagy could be largely reversed by 3-MA pre-incubation (Figure 4B). Furthermore, we also explored whether 10 μg/μl pachyman induced cell autophagy via PI3K/Akt/mTOR signaling pathway. Hence, H9c2 cells were pre-incubated with 10 μM 3-MA and our data showed that 3-MA sig-

nificantly suppressed the protein level of Beclin11 and LC3II/LC3I (Figure 4C). More importantly, pachyman-induced up-regulation of Beclin11 and LC3II/LC3I could be largely reversed by 3-MA pre-incubation (Figure 4C). It is well known that inhibition of the Class I PI3K is a great strategy for cancer therapy and suppression of Class I PI3K induces cancer cell autophagy [12, 13]. Here, we propose that pachyman is a class I PI3K inhibitor. To elucidate the underlying mechanism by which pachyman induced autophagy via class I PI3K signaling pathway, further study is necessary.

### Discussion

Pathological hypertrophy of cardiac muscle is a most common cause of heart failure among patients [14, 15]. A specific feature of pathological cardiac hypertrophy refers to the enlargement of myocytes resulting in enhanced ventricular mass, which is often secondary to increased pressure or volume overload [10, 16, 17]. Furthermore, it is characterized by upregulation of fetal genes, including ANP and β-MHC [17]. To explore the molecular basis of pathological myocyte hypertrophy, we established a cellular cardiac hypertrophy model using AngII treatment.

First, we evaluated whether AngII is effective to establish cardiac hypertrophy cell model. Our data showed that H9c2 cell surface area was

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gradually increased after AngII treatment for 24 h and 48 h. Furthermore, the mRNA levels of ANP and  $\beta$ -MHC, two important molecular markers related to cardiac hypertrophy, were increased after AngII exposure. These data indicated the successful establishment of cellular cardiac hypertrophy model with AngII.

The above model promotes us to explore whether pachyman, an important polysaccharides isolated from mushroom, is protective for cardiac hypertrophy. Interestingly, we found that pachyman gradually decreased cell surface area in a dose-dependent manner. Furthermore, qPCR analysis indicated that 10  $\mu\text{g}/\mu\text{l}$  pachyman incubation decreased the mRNA levels of ANP and  $\beta$ -MHC, indicating the protective role of pachyman in heart failure.

It is reported that accelerated protein synthesis is necessary for cellular growth during hypertrophy, and decreased protein quality control quality results in additional pathologies [18, 19]. Besides, cardiac hypertrophy may lead to reduced ventricular function via inducing misfolded and toxic proteins accumulation [20]. Furthermore, increasing evidence suggests that autophagy exerts a deep role in myocyte protein homeostasis during hypertrophy and failure [21]. Thus, we analyzed the effects of pachyman on H9c2 cell autophagy. GFP-LC3 transfection indicated that pachyman treatment induced prominent autophagic dots in H9c2 cells with or without AngII. Meanwhile, western blot assay demonstrated that preincubation of 10  $\mu\text{g}/\mu\text{l}$  pachyman significantly increased the ratio between LC3II/LC3I and the expression of Beclin1 even in the presence of 5  $\mu\text{M}$  AngII. These data demonstrated that pachyman increased cell autophagy in H9c2 cell myocyte hypertrophy model induced by AngII.

Then, we try to explore the underlying mechanism by which pachyman induces H9c2 cell autophagy. PI3K is activated by receptor tyrosine kinases, thereby regulating the downstream signaling, including cytoskeleton organization, cell growth, and apoptosis [22, 23]. Activation of PI3K is shown to result in the enhancement of protein synthesis and cell size [24, 25]. PI3Ks are important membranous lipid kinases that induce the production of PI(3, 4, 5)P<sub>3</sub> and function as a key second messenger [13]. Based on the structure and function, PI3Ks can be divided into 3 classes. Of them, Class I PI3K is constituted of both regulatory and catalytic subunits (PI3K $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) and

enhances cell survival [12]; In contrast, Class III PI3K is composed of only single member, PI3K $\text{C}3$  or Vps34, which is widely reported to activate cell autophagy [13, 26]. Here, we found that pachyman treatment decreased the phosphorylation levels of p-PI3K, p-Akt, p-mTOR, and p-S6. More importantly, we found that pachyman-induced H9c2 cell autophagy could be largely abolished by 3-MA incubation, a class III PI3K inhibitor, indicating the suppression of class I PI3K/Akt/mTOR signaling by pachyman.

In summary, *in vitro* study shows that pachyman may serve as a protective agent for cardiac hypertrophy via activating autophagy through suppressing the Class I PI3K/Akt/mTOR signaling pathway. The above findings indicate that pachyman could be used as an effective agent for the prevention and therapy of heart failure.

### Disclosure of conflict of interest

None.

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