Cardioprotective effect of Cinnamamide derivative compound 10 against myocardial ischemia-reperfusion through regulating cardiac autophagy via Sirt1

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Abstract

Background and Purpose: Our previous research discovered that cinnamamide derivatives are a new type of potential cardioprotective agents myocardial ischemia-reperfusion (MIR) injury, among which Compound 10 exhibits wonderful beneficial action in vitro. However, the exact mechanism of Compound 10 still needs to be elucidated. Experimental Approach: The protective effect of Compound 10 was determined by detecting the cell viability and LDH leakage rate in H9c2 cells subjected to H2O2. Alterations of electrocardiogram, echocardiography, cardiac infarct area, histopathology and serum myocardial zymogram were tested in MIR rats. Additionally, the potential mechanism of Compound 10 was explored through PCR. Network pharmacology and Western blotting was conducted to monitor levels of proteins related to autophagic flux and mTOR, autophagy regulatory substrate, induced by Compound 10 both in vitro and in vivo, as well as expressions of Sirtuins family members. Key Results: Compound 10 significantly ameliorated myocardial injury, as demonstrated by increased cell viability, decreased LDH leakage in vitro, and declined serum myocardial zymogram, ST elevation, cardiac infarct area and improved cardiac function and microstructure of heart tissue in vivo. Importantly, Compound 10 markedly enhanced the obstruction of autophagic flux and inhibited excessive autophagy initiation against MIR by decreased P-mTOR and increased LAMP2. Furthermore, Sirt1 knockdown hindered Compound 10’s regulation on mTOR, leading to interrupted cardiac autophagic flux. Conclusions and Implications: Compound 10 exerted cardioprotective effects on MIR by reducing excessive autophagy and improving autophagic flux blockage. Our work would take a novel insight in seeking effective prevention and treatment strategies against MIR injury. Keywords: Myocardial ischemia-reperfusion; Cinnamamide derivatives; Autophagic flux; Sirt1; mTOR;
Cardioprotective effect of Cinnamamide derivative compound 10 against myocardial ischemia-reperfusion through regulating cardiac autophagy via Sirt1

Running title: Compound 10 improves cardiac autophagic flux through Sirt1 in MIR

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What is already known

- Myocardial ischemia-reperfusion (MIR) is characterised by disorders of autophagy involving excessive autophagy initiation and blocked autophagic flux.
- Autophagy is closely related to oxidative stress.
- Cinnamamide derivatives possess anti-oxidative ability in myocardial ischemia-reperfusion.

What this study adds

- Cinnamamide derivative compound 10 has beneficial effects on cardiomyocytes’ survive against MIR \textit{in vitro} and \textit{in vivo}.
- Compound 10 improved autophagic flux blockage and inhibited excessive autophagy initiation \textit{in vitro} and \textit{in vivo}.

Clinical significance

- Cinnamamide derivative compound 10 exerts protective and therapeutic value in MIR.
Author Contribution:

Yangchao Zhao and Bo Wei designed and supervised the study and revised the paper.

Wenhua Xue, Jingjing Liu and Xueli Xu performed the experiments and wrote the paper.

Chengxin Chen analysed the data.

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Data availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Declaration of transparency and scientific rigour:

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.
**Conflict of Interest:** The authors declare no conflict of interest.
Abstract (250 words)

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Experimental Approach: The protective effect of Compound 10 was determined by detecting the cell viability and LDH leakage rate in H9c2 cells subjected to H$_2$O$_2$. Alterations of electrocardiogram, echocardiography, cardiac infarct area, histopathology and serum myocardial zymogram were tested in MIR rats. Additionally, the potential mechanism of Compound 10 was explored through PCR. Network pharmacology and Western blotting was conducted to monitor levels of proteins related to autophagic flux and mTOR, autophagy regulatory substrate, induced by Compound 10 both in vitro and in vivo, as well as expressions of Sirtuins family members.

Key Results: Compound 10 significantly ameliorated myocardial injury, as demonstrated by increased cell viability, decreased LDH leakage in vitro, and declined serum myocardial zymogram, ST elevation, cardiac infarct area and improved cardiac function and microstructure of heart tissue in vivo. Importantly, Compound 10 markedly enhanced the obstruction of autophagic flux and inhibited excessive autophagy initiation against MIR by decreased P-mTOR and increased LAMP2. Furthermore, Sirt1 knockdown hindered Compound 10’s regulation on mTOR, leading to interrupted cardiac autophagic flux.
Conclusions and Implications: Compound 10 exerted cardioprotective effects on MIR by reducing excessive autophagy and improving autophagic flux blockage. Our work would take a novel insight in seeking effective prevention and treatment strategies against MIR injury.

Keywords: Myocardial ischemia-reperfusion; Cinnamamide derivatives; Autophagic flux; Sirt1; mTOR;
**Abbreviations:**

- AAR: area at risk
- ADV: adenoviral vector
- AST: aspartate transaminase
- ATCC: american type culture collection
- CETSA: cellular thermal shift assay
- CAT: catalase
- CK-MB: creatine kinase – muscle and brain (subunits)
- ECG: electrocardiogram
- EF: ejection fraction
- FS: fractional shortening
- GSH: glutathione
- HE: hematoxylin-eosin
- $\text{H}_2\text{O}_2$: hydrogen peroxide
- LAD: left anterior descending coronary artery
- LDH: lactate dehydrogenase
- LUT: luteolin
- LV dp/dtmax: maximum rates of developed left ventricular pressure
- LV dp/dtmin: minimum rates of developed left ventricular pressure
- LVEDP: left ventricular end-diastolic pressure
- LVSP: left ventricular systolic pressure
- MDA: malondialdehyde
MIR: ischemia reperfusion injury

MOI: multiplicity of infection

SOD: superoxide dismutase

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
1. Introduction

Because of the high incidence rate and mortality, cardiovascular disease has emerged as the leading cause of death worldwide. By 2030, the World Health Organization projects that 25 million people will have died from cardiovascular disease (Y. Tian et al., 2017). Among them, ischemic heart disease (IHD) is the main global cause of death and disability worldwide (Song et al., 2017). While prompt percutaneous coronary intervention (PCI) or thrombolytic therapy for myocardial revascularization is the most effective treatment to save the endangered myocardium in acute myocardial infarction, subsequent myocardial ischemia-reperfusion (MIR) injury invariably results in the loss of a significant number of cardiomyocytes, increasing the center of gravity dysfunction (Hausenloy & Yellon, 2013; Turer & Hill, 2010). Therefore, reperfusion injury will weaken the beneficial effect of reperfusion treatment on patients with myocardial infarction, and limiting MIR injury can improve the prognosis of patients with acute myocardial infarction (Heusch, 2013). According to recent research, MIR injury is now closely linked to oxidative stress, autophagy and inflammation (Ma, Wang, Chen, & Cao, 2015). Whereas, effective therapeutic strategies targeting these pathological mechanism against MIR are very limited in clinic.

Cinnamide is a unique structure that is found in many natural products. Its molecular structure, containing functional groups, such as acylamin, amino, and cinnamyl, is useful for creating novel therapeutic compounds with possible pharmacological effects (Gaikwad, Nanduri, & Madhavi, 2019). As a result, cinnamide is frequently employed in the medical field. The inhibition of carbonyl reductase I
(CBRI) by cinnamamide derivatives with a 4-hydroxypiperidine moiety has been shown to protect cardiomyocytes from the myocardial damage brought on by doxorubicin (Koczurkiewicz-Adamczyk et al., 2022). Other research has demonstrated that the trans-cinnamamide 1 and cis-cinnamamide 2, extracted from pepper, significantly reduce platelet aggregation generated by arachidonic acid and platelet activating factor in rabbits, indicating that cinnamamide has potential cardiovascular protective effect on myocardial infarction (C. Y. Li et al., 2007). Furthermore, further reports suggest that cinnamamide derivatives may have cardioprotective properties on acute MIR injury in a rabbit model (Fancelli et al., 2014). Studies have discovered that cinnamamide-barbituric acid derivatives exhibit high antioxidant activities, excellent free radical scavenging activity, and considerable protective benefits against \( \text{H}_2\text{O}_2 \) induced injury in HTT2 cells (Chen et al., 2017). Based on the above research, our group previously combined trans- and cis-cinnamamide to produce cinnamamide derivative intermediate A with good antioxidant property, which could further convert to cinnamamide-barbituric acid derivative \( 7w \) after nuclear fusion cyclization of barbituric acid with strong cardioprotective activity, as demonstrated by increased cell viability in cardiomyocytes subjected to \( \text{H}_2\text{O}_2 \) and improved cardiac function in MIR rats (Wei et al., 2022). 17 cinnamamide-barbituric acid derivatives were optimized and synthesized on the basis of compound \( 7w \). Through the determination of their beneficial activity, we discovered that Compound 10 has an obvious cardioprotective effect \textit{in vitro} (Shi et al., 2022). However, the underlying mechanism responsible for the cardioprotective action of cinnamamide-barbituric acid derivative 10 remain elusive.
Whether compound 10 can ameliorate myocardial injury by regulating oxidative stress and autophagy is worth exploring.

Herein, we aimed to elucidate the potential actions of compound 10 on MIR through oxidative stress, autophagy and inflammation, applying MIR rat model in vivo and H2O2 treated H9c2 cardiomyocytes in vitro. We demonstrated that compound 10 could reduce excessive autophagy and improve autophagic flux blockage against MIR via mTOR and LAMP2 by Sirt1.

2. Materials and methods

2.1. Materials

Compound 10 was synthesized as our previous work (Shi et al., 2022). Metoprolol was purchased from TOPSCIENCE company (Shanghai, China). Evans blue, and triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO). Biochemical assay kits of creatine kinase-MB (CK-MB, H197-1-1), lactate dehydrogenase (LDH)(A020-2-2), aspartate aminotransferase (AST, C010-2-1), superoxide dismutase (SOD, A001-3-2), catalase (CAT, A007-1-1), malondialdehyde (MDA)(A003-1-1) and glutathione (GSH, A006-2) were purchased from Nanjing Jiancheng (Nanjing, China). Terminal deoxynucleotidyl transferase-mediated DUTP Nickell End Labeling (TUNEL, G002-1-2) Fresh Red Blood Cell Apoptosis Detection Kit was supplied by Vazyme Biotech (Nanjing, China). BCA Reagent test kit was obtained from Solebao Technology Co. Primary antibodies used were monoclonal against the oxidative stress proteins Sirt1 (Cell Signaling Technology cat. #9475, RRID:
AB_2617130), Sirt2 (Cell Signaling Technology Cat# 2313, RRID: AB_2285974), Sirt3 (Cell Signaling Technology Cat# 2627, RRID: AB_2188622), Sirt4 (Santa Cruz Biotechnology Cat# sc-66269, RRID: AB_1129464), Sirt5 (Cell Signaling Technology Cat# 8779, RRID:AB_2797663), Sirt6 (Cell Signaling Technology Cat# 12486, RRID:AB_2636969), Sirt7 (Cell Signaling Technology Cat# 5360, RRID:AB_2716764), LC3II/I (Novus Cat# NBP1-78961SS, RRID:AB_11032506), Beclin1 (US Biological Cat# B0981-23K, RRID:AB_2233153) P62 (Cell Signaling Technology Cat# 88588, RRID:AB_2800125), ATG5 (Cell Signaling Technology Cat# 12994, RRID:AB_2630393), LAMP2 (Cell Signaling Technology Cat# 49067, RRID:AB_2799349), Rab7 (Cell Signaling Technology Cat# 9367, RRID:AB_1904103), mTOR (Cell Signaling Technology Cat# 2983, RRID:AB_2105622), P-mTOR (Santa Cruz Biotechnology Cat# sc-293133, RRID:AB_2861149). All the other reagents used were of analytical grade. Quantification of protein bands was performed using the Image J program (RRID:SCR_003070).

2.2. Cell culture and treatment

H9c2 cell (ATCC Cat#CRL-1446, RRID: CVCL_XE65) were purchased from the American Type Culture Collection (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) with 10% FBS (GIBCO, Carlsbad, California, USA) and 100IU·mL\(^{-1}\) penicillin and 100 μg·mL\(^{-1}\) streptomycin at 37 °C in a humidified atmosphere of 5% CO\(_2\). Cells were fed every 2–3 days and sub-cultured when they reached 70–80% confluence.
2.3. Compound 10 and H$_2$O$_2$ treatment

The effect of Compound 10 was firstly investigated in H9c2 cells treated with H$_2$O$_2$ as described (Zhang et al., 2021). Briefly, when cells reached 70–80% confluence, Compound 10 was dissolved in cell culture media containing 0.1% DMSO. For the experiments performed in the presence of Compound 10, the compound was added to cells, 24 h prior to hydrogen peroxide treatment, followed by incubation for another 1.5 h. Cells of the control group in vitro were treated with DMSO at 0.1% (v/v). The exact group size for each experimental group in vitro is 5.

2.4. Determination of cell viability

Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay as previously described (Yu Tian et al., 2017). Briefly, H9c2 cells were plated on 96-well plates at a density of 5×10$^3$ cells/well and incubated overnight. To evaluate the protective effect of Compound 10, H9c2 cells were cultured with different concentrations of Compound 10 followed with exposure of 300 μM H$_2$O$_2$ for 1.5 h. On the other hand, after treatment (with or without Compound 10 and Chloroquine (CQ, 20 μM, MedChem Express, USA)), H9c2 cells in different groups were treated with MTT solution and incubated at 37 °C in the dark for 4 h. The model group was cultured under the same conditions, while the control group cells were treated with the same volume of phosphate buffer solution (PBS). Add 20 μL to each hole after a specified time MTT (5 mg/mL), and cultured cells at 37 °C for 4 h. Then, abandon the medium containing MTT, and dissolve the colored nail cream crystal in 100 μL dimethyl sulfoxide (DMSO). Measure the absorption value at 570 nm. The
survival rate of H9c2 cells in each well was expressed as a percentage of control cells.

2.5. Lactate Dehydrogenase (LDH) Measurement

The lactate dehydrogenase (LDH) leakage assay was used to assess cytotoxicity after different treatments. The LDH release rate was measured using the cytotoxicity detection kit, according to the manufacturer’s instructions (C0016, Beyotime, Shanghai, China) and quantified by absorbance at 490 nm using a BioTek plate reader. Results were normalized to the control (CON) group, the amount of LDH release of which was considered as 100%.

2.6. Adenoviral-mediated gene transfer

Antisense Sirt1 adenoviruses (Ad. Sirt1-AS) or control Ad. GFP were obtained from Biocan BioTECH (Shenzhen, China). Adenoviruses were amplified in HEK293 cells, purified with ViraKit from Virapur and tittered, according to the standard procedure of AdenoXTM rapid titer kit from BIOMIGA. After 2 h of plating, H9c2 cells were infected with antisense Sirt1 adenoviruses (Ad. Sirt1-AS) or control Ad. GFP at a multiplicity of infection (MOI) of 400 for 2 h before the addition of a suitable volume of complete DMEM medium. The efficiency of adenoviral gene transfection was detected in cultured H9c2 cells by immunofluorescent signal through fluorescence microscopy (Nikon Eclipse Ti–S, Nikon Ltd, Japan). Nearly 100% of H9c2 cells appeared infected at 400 MOI by 48 h. The cell phenotype and morphology remained similar among non-infected and adenoviral-infected groups after 48 h of infection. The cells were then treated with Compound 10 or H$_2$O$_2$ for indicated time, washed with PBS and harvested for quantitative immunoblotting, or used in the experiments outlined in
the results.

2.7. qRT-PCR

The RNA-easy isolation reagent (Vazyme Biotech Co., Ltd, Nanjing, China) was used to isolate total RNA from cells. HiScript II Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd, Nanjing, China) was employed for reversely transcribing total RNA to cDNA. qRT-PCR was carried out based on the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China) via QuantStudio 6 Flex Real-Time PCR Systems (ThermoFisher, CA, USA). Sangon Biotech Co. Ltd. (Shanghai, China) was responsible for synthesizing PCR primers (Table S1). Data of the samples were normalized to GAPDH. In addition, the relative mRNA expression was determined by equation $2^{-\Delta\Delta Ct}$.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>FP sequence (5’-3’)</th>
<th>RP sequence (3’-5’)</th>
</tr>
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<td>GAPDH</td>
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<tr>
<td></td>
<td>GACC</td>
<td>CG</td>
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<tr>
<td>SQSTM/P62</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>AAATG</td>
<td>TGAG</td>
</tr>
</tbody>
</table>
2.8. Evaluation of fluorescent LC3 puncta

Cells cultured on coverslips were transfected with adenovirus of tandem fluorescent mRFP-GFP-LC3 (Hanbio, Inc., Shanghai, CN) (MOI = 80), a specific marker for autophagosome formation. After adenoviral transfection for 48 h, cells were fixed with 4% paraformaldehyde after treatment with H$_2$O$_2$ or Compound 10. Images of the cells were obtained from the confocal laser scanning microscope (Nikon, A1 PLUS, Tokyo, Japan) and analyzed using the Image J Software. Cells were detected
with green (GFP) or red (mRFP) fluorescence. Autophagosomes are yellow puncta and autolysosomes are only red puncta in merged images due to the acidic lysosomal compartment. Autophagic flux was determined by increased percent of only red puncta in the merged images. The transfection efficiency was more than 90%, and the subsequent transfection-induced cell death was less than 10%.

2.9. Molecular docking studies

To explore the possible binding mode of protein Sirt1 and compound Compound 10, molecular docking between them was performed using MOE software. The crystal structure of Sirt1 complexing with its classic resveratrol (PDB ID = 4i5i) was obtained from the RCSB Protein Data Bank (http://www.pdb.org).

2.10. Cellular thermal shift assay (CETSA)

CETSA was performed according to previous research. Cells were seeded in cell culture dishes (100 mm) and treated with 20 μM Compound 10 or 1% DMSO for 4 h at 37 °C. In short, cells were collected and heated individually at different temperatures (42, 43, 46, 50, 55, 59, 62°C) for 10 min. Then, the samples were centrifuged, and the obtained cells were analyzed by Western blotting.

2.11. Animals

All animal care and experimental studies were approved by the Ethical Committee of Zhengzhou University (yxyllsc20220068). Animal studies were reported in compliance with the ARRIVE guidelines (Percie et al., 2020). Specific pathogen-free (SPF) male Sprague-Dawley rats weighing 200-250g, 7-8 weeks, were obtained from the Henan Provincial Laboratory Animal Center (NO. 410975221100019174,
Zhengzhou, China). All animal experiments were conducted in accordance with the guidelines of the Chinese Council on Animal Care. Rats were housed in groups of 3 to 4 per cage under standard circadian light conditions (12/12 h) at a controlled ambient temperature of 25±2°C for 7 days prior to the experiment. Make every effort to minimize animal distress and the number of animals required to obtain reliable results according to the rules of replacement, improvement, or reduction (3Rs).

2.1.2. Experimental design

After 7 days of domestication, rats were randomly divided into 6 groups (n=12): Sham, MIR, Compound 10 (5mg·kg⁻¹)+MIR (Compound10L+MIR), Compound 10 (10mg·kg⁻¹)+MIR (Compound10M+MIR), Compound 10 (20mg·kg⁻¹)+MIR (Compound10H+MIR) and metoprolol (positive control)+MIR (Met+MIR). Sham and MIR rats were intraperitoneally injected with 1 mL of normal saline 0.01% DMSO. Different concentrations of Compound 10 were directly dissolved in saline. Compound 10 was intraperitoneally injected at 5, 10, and 20 mg·kg⁻¹ for 14 days before operation. The positive control group received intraperitoneal injection of metoprolol (Met) 20mg·kg⁻¹ for 14 days before operation.

2.1.3. Preparation of rat MIR model

Sprague Dawley rats were anesthesized with 4% isoflurane in a 2 liter plexiglass box, endotracheally intubated and mechanically ventilated with 80% oxygen and 20% carbon dioxide. Depth of anesthesia was evaluated by corneal reflex and withdrawal reflex. Then, a thoracotomy was performed between the 3 and 4 intercostal region. The left anterior descending coronary artery and tubule were ligated 2-3 mm from the tip of
the left auricle with a 5-0 wire. After 30 min of ischemia, the sutures were gently unwound and the tube was removed (reperfusion began). Close the skin and remove the endotracheal tube. Rats in Sham operation group underwent thoracotomy, while the left anterior descending coronary artery was only threaded without ligating. ECG was used to monitor the heart rate to ensure the success of LAD ligation, and the life status of the rats during surgery was always concerned. The rats' body temperature was maintained at 37°C through the thermal blanket during the operation. Metoprolol was employed as the positive control, which has been recognized to be able to reduce infarct size and increase left ventricular ejection fraction (Xiao et al., 2019).

2.14. Electrocardiogram (ECG)

Electrocardiograms (ECG) detected the types with alteration (ST-segment depression or elevation). ECG was recorded in anesthetized rats and calculated as lead II ECG through RM6240 multi-channel physiological signal acquisition and processing system (Chengdu, China). Record the change type (ST segment elevation or depression) of the experimental rats.

2.15. Echocardiography

A Vevo 2100 echocardiogram containing an in vivo imaging system (FUJIFILM Visual Sonics, Toronto, Canada) was used for the echocardiography. Under anesthesia, the chest of rat was shaved, and two-dimensional long axis images were captured for end-diastolic and end-systolic volume measurements. The left ventricular ejection fraction (EF%) and fractional shortening (FS%) were automatically calculated and recorded by the echocardiographic system. Each parameter was evaluated by
calculating the average of five cardiac cycles.

2.16. Hemodynamic measurements

The cardiac function of rats after 24 h of reperfusion was also evaluated by hemodynamic measurements by anaesthesia with 4% isoflurane. A catheter (polyethylene 90) was filled with 500 U/ml heparinized saline, advanced in left ventricle through carotid artery, and finally related to the RM6240 multi-channel physiological signal acquisition and processing system. In addition, the heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), as well as contraction and relaxation rates (LV dP/dt\text{max} and LV dP/ dt\text{min}) were continuously recorded.

2.17. Blood and heart tissue preparation

At the indicated time points, rats were euthanatized by overdose anesthesia with pentobarbital sodium (150 mg/kg, i.p.) and the blood and heart samples were extracted. Serum was obtained from blood collected in dry test tubes without anticoagulant. Heart tissues were excised immediately or dissected quickly on ice for evaluating the infarct size, biochemical assays or further detection, respectively.

2.18. Cardiac infarct area measurement

Measurements of area at risk and infarct size were performed as described previously(Zhang et al., 2021). Briefly, hearts were perfused with 1% Evans’ blue dye (Sigma-Aldrich, St. Louis, MO) to delineate the ischemic area at risk (AAR), and then were quickly frozen at −20 °C for 30 min. All sections were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Co., St Louis, Mo) buffer (pH 7.4) at 37 °C
in the dark to determine the unstained necrotic region within the ischemic risk zone. The areas were segmented and assessed digitally by employing the Image-Pro Plus 6.0. The cardiac infarct size, which was stained white or pale, was expressed to be the infarct myocardium-to-left ventricular area ratio×100%.

2.19. Biomarker indexes in serum and heart tissue

Activities of CK-MB, LDH, GSH, AST, CAT, SOD and MDA in serum were assayed using commercial kits purchased from Jiancheng Institute of Biotechnology (Nanjing, China).

2.20. TUNEL staining of heart section

The heart paraffin sections were fabricated and dyed by adopting the TUNEL apoptosis detection kit (KeyGen Biotech, Nanjing, China) for revealing apoptotic cells, following the manufacturer’s instructions. The TUNEL represented the ratio of positive cells to total cells. Apoptotic nucleuses were visualized with light microscopy or fluorescent microscopy. The experiment was repeated on five different sections for each specimen. Ten random fields (×400) per section were analyzed. Then the average percentage of apoptotic cell was calculated.

2.21. Heart histopathological examination

Heart tissues were fixed in 10% buffered formalin and embedded in paraffin. For the histological assessment, paraffin embedded tissue sections of heart (4 μm) were stained with hematoxylin-eosin (H&E), and examined microscopically (× 200).

2.22. Western blotting

Western blotting was performed as described in our previous studies (Cui et al.,
2021). For cell samples, cell lysates were obtained using commercial RIPA lysis buffer (Beyotime, China). For cardiac samples, they were homogenized with commercial RIPA lysis buffer. The protein content was determined by BCA kit (Solobo, China). Then, proteins were separated by SDS-PAGE and transferred to PVDF membranes. Western blots were blocked in 5% nonfat dry milk-TBS-0.1% Tween 20 for 2 h. Then, the membrane was incubated with the primary antibody overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody rabbit or mouse antibody (1:10,000; Cell Signaling Technology Co., Ltd, MN, USA) was incubated for 2 h. Immunoreactivity was detected by a gel imaging system (Protein Simple, Santa Clara, California, USA) and enhanced chemiluminescence detection reagent super Signal West Pico chemiluminescence reagent (34,079, Pierce, Thermo Scientific, Rockford, IL, USA). GAPDH (Cell Signaling Technology Cat#5174, RRID: AB_10622025) was used as a loading control. For calculation of relative changes in protein expression, values of individual samples were divided by the mean value of samples from the CON, Ad.GFP+CON or Sham group.

2.23 Data and statistical analysis

Data analysis was performed blindly whenever it was possible. All data are represented as individual values with mean ± SD. The one-way ANOVA with Tukey's post hoc test or nonparametric Kruskal–Wallis test followed by the Bonferroni test (from GraphPad Prism 5.0) were used for multi-group comparison. Results were considered statistically significant at P < 0.05. The statistical analysis was undertaken only for studies where each group size was at least n=5. Data were normalised and
results were expressed as a percentage of the results obtained in control (CON), Sham or Ad.GFP+CON conditions for Western blotting experiments and simultaneous measurement of cell viability experiments.

3. Results

3.1. Compound 10 inhibited myocardial injury in H9c2 cells subjected to H2O2

Initially, we selected cell viability and lactate dehydrogenase (LDH) leak assay to evaluate the effect of Compound 10 on cell viability in H2O2 treated H9c2 cells. We found that H9C2 cells did not produce significant cytotoxicity after 24 hours of pretreatment with Compound 10 up to 80 μM (Fig. 1A). Therefore, Compound 10 concentrations equal to or less than 20 μM and H2O2 (300 μM) were used in the following tests. Pretreatment with Compound 10 (5, 10, 20 μM) for 24 h significantly increased the cell viability of H9c2 cells exposed to H2O2 (Fig. 1B). The membrane integrity was measured by calculating the LDH leakage rate. Relative to the control cells, H2O2 exposure markedly elevated the release rate of LDH by 2.83 folds. Compound 10 significantly reduced LDH release rate in a dose-dependent manner, compared with H2O2 group (Fig. 1C). Additionally, compared with the control group, H9c2 cells morphologically atrophied and died after H2O2 treatment. While H2O2-induced damage was significantly recovered by pretreatment with Compound 10 in a dose dependent manner (Fig. 1D).

3.2. Compound 10 ameliorated myocardial injury against MIR in vivo
In vivo, we established a rat myocardial ischemia-reperfusion model by ligating the left anterior descending coronary artery (30 min) and then reperfusion for 24 h, and evaluated the effect of Compound 10 on MIR in vivo. During the whole process of MIR, ECG was monitored and recorded throughout the whole process. After 24 h of reperfusion, the ST segment was elevated continuously in the MIR group, while the elevation of the ST segment was significantly improved in the Compound 10 treated rats (Fig. 2A). In addition, the infarct size of a single rat heart was evaluated by Evans blue-TTC double staining (Fig. 2B). And there was no significant statistical difference in risk areas among groups (Fig. 2C). Additionally, increased cardiac infarct size was observed in MIR rats up to 80.87% compared to sham group, however, compared with MI rats, pretreatment with Compound 10 (5, 10 or 20 mg·kg⁻¹) significantly and dose-dependently reduced the infarct size to 72.59%, 52.93% and 44.20% (Fig. 2D).

In addition, six sensitive biochemical indexes in serum were analyzed after 24h reperfusion (Fig. 2E-J). The activities of serum CK-MB and LDH in MIR injury group were significantly higher than those in Sham group, as significantly increased by 2.58 and 1.77 folds, respectively. In addition, Compound 10 (5, 10 or 20 mg·kg⁻¹) pretreatment reversed such alterations in myocardial zymogram in a dose-dependent manner (Fig. 2E and F). And, the serum CAT, GSH and SOD activities or concentrations in the MIR group were significantly declined compared to those in the Sham group, while the MDA levels was markedly augmented (Fig. 2G-J). Notably, Compound 10 (20 mg·kg⁻¹) increased serum CAT, GSH and SOD activities or concentrations to 1.75, 2.25 and 1.33 folds, respectively, and decreased MDA levels to
only 52.31% to those in MIR rats (Fig. 2G-J). Compound 10 (5 or 10 mg·kg$^{-1}$) also obviously reversed these changes in biochemical indexes. Moreover, compared with the MIR group, Compound 10 (5, 10 or 20 mg·kg$^{-1}$) indeed improved the contents or activities of MDA, CAT, SOD, AST in heart tissues in a dose-dependent manner (Fig. 2K-N).

3.3. Compound 10 improved cardiac function against MIR

In vivo

Microscopic examination of heart tissue sections from different experimental groups was highly differentiated (Fig. 3A). In the Sham group, the myocardial cell membrane was intact, the myocardial fiber was normal, and there was no myocardial infarction, inflammatory infiltration and myocardial necrosis. Compared with the Sham operation group, there were leukocyte infiltration, large area myocardial necrosis and severe edema in the MIR group. It was worth noting that Compound 10 treatment alleviated these pathological changes dose-dependently (Fig. 3A). Left ventricular function was evaluated by echocardiography (Fig. 3B). Compared with the sham operation group, the cardiac function of the MIR group was significantly decreased by 54.55% in EF (Fig. 3C) and 34.82% in FS (Fig. 3D). However, the EF% and FS% of Compound 10+MIR (20 mg·kg$^{-1}$) were significantly increased to 87.71% and 57.56%, respectively (Fig. 3C and D). Compared with the MIR group, the hemodynamic function recovery also confirmed that Compound 10 indeed dramatically improved the cardiac function, as demonstrated by significantly decreased LVSP (Fig. 5F), increased
LVEDP (Fig. 3F), and decreased maximum and minimum pressure rates of left ventricle (LV dp/dt_max and LV dp/dt_min) (Fig. 3G). It was worth noting that preconditioning with Compound 10 expectedly restored these changes to near normal values, indicating that Compound 10 possessed strong cardioprotective effect against MIR injury in rats in vivo. As depicted in Fig. 3H and I, compared with the Sham-operated group, the number of TUNEL-positive myocardial nuclei in MIR group was significantly increased to 58.07%, and obvious red apoptotic cells appeared in MIR heart tissue. Such index was markedly decreased in Compound 10 treatment group in a dose-dependent manner.

3.4. Compound 10 exerted cardioprotective action via regulating autophagy in H9c2 cells subjected to H2O2

In order to explore the mechanisms underlying beneficial actions of Compound 10, expressions of mRNA of key genes related to inflammation, autophagy, and oxidative stress were measured in H9c2 cells suffered from H2O2-induced injury. Notably, compared with the CON cells, mRNA levels of autophagy related genes (i.e., LC3II/I, Beclin1, P62, ATG5, LAMP2 and Rab7), stress induced anti-oxidant genes (i.e., Nrf2, HO-1) and inflammatory response related genes (i.e., NLRP3, ASC, IL-6) in H9c2 cells exposed to H2O2 were significantly up-regulated, and SOD was markedly decreased (Fig. 4A-G). However, Compound 10 pretreatment only dramatically reversed the abnormally elevated mRNA expressions of autophagy related genes induced by H2O2 in a dose-dependent manner (Fig. 4A-F).

The target components were further screened by network pharmacology according
to OB>40% and DL>0.18. 485 and 20159 targets were obtained for Compound 10 and MIR respectively. Use the "jvenn" tool (Bardou et al., 2014) to obtain 436 key targets to extract the intersection of components and disease targets (Fig. 4H). Afterwards, PPI network is used to summarize the interaction between Compound 10 target and MIR model (Fig. 4I). Import the PPI network into the Cytoscape 3.8.1 software, analyze the network topology attributes, and select 20 common targets as potential targets of Compound 10 treatment MIR according to the degree value. Then it was enriched and analyzed by GO and KEGG functions (Fig. 4J-K) only to find that Compound 10 was involved in multi-signaling pathways by regulation of autophagy, including cancer signal pathway, cAMP signal pathway, Alzheimer disease signal pathway and so on.

To further monitor the autophagic flux, tandem fluorescent mRFP-GFP-LC3 was transfected into H9c2 cells (Ad-LC3-H9c2). The control Ad-LC3-H9c2 showed basal autophagy with few autolysosomes and autophagosomes. However, Ad-LC3-H9c2 treated with H2O2 were observed accumulated autophagosomes and decreased autolysosomes, which indicated excessive autophagy initiation or suspended autophagosome clearance (i.e., excessive autophagy or autophagic flux blockage) in cardiomyocytes. In the Ad-LC3-H9c2 cells treated with Compound 10 (10 μM) alone, the number of autophagosomes significantly declined, and autolysosomes markedly elevated, compared to the CON group. Importantly, in Compound 10+H2O2, dramatically increased autolysosomes and decreased autophagosomes were observed, relative to those in H2O2 only exposure cells (Fig. 4L-M). These data suggested that Compound 10 treatment could indeed enhance cardiac autophagic flux by inducing the
consumption of autophagosomes by improving autolysosomes.

### 3.5. Compound 10 decreased excessive autophagy initiation and enhanced autophagic flux blockage *in vitro*

Furthermore, expressions of autophagic flux and autophagy initiation related proteins were examined in each experimental group (Fig. 5A). Compared with the CON cells, LC3II/I, Beclin1, P62, ATG5 and Rab7 in the H$_2$O$_2$ group increased by 2.79, 3.26, 2.16, 1.30 and 2.64 folds, respectively, and LAMP2 was decreased by 89.77% (Fig. 5B-G). Compared with the model group, pretreatment with Compound 10 (20 μM) significantly reduced LC3II/I, Beclin1, P62, ATG5, Rab7 in H9c2 cells to 40.59%, 28.27%, 41.43%, 23.33%, 35.05%, respectively, and significantly increased LAMP2 content by 12.54 folds (Fig. 3B-G). Compound 10 (5 or 10 μM) also markedly reversed those abnormal alterations induced by myocardial injury *in vitro*. mTOR levels in every group showed non-significant alteration. In addition, relative to the CON cells, it was found that P-mTOR, as autophagy regulatory substrate, in H$_2$O$_2$ group was significantly reduced to 10.22%, indicating the excessive autophagy initiation during H$_2$O$_2$ exposure. However, the content of P-mTOR in Compound 10 (5, 10, 20μM) pre-incubated group was elevated to 5.22, 5.36, 6.19 folds, respectively, as compared to the H$_2$O$_2$ cells (Fig. 5H-J), suggesting the inhibitory effect of Compound 10 on excessive autophagy induced by H$_2$O$_2$.

Additionally, chloroquine (CQ), an autophagosome–lysosome fusion inhibitor, was administered in cardiomyocytes to explore the role of Compound 10 in autophagic
flux blockage. Compared with cells treated with H\textsubscript{2}O\textsubscript{2} alone, CQ (20 μM) treatment further reduced the cell viability induced by H\textsubscript{2}O\textsubscript{2}. Pretreatment with Compound 10 alone for 24 h significantly increased the cell viability of H9c2 cells injured by H\textsubscript{2}O\textsubscript{2} (Fig. 5K). Our data showed that there was no significant difference in cell viability between H\textsubscript{2}O\textsubscript{2} cells, which are grouped in the presence or absence of CQ. However, CQ (20 μM) significantly reversed the beneficial effect of Compound 10 on cell viability in H9c2 injured by H\textsubscript{2}O\textsubscript{2}. Expressions of autophagy related proteins were further detected with or without CQ. Fig. 3M-P pictured that, compared with H\textsubscript{2}O\textsubscript{2}, the levels of LC3II/I and P62 in H\textsubscript{2}O\textsubscript{2}+CQ co-treatment group had no significant difference between the two groups. However, CQ eliminated the beneficial effect of Compound 10 on autophagic flux in H9c2 cells treated with H\textsubscript{2}O\textsubscript{2}, indicating that Compound 10 played a cardioprotective role depending on enhancing autophagic flux.

3.6. Compound 10 decreased excessive autophagy initiation and enhanced autophagic flux blockage in vivo

In order to further verify the protective mechanism of Compound 10 against MIR injury, the effect of Compound 10 on autophagic flux in vivo was further investigated. In addition, the expression of autophagic flux related proteins was detected after H\textsubscript{2}O\textsubscript{2} was applied to cardiomyocytes (Fig. 6A-G). Compared with the Sham rats, LC3II/I, Beclin1, P62, ATG5 and Rab7 in the MIR group increased by 3.72, 2.54, 1.39, 1.40 and 1.21 folds, respectively, and LAMP2 decreased to only 9.25% (Fig. 6B-G); Relative to the MIR group, Compound 10 (20 mg·kg\textsuperscript{-1}) pretreatment significantly reduced LC3II/I,
Beclin1, P62, ATG5 and Rab7 to 33.06%, 34.78%, 30.43%, 23.33% and 35.05% of those in MIR rats, respectively, and markedly increased cardiac expression of LAMP2 by 11.19 folds (Fig. 6B-G). Compound 10 (5 or 10 mg·kg⁻¹) also markedly reversed those abnormal alterations induced by myocardial injury in vivo. Furthermore, cardiac P-mTOR and mTOR were also detected. Cardiac mTOR was observed as nonsignificant difference among all experimental animals. Notably, data depicted that MIR indeed induced remarkably declined cardiac P-mTOR, relative to the Sham group, suggesting excessive autophagy initiation during MIR. Whereas, Compound 10 administration significantly elevated cardiac levels of P-mTOR in a dose-dependent manner against MIR in vivo.

3.7. Compound 10 upregulated cardiac Sirt1 other than the other sirtuins family members in vitro and in vivo

Given that Sirtuins have been reported to play an important role in the cardiac autophagy, the changes in expressions of all Sirtuins family members (including 1-7) were examined against myocardial injury in vitro and in vivo (Fig. 7A-F). In H₂O₂ group, we found that the level of Sirt1 decreased to only 34.33%, as compared with CON group (Fig. 7A and B). Meanwhile, cardiac Sirt1 was markedly declined in MIR rats, relative to the Sham animals (Fig. 7D and E). Expressions of some sirtuins family members also showed significant decrease, including Sirt2,3 and 4 in vitro, and Sirt2 and 3 in vivo. However, Compound 10 only dose-dependently and dramatically elevated expression of Sirt1 in vitro and in vivo.
The interaction between Compound 10 and Sirt1 was also explored through CETSA analysis. Compared with DMSO treated cells, Sirt1 was relatively stable between 42 °C and 62°C in Compound 10 treated cells (Fig. 7G-H). However, in DMSO treated cells, Sirt1 was degraded at 59°C. These results indicated that Compound 10 could indeed increased the thermal stability of Sirt1 in cardiomyocytes.

Additionally, crystal structure of Sirt1 and its classical composite (PDB ID=4i5i) from RCSB protein database (https://www.rcsb.org/structure/4I5I) was obtained for molecular docking. Docking with ligand compound Compound 10, 20 positions of the ligand are written and checked, and then the interaction analysis between the binding pocket of Sirt1 and the position of Compound 10 ranking top was conducted (Fig. 7I-J). These compute based calculations and findings further confirmed the interaction between Compound 10 and Sirt1. A π-π bond between one end of Compound 10 and Phe434 of Sirt1. On the other hand, 1,3-dimethyl Barbituric acid of Compound 10 formed π-π bond with Phe273, which was the main site of interaction.

3.8. Compound 10 regulated cardiac autophagy by mTOR and Rab7 via Sirt1

In order to demonstrate the role of Sirt1 in the cardioprotection of Compound 10 against myocardial injury, Ad.GFP or Ad.Sirt1-AS infected H9c2 cells for 48 h were employed (Fig. 8A), and the level of Sirt1 was checked. In the cells infected with Ad. Sirt1-AS, the Sirt1 level decreased by 47.67% compared with the Ad.GFP+CON group (Fig. 8B). However, relative to Ad.GFP+CON cells, the cell viability in Ad.GFP+H₂O₂
group dramatically decreased to only 25.54%. Relative to Ad.GFP+H2O2 only group, Compound 10 (5,10, 20 μM) treatment in Ad.GFP transfected cells dose-dependently elevated cell viability to 52.87%, 75.21% and 101.68%. Importantly, knockdown of Sirt1 markedly blocked those beneficial effects of Compound 10, as demonstrated by the cell viability in Ad. Sirt1-AS+Compound 10 (5, 10, 20 μM)+H2O2 to only 31.80%, 31.75% and 33.61%, respectively (Fig. 8D). At the same time, the leakage rate of LDH also showed a significant upward in Ad.GFP+H2O2 and Ad.Sirt1-AS+H2O2, compared to the Ad.GFP+CON and Ad.Sirt1-AS+CON, respectively (Fig. 8E). Compound 10 markedly declined LDH leakage in a dose-dependent manner in Ad.GFP transfected cells. However, knockdown of Sirt1 abolished these actions, as demonstrated by dramatically elevated LDH leak rate in Ad.Sirt1-AS+Compound10H (5, 10 or 20 μM)+H2O2, relative to the Ad.GFP+Compound10 H (5, 10, or 20 μM)+H2O2, accordingly and respectively.

Finally, autophagy and autophagic flux related proteins were detected in cells transfected with Ad.GFP or Ad.Sirt1-AS (Fig. 8F-L). In Ad.GFP transfected CON cells, H2O2 significantly increased the expression of LC3II/I protein by 1.85 folds, Beclin1 by 1.71 folds, P62 by 1.52 folds, ATG5 by 1.55 folds, and Rab7 by 1.32 folds, while the expression of LAMP2 protein was reduced by 33.68%. However, pretreatment with Compound 10 (20 μM) significantly reduced LC3II/I (56.88%), Beclin1 (68.67%), P62 (38.81%), ATG5 (55.50%) Rab7 (60.84%) in H9c2 cells (Fig. 8F-L). These alterations showed that Compound 10 indeed promoted the degradation of autolysosomes.
especially for LAMP2, indicating that Sirt1 played an important role in the regulatory effect of Compound 10 on cardiac autophagic flux via LAMP2.

In addition, we also monitored changes in mTOR in the experimental group (Fig. 8M). Compared with Ad.GFP+H2O2 group, Ad.Sirt1-AS+H2O2 group showed no significant change in P-mTOR. But, after Compound 10 was applied, Ad. Sirt1-AS significantly inhibited the increase of P-mTOR, with an inhibition rate of 41.32% (Fig. 8N-O).

4. Discussion

In this study, our results showed for the first time that the cinnamamide derivative Compound 10 reduced the size of infarction and improved the cardiac function caused by MIR injury. Compound 10 played an important role in the reversing excessive cardiac autophagy initiation and blocked autophagic flux in vitro and in vivo via the activation of Sirt1/mTOR and LAMP2 signaling pathway.

In patients with myocardial infarction (MI), the treatment option to reduce acute myocardial ischemic injury and limit the size of myocardial infarction is to use thrombolytic therapy or primary PCI for timely and effective myocardial reperfusion(Song et al., 2017). However, the process of reperfusion can induce cardiomyocyte death, which is called myocardial reperfusion injury. At present, there is no effective treatment to prevent myocardial reperfusion injury. Therefore, the new treatment strategy to prevent reperfusion injury may improve the clinical outcome of patients with myocardial infarction.
Cinnamamide derivatives have been proved to be effective anti-oxidative and anti-inflammatory derivatives (Shi et al., 2022). However, whether the cinnamamide derivative Compound 10 plays a protective role against myocardial ischemia reperfusion injury is unclear. In this study, we found that pretreatment with Compound 10 (5, 10, 20 μM) had a strong cardioprotective effect, which showed that it significantly increased the viability of H9c2 cells injured by H2O2, markedly reduced the leakage rate of LDH, and improved cell morphology. In addition, compared with MIR rats, pretreatment with Compound 10 (5, 10, 20mg·kg⁻¹) significantly improved the stability of myocardial cell membrane, decreased the level of serum myocardial enzyme map and improved the changes of cardiac histopathology. Encouragingly, we found that Compound 10 not only alleviated the systolic dysfunction of the left ventricle, but also reduced MIR induced myocardial infarction in vivo. Of importance, the cardioprotective action of Compound 10 was greater than that of Met both in vitro and in vivo, the positive control clinically used for MI and MIR patients. Therefore, the pre-administration of Compound 10 indeed possessed a strong cardioprotective effect against MIR injury in vitro and in vivo.

Autophagy, a cellular process associated with the degradation of damaged or unnecessary proteins and organelles (Ma et al., 2015), is an indispensable part of biological health, and the disturbance in autophagic flux either aggravates or alleviates various pathological changes of various human diseases (Eisenberg, Abdellatif, Ljubojević-Holzer, & Sedej, 2022). In the heart, autophagy plays a key role in response to pathological stimuli, including cardiac hypertrophy, heart failure and MIR. It is called
a double-edged sword in heart protection (Ma et al., 2015; Zhang et al., 2021). In the basic state, autophagy is helpful for cell recovery during transient myocardial ischemia. However, the subsequent reperfusion can lead to excessive autophagy initiation, which is a cytotoxic effect and can promote the excessive degradation and self digestion of important cell components, known as autophagy or type II programmed cell death (Wu et al., 2018). Therefore, it is necessary to regulate the excessive autophagy activation and impaired autophagic flux through drug intervention to fight against common cardiovascular diseases (Abdellatif, Ljubojevic-Holzer, Madeo, & Sedej, 2020; C. Li et al., 2022). Notably, caffeic acid (3,4-dihydroxycinnamic acid) modified on the structure of cinnamic acid also has antioxidative (Kassa, Whalin, Richards, & Alayash, 2021), anti-inflammatory (Zielińska et al., 2021) and immunomodulatory effects (Tada et al., 2018). Recently, it was found that caffeic acid can regulate autophagy to improve diabetes nephropathy in rats (Matboli et al., 2017). To explore the mechanism underlying Compound 10’s beneficial action on cardiomyocytes, real-time PCR was conducted to detect mRNA levels of key genes related to main pathological factors of MIR injury, including oxidative stress, inflammation and autophagy. Data depicted that Compound 10 markedly and dose-dependently regulated autophagy proteins. To monitor the potential effect of Compound 10 on autophagy initiation and autophagic flux, adenovirus transfection with tandem fluorescent mRFP-GFP-LC3 were used to observe the changes in the number of autophagosomes and autophagic lysosomes in different groups, as well as the autophagic flux related protein expressions demonstrated by Western blotting.
Beclin1, a tumor suppressor gene and a key protein for regulating autophagy, participates in the formation of autophagosome membranes to induce autophagy (Cheng et al., 2017). ATG5 (Sheng et al., 2018) and Rab7 (Kuchitsu & Fukuda, 2018) play an important role in the formation of autolysosomes, and excessive autophagy and blocked autophagic flux will be triggered by enormous accumulation of these two proteins. LAMP2 is involved in mediating the degradation of lysosomes, and lack of LAMP2 may lead to lysosomal accumulation and prevent the generated autophagosomes from binding to them, leading to cardiomyopathy (Alcalai et al., 2021). LC3 is the main component protein of autophagosome formation and a regulator of autophagy (Zielińska et al., 2021). In addition, P62, an important regulator and receptor of autophagy, is negatively correlated with the flux of autophagy (Y. Li et al., 2020). LC3II/I and P62 are markers for monitoring autophagy activation (Zielińska et al., 2021). In the present study, Compound 10 could significantly reduce the accumulation of autophagosomes caused by excessive autophagy activation induced by MIR, as demonstrated by dose-dependently reduced cardiac LC3 II/I, Beclin1, P62 and elevated LAMP2 in cardiomyocytes suffered to H₂O₂ exposure and rats subjected to MIR injury, respectively. Furthermore, CQ, a lysosomal inhibitor, was employed to evaluate the effect of Compound 10 pretreatment on autophagic flux in vitro. It was found that CQ treatment dramatically increased the LC3 II/I ratio and P62 level, suggesting that autophagic flux was blocked and aggravated myocardial cell death. Noteworthy, the combination of Compound 10 and CQ remarkably improved the autophagic clearance and cell viability in cardiomyocytes subjected to H₂O₂ treatment. Meanwhile, mTOR
is the most negative feedback regulatory substrate for autophagy (Amaravadi et al., 2011). Next, changes of autophagy-regulatory substrate P-mTOR and mTOR were detected in vitro and in vivo, and the results showed that the level of P-mTOR and mTOR in the H2O2 or MIR group was significantly reduced, respectively, suggesting that excessive autophagy initiation occurred, which adversely affected the normal autophagy flow. However, Compound 10 exhibited a dose-dependent elevated levels of P-mTOR, indicating that Compound 10 had an inhibitory effect on the excessive formation of autophagy. These results indicated that the cardioprotective effect of Compound 10 was dependent on its effect on the recovery of impaired autophagic flux.

Class III NAD dependent deacetylase sirtuins can regulate autophagy of many cells in response to cell stress. The sirtuins family (Sirt1-7) also plays an important role in cardiovascular disease (Lee & Im, 2021). In our study, it was shown that only Sirt1 was significantly increased in MIR rats and cells injured by H2O2, and that Compound 10 dose-dependently elevated Sirt1 expression in vitro and in vivo. Moreover, it was exciting to learn that Sirt1 could play a protective role in the heart by influencing autophagy (Kim, Mondaca-Ruff, Singh, & Wang, 2022), and activation of Sirt1 would also enhance autophagic flux, thereby reducing MIR induced cardiac damage (Lee & Im, 2021). In this study, we found that Compound 10 could bind with Sirt1 by molecular force through molecular docking research, and that Compound 10 increased the thermal stability of Sirt1 in cardiomyocytes.

Finally, antisense adenovirus containing the Sirt1 gene or Ad.GFP was transfected in H9c2 cells to explore the role of Sirt1 in regulatory effect of Compound 10 on cardiac
autophagy. Compared with the Ad.GFP+CON group, the expression of LC3II/I, P62, ATG5 and Beclin-1 significantly increased in the Ad.GFP+H2O2 cells, as well as markedly decreased Rab7 and P-mTOR expressions. Compound 10 pre-incubation reversed the above phenomenon, thereby reducing the accumulation of autophagosomes and increasing the binding of autophagic lysosomes, and improving the obstruction of autophagic flux. However, the above-mentioned protective effects of Compound 10 on cardiomyocytes were dramatically blocked by knockdown of Sirt1. These observation further indicated Sirt1 indeed play a key role in ameliorating excessive autophagy initiation and improving impaired autophagic flux induced by Compound 10.

Taken together, Compound 10 exerted cardioprotective effects on MIR by reducing excessive autophagy via downregulation of P-mTOR and improving autophagic flux blockage by upregulating of LAMP2 through Sirt1. Our study provides insights for cinnamamide derivatives to protect cardiovascular diseases by inhibiting excessive autophagy, and also provides new ideas for Cinnamamide derivatives’ translation in drug discovery.

5. Author declaration

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the Plos Medicine guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.
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