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ORIGINAL ARTICLE

Cyclic peptide RD808 reduces myocardial injury induced by β_1-adrenoreceptor autoantibodies

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Abstract

Autoantibodies against the second extracellular loop of β_1-adrenergic receptor (β_1-AA) have been shown to be involved in the development of cardiovascular diseases. Recently, there has been considerable interest in strategies to remove these autoantibodies, particularly therapeutic peptides to neutralize β_1-AA. Researchers are investigating the roles of cyclic peptides that mimic the structure of relevant epitopes on the β_1-AR-EC_{II} in a number of immune-mediated diseases. Here, we used a cyclic peptide, namely, RD808, to neutralize β_1-AA, consequently alleviating β_1-AA-induced myocardial injury. We investigated the protective effects of RD808 on the myocardium both in vitro and in vivo. RD808 was found to increase the survival rate of cardiomyocytes; furthermore, it decreased myocardial necrosis and apoptosis and improved the cardiac function of BalB/c mice in a β_1-AA transfer model. In vitro and in vivo experiments showed that myocardial autophagy was increased in the presence of RD808, which might contribute to its cardioprotective effects. Our findings indicate that RD808 reduced myocardial injury induced by β_1-AA.

Keywords β_1-Adrenergic receptor · Autoantibody against the second extracellular loop of β_1-adrenergic receptor · Myocardial injury · Cyclic peptide RD808

Introduction

Cardiovascular diseases (CVD) have become an important health challenge in recent years. The burden and mortality of CVD, including hypertension [1], atherosclerosis [2], ischemia–reperfusion injury [3], and heart failure [4], remains at a high level worldwide; however, the underlying mechanisms are not yet completely clear. In several studies, autoantibodies against G-protein-coupled receptor (GPCR-AABs) have been attributed to the progression of cardiovascular disease [5–7]. More recently, researchers have demonstrated that β_1-adrenoceptor autoantibody (β_1-AA), a classic member of the GPCR-AAB family, is a significant pathogenic driver in CVD. In a previous study, we demonstrated that the long-term existence of β_1-AA led to cardiomyocyte damage, cardiac dysfunction, and changes in heart structure by either β_1-AR-EC_{II} peptides [8] or β_1-AA immunization [9]. Other findings have confirmed that β_1-AA-positive heart diseases were the pathophysiological consequences of β_1-AA overstimulation [10–12]. Taken together, these results provide evidence pointing to the involvement of β_1-AA in the development of CVD. Consequently, there is an urgent need for targeted therapy.

Inhibiting the pathogenic role of β_1-AA include immunoadsorption (IA) to remove β_1-AA from the patients' serum or by neutralizing the antibodies in vivo [13]. Despite the apparent efficacy of IA in dilated cardiomyopathy (DCM) and Chagas' cardiomyopathy [14, 15], its high cost and complex procedure limit its utility. Therefore, only a minority of CVD patients benefit from IA, and alternative methods that

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can neutralize β_1-AA could largely facilitate treatment of β_1-AA-positive diseases.

Recently, there has been considerable interest in the use of cyclic peptides to neutralize β_1-AAs in vivo due to the unique advantages of this method both in terms of biological stability and safety [16, 17]. Jahns et al. [18] reported that in rats with β_1-AA-induced heart failure, the symptoms were effectively attenuated after treatment with cyclic peptides derived from the second extracellular loop of the β_1-adrenergic receptor (β_1-AR-EC_{II}). In another study by the same researchers [19], the use of cyclic peptides was shown to be an effective method to neutralize β_1-AAs for treating β_1-AA-positive diseases. In spite of several valid studies in β_1-EC_{II}-CP (cyclic peptides derived from β_1-AR-EC_{II}), smaller molecules might be necessary because of its supposed lower immunogenicity. In the present study, we described a new cyclic peptide, RD808, with head-to-tail amide bond cyclization, and explored its cardioprotective effects and possible mechanisms.

Materials and methods

Animals

A total of 36 male BalB/c mice (weighing 20–22 g), and fifteen 0–3-day-old Sprague–Dawley (SD) rats (male and female, weighing 4–6 g) were purchased from Vital River, license: SCXK (Beijing), 2012-0001. The experimental procedures were compliant with the Guidelines for the Care and Use of Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of Capital Medical University (Permission Number AEE-2015-097). Until the experiments were initiated, the mice were housed in a temperature-controlled room with a 12-h light/dark cycle and controlled humidity and temperature, with food and water provided ad libitum. Then, 0- to 3-day-old newborn SD rats were used to obtain neonatal rat cardiomyocytes (NRCMs).

A total of 36 healthy adult male BalB/c (SPF grade) mice were used in this study. The mice were randomly divided into one of the following six groups ($n = 6$ per group): (1) vehicle group; (2) β_1-AA group: the mice received 10 μg g^{-1} of β_1-AAs via tail vein injection every 2 weeks; (3) prevention study: the mice received intravenous application of RD808 with β_1-AA from the start of the experiment; (4) therapy study: the mice received 2 μg g^{-1} RD808 1 month after β_1-AA administration was initiated; (5) metoprolol group: the mice received 20 mg kg^{-1} day^{-1} metoprolol via intragastric administration throughout the experiment to prevent cardiac dysfunction; (6) RD808 group: the mice in this group received 2 μg g^{-1} RD808 via tail vein injection. Animals received β_1-AA or RD808 via tail vein injection every two weeks until 8 weeks. All BalB/c mice were euthanized by decapitation at 8 weeks, as suggested by the AVMA Guidelines on euthanasia.

Preparation of β_1-AA

Monoclonal antibodies against β_1-AR-EC_{II} were prepared by hybridoma cell fusion. The hybridoma cell line was cloned and selected for culture. Then, 1×10^6 hybridoma cells were transferred into the abdominal cavity of mice, and ascites was collected after 10–14 days. Finally, a MAbTrap Kit (GE Healthcare, Stockholm, Sweden) was used for affinity assessment and purification of ascitic IgG.

Synthesis of RD808

All peptides used in the present study were produced in Hangzhou Peptide Biochem Co., Ltd (Zhejiang, China). As β_1-AA binds to the epitope at β_1-AR-EC_{II}, six databases were used to analyze the precise region of β_1-AR-EC_{II} targeted by β_1-AA. Subsequently, the cyclic peptide was synthesized using a solid phase method, according to the amino acid sequence of analyzed epitope. 2-CTC resin and Fmoc-Asp(OtBu)-OH were incorporated in anhydrous dichloromethane and DIEA, respectively. MeOH was utilized to seal the 2-CTC resin, and washed three times with DMF. The Fmoc group was removed using 20% piperidine in DMF, and washed five times with DMF. Fmoc-Asn(Trt)-OH and HoBt were then dissolved in DMF, and DIC was added for 10 min. Currently, the above mixtures were allowed to react with nitrogen, and the reaction was stopped when the result was negative. Then, the resultant mixture was washed with DMF, after which DMF was removed with vacuum aspiration. The linear peptide resin (H-Arg(pbf)-Arg(pbf)-Cys(Trt)-Tyr(tBu)-Asn(Trt)-Asp(OtBu)-CTC) was obtained according to the above steps, and the procedure was repeated to link the amino acids (Fmoc-Tyr(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(pbf)-OH, Fmoc-Arg(pbf)-OH). The linear peptide resin was then dissolved in TFA/DCM, and the resin was removed by filtration. After removing the resin, the filtrate was concentrated by flushing with water, acid, and saturated salt water in that order. Crude linear peptide was achieved after the filtrate was dried. To introduce the cyclic peptide, the crude linear peptide was reacted with HoBt, EDC, and DIEA in DCM at room temperature, and the amide bond was cyclized. Crude cyclic peptides were dissolved in lysate (TFA:TIS:H_2O) for 3 h, precipitated by methyl tert-butyl ether precipitate, and the precipitate was obtained by centrifugation and washing. The cyclic peptide was achieved after purification by high-performance liquid chromatography and mass spectrometry, with a purity of > 98% (Figure S1).

Enzyme-linked immunosorbent assay (ELISA)

RD808 was dissolved in 10 μg mL^{-1} Na_2CO_3 solution (0.1 mol L^{-1}, pH 11.0), and used to coat the wells of a 96-well plate overnight at 4°C. The plate was blocked with 1% bovine serum albumin (BSA) buffer (1 g BSA, 0.1 mL Tween-20 in 100 mL phosphate-buffered saline (PBST), pH 7.4) for 1 h at 37 °C. After the plates were washed three times with PBST, 50-μL solutions containing 5 μL of sample (β_1-AA-negative mouse IgG, Sigma, America; rabbit anti-β_1-AR antibodies, epitope: aa394–408, Abcam, San Francisco, CA, USA; β_1-AA from ascitic IgG; mouse serum) and 45 μL of PMT was added to each well. The wells were incubated for 1.5 h at 37°C. After washing three times, 5 μL of biotinylated goat anti-mouse IgG antibodies (1 μL of antibodies diluted in 3999 μL PMT; Zhong Shan Jin Qiao, China) was added to the wells and incubated for 1 h at 37°C. Then, streptavidin–peroxidase conjugate (1:3000, Vector, CA) was added to the wells and incubated at 37°C for 1 h. Finally, 2,2-azino-di(3-ethylbenzothiazoline) sulfonic acid (ABTS, Sigma, San Francisco, CA, USA) and H_2O_2 (Roche, Basel, Switzerland) substrate buffer were added to the plates, and the reaction mixture was allowed to react in the dark for 0.5 h at 37 °C.

To detect serum β_1-AA levels, the wells of a 96-well plate were coated with a synthetic peptide corresponding to the β_1-AR-EC_{II} sequence. The serum ratio was 1:10, and other procedures were performed as described above.

Isolation and culture of NRCMs

Cardiomyocytes were isolated from neonatal rat hearts (0- to 3-day-old newborn SD rats) using trypsin and collagenase [30 mg trypsin powder, porcine 1:250 (Sigma), 20 mg collagenase type 2 (Worthington) in 50 mL phosphate-buffered saline (PBS) buffer]. After the fragments were completely digested, the cell suspension was collected and centrifuged at 1000 rpm for 10 min. Then, the precipitated cells were inoculated in culture medium and incubated with 5% CO_2 and 95% air for 1.5 h at 37°C. The supernatant was then centrifuged again to obtain NRCMs.

Surface plasmon resonance (SPR)

SPR experiments were carried out using Biacore T200 (GE Healthcare, Chicago, IL, USA). All SPR-based materials were purchased from GE Healthcare (Chicago, IL, USA). β_1-AA was diluted in 10 mmol L^{-1} sodium acetate buffer at pH 5.5 and immobilized on an SPR chip (Sensor chip CM5, GE Healthcare, Chicago, IL, USA) using an amine coupling kit. Approximately 2655RU of immobilized proteins were obtained. Interaction analysis was carried out using HBS-EP as a running buffer. Six concentrations of RD808 ranging from 38.75 μmol L^{-1} to 1.24 mmol L^{-1}) were injected using the "Kinetics/Affinity" program. A flow cell without immobilized protein served as a nonspecific binding control. The chip surface was regenerated after each cycle by injecting with 10 mmol L^{-1} regeneration buffer (glycine–HCl pH 1.5) for 30 s, and washing with running buffer for 150 s before the next cycle. Affinity (K_D) was determined using the "steady state affinity" model in the Biacore T200 evaluation software version 2.0.

Western blot

Phospho-PKA, Cleaved Caspase-3 and autophagy-related proteins in NRCMs were measured by Western blot. Total proteins were obtained from NRCMs using cell lysis buffer and then quantified with BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Protein loading (20 μg for cells, 60 μg for heart tissues) was subjected to 12% SDS-PAGE and then transferred onto a PVDF membrane. Primary monoclonal antibodies (rabbit anti-phospho-PKA, phospho T197, 1:1000, Abcam, San Francisco, CA, USA; mouse anti-β-actin monoclonal, 1:1000, Santa Cruz, Santa Cruz, CA, USA; rabbit anti-Cleaved Caspase-3, 1:1000, Cell Signaling Technology, Boston, MA, USA) were incubated at 4 °C overnight. Binding of specific antibody was detected with a horseradish peroxidase-conjugated anti-rabbit IgG or peroxidase-conjugated anti-mouse IgG at a dilution of 1:3000 for 1 h (Zhongshan Golden Bridge Biotechnology, China). After washing with TBST three times, ECL Plus substrate (Thermo Scientific, Waltham, MA, USA) was applied to the blot. The images were captured by a gel documentation system (ChemiDoc XRS + system, Bio-Rad, Hercules, CA, USA) and the optical density of protein bands was analyzed using gel software Image Lab 3.0.

Cell survival assay

CCK-8 is a highly sensitivecolorimetric assay for the determination of the number of viable cells in cell proliferation or toxicity tests. Here we measured the cell survival using CCK-8 assay (DOJINDO, Japan) according to the manufacturer's instructions. NRCMs were seeded in 96-well plate at 100 μL^{-1} and cells were challenged with different treatments. Subsequently, 100 μL CCK-8 mixture, containing 10 μL CCK-8 solution and 90 μL DMEM medium, was added to the plate, then the cells were incubated for 2 h at 37°C. Sample absorbance was measured by a microplate reader at the absorbance 450 nm.

LDH assay

NRCMs were challenged with different treatments consistent with the CCK-8 experiment, and LDH release in

supernatant was measured to assess the necrosis. Cells in 96-well plates were centrifuged at 400*g* for 5 min, with 100 μL culture medium taken into 60 μL react solutions [20 μL lactic acid solution + 20 μL INT solution (1×) + 20 μL enzyme solution, Beyotime Biotechnology, China]. Then the plates were placed at room temperature in dark for 30 min and the absorbance was measured at 490 nm.

cAMP assay

cAMP levels in NRCMs were measured using radioimmunoassay. The samples were tested according to the cyclic adenosine monophosphate radioimmunoassay kit [cAMP [^{125}I] RIA (RK-509)].

Doppler ultrasound for cardiac function

After 8 weeks different treatment, each group of mice was arranged to perform Doppler ultrasound to evaluate the left ventricular function. Echocardiograms were obtained from anesthetized mice (1% isoflurane, inhalation anesthesia) with a Vevo2100 system (Visual Sonics Inc., Canada). Briefly, M-mode and B-mode tracings were employed using a MS-400 probe. Left ventricular ejection fraction (EF) and fractional shortening (FS) were calculated to assess cardiac function in mice. Left ventricular anterior wall end-diastolic thickness (LVAW; d) and left ventricular end-diastolic volume (LV, Vol; d) were measured to assess the structure of left ventricle.

Detection of autophagic flux

Autophagic flux was evaluated by fluorescent LC3 puncta after NRCMs were transfected with adenovirus tandem fluorescent mRFP-GFP-LC3 (Ad-tf-LC3, Hanbio, China). First, NRCMs were cultured on coverslips of a 24-well plate for 3 days. Then, the cells were transfected with mRFP-GFP-LC3 at a multiplicity of infection of 20 for 24 h. Then, the cells were washed three times with PBS and fixed with 100% methanol (at – 20 °C) for 20 min. Finally, NRCMs were prepared with anti-fade fluorescence mounting medium, and detected by confocal fluorescence microscopy. The number of green fluorescent protein (GFP) and monomeric red fluorescent protein (mRFP) punctuation per cell was analyzed using Image J program to reflect the autophagic flux. Green fluorescence in the cytoplasm was presented by GFP, which was invisible in autolysosomes with acidic pH, while mRFP showed red fluorescence. Yellow dots were the merging of red and green fluorescence, and reflected autophagosomes.

Serum assays

After the completion of Doppler ultrasound, blood samples were collected from the mice using the eye-catching method. Blood-containing tubes were placed for 3 h at 4 °C, and centrifuged for 10 min at 3000 rpm for 4°C. The supernatant, which was the serum, was used for the detection of biochemical indicators using Toshiba 120 automatic biochemical analyzer.

Statistical analysis

All data were presented as the mean values ± standard deviation, except for cardiac function indicators, which were shown as the mean values ± standard error. SPSS v 13.0 was used for all statistical analyzes. One-way analysis of variance was used to compare the mean values between groups. A P value of < 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism 5.00 (San Diego, CA, USA).

Results

1. Binding between β_1-AA and RD808

   RD808 is a cyclic peptide derived from the β_1-AR-EC_{II} consisting of six amino acids, synthesized by solid phase peptide synthesis (Fig. 1a). SPR is an optical method to measure the interaction between molecules. In our experiment, monoclonal antibodies against β_1-AR-EC_{II} (β_1-AA) prepared by hybridoma cell fusion technique were immobilized on a CM5 chip. Steady-state analysis program was used to analyze the binding characteristics between autoantibodies and RD808. As shown in the results, the affinity was 7.763×10^{-4} M, the R_{max} was 259.2, and the Chi square value was 42.6 RU. These data indicated that β_1-AA and RD808 might bind to each other directly (Fig. 1b). The above results were then compared with the chromogenic methods of ELISA (Fig. 1c), confirming that RD808 has a moderate binding affinity to β_1-AA. Negative control: β_1-AA-negative mouse IgG (Sigma, San Francisco, CA, USA); positive control: rabbit anti-β_1-AR antibodies, epitope: aa394–408 (Abcam, San Francisco, CA, USA).
2. RD808 decreased the agonist-like effects stimulated by β_1-AA

   Similar to classical β_1-AR agonists, β_1-AA can induce receptor-mediated signal cascades, leading to an increase in beating frequency, cAMP levels, and p-PKA levels. In this study, the β_1-AR blocker metoprolol (Met) was added to NRCMs (10^{-6} mol L^{-1}) 30 min before β_1-AA treatment (10^{-7} mol L^{-1}). RD808 (10^{-6} mol L^{-1}), and incubated for 30 min at 37 °C. NRCMs were found

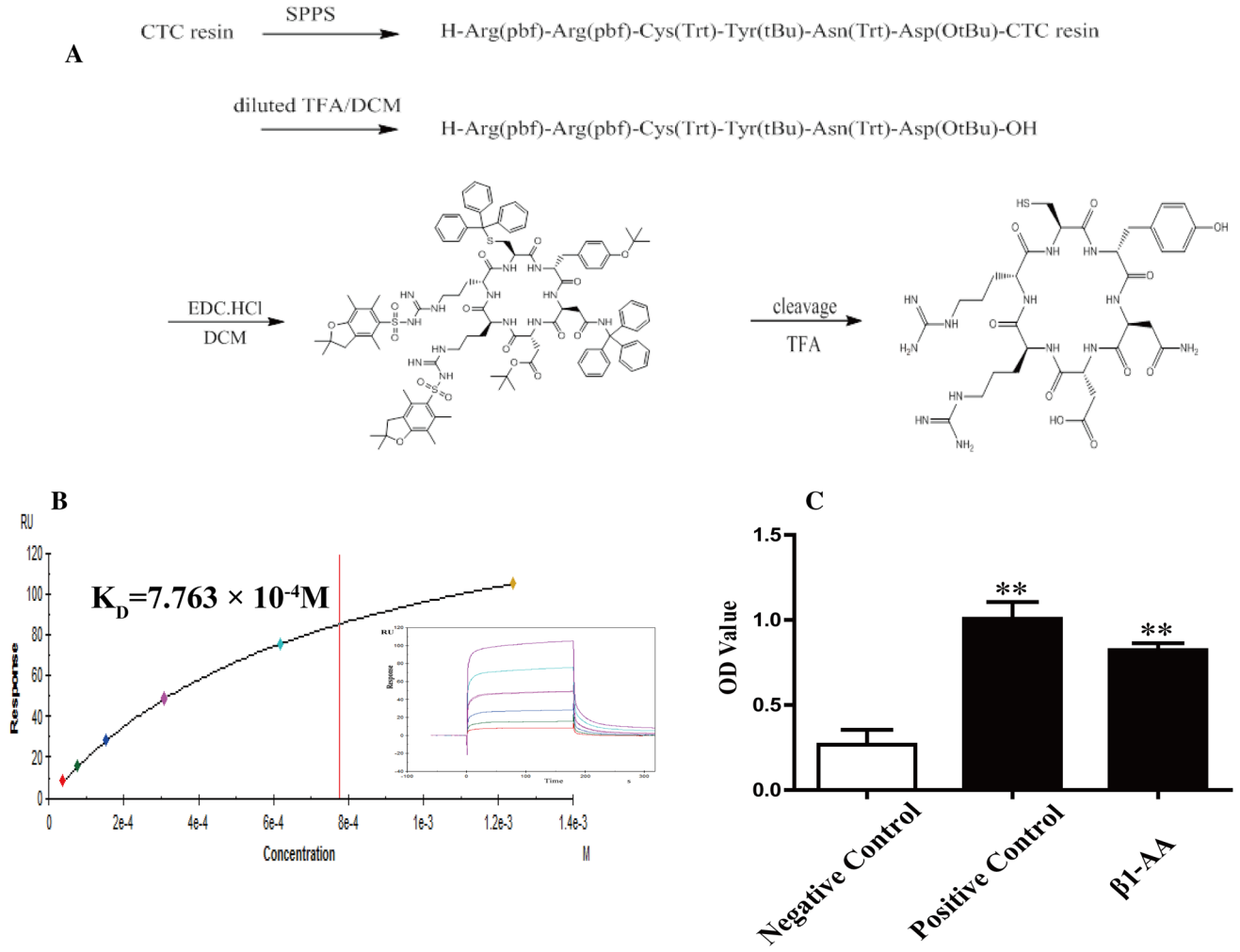


Figure 1 Binding between β_1-AA and RD808. **a** Chemical route for synthesis of RD808. (B) Affinity (KD) between β_1-AA and RD808. Concentration series of cyclic peptide (38.75 μmol L^{-1} to 1.24 mmol L^{-1}) were run over β_1-AA and used to calculate the affinity through steady-state analysis. The affinity of RD808 for β_1-AA was 7.763×10^{-4} M. **c** ELISA was used to analyze the combination between β_1-AA and RD808. Negative control: β_1-AA-negative mouse IgG, Sigma, America; positive control: rabbit anti-β_1-adrenergic receptor antibody, epitope: aa394–408, Abcam, America. Data were expressed as mean values ± SD. $^{**}P < 0.01$ vs. control group, $n = 4$

to have a reduced chronotropic response in the presence of Met or RD808, compared with β_1-AA alone, resulting in a higher beating rate (Fig. 2a). Likewise, intracellular cAMP levels were lower in the presence of Met or RD808 (Fig. 2b) and the p-PKA level was consistent with changes in the intracellular cAMP level (Fig. 2c). Taken together, our results suggested that RD808 significantly decreased the agonist-like effects induced by β_1-AA.

3. RD808 pretreatment prevented the myocardial damage induced by β_1-AA in vitro

We determined whether RD808 exerted cytoprotective effects against β_1-AA overstimulation. As a pathological factor in the myocardial damage process, β_1-AA significantly reduced the cell survival rate of cardiomyocytes in our experiment. The cell survival rate was significantly higher in the presence of RD808 than in its absence (Fig. 3a). A similar result was obtained for LDH release in the cardiomyocyte supernatant (Figure S3), indicating that RD808 inhibited myocardial necrosis. Cleaved caspase-3, an indicator of cell apoptosis, was detected in our experiment. Cleaved caspase-3 protein levels showed a significant decline after RD808 treatment (Fig. 3b).

4. Cardiac dysfunction was ameliorated with RD808 treatment in vivo

Cardiac function declined at 8 weeks according to our previous animal model. In this ISO-induced chronic heart failure model, we followed changes in LVEF over a 16 week period (Figure S2). In mice models with 8 weeks transfer of β_1-AA, the β_1-AA serum titers declined significantly after RD808 administration

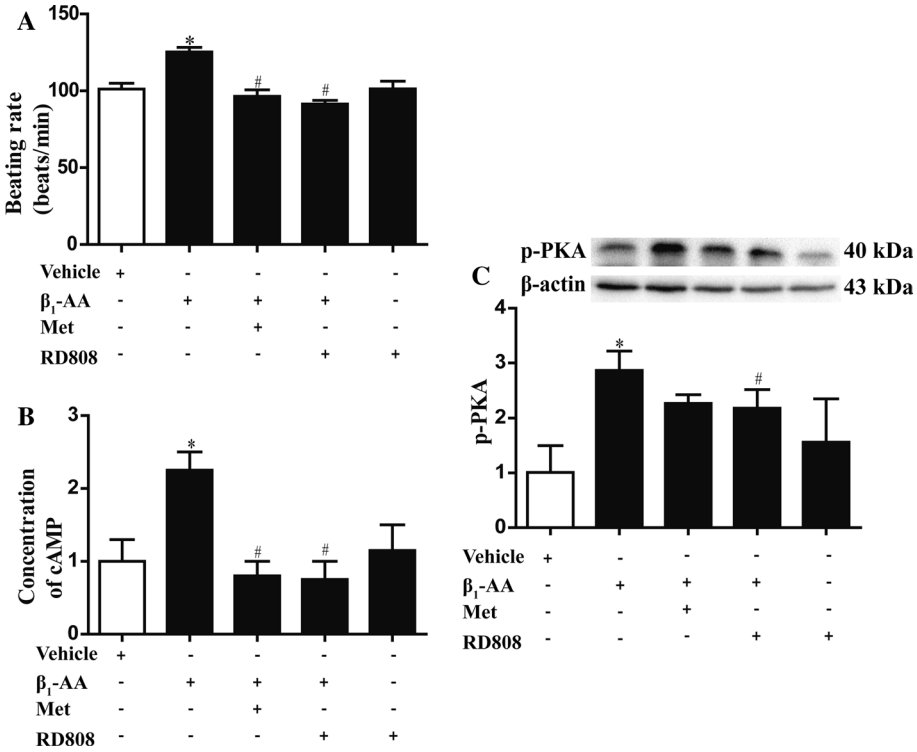


Figure 2 RD808 decreased the agonist-like effects stimulated by β_1-AA. **a** Beating rate of NRCMs. **b** The level of cAMP in NRCMs was measured by radioimmunoassay. **c** The level of p-PKA expression was measured by Western blot. β_1-AA and the peptide were co-incubated for 1h at 37 °C, then supernatants were collected to treat NRCMs. Data were expressed as mean values ± SD. $^{*}P<0.05$ vs. vehicle group, $^{\#}P<0.05$ vs. β_1-AA group, $n=6$

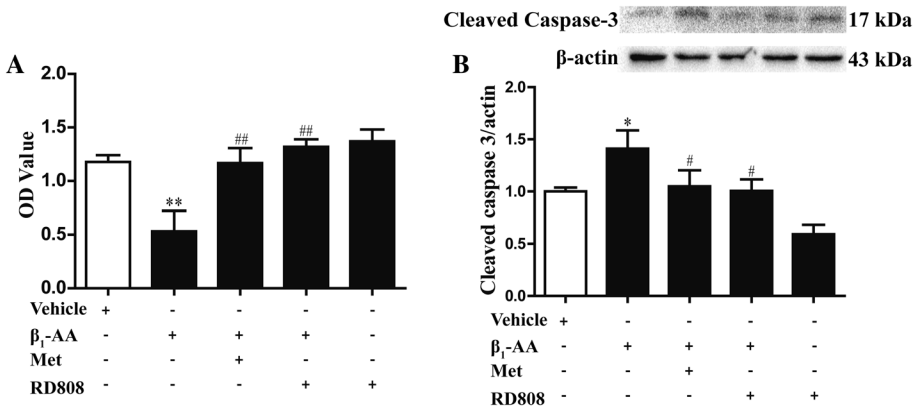


Figure 3 RD808 pretreatment prevented the myocardial damage induced by β_1-AA in vitro. NRCMs injuries were measured with survival rate (**a**) and the expression of cleaved caspase-3 (**b**). After treated with RD808, NRCMs injuries were ameliorated compared with that in β_1-AA alone group. Data are expressed as mean values ± SD. $^{*}P<0.05$ $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ $^{\#\#}P<0.01$ vs. β_1-AA group, $n=6$

in either the prevention study or therapy study group, compared with the β_1-AA group (Fig. 4a). Mice that had received β_1-AA developed cardiac dysfunction and centripetal hypertrophy, manifested as reduced left ventricular ejection fraction, short axis shortening, end-diastolic volume, and increased wall thickness (Fig. 4b, c). RD808 administration could reverse these early-stage changes of cardiac dysfunction in either the prevention study or therapy study group. Contrary to some previous reports, metoprolol did not show a clear protective effect as a control drug. Simultaneously, the prevention study group of RD808 exhibited decreased levels of serum CK–MB, a biomarker of myocardial damage or necrosis [20], compared with the β_1-AA alone group (Fig. 4d). Moreover, the cleaved caspase-3 level was significantly lower in the RD808 group than in the β_1-AA alone group (Fig. 4e). Based on these results, we proposed that RD808 plays a role in improving cardiac function in the long-term presence of β_1-AA in vivo.

5. RD808 elevated autophagy against β_1-AA-induced injury

It has been reported that autophagy might be involved in cardioprotection, while reduced autophagy is considered to be involved in cardiovascular diseases [9]. To explore the possible mechanism involved in cardioprotection against the β_1-AA of RD808, Western blot analysis was used to assess the level of autophagy-related proteins in heart tissue and cardiomyocytes. As shown in Fig. 5a, the ratio of LC3-II-to-LC3-I and the Beclin-1 expression level were both

significantly higher following RD808 administration in the prevention and therapy study groups, indicating that RD808 may play a role in regulating myocardial autophagy.

Moreover, similar effects were observed in vitro. After treatment with β_1-AA in the presence of RD808, the ratio of LC3-II-to-LC3-I increased (Fig. 5b). To further assess autophagic flux, cardiomyocytes were transfected with mRFP-GFP-LC3 and the number of puncta was determined. The cells were treated with β_1-AA (10^{-6} mol L^{-1}) with or without RD808 (10^{-5} mol L^{-1}) for 48 h. Fluorescence images revealed that RD808 increased the percentage of red and yellow fluorescence compared to β_1-AA alone, indicating that both autolysosomes and autophagosomes were significantly increased (Fig. 5c). This result was consistent with the level of autophagy-related proteins. In addition, to determine the cytoprotective effect of autophagy, the survival rate of NRCMs was assessed by CCK-8. Our results showed that the reduced survival rate of NRCMs was alleviated when cells were pretreated with the specific mTOR inhibitor rapamycin (RAPA), which can accelerate autophagy (Fig. 5d).

Discussion

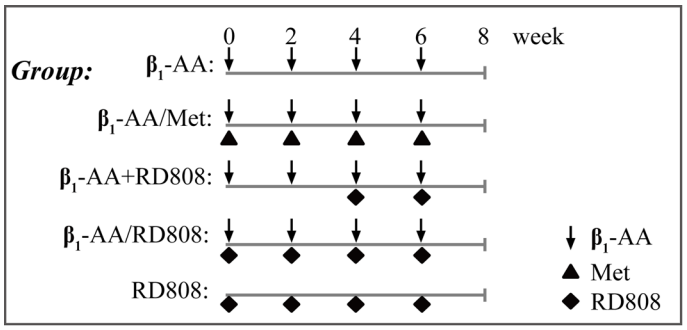
Together, our findings demonstrated the protective effect of RD808 against β_1-AA-induced myocardial injury. RD808 is a new small cyclic peptide derived from β_1-AR-EC_{II}, which aims to neutralize β_1-AA both in vitro and in vivo. This study confirms previous data on the possible beneficial effects of therapeutic peptides in β_1-AA-positive CVD.

Studies have reported that prolonged β_1-AR activation leads to pathological myocardial injury [21–23], which can be induced by β_1-AR agonists [24, 25]. Other studies as well as ours have demonstrated that long-term exposure to β_1-AA can lead to cardiomyocyte damage, cardiac dysfunction, and changes in heart structure. Matsui et al. reported that rabbits immunized with the peptide corresponding to β_1-AR-EC_{II} developed cardiomyocyte damage and changes in heart structure [10]. Wang et al. reported a similar investigation in which β_1-AA from purified IgG with β_1-AA-positive rats were transferred into rats, which then developed cardiac dysfunction [9]. Based on previous reports, we transferred β_1-AA into mice with or without RD808 to clarify whether the latter could play a cardioprotective role against β_1-AA-induced injury. Two different RD808 treatment groups (prevention study and therapy study) were conducted as described previously [19]. In the models with 8-week exposure to β_1-AA, mice developed the symptoms of cardiac dysfunction and presented with declined left ventricular ejection fraction and fractional shortening; meanwhile, these phenomena were alleviated when the mice were treated with RD808 in the prevention and therapy groups. As shown in Fig. 4a, these changes may be related to the decreased serum titers of β_1-AA in the presence of RD808. DCM is the final result of myocardial damage and a major cause of heart failure [10, 26]. Regarding the role of β_1-AA in the pathological process of cardiac dysfunction, we deemed that early-stage changes in cardiac function occurred in the form of reduced pump function and concentric hypertrophy in the second month. The initial phenotype of cardiac hypertrophy may be a response to the activation of β_1-AR by β_1-AA. However, most researchers have focused on the later stages of cardiac dysfunction. The mice in an unpublished study by our group developed severe DCM and heart failure 9 months after transfer of β_1-AA. Similar results were presented by Jahns et al. in a rat heart failure model [12, 27].

Considering the current application of cyclic peptides, comparable studies have been reported. For example, more than 60% of cAMP production in β_1-AR signal transduction was blocked 24 h after the rats were given β_1-EC_{II}-CP, and ventricular dilatation improved after administration of β_1-EC_{II}-CP for 12 months [28]. Hong et al. reported that the atrial effective refractory period was prolonged in a rabbit tachycardia model that received RI peptide, which mimics the functional epitope RCYNDPKCCD in β_1-AR-EC_{II} [29]. Recently, a cyclic peptide termed COR-1 corresponding to β_1-AR-EC_{II} has also been introduced for its potential application in heart failure treatment without obvious side effects. In vitro, after incubation with β_1-AA and application of specific peptides, more than 80% of cAMP production and the beating rate of the cells was reduced [18, 30]. In this study, our finding supported the viewpoint that β_1-EC_{II}-CP, which neutralizes β_1-AA in vivo, is a promising method for treating β_1-AA-positive patients.

Cyclic peptides have a high bioavailability, low immunogenicity, and small molecular weight. However, several cyclic peptides corresponding to β_1-AR-EC_{II} have been discussed and shown to have excellent potential. In the present study, we used RD808 due to its advantages of smaller molecular weight compared with other peptides [31]. Although the binding affinity between RD808 and β_1-AA was lower than that of the β_1-AR-EC_{II} peptides to β_1-AA, this result also demonstrates that macromolecular protein (β_1-AA) was weakly combined with small molecule (RD808).

RD808 showed rapid binding to and fast dissociation from β_1-AA, reflecting a concerned issue currently about drug pharmacokinetics. Notably, as other studies have shown, cyclic peptides possess a short half-life, and the time to the maximal concentration is usually only several minutes [32]. At present the pharmacokinetic changes after RD808 administration in vivo are not clear yet. We measured the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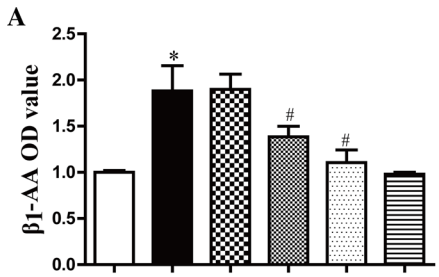
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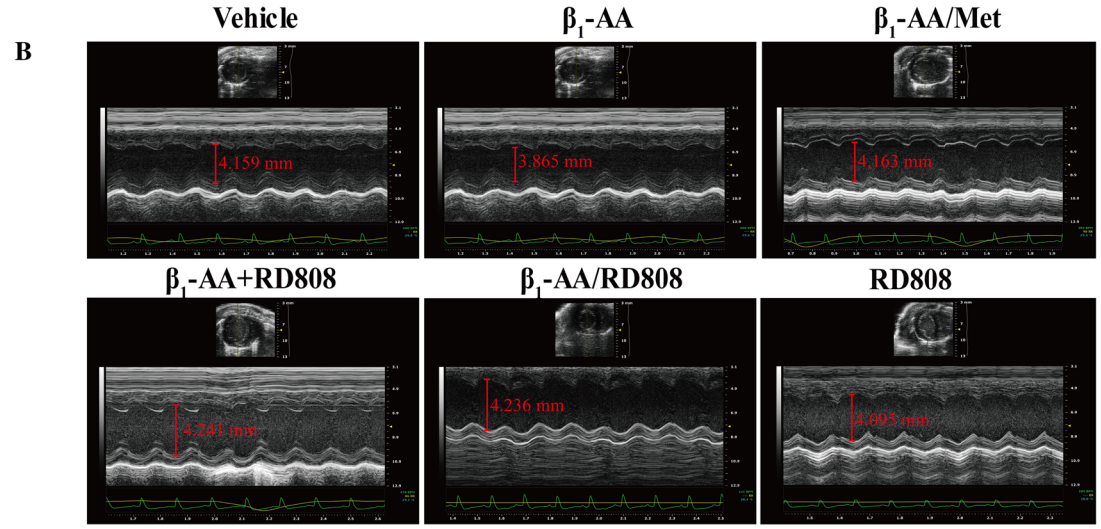
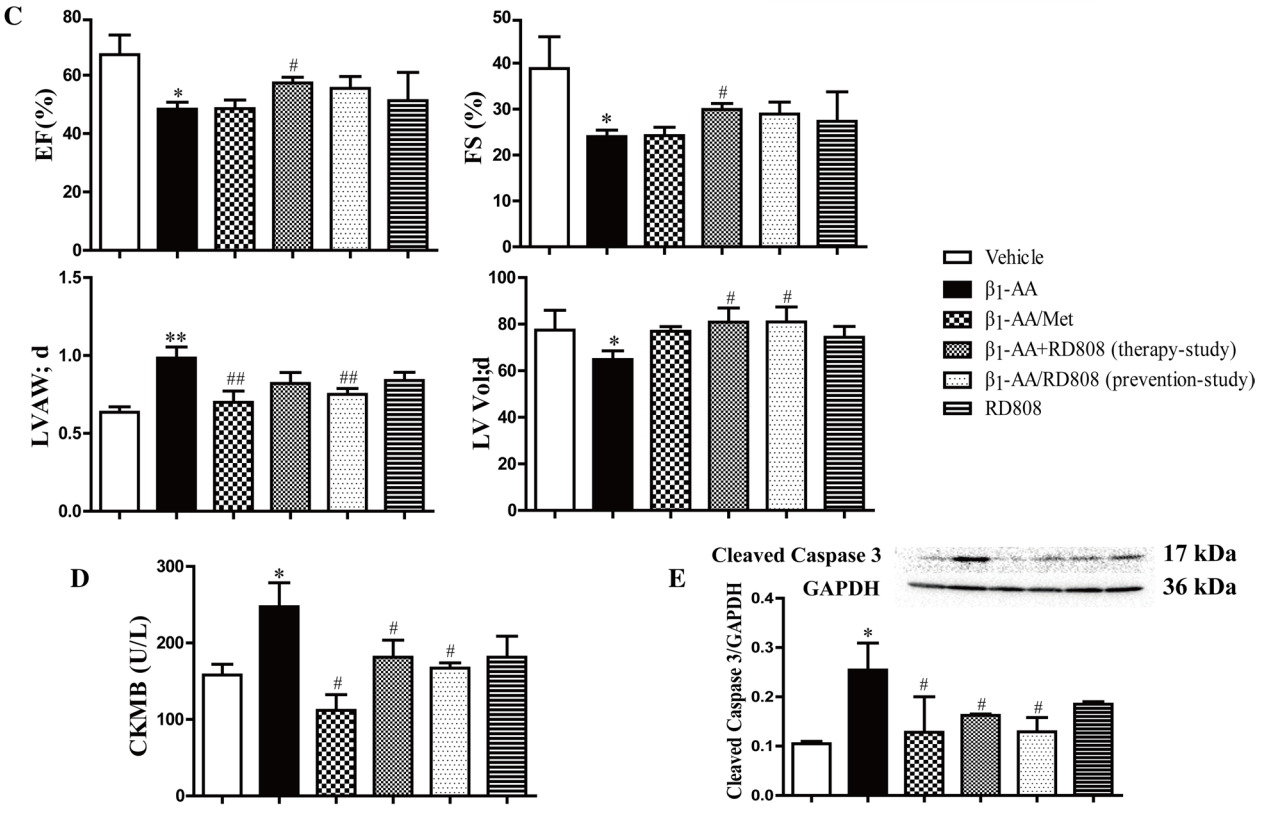

B
Vehicle
β1-AA
β1-AA/Met
4.159 mm
3.865 mm
4.163 mm
β1-AA+RD808
β1-AA/RD808
RD808
4.241 mm
4.236 mm
4.095 mm


C
EF(%)
FS (%)
LVAW; d
LV Vol;d
Vehicle
β1-AA
β1-AA/Met
β1-AA+RD808 (therapy-study)
β1-AA/RD808 (prevention-study)
RD808

D
CKMB (U/L)

E
Cleaved Caspase 3
GAPDH
17 kDa
36 kDa
Cleaved Caspase 3/GAPDH

◂**Figure 4** β_1-AA-induced cardiac dysfunction was ameliorated with RD808 treatment in vivo. Met was given as a control drug in the prevention of cardiac dysfunction and RD808 was administered in the manner of prevention study or therapy study. **a** Serum titers of β_1-AA in different groups. Data were expressed as mean values ±SD. $^*P<0.05$ vs. vehicle group, $^{\#}P<0.05$ vs. β_1-AA group, n = 6. **b** Representative images of echocardiogram. **c** EF, FS, LVAW, d and LV Vol, d changes in each group. Data were expressed as mean values ± SE. $^*P<0.05$ $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ $^{\#\#}P<0.01$ vs. β_1-AA group, $n=6$. **d** Level of serum CK–MB and **e** expression of cleaved caspase-3 were significantly lower than that in β_1-AA alone group. Data are expressed as mean values ± SD. $^*P<0.05$ vs. vehicle group, $^{\#}P<0.05$ vs. β_1-AA group, $n=6$

RD808 concentration in plasma after a single intravenous administration of 1 h or 2 h in mice; however, almost no signal was detected. This is the main limitation of this study. To prolong the half-life and improve the bioavailability, the structure of cyclic peptides may be improved in the form of retro-inverso or liposomal packaging for the purpose of metabolic stability. Therefore, in the future, we will not only try to clarify the pharmacokinetic changes in vivo after cyclic peptide administration but also improve the cyclic peptide structure for improved bioavailability.

In terms of therapeutic peptides, safety is an essential standard to evaluate a new drug. More recently, the safety of the new cyclic peptide COR-1, whose structure mimics that of the relevant epitope on β_1-AR-EC_{II}, has been verified in humans [28]. COR-1 was safe, well-tolerated, and concentration-dependent, and there are no drug-related adverse events and significant changes in toxicity. Although the therapeutic effect and safety of these cyclic peptides have been confirmed, molecular weight is still a core potential in drug development. Consequently, security evaluation was carried out to prove whether or not our small molecule cyclic peptide was safe. Our results indicated that there were no significant differences after RD808 treatment in either cardiomyocyte survival or LDH level in vitro (Figure S4). Additionally, there were no obvious abnormal parameters in either liver or kidney function even when the mice received RD808 for 8 weeks in vivo (Table S1). It can be seen that this small molecule peptide might offer some therapeutic potential in β_1-AA-positive diseases.

Ultimately, the mechanism underlying RD808-mediated cardioprotective effects against β_1-AA may be associated with autophagy regulation. Autophagy is a major intracellular degradation process, including abnormal proteins and cleaning of damaged organelles for cellular homeostasis, maintained at an appropriate level under normal physiological conditions. Myocardial autophagy is impaired in most forms of heart disease, and excessive and uncontrolled activation to autophagy leads to autophagic cell death [33, 34]. Despite the above-mentioned studies, the mechanisms by which autophagy exerts cardioprotection are still under research [35, 36]. It has been reported that myocardial autophagy is triggered during heart failure [37] and myocardial ischemia [38], resulting in cell death in the form of apoptosis, which is the bidirectional regulation of autophagy, has been observed in different disease stages and organs. Regarding the role of autophagy insufficiency in cardiac dysfunction, our previous study showed that decreased myocardial autophagy contributed to cardiomyocyte death followed by cardiac dysfunction in long-term β_1-AA transfer models [9]. Furthermore, another research by Wang et al. showed that the decline in autophagy was involved in β_1-AA-induced cardiomyocyte apoptosis both in myocardial tissues and neonatal rat cardiac myocytes (NRCMs) [39]. In this study, the expression of autophagy-related proteins and autophagy flux level in cardiomyocytes was significantly attenuated in the presence of β_1-AA, while RD808 treatment reversed these changes by probably promoting myocardial autophagy. Consistent with the above-mentioned findings, similar data were demonstrated in vivo experiments in the prevention study as well as therapy study groups, implying that accelerating autophagy may play a role in the RD808-mediated cardioprotective effects against β_1-AA.

In conclusion, this study indicated that the new cyclic peptide RD808 with smaller molecule weight reduced the myocardial injury induced by β_1-AA, and accelerating autophagy might play a role in RD808-mediated cardioprotective effects.

Limitations

In our study, there are some limitations which need to be addressed in the next study. First, we only observed that immunized animals developed hypertrophy in early-stage myocardial damage during the pathological progression of heart failure. Second, in vivo experiments were stopped too early to draw sound conclusions, and the changes in cardiac dysfunction model with transfer of β1-AA were not continuously tested, which needs to be assessed in future studies. Third, RD808 in the therapy study was only administered two times, an assessment of the efficacy of cyclic peptide on β1-AA-mediated cardiac dysfunction also requires a long period of continuous application. Fourth, as a control drug in our mouse-model for cardiac dysfunction, metoprolol should have been administered not only in a preventive but also in a therapeutic manner, as done for RD808. Last not

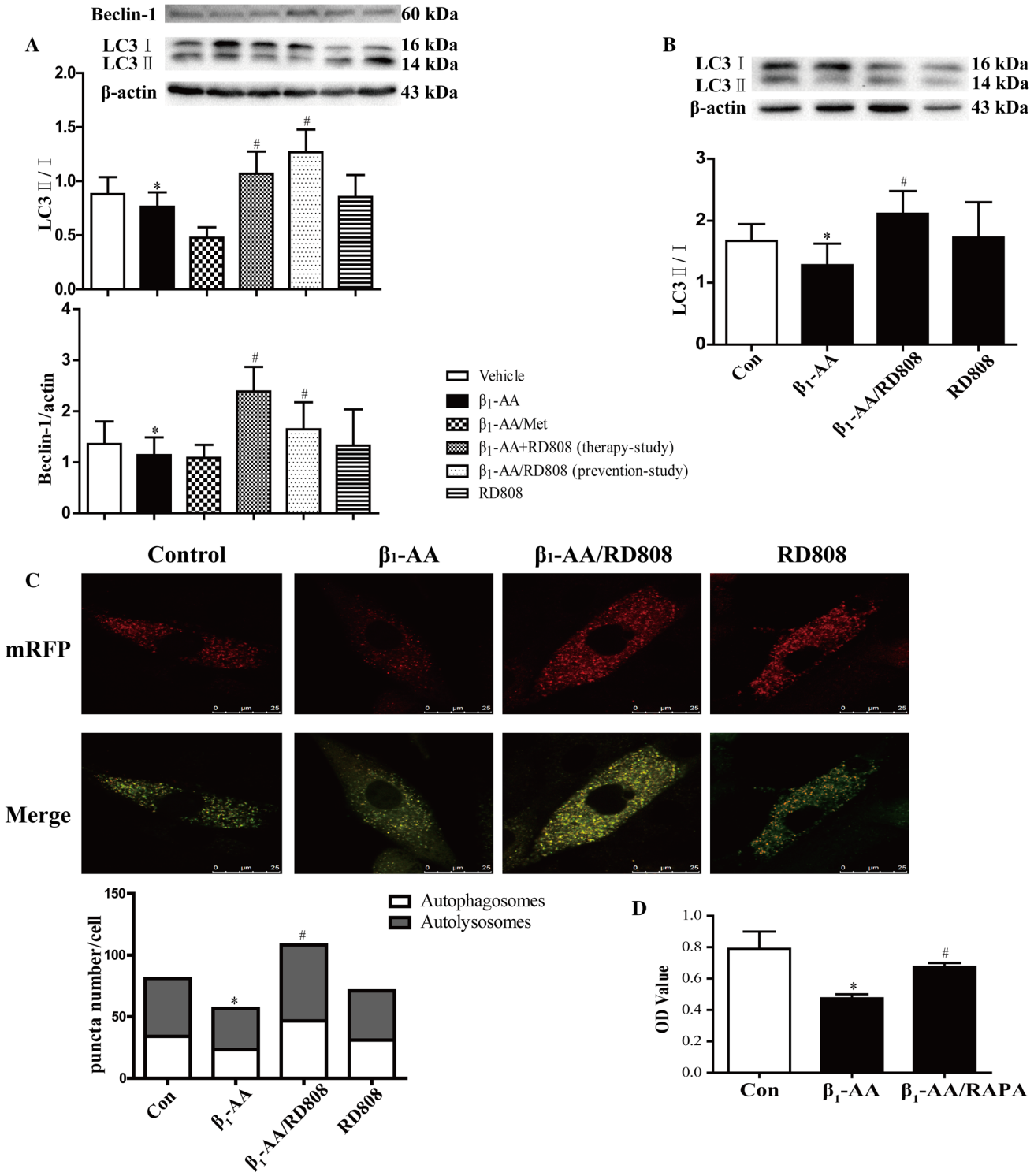


Figure 5 RD808 elevated autophagy against β_1-AA-induced injury. **a** The ratio of LC3-II-to-LC3-I and the Beclin-1 expression level were both significantly higher following RD808 administration in the prevention and therapy study groups than that in β_1-AA alone group. **b** Expression of LC3 in NRCMs. **c** NRCMs were transfected with mRFP-GFP-LC3 for 24 h. The mean puncta numbers of autophagosomes and autolysosomes were represented by yellow and red dots per cell, respectively. **d** Survival rate of NRCMs detected with CCK-8. $^{*}P<0.05$ vs. vehicle group, $^{\#}P<0.05$ vs. β_1-AA group, $n=6$

least, detailed pharmacokinetic in vivo-data on the applied RD808 were unfortunately not obtained in the frame of the present study.

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Author contributions Conceived and designed the experiments: HL, WW. Performed the experiments: YD, YB, SZ, WX, JX, YZ, HY, NC. Analyzed the data: YD, YW. Contributedreagents/materials/analysis tools: SZ. Contributed to the writing of the manuscript: YD.

Conflict of interest The authors declare the absence of any competing interest.

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