Bicyclol Attenuates Obesity-induced Cardiomyopathy via Inhibiting NF-κB and MAPK Signaling Pathways

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Abstract

Purpose: Schisandra is a well-known traditional Chinese medicine in East Asia. As a traditional Chinese medicine derivative with Schisandra chinensis as raw material, bicyclol is well known for its significant anti-inflammatory effect. Chronic inflammation plays an significant part in obesity-induced cardiomyopathy. Our purpose was to explore the effect and mechanism of bicyclol on obesity-induced cardiomyopathy.

Methods: Mice fed with a high-fat diet (HFD) and cardiomyocytes stimulated by palmitic acid (PA) were used as models of obesity-related cardiomyopathy in vivo and in vitro, respectively. The therapeutic effect of bicyclol on pathological changes such as myocardial hypertrophy and fibrosis was evaluated by staining cardiac tissue sections. PCR was used to detect inflammatory factors in H9c2 cells and animal heart tissue after bicyclol treatment. Then, we used western blotting to detect the expression levels of myocardial hypertrophy related protein, myocardial fibrosis related protein, NF-κB and MAPK pathways.

Results: Our results indicated that bicyclol treatment significantly alleviates HFD-induced myocardial inflammation, fibrosis and hypertrophy by inhibiting the MAPK and NF-κB pathways. Similar to animal level results, bicyclol could significantly inhibit PA-induced inflammation and prevent NF-κB and MAPK pathways to be activated.

Conclusion: Our results showed that bicyclol is expected to become a potential drug to treat obesity-induced cardiomyopathy.

1. Introduction

Obesity is an increasingly serious global health problem[1]. According to the report, 1.5 billion adults are considered overweight, of which more than 500 million are defined as obese[2]. Long-term obesity can increase the risk of cardiovascular diseases[3]. The development of obesity-induced chronic, low-grade inflammatory states is the key in promoting systemic metabolic disorders and cardiovascular disease[4]. As we all know, obesity could stimulate adipose tissue to release inflammatory mediators, leading to an inflammatory state and oxidative stress[5]. The increasing number of research results suggests that ventricular remodeling caused by long-term infiltration of inflammatory factors eventually leads to cardiac disease and is associated with cardiac dysfunction, arrhythmia, and adverse outcomes[6]. Therefore, inhibition of these inflammatory cytokines related classical pathways has become a significant strategy for the treatment of obese cardiomyopathy.

In recent years, researchers have paid more and more attention to the therapeutic effects of natural compounds and their derivatives on obese cardiomyopathy. Schisandra, the dry ripe fruit of *Schizandra chinensis (Turcz.) Baill*, is a traditional herb distributed in Asian countries, especially in China[7]. It is reported that it has a variety of biological activities, including anti-inflammatory, antidepressant, antioxidant, anti-osteoporosis and hypoglycemic effects[8–12].

Bicyclol (Bic, in Fig. 1A), an approved drug in China, is one kind of Chinese medicine derivative with Schisandra chinensis as raw material[13]. Recent studies have found that it is a drug with anti-tumor, anti-fibrosis and anti-inflammatory effects[14–16]. Among them, the anti-inflammatory effect is particularly outstanding. Bic reduces liver inflammation caused by hepatitis C virus infection by inhibiting the activation of MAPK and NF-κB pathways[17]. Another study found that Bic treated ischemic stroke in rats by inhibiting the expression of inflammatory related proteins TLR4, TRAF6 and NF-κB[18]. It's worth noting that a recent study found that Bic plays a cardioprotective role by reducing oxidative stress induced by ischemia-reperfusion[19]. This indicated that Bic has a potential myocardial protective effect in obesity-related cardiomyopathy. However, the mechanism of action of Bic in obesity-related cardiomyopathy is still unclear.

Thus, in our study, we aim to investigate the effect of Bic on obese cardiomyopathy and its mechanism. Results of the current study indicated that Bic can treat obesity-induced cardiomyopathy via inhibiting the NF-κB pathway and MAPK pathway. Therefore, Bic may be a promising potential drug to treat obesity-induced cardiomyopathy.

2. Materials And Methods

2.1. Reagents

The bicyclol with a purity of 99.7% (batch number: 101044–201001) was from China Institute of pharmaceutical and biological products identification. HFD and standard diet were from MediScience Diets Co. Ltd (Yangzhou, China). Palmitic acid (C16:0) were purchased from Sigma (St. Louis, MO, USA). The assay kits for low-density lipoprotein (LDL), transgender (TG) and total cholesterol (TCH) were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). P38 inhibitor SB203580 (T1746), MEK1/2 inhibitor U0126 (T21332) and JNK1/2/3 inhibitor SP-600125 (T3109) were purchased from Topscience Biochem Technology (Shanghai, China). NF-κB inhibitor Bay 11-7082 (HY-13453) was obtained from MedChemExpress (Shanghai, China). Antibody p-P65 (3033S), P65 (8242S), p-P38 (4631S), P38 (8690S), ERK (4695S), p-ERK (4370S), JNK (9252S) and p-JNK (4668S) were obtained from Cell Signaling Technology (Danvers, MA). COL-1 (ab34710), TGF-β1 (ab50967) and β-MyHC (ab50967) were from Abcam BioTech (Beijing, China). GAPDH (MB001) was from Bioworld (Nanjing, China). Bic was dissolved with CMC-Na (1%) in vivo studies and dimethyl sulfoxide (DMSO) in vitro studies. SB203580, U0126, SP-600125 and Bay 11-7082 was formulated with DMSO for in vitro studies. The inhibitor mixture is composed of SB203580, U0126, SP-600125 and Bay 11-7082 with the same concentration.

2.2. Cell culture

We purchased H9c2 cells from Cell Resource Center (Shanghai, China). A mixture of DMEM medium (Gibco, Germany) with 10% FBS was used to culture the cells. In order to ensure that the cells maintain normal growth and biological process during the experiment, the cells were cultured in a 37℃ incubator containing 5% CO_2.

2.3. Animal experiments

Mice feeding and option processes were approved by the Welfare Committee of Wenzhou Medical University (Approved ID: wydw2021-0107). We obtained two-month-old male C57BL / 6 mice from the animal center of Wenzhou Medical University. The mice were randomly divided into four groups: (I) Control group; (II) HFD group; (III) HFD + Bic(50 mg/kg) group; (IV) HFD + Bic(100 mg/kg) group. The control mice were fed a standard diet. The other three obesity groups of mice were induced with HFD. There were 6 mice in each group. From the 4th month, the mice in the Bic treatment groups were given the corresponding dose of Bic by intragastric injection every 2 days. Weight was recorded weekly.

2.4. Histological analyses

The heart tissue was immersed in formalin for tissue fixation, and then made into 5μM tissue section. According to the corresponding instructions, we stained the heart tissue sections with hematoxylin eosin (H & E), Masson and Sirius red respectively. We used a microscope equipped with a camera to obtain the corresponding representative images.

2.5. Real-time quantitative PCR

First, we used Trizol to extract total mRNA. Subsequently. Then we used the primescript RT Kit (Takara) for the reverse transcription process of mRNA. QuantStudio ® 3 RT PCR (Applied Biosystems) was used for PCR analysis. Primers used in the PCR were obtained from SANGON biotech (Shanghai, China) (supplementary table S1). In the analysis, the expression levels of mRNAs will be standardized with the expression level of *Actb*.

2.6. Western blot

We prepared cell and tissue protein samples with lysates. Boil samples for 10 minutes. After adding to 10% sodium dodecyl sulfate polyacrylamide gel, electrophoresis was performed at 120 V voltage until the protein marker was clearly separated. The protein was then transferred from the gel to the nitrocellulose filter membrane at 300 mA current for 90 minutes. The buffer was blocked with 5% BSA for 1 hour. The corresponding antibodies were incubated at 4°Covernight. Incubate with the corresponding secondary horseradish peroxidase binding antibody (Beyotime, Shanghai, China) in a slow shaker for 2 hours. The bands were observed and photographed by exposure operation. Image J software was used for protein quantitative analysis.

2.7. Assessment of heart function and myocardial injury

We centrifuged the blood at 3000 rpm until it stratified. The serum was collected for biological testing. The level of LDH and CK-MB were separately measured by LDH assay kit and CK-MB assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). To compare the cardiac function of mice in each group, we used transthoracic echocardiography to evaluate and analyze the cardiac function data of mice in each group.

2.8. Cell viability assay and cell death detection

H9c2 cells were evenly distributed on the Petri dish with a quatity of 5×10^3 cells in each well. After the cells adhered to the wall, the corresponding concentration of drug was added to the culture plate for 12 hours. Then we add 10 μM of CCK-8 solution (Beyotime, Shanghai, China) to each well. Continue to incubate in the incubator for 1h. We measured and recorded the absorbance of each well at 450 nm for subsequent analysis.

2.9. Fluorescence immunocytochemistry for NF-κB p-65 translocation

We used 4% paraformaldehyde to treat the cell samples for 15 minutes. Wash the samples with PBS buffer for 3 times. Then we used 0.25% Triton to incubate the samples for 15 minutes and washed again for 3 times. Subsequently, the cell samples were treated with 5% BSA blocking solution prepared from PBS buffer at 37 ℃ for 1 hour. P65 antibody was added and cultured overnight at 4 ℃, then fluorescent secondary antibody was added and cultured at 37 ℃ for 1 hour. Add anti fluorescence quencher containing dpai. Observed and recorded under Leica microscope.

2.10. Statistical analysis

We used mean ± SEM to describe the experimental data. In order to test the significant difference between groups, we applied students' t-test in our study. The analysis process was completed by graphpad Pro prism 8. The p value < 0.05 was used as the statistical standard of significant difference.

3. Results

3.1 Bic alleviates cardiac dysfunction and myocardial injury caused by HFD

To investigate the role of Bic in obesity-induced cardiomyopathy, HFD + Bic groups were received the corresponding dose of Bic. The weight gain of the HFD group was significantly more than that of the control group. Bic treatment had no significant effect on body weight in the HFD group(Fig. 1B). However, it was worth noting that the ratio of heart weight to tibial length of HFD mice decreased significantly after Bic treatmen(Fig. 1C). To measure the cardiac function of mice in each group, we used transthoracic echocardiography to evaluate and analyze the EF%, FS%, LVAW;d and LVPW;d (Fig. 1D-H). Our outcome indicated that a high-fat diet could induce cardiac dysfunction and ventricular wall hypertrophy. However, Bic treatment can significantly improve cardiac function and reduced ventricular wall thickening. We tested serum LDH and serum CK-MB to detect the protection of bicyclol on myocardium(Fig. 1I-J). The data showed that Bic can reverse the increase of serum LDH and serum CK-MB caused by HFD. Besides, we found that Bic treatment could reduce serum LDL, TCH and TG in HFD-induced obese mice, suggesting that Bic has a certain lipid-lowering effect (Figure S1).

3.2 Bic can ameliorate myocardial fibrosis and myocardial hypertrophy in HFD mice

We found that Bic administration significantly alleviated HFD-induced myocardial hypertrophy by gross examination (Fig. 2A).The pathological changes of heart tissues in each group were evaluated by H & E staining (Fig. 2B). Obvious myocardial fibers disorganization was observed in HFD group. However, the images showed that Bic can significantly improve the disorganization and thickening of myocardial fibers. The results of Sirius red and Masson staining indicated that the content of collagen in mouse heart increased significantly after HFD treatment(Fig. 2C-D). In the groups that were treated with Bic, especially the 100 mg/kg treatment group, myocardial fibrosis was significantly reduced. After that, we detected the protein expression of β-MyHC, COL-1, TGF-β1 and ANP, which are highly expressed in cardiac hypertrophy and myocardial fibrosis (Fig. 2E). We conducted three independent experiments. The quantitative detection of bands showed that Bic treated groups can decrease the expression of β-MyHC, COL-1, TGF-β1 and ANP at the protein levels (Fig. 2F). Moreover, we used PCR to test the mRNA levels of Myh7, Col1a1, Tgfb1 and Nppa (Fig. 2G). The outcomes indicated that Bic treatment could significantly ameliorate these changes at the transcript level caused by HFD.

3.3 Bic reduces myocardial inflammatory injury by inhibiting the activation of NF-κB and MAPK signaling pathways in the heart of HFD mice

It is well known that long-term cardiac inflammation usually causes myocardial fibrosis[20]. And IL-1β, IL-6 and TNF-α are common factors in inflammation, which were significantly overexpressed in HFD-induced cardiomyopathy[21]. Therefore, we tested transcriptional levels of these inflammatory factors in cardiac tissue. Our results showed that both of two doses of Bic can inhibit inflammation caused by HFD (Fig. 3A). MAPK and NF-κB pathways were classic in the regulation of inflammation. Therefore, we detected the protein levels of pathways-related proteins (Fig. 3B). Three independent experiments were used to quantify the protein of the bands and make statistics. We found that two doses, especially the high dose group, could significantly inhibit the activation of these pathways (Fig. 3C).

3.4. Bic protects against myocardial inflammatory injury by inhibiting the activation of NF-κB and MAPK signaling pathway in vitro

First, we tested the influence of Bic on H9c2 cells with CCK8 assay kit, and the result showed that treatment with Bic concentration less than 20 μM had almost no effect on H9c2 cell viability (Figure S2). To explore the inhibiting effect of Bic on inflammatory factors in-vitro, H9c2 cells were pretreated with or without Bic and then stimulated with 200 μM of palmitic acid (PA) (Fig. 4A). We found that inflammatory cytokines were significantly increased after PA stimulation in vitro. However, both doses of Bic treatment groups inhibited the transcriptional expression levels of these inflammatory factors. After that, western blot and cellular immunofluorescence were used to verify the inhibitory effect of Bic on the MAPK and NF-

κB pathways in vitro (Fig. 4B-E). Western blots showed that Bic significantly inhibited the activation of the pathways. It reduced the phosphorylation of pathway-related proteins, such as P65, P38, JNK, ERK, and restored the content of IκBα. Three separate experiments were conducted, and the strip quantitative data were statistically analyzed. The cell immunofluorescence images showed that the intensity of green fluorescence signal in the nucleus of PA group was significantly increased, which indicated that PA activateed intracellular NF-κB pathway, which significantly increased the process of p65 nuclear transfer. However, after pretreatment of PA group with Bic, the intensity of green fluorescence signal in the nucleus decreased significantly. These outcomes also showed that Bic inhibited PA-induced activation of signaling pathways in vitro. To further test the effect of Bic on these two pathways, we took the inhibitor mixture group as the positive control group (Fig. 4F). Interestingly, there was no significant difference between the Bic treatment groups added with or without inhibitor mixture. This suggests that Bic reduces PA-induced inflammation mainly by inhibiting NF-κB and MAPK pathways.

4. Discussion

We first took HFD-induced obese mice as an in vitro model of obese cardiomyopathy. In the group treated with Bic, it could significantly reverse myocardial inflammation, myocardial fibrosis and myocardial hypertrophy caused by HFD, and finally improve cardiac function. Bic inhibited NF-κB and MAPK pathways to achieve the above therapeutic effects. Subsequently, we used PA-stimulated H9c2 as an in vitro model of obese cardiomyopathy to further verify that Bic could inhibit the NF-κB and MAPK pathways to inhibit the PA-induced inflammation.

Obesity is a chronic disease. At the beginning, adipose tissue will produce local inflammation, which leads to long-term low-grade inflammation throughout the body[22]. These pathological changes will cause multiple organs to be involved and eventually lead to organ dysfunction[23, 24]. It is noteworthy that studies in recent years have shown that obesity-induced chronic inflammation was related to various cardiovascular diseases. Chronic inflammation caused by obesity existed in the development of myocardial inflammation, myocardial fibrosis, and myocardial hypertrophy. which will eventually lead to myocardial dysfunction[25, 26]. Inhibition of inflammation has become an effective strategy in the treatment of obese cardiomyopathy. As previously mentioned, Bic, a traditional Chinese medicine derivative with Schisandra chinensis as raw material, is an approved clinical drug to treat chronic hepatitis [27]. In addition, it is worth noting that Bic have anti-inflammatory effects in other inflammation-related models. Zhang et al found that Bic could regulate the oxidative stress to protect the rat brain from focal ischemia[28]. Luo et al found that Bic can reduce TNF-α and IL-1β to alleviate acute lung injury.[29]. Therefore, we believe that Bic may be a potential effective drug for the treatment of obesity-induced cardiomyopathy

It is known to all that the free fatty acid would generally increase in overweight people, in which the proportion of saturated fatty acid palmitate (C16:0) was the highest[30, 31]. PA is often used as an inducer for obese cardiomyopathy model in vitro, which can cause inflammation, fibrosis and myocardial damage in vitro[32, 33]. In previous studies, we found that PA induces the inflammation of

cardiomyocytes by directly binding Toll-like receptor 4 (TLR4) accessory protein myeloid differentiation 2 (MD2)[34]. As the classic TRL4-related pathways, NF-κB and MAPK pathways were significantly activated by PA. Therefore, inhibiting the activation of these pathways provides important ideas for the treatment of obese-induced cardiomyopathy. Studies have found that a variety of drugs can improve obese cardiomyopathy by reducing the activation of these two pathways. Hinokinin alleviates HFD-induced cardiac injury by restraining the MAPK pathway[35]. Curcumin analogue C66 could inhibit JNK-mediated NF-κB and MAPK pathways for treating obesity induced cardiomyopathy[36]. As expected, in our study, PA could activate the pathway of H9c2 cells and make them produce inflammatory factors. However, Bic can reverse the activation of pathway and reduce the level of myocarditis. In vivo, Bic can also improve myocardial fibrosis, myocardial hypertrophy and myocardial injury caused by inflammation.

It is well known that in addition to inflammation, there are many other biological processes also involved in the occurrence of obese cardiomyopathy. Oxidative stress is one of the most significant mechanisms in the process of inflammation, fibrosis and hypertrophy caused by obese cardiomyopathy. It is worth noting that Bic has been reported to have antioxidant stress effects[17]. In addition, in obese cardiomyopathy, in addition to NF-κB and MAPK pathways, Jak2 / Stat3 pathway and Tgf-1β/ Smad3 pathway were also involved[37]. Therefore, the deficiency of this study is that it did not explore the effect of Bic on oxidative stress in obese cardiomyopathy.

5. Conclusion

In general, our study found that Bic can alleviate the inflammation of obesity-induced cardiomyopathy by inhibiting NF-κB and MAPK pathways in vivo and in vitro. Importantly, as a chronic inflammation, obese-induced cardiomyopathy needs long-term treatment. Nowadays, there are few drugs for the treatment of obese cardiomyopathy, and most drugs are not used in clinical treatment. The safety and therapeutic effect of these drugs need to be verified. Bic has the characteristics of small toxic and side effects and strong anti-inflammatory effect, which is suitable for long-term use by clinical patients. Our results extend the clinical indications of Bic. Unfortunately, our study has not explored the target of Bic in the treatment of obese-induced cardiomyopathy and its effect on other signaling pathways. We will further explore these problems in future research. In summary, our results show that Bic is expected to become a potential drug to treat obesity-induced cardiomyopathy.

Abbreviations

BSA, Bovine serum albumin; CCK-8, Cell counting kit; CK-MB, creatine kinase-MB; DMSO, dimethyl sulfoxide; EF, ejection fraction; FS, fractional shortening; HFD, high-fat diet; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LVAW;d, left ventricular anterior wall; diastolic; LVPW;d, left ventricular posterior wall; diastolic; LDL, low-density lipoprotein; MD2, myeloid differentiation 2; PA, palmitic acid; TLR4, toll-like receptor 4; TG, transgender; TCH, total cholesterol

Declarations

Data Availability

All the data in this study are available upon reasonable request from the corresponding author.

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Author contributions

Weijian Huang, Guang Liang and Gaojun Wu contributed to the literature search and study design. Guang Liang, Yanghao Chen, and Bozhi Ye participated in the drafting of the article. Yanghao Chen, Wante Lin, Lingfeng Zhong, Zimin Fang, Bozhi Ye, and Zhe Wang carried out the experiments. Bozhi Ye and Gaojun Wu revised the manuscript. Weijian Huang, and Nipon Chattipakorn contributed to data collection and analysis.

Code availability

Not applicable.

Consent for publication

Not applicable.

Consent to participate

Not applicable.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics Statement

All animal experiments were in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Welfare Committee of Wenzhou Medical University (Approved ID: wydw2021-0107).

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Figures

Figure 1

Bic alleviates cardiac dysfunction and myocardial injury caused by HFD. (A) Bicyclol structure. (B) Body weight gain during a 24-week period. (C) Heart weight. (D-H) Results of transthoracic echocardiography in mice. (I-J) Serum levels of CK-MB and LDH in mice from each group. N =6. Control group vs HFD group: “ns” indicates $p > 0.05$, “#” indicates $p < 0.05$, “##” indicates $p < 0.01$ and “###” indicates $p < 0.001$. HFD + Bic groups vs HFD group: “ns” indicates $p > 0.05$, “*” indicates $p < 0.05$, “**” indicates $p < 0.01$ and “***” indicates $p < 0.001$.

Figure 2

Bic treatment can ameliorate myocardial fifibrosis and myocardial hypertrophy in HFD mice.

Gross morphological image of heart Representative’s images of staining for (B) H&E, (C) Sirius red and (D) Masson trichrome (400X amplification). (E) The protein expression of β-MyHC, COL-1, TGF-β1 and ANP in heart tissue were examined by Western blotting. (F) Band quantification for β-MyHC, COL-1, TGF-β1 and ANP. The relative contents of protein are compared with the GAPDH. (G) Effects of Bic on the mRNA levels of Myh7, Col1a1, Tgfb1 and Nppa in mouse heart tissues were detected by real-time qPCR assay. N =6. Control group vs HFD group: “ns” indicates $p > 0.05$, “#” indicates $p < 0.05$, “##” indicates $p < 0.01$ and “###” indicates $p < 0.001$. HFD + Bic groups vs HFD group: “ns” indicates $p > 0.05$, “*” indicates $p < 0.05$, “**” indicates $p < 0.01$ and “***” indicates $p < 0.001$(A)

Figure 3

Bic reduces myocardial inflammatory injury by inhibiting the activation of NF-κB and MAPK signaling pathways in the heart of HFD mice.

The mRNA levels of IL-1β, IL-6 and TNF-α in heart tissues were detected by real-time qPCR assay. (B-C) Immunoblotting and band quantitative results for p-P65, P65, p-P38, P38, p-JNK, JNK, p-ERK, ERK. N =6. Control group vs HFD group: “ns” indicates p > 0.05, “#” indicates p < 0.05, “##” indicates p < 0.01 and “###” indicates p < 0.001. HFD + Bic groups vs HFD group: “ns” indicates p > 0.05, “*” indicates p < 0.05, “**” indicates p < 0.01 and “***” indicates p < 0.001. (A)

Figure 4

Bic protects against myocardial inflammatory injury by inhibiting the activation of NF-κB and MAPK signaling pathways in vitro.

(A) H9c2 cells were pretreated with 5,10 μM Bic for 1 hour, stimulated with 200 μM PA for 12 hours and the mRNA level of IL-1β, IL-6 and TNF-α are performed using real-time qPCR. (B-D) H9c2 cells were pretreated with 5,10 μM Bic for 1 hour, stimulated with 200 μM PA for 1 hour. Bic reduced PA-induced activation of NF-κB signaling pathway and MAPK signaling pathway. (E) Immunoblotting band quantification of these two pathways. (F) H9c2 cells were pretreated with 10 μM Bic or 5 μM inhibitor mixture or 10 μM Bic + 5 μM inhibitor mixture for 1 h and then exposed to 200 μM PA for 12 h, and the mRNA level of IL-1β, IL-6 and TNF-α were performed using real-time qPCR. N =3. Control group vs PA group: “ns” indicates p > 0.05, “#” indicates p < 0.05, “##” indicates p < 0.01 and “###” indicates p < 0.001. PA + Bic groups vs PA group: “ns” indicates p > 0.05, “*” indicates p < 0.05, “**” indicates p < 0.01 and “***” indicates p < 0.001.

Figure 5

Schematic diagram of the results of this study.

PA induces the activation of MAPK and NF-κB pathways in H9c2 cells by directly binding to MD2. Activated these two pathways lead to induce myocardial inflammation, myocardial fibrosis, hypertrophy, and dysfunction. Bic treatment can block these pathways and alleviate myocardial inflammation, fibrosis and dysfunction.

Supplementary Files

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- SupplementaryFiles20220408.docx