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Original Article

High content screening identifies licoisoflavone A as a bioactive compound of Tongmaiyangxin Pills to restrain cardiomyocyte hypertrophy via activating Sirt3

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ABSTRACT

Background: : Cardiac hypertrophy is a prominent feature of heart remodeling, which may eventually lead to heart failure. Tongmaiyangxin (TMYX) pills are a clinically used botanical drug for treating multiple cardiovascular diseases including chronic heart failure. The aim of the current study was to identify the bioactive compounds in Tongmaiyangxin pills that attenuate cardiomyocytes hypertrophy, and to investigate the underlying mechanism of action.

Methods and Results: : The anti-hypertrophy effect of TMYX was validated in isoproterenol-induced cardiac hypertrophy model in C57BL/6 mice. After TMYX treatment for 2 weeks, the heart ejection fraction and fractional shortening of the mice model was increased by approximately 20% and 15%, respectively, ($p < 0.05$). Besides, TMYX dose-dependently reduced the cross section area of cardiomyocytes in the angiotensin-II induced hypertrophy H9c2 model ($p < 0.01$). Combining high content screening and liquid chromatography mass spectrometry, four compounds with anti-cardiac hypertrophy effects were identified from TMYX, which includes emodin, licoisoflavone A, licoricone and glyasperin A. Licoisoflavone A is one of the compounds with most significant protective effect and we continued to investigate the mechanism. Primary cultures of neonatal rat cardiomyocytes were treated with a hypertrophic agonist phenylephrine (PE) in the presence or absence of licoisoflavone A. After 48 h of treatment, cells were harvested and mitochondrial acetylation was analyzed by western blotting and Image analysis. Interestingly, the results suggested that the anti-hypertrophic effects of licoisoflavone A depend on the activation of the deacetylase Sirt3 ($p < 0.01$). Finally, we showed that licoisoflavone A-treatment was able to decrease relative ANF and BNP levels in the hypertrophic cardiac cells ($p < 0.01$), but not in cells co-treated with Sirt3 inhibitors (3-TYP) ($p > 0.05$).

Conclusion: : TMYX exerts its anti-hypertrophy effect possibly through upregulating Sirt3 expression. Four compounds were identified from TMYX which may be responsible for the anti-hypertrophy effect. Among these compounds, licoisoflavone A was demonstrated to block the hypertrophic response of cardiomyocytes, which required its positive regulation on the expression of Sirt3. These results suggested that licoisoflavone A is a potential Sirt3 activator with therapeutic effect on cardiac hypertrophy.

Abbreviations: Ang ii, angiotensin-II; d, diastole; EF, ejection fraction; FS, fraction shortening; HCS, high-content screening;; IHC, immunohistochemistry; ISO, isoproterenol, LC-MS, liquid chromatograph-mass spectrometer; LVPW, left ventricular posterior wall depth; LVID, left ventricular diameter; LV Vol, left ventricular volume; PE, phenylephrine; s, systolic;Sirt3, Sirtuin3 TAC, transverse aortic constriction; TCM, Traditional Chinese medicine; TMYX, Tongmaiyangxin Pills; 3-TYP, the selective Sirt3 inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine

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[1] These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Introduction

Cardiovascular disease is the leading cause of morbidity and mortality throughout the world. Cardiac remodeling is an alteration process of both the structure and the physiological function of the heart, caused by changes of cardiac cells. In its early stage, cardiac remodeling is an adaptive process that allows the heart to maintain or increase its hemodynamic performance (Frey and Olson, 2003). However, this condition soon declines toward a maladaptation process which may lead to heart failure (Cohn et al., 2000; Zhan-Peng and Da-Zhi, 2014; Liu et al., 2015). Hypertrophy is a prominent feature of the remodeled heart, which is characterized by an increase in the thickness of the ventricular wall, due to cardiomyocyte enlargement, cell death and cardiac fibrosis. Sustained cardiac hypertrophy is often associated with significantly increased risks of heart failure and malignant arrhythmia (Eiki and Kass, 2007; Hill and Olson, 2008).

Traditional Chinese medicine (TCM) has been reported as an important resource for alternative and complementary therapy in cardiovascular diseases in China and many other Asian countries (Yu et al., 2019). Tongmaiyangxin (TMYX) pills are synthesized based on a classic TCM prescription, which has been approved to treat coronary heart disease by the National Medical Products Administration of China. TMYX consists of 11 herbs and displays various pharmacological effects, including myocardial protection (Wang et al., 2011), anti-inflammation (Tao et al., 2015), and anti-epithelial-mesenchymal transition (Liu et al., 2016). In our previous study, we identified six active compounds of TMYX with dose-dependent anti-inflammatory properties by high-resolution mass spectrometry coupled with chemometric methods (Tao et al., 2015). Clinical studies suggested that TMYX can also prevent the process of heart failure, and is effective in treating premature ventricular contraction (Ke et al., 2010; Wen-Ping et al., 2014; Peng et al., 2016). However, whether TMYX has a therapeutic effect in cardiac hypertrophy is still unclear.

In the present study, we investigated the effects and functional mechanism of TMYX in cardiac hypertrophy. The anti-hypertrophy effects of TMYX were firstly evaluated in the isoproterenol (ISO) treated cardiac hypertrophy model in mice. Next, a rapid and reliable High-Content Screening (HCS) assay was applied to screen the anti-hypertrophy active compounds from TMYX, using angiotensin II (Ang II) induced hypertrophic H9c2 cardiac cells. As a result, eight compounds were identified by LC-MS, and the anti-hypertrophy effects of four compounds were subsequently validated. Through identifying anti-hypertrophy compounds from TMYX, it is of great interest to identify licoisoflavone A as a Sirt3 activator, which was further validated on phenylephrine (PE)-induced cardiac hypertrophy model.

Materials and methods

Reagents and chemicals

Tongmaiyangxin Pills (TMYX, batch number A107650) were obtained from LeRenTang pharmaceutical factory (Tianjin, China). Angiotensin II (Ang II) was purchased from Aladdin. Isoprenaline hydrochloride (ISO) and Phenylephrine (PE) was ordered from Sigma. The selective Sirt3 inhibitor 3-(1H-1,2,3-triazol-4-yl)pyridine (3-TYP; CAS: 120,241-79-4) was purchased from MedChemExpress. Glycycoumarin, Licoisoflavone A, Licoricone, Glycyrol, Glyasperin A, Licorisoflavan A were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Glycyrrhizic acid and Emodin were purchased from Shanghai Winherb Medical Technology Co., Ltd. Captopril was obtained from Meilunbio. The purity of all the compounds investigated were more than 98%. Sirt3 (D22A3) Rabbit mAb and Alexa Fluor 488 Phalloidin were purchased from Cell Signaling Technology. Anti-SOD2/MnSOD (acetyl K68) antibody was obtained from Abcam. Sirt3 Antibody Rabbit Polyclonal (10,099-1-AP) was purchased from Proteintech. Deionized water was prepared with an Elga PURELABflex system (ELGA LabWater, UK).

Animals

Eight-week-old male C57BL/6 mice weighing 20 g ± 5 g were purchased from Shanghai Slac Laboratory Animal Technology Co., China. Experiments involving live animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised1996) and were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Isoproterenol (ISO)-induced cardiac hypertrophy model in mice

Eight-week-old male C57BL/6 mice were randomly divided into the ISO group and the vehicle (saline) group, and received 5 mg/kg/day ISO or saline for 14 days by subcutaneous injection. According to the results of transthoracic echocardiography, those mice with myocardial hypertrophy or cardiac failure were further divided into the model group and the treatment group. TMYX extracts were mixed with 0.5% CMC−Na solution into suspensions, by gavage at a dose of 2 g/kg/day for 14 days in the treatment group, and the heart function was evaluated by transthoracic echocardiography. Subsequently, the animals were sacrificed and the hearts were collected for further examination.

Hemodynamics assay

Mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg). The right common carotid artery was isolated and a 1.4F Mikro-Cath™ diagnostic pressure catheter (Millar, USA) was inserted into the left ventricle through the artery. The left ventricular systolic pressure (LVSP) and the max rate of rise of left ventricular pressure (± dp/dt) were recorded using a Power Lab 8 data collecting system (ADInstruments Co., Australia).

Histological examination and immunohistochemistry (IHC) analysis

Cardiac structure was analyzed by hematoxylin-eosin staining of heart sections. Fibrosis was detected with Masson's trichrome staining kit and the experimental procedure was performed according to the manufacturer's protocol. All sections were observed under an inverted microscope. The cross-sectional area of cardiac myocytes was measured in transverse sections of the left ventricle after staining of the plasma membrane with rhodamine-labeled wheat germ agglutinin (WGA). IHC assay for Sirt3 was performed on 4 μm formalin-fixed, paraffin-embedded heart sections of TAC surgery specimens, with Sirt3 antibody (CST, 10,099-1-AP)(1:100) staining prior to hematoxylin counterstaining. For each slice, 5 high-power fields were randomly selected for analysis.

Cell culture and high-content screening assay

The rat cardiomyocyte cell line H9c2 was obtained from Nanjing Beretti Biological Technology Co., Ltd and cultured in high glucose DMEM with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were seeded in 96-well plates at a density of 2000 cells for 1 day. Then, the medium was replaced with fresh serum-free medium with samples and Ang II (100 nM) alone or in combination for another 2 days.

Primary cultures of cardiac myocytes were prepared from neonatal rat hearts. In brief, hearts were removed from 1- to 3-day-old pups (Sprague-Dawley rats, either sex) and kept in cold PBS. Ventricles were cut into 1 mm^3 pieces and digested using Neonatal Heart Dissociation Kit (MACS, Germany). The digested solution was collected with the cannula-syringe avoiding to avoid tissue chunks and mixed with 10 ml FBS (100%). These steps were repeated three times till no tissue chunks

are visible. Tissue digest was spun and the pellet was dissolved in DMEM with 10% FBS. Cells were pre-plated for 0.5 h to remove fibroblasts, and unattached cardiomyocytes in suspension were collected and plated in fibronectin-coated culture plates. Cardiomyocytes cultures were used after 24 h of plating.

Cell monolayers were washed with PBS to remove dead cells prior to immobilization with cold 4% paraformaldehyde. After fixing for 30 min, the cells were permeabilized with 0.1% Triton X-100 for 10 min, and then incubated with a mixture of Alexa Fluor 488 Phalloidin (1:20) and Hoechst (1:1000) at room temperature for 10 min. In Figures 2, 3 and 5, the stained samples were then subjected to Leica DMI 3000 B, using a 63 × objective. Images were analyzed for cell size using Image Pro Plus 6.0 software (Media Cybernetics).In Fig. 6, images were acquired using an ImageXpress Micro® Confocal High-Content Imaging System (Molecular Devices), with a 40 × Plan Fluor objective. Images were analyzed using the analysis module of MetaXpress® High-Content Image and Analysis Software (Molecular Devices). Then, Count Nuclei, Cell Scoring application modules were used for cell count and average cell area assessment.

Preparation of TMYX solutions

The process of fraction preparation of TMYX was described in our previous publication (Liu et al., 2016). Briefly, Tongmaiyangxin Pill was obtained from LeRenTang pharmaceutical factory (Tianjin, China). It is composed of eleven Chinese medicinal herbs: Radix Rehmanniae, Caulis Spatholobi, Radix Glycyrrhizae, Ramulus Cinnamomi, Radix Ophiopogonis, Radix Polygoni multiflori preparata, Asini Corii colla, Fructus Schisandrae, Radix Codonopsis, Capapax et Plastrum Testudinis, and Fructus Jujubae. The preparation process of TMYX Pills was in accordance with the national standard preparation method (Ministry of Health of China, 2015).

Screening of anti-hypertrophy fractions from TMYX

To prepare fractions for further bio-assays and LC-MS analysis, we used preparative chromatography to isolate TMYX pills extracts. Briefly, TMYX Pills (50 g) was finely grounded using a mortar and homogenized in methanol (500 ml), then extracted with a sonicator (KQ-250B, Kunshan ultrasonic instrument co., LTD) for two times (50 min for each time). The extracts were filtered using a Büchner funnel and removed under reduced pressure with a rotary evaporator to 20 ml. The concentrates were centrifuged for 5 min at 6000 × g. Finally, the supernatant was filtered through 0.22 μm for further experiments.

Separation experiments comprised of the following steps: firstly, the extracts of TMYX Pills were loaded onto an ODS column (YMC Co., Ltd. Tokyo, Japan), the column was rinsed with H_2O firstly; and then eluted with methanol. Methanol elution portion was subjected to preparative HPLC to obtain fractions. The preparation of fractions was performed on a 1200 series LC system (Agilent, Palo Alto, CA, USA) with Zorbax SB-C18 column (21.2 × 250 mm, 7 μm, Agilent Technologies, MD, USA). The mobile phase consisted of 0.1% aqueous formic acid (phase A) and acetonitrile (phase B) at a flow rate of 10 ml/min. The elute gradient was as follows: 0–60 min, 5% − 90% B; 60–72 min, 90% − 95% B; 72–82 min, 95% B. Fractions were collected every three minutes, from the start to the seventy-two minute, named TM1-TM24 in sequence. Except for three fractions (TM1, TM19, TM24) with rare amounts, All the fractions were concentrated to less than 5 ml under reduced pressure and freeze-dried. For the evaluation of anti-hypertrophy activity, the fractions were chosen and dissolved in DMSO to a concentration of 50 mg/ml.

Compounds analysis by LC-MS

The identification of the fractions of TMYX was reported in previous work (Liu et al., 2016). The constituents of active fractions from TMYX were characterized by LC-MS. Chromatography was carried out on an Agilent ZORBAX SB-C18 column, the mobile phase was 0.05% formic acid-water (A) and acetonitrile (B). The gradient procedure is as follows: 0–20 min, 10% − 30% B; 20–40 min, 30% − 35% B; 40–60 min, 35% − 50% B; 60–80 min, 50% − 80% B; 80–90 min, 80% B; 90–91 min, 80% − 95% B; 91–95 min, 95% B. The parameters of the mass spectrometry were as follows: sheath gas flow, 18 l/min; auxiliary gas flow, 6 l/min; capillary temperature, 350 °C. The parameters of the negative mode were ion source, 3 kV; source current, 80 μA; capillary voltage, − 15 V; tube lens offset voltage, − 30 V. The parameters of the positive mode were ion source, 4 kV; source current, 80 μA; tube lens offset voltage, 25 V; capillary voltage, 19 V.

The accurate mass measurement was determined by AB SciexTripleTOF™5600$^+$ (AB Sciex Pte. Ltd., USA). The chromatography conditions were the same as the LC-MS analysis described above. The operating parameters were as follows: gas pressure, 25 psi; nebulizer pressure, 60 psi; auxiliary gas pressure, 60 psi; ion spray voltage, − 4.5 kV; declustering potential, 100 V; source temperature, 100 V.

Western blot

To investigate the effect of licoisoflavone A on Sirt3 protein expression, neonatal rat cardiomyocytes were treated for 24 h in serum-free DMEM, and were then exposed to licorisoflavan A (15 μM) in the presence of phenylephrine (PE) (100 μM) for another 48 hr. Cardiomyocytes were scraped and lysed in cell lysis buffer containing RAPI and PMSF. Antibodies Sirt3, MnSOD (Cell Signaling, 1:1000) and MnSOD (acetyl K68) (Abcam, 1:500) were used for western blot. The bolts were visualized by ECL chemiluminescence reagent.

Quantitative real-time PCR analysis

Total RNA from each frozen heart was isolated using QIAGEN RNeasy Mini Kit according to the manufacturer's instructions. The total RNA concentration was determined using a NanoDrop 2000 spectrophotometer. Real-time reverse transcriptase PCR was performed using 1 μg of total RNA for the QuantiTect Reverse Transcription Kit and QuantiFast SYBR Green PCR Kit with primers. The expression of GAPDH was determined as an internal control. The cycling protocol need to hatch at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Relative target gene mRNA expression levels was normalized to GAPDH.

Primer sequences of genes used for RT-PCR analysis are given in Table 1.

Statistical analysis

The data are expressed as mean ± SEM. Parameter comparisons between groups were made with one-way ANOVA analysis of variance. GraphPad prism 7 software (GraphPad Software, USA) was used to carry out statistical analysis. $p < 0.05$ was considered statistically significant.

Table 1
Sequences of primers used for RT-RCR analysis.

Primer Name	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
ANF	GGGAAGTCAACCCGTCTCA	GGCTCCAATCCTGTCAATCC
BNP	AAGCTGCTGGAGCTGATAAGA	GTTACAGCCCAAACGACTGAC
GAPDH	TCCACCACCCTGTTGCTGTAGC	TGGAAAGCTGTGGCGTGATG

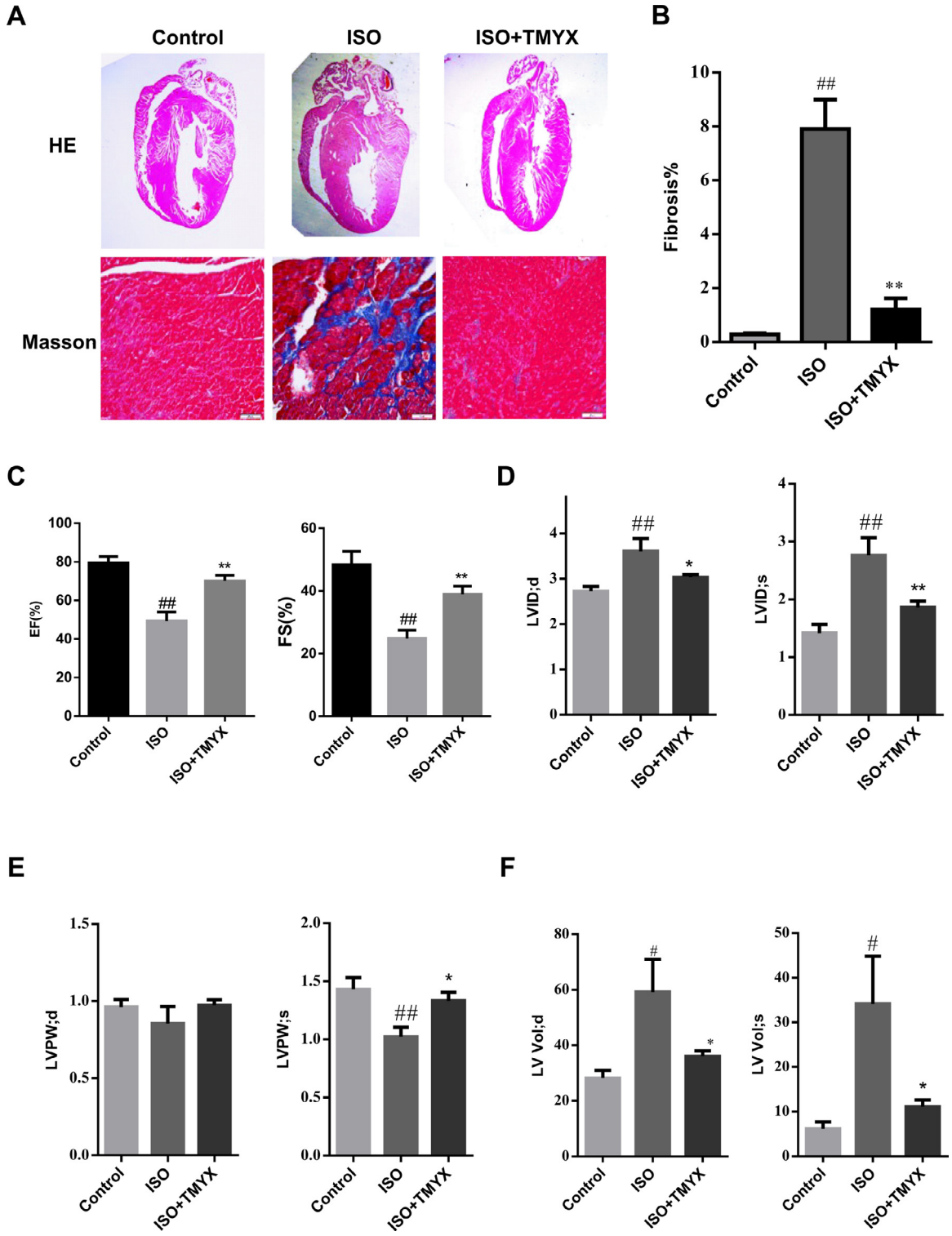

Fig. 1. TMYX attenuates pre-established heart failure in mice. (A) H.E-stained sections of whole hearts of different groups of mice (6.5 ×) and the sections of hearts stained with Masson's trichrome to detect fibrosis (400 ×). (B) Quantification of cardiac fibrosis in different groups of mice, $n = 4$. (C, left) Echocardiographic measurements of ejection fraction, (C, right) fractional shortening, (D) left ventricular diameter, (E) left ventricular posterior wall thickness and (F) left ventricular volume in control, ISO, ISO treated with TMYX, $n = 9–10$ (d: end diastolic, s: end systolic).

Results and discussion

TMYX rescued cardiac fibrosis and heart failure in ISO-treated mice model

The heart hypertrophy mice model was established by a 2-week ISO stimulation, and was then treated with TMYX for the following 2 weeks. Masson's trichrome staining results showed that the level of cardiac fibrosis was increased for nearly 7-fold after ISO treated, compared with the control mice. In contrast, TMYX treatment improved the heart structure and markedly reduced the accumulation of interstitial fibrosis (Fig. 1A and B). We next examined the indices of heart function in the three groups. As anticipated, both the levels of ejection fraction and

fractional shortening were largely reduced in the ISO model group. Moreover, the diameter and the volume of left ventricle were increased in the ISO model group, accompanied with decreased left ventricular posterior wall thickness by the end of the systolic stage, indicating the development of heart failure in mice after ISO stimulation. However, TMYX treatment effectively reversed all of the above pathological alterations caused by ISO and almost completely restored the heart function (Fig. 1C–F).

TMYX inhibits cardiac hypertrophy in ANG II stimulated cardiomyocytes

Cardiac hypertrophy is characterized by an increase in the size of cardiomyocytes. HCS has become an efficient and reliable technique which measures complex phenotypic outcomes (Singh et al., 2014), by integrating the investigation approach of cell biology with image acquisition, processing, and analysis. Thus, we applied an HCS assay to evaluate the degree of cardiac hypertrophy in Ang II-stimulated H9c2 cardiomyocytes model, with or without TMYX treatment. Angiotensin II (Ang II) plays an important role in the pathogenesis and progression of cardiac hypertrophy and heart failure, and is widely used as a model drug to induce cardiac hypertrophy (Sadoshima et al., 1993). Markedly, the diameter and length of cardiomyocytes were significantly increased after stimulated by Ang II, and the average cross section area of cardiomyocytes was also increased by about 50%, compared with the control group. Nevertheless, TMYX reduced the cellular size of Ang II cardiomyocytes in a dose-dependent manner ($p < 0.01$). The anti-hypertrophy effect of TMYX at 50 μg/ml was even better than that of Captopril (Romankiewicz et al., 1983), which is an angiotensin converting-enzyme inhibitor used to treat congestive heart failure (Fig. 2Aand B). No significant difference was detected for the cross-section area of cardiomyocytes among the control group, captopril treated group and the TMYX treated group (Fig. S1).

Screening active compounds with anti-hypertrophy effects by HCS combined with LC-MS

We next combined the HCS assay with the LC-MS method to screen the anti-hypertrophy activity fractions in TMYX. 21 fractions with sufficient quantities were screened by HCS. As a result, five active fractions were identified in the screening (Fig. 3B), and the representative fluorescent images of active fractions were shown in Fig. 3A. Afterwards, the chemical constituents in the active ingredients were determined by the LC-MS analysis (Fig. 4). We focused on those compounds that are shared by two or more active fractions, assuming that they are more likely to possess the anti-hypertrophy effect. The identified compounds from active ingredients were shown in Table 2. By this approach, eight potentially active compounds were selected for further research, including glycyrrhizic acid (25 μM), glycycoumarin, licoisoflavone A (5 μM) , licoricone (5 μM) , glycyrol (1 μM) , glyasperin A (5 μM) , licorisoflavan A (5 μM) and emodin (5 μM) .

To confirm whether the eight compounds have anti-hypertrophy effect, HCS assay was applied to compare the changes in the size of cardiomyocytes between the model group and the groups treated with different compounds. Notably, glyasperin A, emodin, licoricone, and licoisoflavone A were able to significantly decrease the size of Ang II stimulated cardiomyocytes, by about 40%, 50%, 30%, and 46% respectively (Fig. 5A and B). Among them, emodin and licoisoflavone A are the two compounds with most significant effects. The mechanism of emodin will be investigated in our further work, and here we continued to investigate the mechanism of licoisoflavone A.

Licoisoflavone A-mediated up-regulation of Sirt3 blocks the cardiac hypertrophic response

Previous studies suggested that Sirt3 is an important member in the Sirtuins family and has been indicated as a positive regulator of cardiac hypertrophy (Sundaresan et al., 2009). Consistently, in the ISO-stimulated mice model, we found that the expression of Sirt3 was significantly down-regulated in the ISO group, but was greatly up-regulated after TMYX treatment (Fig. 6A and B).

Therefore, we asked whether licoisoflavone A protects cardiomyocytes from developing hypertrophy through regulating Sirt3. In order to test this, cardiomyocytes were treated with a hypertrophic agonist phenylephrine (PE), with or without the presence of licoisoflavone A (Fig. S2). After 48 h of treatment, cells were harvested and mitochondrial acetylation was analyzed by western blotting. PE-treated cardiomyocytes showed downregulated Sirt3 expression, which was increased in licoisoflavone A-treated cardiomyocytes (Fig. 6C and D). To test whether increased Sirt3 expression were associated with its increased activity, we analyzed the acetylation status of the Sirt3 substrates, MnSOD, using antibodies which specifically detect MnSOD acetylation at K-68. Consistently, we observed reduced acetylation of MnSOD following licoisoflavone A treatment, suggesting increased activity of Sirt3 (Fig. 6C and E).

Finally, we inquired whether the regulatory effect of licoisoflavone A on Sirt3 activity is required for its anti-hypertrophy effect. Increased expression of hypertrophic indicators (ANF and BNP) and cellular size was detected after PE administration, which were significantly inhibited by licoisoflavone A treatment (Fig. 6F–I). Importantly, co-treatment of licoisoflavone A with the selective Sirt3 inhibitor 3-TYP almost completely blocked the protective effects of licoisoflavone A on cardiac hypertrophy (Fig. 6F–I). Taken together, the above evidence

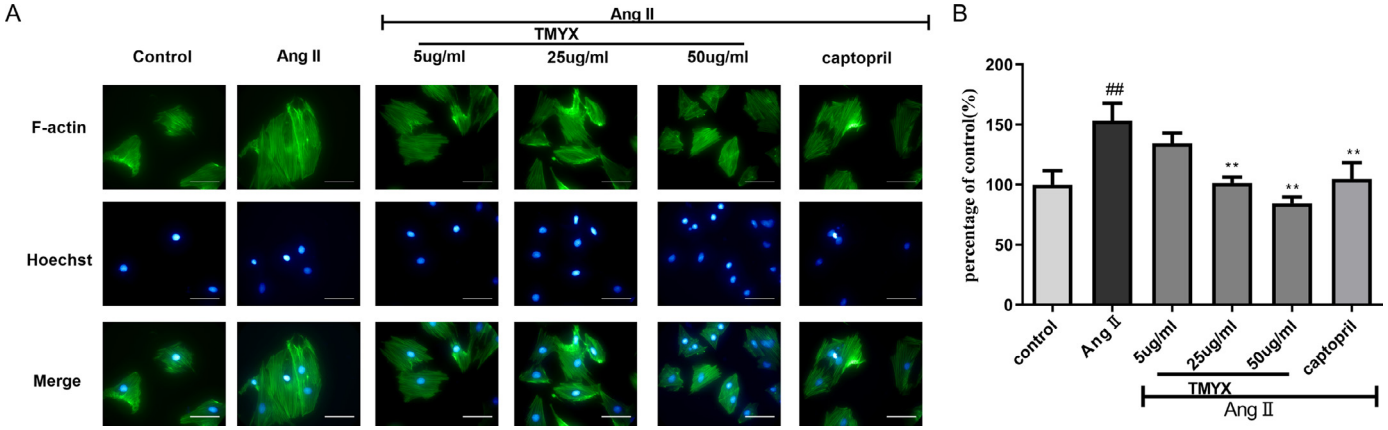

Fig. 2. The anti-hypertrophy effect of TMYX. (A) The representative fluorescent images of cardiomyocyte with different treatment (green: phalloidin, blue DAPI), scale bar = 100 μm. (B) The relative area corrected by nucleus and normalized to control., $n = 12$. Captopril as the positive drug. $^{\#\#}p < 0.01$ *vs.* Control, $^{**}p < 0.01$ *vs.* Ang II. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

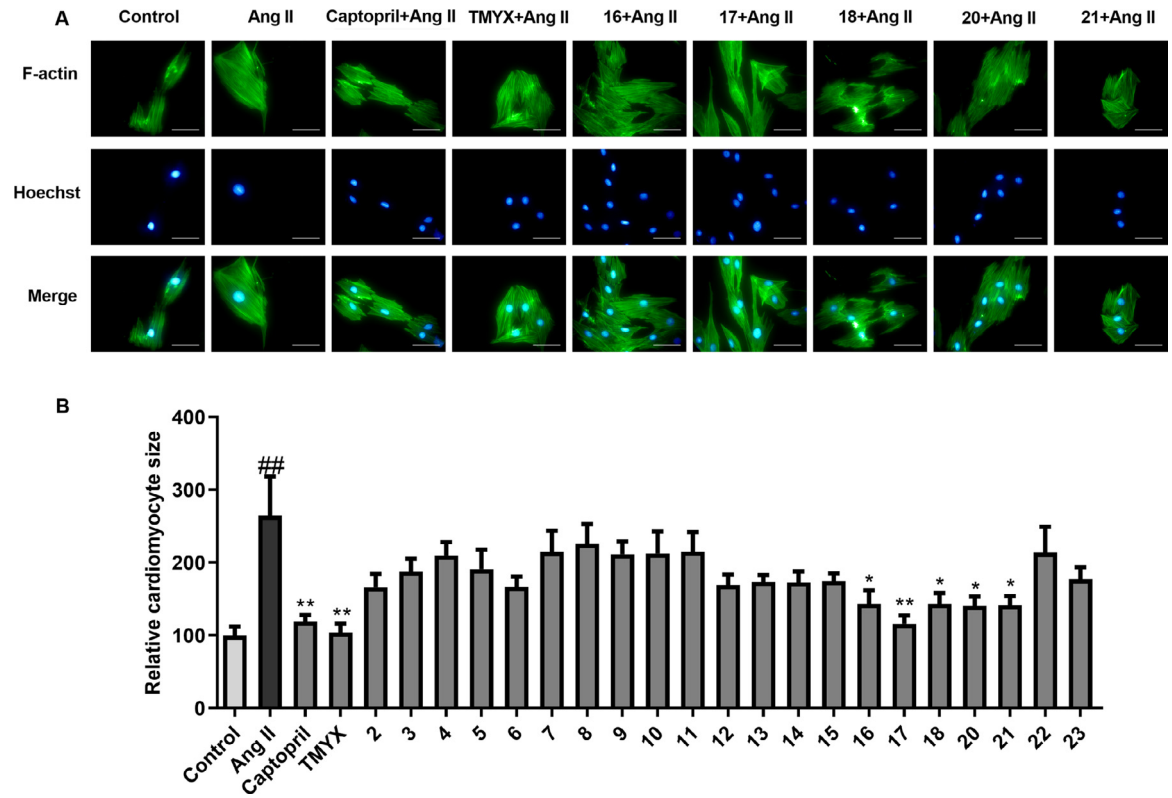

Fig. 3. Screening active fraction of anti-hypertrophy from TMYX. (A) The representative fluorescent image of active fractions. scale bar = 100 μm. (B) The relative area corrected by nucleus, the numbers of 2–18 and 20–23 represent the fractions of TMYX, $##p < 0.01$ *vs.* Control, $**p < 0.01$ *vs.* Ang II, $n = 12$.

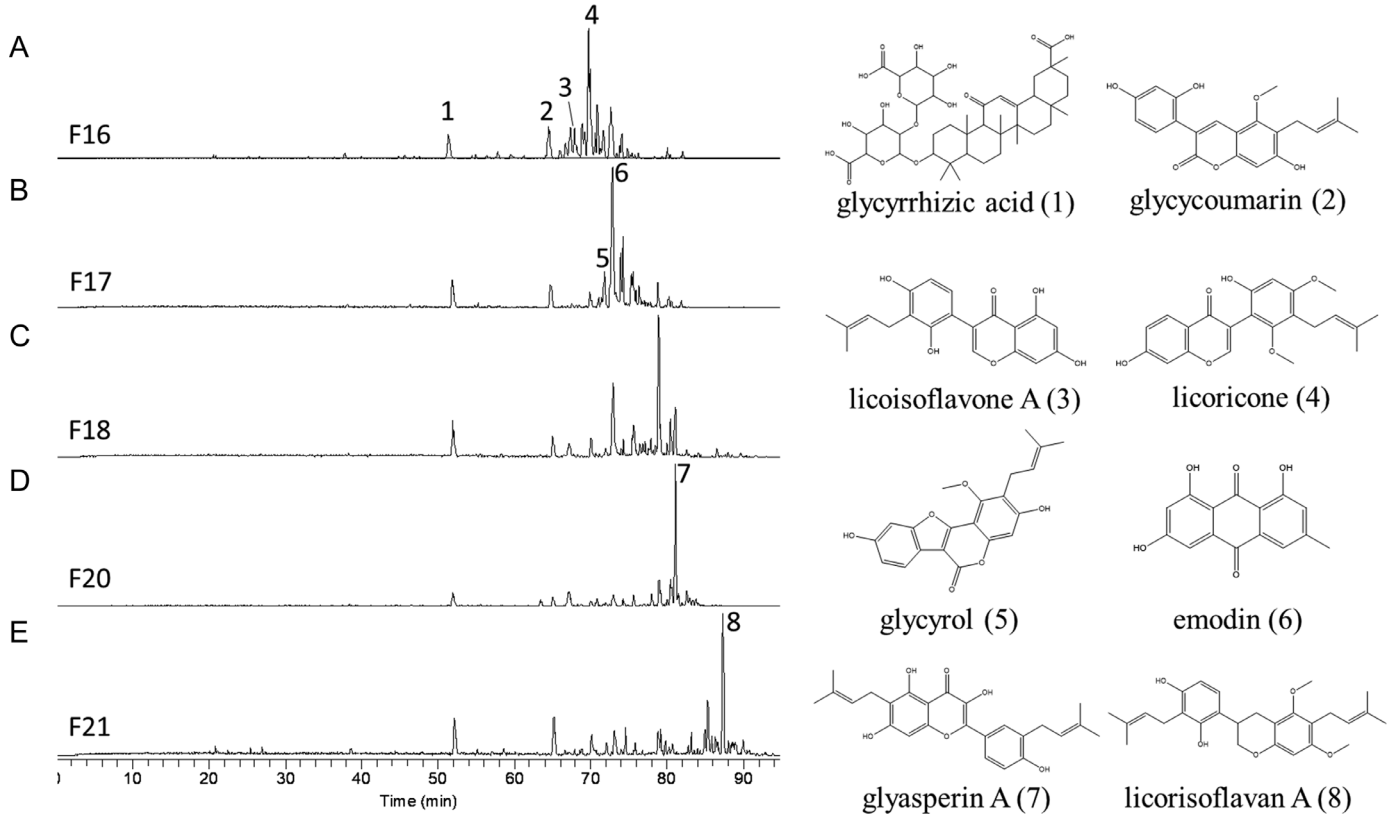

Fig. 4. The chromatogram of fraction 16 (A), 17 (B), 18 (C), 20 (D), 21 (E) from TMYX. Eight compounds were speculated respectively.

Table 2
The identification of compounds from active fractions of TMYX.

Peak no.	tR (min)	Molecular Formula	$[M-H]^-/[M+H]^+$ Detected	Error (ppm)	Identification	Source
1	51.26	$C_{42}H_{62}O_{16}$	821.3956	0.3	Glycyrrhizic acid	*Glycyrrhiza uralensis Fisch*
2	63.83	$C_{21}H_{20}O_6$	367.118	0.9	Glycycoumarin	*Glycyrrhiza uralensis Fisch*
3	69.42	$C_{20}H_{18}O_6$	353.1043	3.5	Licoisoflavone A	*Glycyrrhiza uralensis Fisch*
4	71.05	$C_{22}H_{22}O_6$	381.1356	3.2	Licoricone	*Glycyrrhiza uralensis Fisch*
5	72.53	$C_{15}H_{10}O_5$	269.0452	2.6	Emodin	*Fallopia multiflora (Thunb.) Harald*
6	72.9	$C_{21}H_{18}O_6$	365.1043	3.4	Glycyrol	*Glycyrrhiza uralensis Fisch*
7	81.07	$C_{25}H_{26}O_6$	421.1639	−1.6	Glyasperin A	*Glycyrrhiza uralensis Fisch*
8	87.27	$C_{27}H_{34}O_5$	437.2321	−0.4	Licorisoflavan A	*Glycyrrhiza uralensis Fisch*

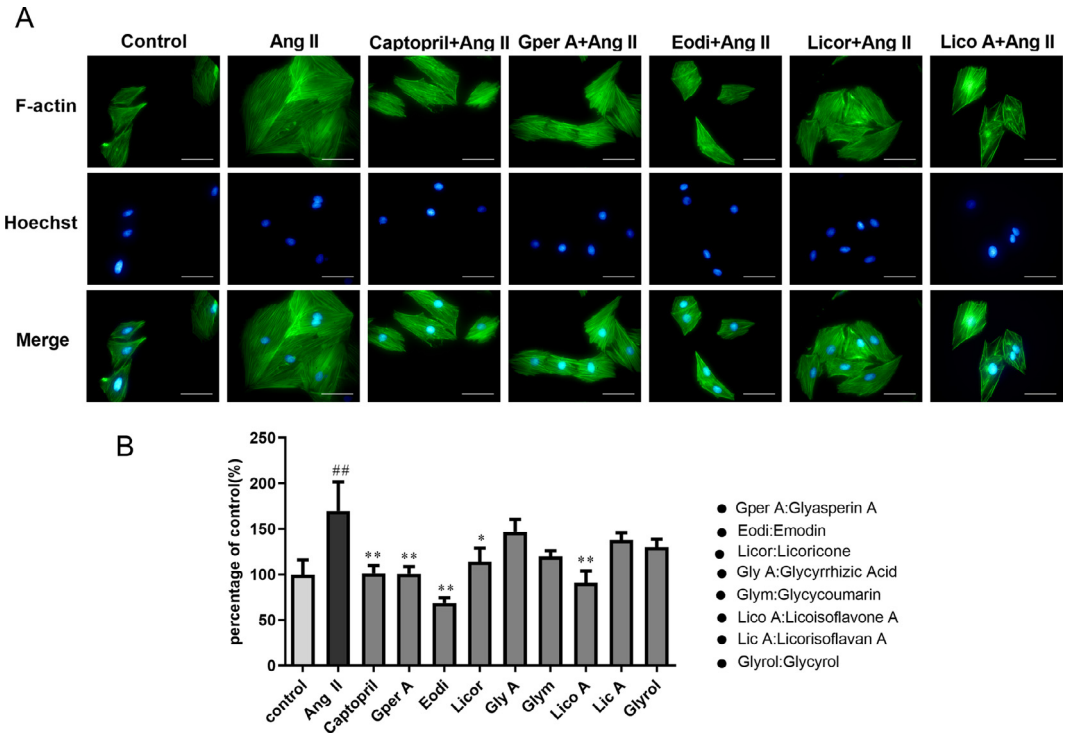

Fig. 5. Screening active compounds of anti-hypertrophy from TMYX. (A) The representative fluorescent images of active components, scale bar = 100 μm. (B) The relative area of different groups corrected by nucleus and normalized to control, $n = 12$, repeat 3 times. $^{\#\#}p < 0.01$ *vs.* Control, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* Ang II.

indicates that licoisoflavone A exerts anti-hypertrophy effect through up-regulating the levels of Sirt3.

Discussion

In the present study, we report that TMYX can inhibit the development of cardiac hypertrophy and heart failure, as well as up-regulating the expression of the anti-hypertrophic protein Sirt3. Cardiac hypertrophy is a prominent pathological feature leading to heart failure. Sirt3 is a member of the Sirtuins family, which is located in the mitochondrial matrix (Romankiewicz et al., 1983) and determines mitochondrial deacetylase activity (Michishita et al., 2005). Sirt3 is a primary deacetylase involved in the deacetylation of acetylated mitochondrial proteins, which take more than 65% of the total mitochondrial proteins, and thus is essential for physiologic function of these proteins (Hebert et al., 2013). Increasing evidence indicated Sirt3 as a key regulator of cardiac hypertrophy. Deficiency of Sirt3 in mice led to the development of hypertrophy, while the overexpression of Sirt3 inhibited the hypertrophic response (Sundaresan et al., 2009). The mechanism towards Sirt3's role in cardiac hypertrophy may involve reduced ROS production (Lang, 2002) or increased autophagy (Li et al., 2016). Supportively, our results also observed a reduction of Sirt3 expression in the hypertrophic mice and cell line. Importantly, both TMYX and licoisoflavone A were able to significantly increase the level of Sirt3, indicating that the anti-hypertrophy effects of these drugs may work through mediating Sirt3 expression.

High-Content Screening (HCS) is a technique based on automated microscopy which enables the measurement of various biomarkers in a single cell or cell organelle, with different fluorescence signals to reflect cellular responses to compounds treatment (Gasparri et al., 2007; Fraietta and Gasparri, 2016). For example, HCS technology is capable to simultaneously measure the nucleus, mitochondrion (Beeson et al., 2010), cytoskeleton, and even the alteration of calcium levels within cells (Kim et al., 2012). Amongst its various applications, HCS greatly facilitates the process of drug screening, especially the screening for effective components in Tradition Chinese Medicine (TCM) or herb medicine, which shows multi-aspect effects through multi-target mechanisms. One of our previous studies has applied the HCS technique to identify inhibitors of epithelial-mesenchymal transition in HK-2 cells (Liu et al., 2016).

Through fraction extraction, compounds analysis and biological assays, we screened out licoisoflavone A, licoricone, licorisoflavan A and emodin from TMYX solution (Fig. S3. and Table S1), which showed anti-hypertrophy effects. Among them, licoisoflavone A, licoricone, licorisoflavan A are derived from Glycyrrhiza uralensis Fisch, and emodin is from Polygonum multiflorum Thunb. Licoisoflavone A,

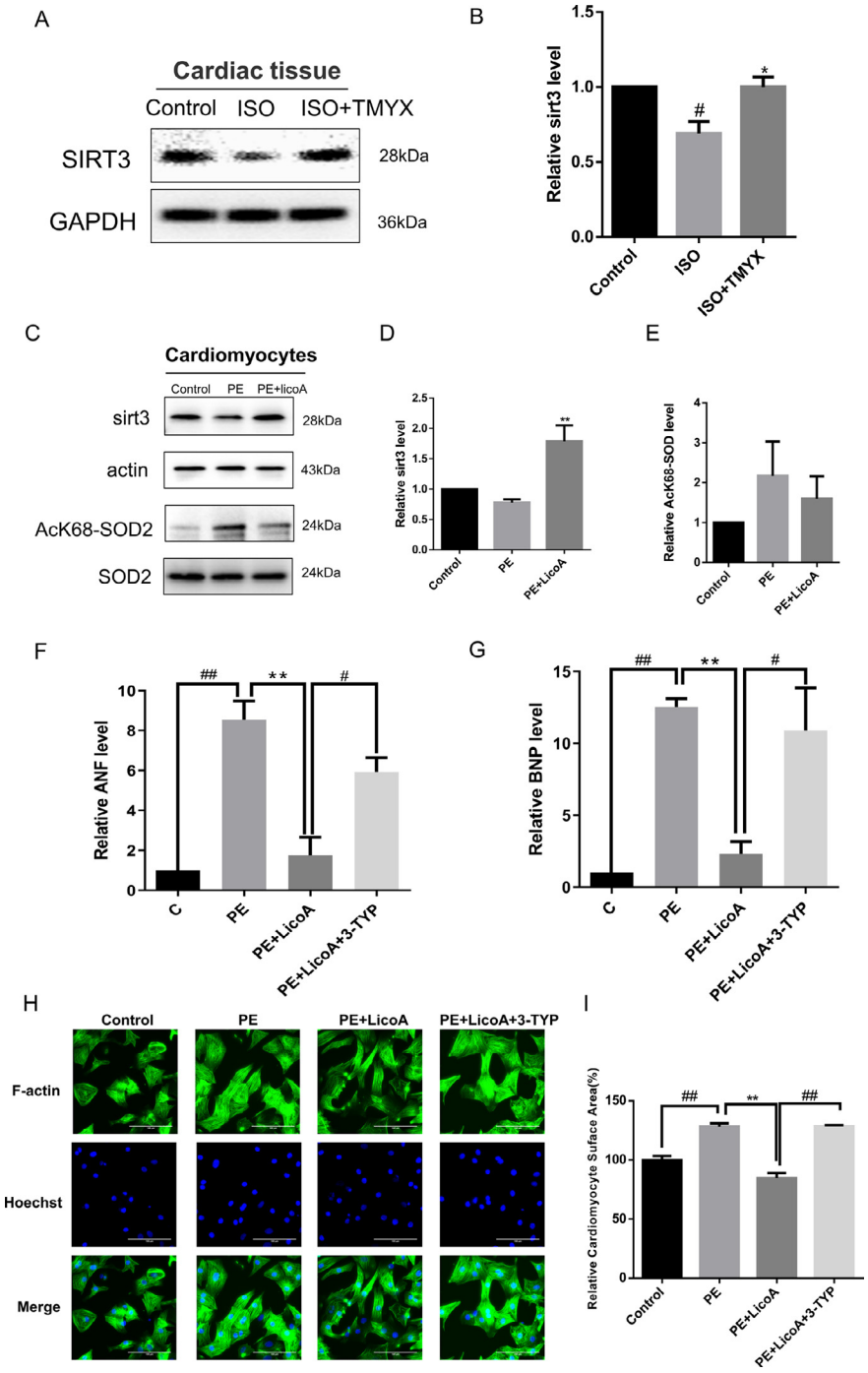

Fig. 6. Western-Blot of Sirt3 protein (A) and relative expression (B) in the heart samples of control, ISO and ISO with TMYX. $^{\#}p < 0.05$, *vs.* Control, $^{*}p < 0.05$ *vs.* ISO. (C–E) represent licoisoflavone A blocks the cardiac hypertrophic response by upregulating Sirt3, SOD2 and AcK68-SOD2 expression with licoisoflavone A treated in the model of hypertrophy. $^{**}p < 0.05$ *vs.* PE (F–G) Quantification of relative ANF and BNP levels in cardiomyocytes treated with Licoisoflavone A in the model of hypertrophy with or without 3-TYP. $^{\#\#}p < 0.01$ PE *vs.* Control, $^{**}p < 0.01$ PE + LicoA *vs.* PE. $^{\#}p < 0.01$ PE + LicoA + 3-TYP *vs.* PE + LicoA (H–I). The representative fluorescent images of Licoisoflavone A. scale bar = = 100 μm $^{\#\#}p < 0.01$ PE *vs.* Control, $^{**}p < 0.01$ PE + LicoA *vs.* PE. $^{\#\#}p < 0.01$ PE + LicoA + 3-TYP *vs.* PE + LicoA.

licoricone, licorisoflavan A belong to flavonoids, which are the main chemical compositions of Glycyrrhiza (Qingying and Min, 2009). It has reported that they had protection effects on hypoxia (Li et al., 2016) and toxidation (Shan et al., 2015). Licoisoflavone A was also reported to have antibacterial and gastroprotective activities (Quesada et al., 2012), but its role in anti-hypertrophy or SIRT3 activation has not been reported. To the best of our knowledge, licoisoflavone A is the third naturally derived SIRT3 activator after honokiol (Pillai et al., 2015) and sesamin (Fan et al., 2017).

A couple of drugs were used in constructing the heart hypertrophy model. In high content screening, we selected Ang II as a model drug to induce cardiac hypertrophy. Ang II plays a vital role in the pathogenesis and progression of cardiac hypertrophy and heart failure, which is characterized by fibrosis, myocyte hypertrophy, cardiac myocyte death, and alterations in metabolism. At present, the mechanism of Ang II-induce-cardiac hypertrophy has been researched relatively clearly. The renin-angiotensin-aldosterone system (RAAS) was involved in cardiac hypertrophy, and drugs were directly inhibiting RAAS in the hypertrophic myocardium and improved cardiac function (Parodi-Rullan et al., 2012). PE (phenylephrine), as another important hypertrophic growth factors for the heart, activated different pathways to induce hypertrophic responses. In the PE-induced- cardiac hypertrophy responses, studies showed that Sirt3 overexpression shuts down most of the signaling pathways leading to hypertrophy by controlling ROS accumulation in the heart (Sundaresan et al., 2009). The mitochondrial ETC is the main source of ROS, and Sirt3 enhances the ability of the mitochondria to cope with ROS in multiple ways. The key superoxide scavenger, Mn superoxide dismutase (SOD2), can reduce superoxide

production and protect against oxidative stress. Sirt3 directly regulates the activity of SOD2 by deacetylation (Qiu et al., 2010; Tao et al., 2010; Dikalova et al., 2017; Xie et al., 2017) . Moreover, Sirt3 can sequester forkhead box O3a(FOXO3A) in the nucleus to increase the transcription of SOD2 and other key genes involved in antioxidation (Sundaresan et al., 2008). In the ISO-stimulated mice model, we found that the expression of Sirt3 was significantly down-regulated in the ISO group compared with control group, but was greatly up-regulated after TMYX treatment. Therefore, we studied the anti-cardiac hypertrophy mechanism of licoisoflavone A in the PE-induced- cardiac hypertrophy. In this study, we characterized hypertrophy of cardiomyocytes by monitoring cell size, protein expression, expression of ANF and BNP. Our data indicate that licoisoflavone A blocks the cardiac hypertrophic response by upregulating Sirt3, but the mechanisms of other compounds remain unclear and worth forward study.

Conclusion

In the present study, we demonstrated that the anti-cardiac hypertrophy effect of TMYX, and its major active component licoisoflavone A, possibly through upregulating the expression of Sirt3. Our findings proved that high content screening combined with pharmacological validation is a feasible and efficient way to identify novel lead compounds from natural products.

CRediT authorship contribution statement

Rui Guo: Data curation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Ningning Liu:** Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Hao Liu:** Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Junhua Zhang:** Conceptualization, Supervision, Writing - review & editing. **Han Zhang:** Investigation, Writing - original draft, Writing - review & editing. **Yingchao Wang:** Data curation, Writing - review & editing. **Mirko Baruscotti:** Conceptualization, Formal analysis, Writing - review & editing. **Lu Zhao:** Supervision, Writing - review & editing. **Yi Wang:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2020.153171.

References

Beeson, C.C., Beeson, G.C., Schnellmann, R.G., 2010. A high-throughput respirometric assay for mitochondrial biogenesis and toxicity. Anal. Biochem. 404 (1), 75–81.
Cohn, J.N., Ferrari, R., Sharpe, N., 2000. Cardiac remodeling–concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an international forum on cardiac remodeling. J. Am. Coll. Cardiol. 35 (3), 569–582.
Dikalova, A.E., Itani, H.A., Nazarewicz, R.R., McMaster, W.G., Flynn, C.R., Uzhachenko, R., et al., 2017. "Sirt3 impairment and SOD2 hyperacetylation in vascular oxidative stress and hypertension. Circ. Res. 121 (5), 564–574.
Eiki, T., Kass, D.A., 2007. Role of oxidative stress in cardiac hypertrophy and remodeling. Hypertension 49 (2), 241.
Fan, D., Yang, Z., Liu, F.Y., Jin, Y.G., Zhang, N., Ni, J., et al., 2017. Sesamin protects against cardiac remodeling via sirt3/ROS pathway. Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. 44 (6), 2212.
Fraietta, I., Gasparri, F., 2016. The development of high-content screening (HCS) technology and its importance to drug discovery. Expert Opin. Drug Discov. 11 (5), 501–514.
Frey, N., Olson, E.N., 2003. "Cardiac hypertrophy: the good, the bad, and the ugly. Annu. Rev. Physiol. 65, 45–79.
Gasparri, F., Ciavolella, A., Galvani, A., et al., 2007. Cell-cycle inhibitor profiling by high-content analysis. Adv. Exp. Med. Biol. 604 (604), 137.
Hebert, A.S., Dittenhafer-Reed, K.E., Yu, W., Bailey, D.J., Selen, E.S., Boersma, M.D., et al., 2013. Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. Mol. Cell 49 (1), 186–199.
Hill, J.A., Olson, E.N., 2008. Cardiac plasticity. N. Engl. J. Med. 358 (13), 1370–1380.
Ke, L., Sun, L., Gao, K.J., 2010. Tongmaiyangxin pill for treatment of ventricular premature in coronary heart disease with deficiency syndrome of both qi and yin: a multi-center clinical trial. Chin. J. Integr. Med. Cardio Cerebrovasc. Dis. 8 (4), 401.
Kim, J.A., Han, E., Eun, C.J., Yu, K.T., Song, J., et al., 2012. Real-time concurrent monitoring of apoptosis, cytosolic calcium, and mitochondria permeability transition for hypermulticolor high-content screening of drug-induced mitochondrial dysfunction-mediated hepatotoxicity. Toxicol. Lett. 214 (2), 175–181.
Lang, D., 2002. Cardiac hypertrophy and oxidative stress: a leap of faith or stark reality? Heart 87 (4), 316.
Li, J., Chen, T., Xiao, M., Li, N., Wang, S., Su, H., et al., 2016. Mouse Sirt3 promotes autophagy in AngII-induced myocardial hypertrophy through the deacetylation of FoxO1. Oncotarget 7 (52), 86648.
Li, L., Liu, N., Dai, X., Yan, H., Ling, Z., Xing, L., et al., 2016. Development of a dual screening strategy to identify pro-angiogenic compounds from natural products: application on Tongmai Yangxin pills. RSC Adv. 6 (116), 115308–115316.
Liu, N., Li, L., Zhu, X., Ling, Z., Feng, J., Hu, Y., et al., 2016. A high content screening assay to identify compounds with anti-epithelial-mesenchymal transition effects from the Chinese herbal medicine Tong-Mai-Yang-Xin-Wan. Molecules 21 (10), 1340.
Liu, X., Xiao, J., Zhu, H., Wei, X., Platt, C., Damilano, F., et al., 2015. miR-222 is necessary for exercise-induced cardiac growth and protects against pathological cardiac remodeling. Cell Metab. 21 (4), 584–595.
Michishita, E., Park, J.Y., Burneskis, J.M., Barrett, J.C., Horikawa, I., 2005. Evolutionarily conserved and nonconserved cellular localizations and functions of human Sirt proteins. Mol. Biol. Cell 16 (10), 4623–4635.
Ministry of Health of China, 2015. Pharmacopoeia of the People's Republic of China. China Medical Science and Technology Press, pp. 1451.
Parodi-Rullan, R., Barreto-Torres, G., Ruiz, L., Casasnovas, J., Javadov, S., 2012. Direct renin inhibition exerts an anti-hypertrophic effect associated with improved mitochondrial function in post-infarction heart failure in diabetic rats. Cell Physiol. Biochem. 29 (5–6), 841–850.
Peng, X.P., Deng, L.M., Si-Liang, W.U., Zhao, B., Liu, Z.H., Ding, J.G., 2016. Clinical research of Tongmai Yangxin decoction in preventing the recurrence of heart failure with yang-deficiency of heart and kidney. Guid. J. Tradit. Chin. Med. Pharm. 10 (25), 770–777.
Pillai, V.B., Samant, S., Sundaresan, N.R., Raghuraman, H., Kim, G., Bonner, M.Y., et al., 2015. Honokiol blocks and reverses cardiac hypertrophy in mice by activating mitochondrial Sirt3. Nat. Commun. 6, 6656.
Qingying, Z., Min, Y., 2009. Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). J. Chromatogr. A 1216 (11), 1954–1969.
Qiu, X., Brown, K., Hirschey, M.D., Verdin, E., Chen, D., 2010. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab. 12 (6), 662–667.
Quesada, L., Areche, C., Astudillo, L., Gutierrez, M., Sepúlveda, B., San-Martín, A., 2012. Biological activity of isoflavonoids from Azorella madreporica. Nat. Prod. Commun. 7 (9), 1187–1188.
Romankiewicz, J.A., Brogden, R.N., Heel, R.C., Speight, T.M., Avery, G.S., 1983. Captopril: an update review of its pharmacological properties and therapeutic efficacy in congestive heart failure. Drugs 25 (1), 6–40.
Sadoshima, J., Xu, Y., Slayter, H.S., Izumo, S., 1993. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 75 (5), 977.
Shan, T., Liang, X.Y., Wang, Y., 2015. Screening of active compounds with myocardial protective effects from Tongmai Yangxin pill. Zhejiang Da Xue Xue Bao Yi Xue Ban 44 (2), 145–153.
Singh, S., Carpenter, A.E., Genovesio, A., 2014. Increasing the content of high-content screening: an overview. J. Biomol. Screen. 19 (5), 640–650.
Sundaresan, N.R., Madhu, G., Rajamohan, S.B., Ayman, I., Gupta, M.P., 2009. Sirt3 blocks the cardiac hypertrophic response by augmenting FoxO3a-dependent antioxidant defense mechanisms in mice. J. Clin. Invest. 119 (9), 2758–2771.
Sundaresan, N.R., Samant, S.A., Pillai, V.B., Rajamohan, S.B., Gupta, M.P., 2008. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. Mol. Cell Biol. 28 (20), 6384–6401.
Tao, R., Coleman, M.C., Pennington, J.D., Ozden, O., Park, S.H., Jiang, H., et al., 2010. Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Mol. Cell 40 (6), 893–904.
Tao, S., Huang, Y., Chen, Z., Chen, Y., Wang, Y., Wang, Y., 2015. Rapid identification of

anti-inflammatory compounds from Tongmai Yangxin pills by liquid chromatography with high-resolution mass spectrometry and chemometric analysis. J. Sep. Sci. 38 (11), 1881–1893.
Wang, Y., Zhang, L., Yang, X., Li, Z., Xing, Y., 2011. Effect of tongmai yangxin pills on inflammatory factors and oxidative stress of cardiomyocyte injury induced by hypoxia. J. Tradit. Chin. Med.
Wen-Ping, X.U., Lai-Lai, L.I., Wang, Y.Y., Meng, X.W., Wang, Y., 2014. Effects of tongmai yangxin pill on myocardial hypertrophy caused by isopropylarterenol. Tianjin J. Tradit. Chin. Med. 108, 1081–1089.
Xie, X., Wang, L., Zhao, B., Chen, Y., Li, J., 2017. SIRT3 mediates decrease of oxidative damage and prevention of ageing in porcine fetal fibroblasts. Life Sci. 177, 41–48.
Yu, Y., Li, Z., Guo, R., Qian, J., Zhang, H., Zhang, J., et al., 2019. Ononin, sec-O-beta-D-glucosylhamaudol and astragaloside I: antiviral lead compounds identified via high throughput screening and biological validation from traditional Chinese medicine Zhongjing formulary. Pharmacol. Res. 145, 104248.
Zhan-Peng, H., Da-Zhi, W., 2014. miR-22 in cardiac remodeling and disease. Trends Cardiovasc. Med. 24 (7), 267–272.