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Liguzinediol protects against cardiac fibrosis in rats *in vivo* and *in vitro*

Xiang Wu[a,1], Xu Qi[c,1], Ying Lu[a], Chao lin[a], Yao Yuan[a], Qing Zhu[a], Qiuyi Yin[a], Wei Li[a], Yu Li[a,b,*], Huimin Bian[a,*]

[a] *Jiangsu Key Laboratory for Chinese Medicine Processing, Collage of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210046, China*
[b] *Department of Preclinical Medicine, Nanjing University of Chinese Medicine, Nanjing 210046, China*
[c] *Department of Respiratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*

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Cardiac fibrosis plays a causal role in the development of heart failure, and anti-fibrotic therapy represents a promising strategy to mitigate heart failure. The purpose of this study was to investigate the effect of a new drug-liguzinediol on cardiac fibrosis of heart failure Male Sprague-Dawley rats (SD) rats and the underlying mechanisms. Liguzinediol was administered to rats that were injected with doxorubicin (Dox) for four weeks. Two weeks later, its effects on cardiac fibrosis were assessed by haematoxylin and eosin (HE) staining and Masson staining. The collagen content was determined by Elisa, and protein expression was detected by western blot *in vitro* and *in vivo*. Liguzinediol decreased cardiac muscle fiber break evidenced by HE staining and it significantly reduced cardiac fibrosis evidenced by Masson staining in DOX-treated rats. In addition, the hydroxyproline level and the ratio of type I/III collagens were also significantly decreased, and western blot assays showed that liguzinediol regulated the balance between matrix matalloproteinases (MMPs) and tissue inhibitor of metalloproteinase (TIMPs) to protect cardiac remodeling *in vivo* and *in vitro*. These data collectively indicated that liguzinediol could protect against cardiac fibrosis in rats. Liguzinediol could be exploited to be a promising candidate for cardiac fibrosis.

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1. Introduction

Cardiac fibrosis induced by Dox has been reported in several long-term studies [1,2]. A single injection with Dox for several weeks could lead to loss of collagen matrix and cardiac remodeling [3,4]. Even after the drug is removed from the circulation and cardiac tissue, mitochondrial dysfunction, cell death and remodeling changes continue to occur [5]. Cardiotoxicity is particularly prevalent in many patients receiving Dox, which leads to cardiomyocyte apoptosis, myocardial fibrosis, cardiomyopathy, arrhythmias and congestive heart failure (CHF) [6]. CHF is associated with extracellular matrix (ECM) remodeling resulting from an imbalance between the synthesis and degradation of ECM collagens [7,8]. Severe degree of cardiac remodeling is associated with an increased risk of morbidity and mortality in patients with heart failure [9,10]. So we chose Dox to establish animal model with cardiac fibrosis.

Changes in ECM structure are causally associated with alterations of left ventricle (LV) function in the progression of heart disease to heart failure [11–13]. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) influence LV structural and functional properties, as they determine ECM turnover and remodeling [14]. Abnormalities in expression of MMPs and TIMPs, particularly involving MMP-2, MMP-9 and TIMP-1, have been characterized in various cardiovascular diseases [13].

Liguzinediol (Fig. 1) is a compound derived by structural modification from the natural active ingredient ligustrazine [15]. Ligustrazine (4-methylpyrazine, TMP) is a traditional Chinese medicine for angina pectoris. Studies have demonstrated that TMP can attenuate renal tubulointerstitial fibrosis [16], and alleviate the development of liver fibrosis in rats [17]. Recent evidence showed that TMP can not only inhibit the proliferation of rat cardiac fibroblasts (CFs), but also decrease the mRNA level and secretion of collagen I in cultured rat CFs, which are increased by angiotensin II (Ang II) [18]. We herein hypothesized that liguzinediol may inhibit cardiac fibrosis. To test this hypothesis, we performed *in vivo*

Abbreviations: Ang II, angiotensin II; CFs, cardiac fibroblasts; CHF, congestive heart failure; DMSO, dimethylsulfoxide; Dox, doxorubicin; ECM, extracellular matrix; Hyp, hydroxyproline; LV, left ventricle; MMPs, matrix matalloproteinases; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; TIMPs, tissue inhibitor of metalloproteinase; TMP, ligustrazine.

* Corresponding authors at: Nanjing University of Chinese Medicine, Xianlin Avenue, Qixia District, Nanjing, Jiangsu, 210023, China.
E-mail addresses: qxliyu@sina.com (Y. Li), hmbian@sina.com (H. Bian).
[1] With equal contributions to the work.

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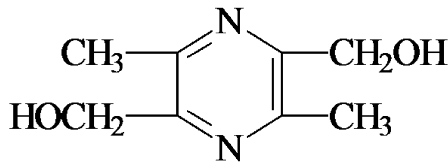

Fig. 1. The structure of liguzinediol.

studies with a rat model of DOX-induced cardiac fibrosis and *in vitro* investigations with CFBs and tried to find out the preliminary mechanisms.

2. Materials and methods

2.1. Materials

Liguzinediol (purity > 99%) was bestowed by Professor Wei Li (Nanjing University of Chinese Medicine). 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Ang II and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (Sigma, USA). Doxorubicin hydrochloride was purchased from Beijing Huafeng United Corporation. All antibodies used in the present study were obtained from Santa Cruz (USA). All other reagents and kits were from commercial suppliers and of standard biochemical quality.

3. Methods

3.1. Animal care

The animal experimental protocols were approved by the local Animal Research Ethics Committee and confirmed to internationally accept ethical standards. Sprague-Dawley rats were obtained from Shanghai Laboratory Animal Center (Shanghai, China). All rats were housed under diurnal lighting conditions and allowed food and water ad libitum.

3.2. Animal model

60 Male Sprague-Dawley rats weighing 250 ± 20 g were randomly divided into control group (normal saline, NS, $n = 8$) and DOX group (2 mg/kg). NS and DOX were given by intraperitoneal injection one time per week. After four weeks, rats in Dox group was divided into 5 groups according to their weight, including DOX group, liguzinediol 5 mg/kg group, 10 mg/kg group, 20 mg/kg group and digitalis 0.0225 mg/kg group. NS and different doses of liguzinediol were given by intragastric administration one time per day. After two weeks, all rats were scarified and the hearts were isolated for HE staining, Masson staining and western blot assays.

3.3. HE staining and Masson staining

Following anesthesia, the hearts were excised and immediately placed in 10% neutral-buffered formalin at room temperature for 24 h after a brief rinse with phosphate-buffered saline. The heart specimen were embedded in paraffin, cut in 5 μm sections and stained with hematoxylin and eosin. In brief, 4 high-power fields (200 × magnification) in each section were randomly selected in theinfarct area. Furthermore, myocardial sections were stained with masson trachoma and fibrosis area in the peri-infarct area was calculated from 10 to 15 random fields (200 × magnification) by dividing the pixels of blue stained area to the total pixels of non-white area in per section using Photoshop CS5 [19,20].

3.4. Collagen secretion

Collagen secretion of rat hearts was detected by ELISA. Type I and III collagens were measured according to the instructions of the rat collagen ELISA kit (Abcam, USA). The level of collagen was calculated according to the A450 value obtained with the Bio-rad automatic detector (Bio-rad, USA). The experiment was performed in triplicate.

3.5. Determination of hydroxyproline content

Hydroxyproline (Hyp) content in heart specimens represented the total amount of collagen in rat hearts, which was quantified to evaluate the degree of cardiac fibrosis using a colorimetric method [21]. The hydroxyproline detection kit was purchased from Nanjing Jiancheng Bioengineering (Nanjing, China). In brief, 100 mg of freeze-dried heart specimens were weighed and tested according to the manufacturer's instructions. At the end of experiments, absorbance of each sample was read at 550 nm using a spectrophotometer. Each sample was analyzed in triplicate.

3.6. Total protein extraction from LV tissue

Frozen hearts were cut into small pieces and homogenized in 0.5 ml of RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris/HCl and 2 mM PMSF, pH 7.4) prior to being transferred into small tubes and rotated at 4 °C overnight. Solubilized proteins were collected after centrifugation at 10000*g* for 30 min. The supernatant was collected and stored at −80 °C. The whole proteins were used to test the expression of MMP-2, MMP-9 and TIMP-1.

3.7. Western blot analysis of proteins in LV tissue

The protein concentration of each sample was quantified using the enhanced BCA Protein Assay Kit (Beyotime Biotechnology, China). Protein lysates from each group were separated by SDS/PAGE and electrotransferred onto a PVDF membrane. The non-specific proteins on membranes were blocked with 5% non-fat dried skimmed milk powder prepared in TBS + 0.1% Tween 20 for 2 h at room temperature. Immunoblotting was then performed using 2 μg/ml rabbit anti-rat MMP-2 polyclonal antibody (Santa Cruz, USA), rabbit anti-human MMP-9 polyclonal antibody (Santa Cruz, USA), rabbit anti-human TIMP-1 polyclonal antibody (Santa Cruz, USA) respectively. Membrane blots were washed and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG antibodies or anti-mouse IgG antibodies. Immunoreactive proteins were then visualized using ECL® plus, a Western blotting detection system (R&D Systems).

3.8. Isolation and culture of cardiac fibroblasts

The cultured CFs used in these experiments were obtained from neonatal Wistar rats as previously described [22], with slight modifications. Briefly, after being treated with 0.25% trypsin solution, fibroblasts were isolated by the removal of myocytes through selective adhesion of nonmyocytes at a 1.5 h pre-plating interval. CFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin (1%v v-1) and foetal bovine serum (10% v/v). CFs at the 3rd or 4th passage were used in the experiments.

3.9. Cell viability assay

MTT (Sigma, USA) assay was used to determine cell viability following the manufacturer's protocols. Briefly, after AngII [18]

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