

## Original Research Article

# Loureirin B attenuates amiodarone-induced pulmonary fibrosis by suppression of TGF $\beta$ 1/Smad2/3 pathway

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### Abstract

**Purpose:** To investigate the therapeutic effect of loureirin B (LB) on amiodarone (AD)-induced pulmonary fibrosis (PF).

**Methods:** Forty-eight male C57BL/6 mice, 8–10 weeks of age, were divided into four groups (n=12). Oral administration of amiodarone hydrochloride (AD) was performed for 4 weeks to induce pulmonary fibrosis. The degree of fibrosis was assessed by Masson staining, while collagen I and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) levels were evaluated by Western blot analysis. ELISA was used to measure the levels of cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in bronchoalveolar lavage fluid (BALF) and lung tissue. Levels of p-Smad2, Smad2, p-Smad3 and Smad3 were determined by western blotting.

**Results:** AD treatment increased the collagen levels and expression levels of collagen I and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in lung tissue and of inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, in both bronchoalveolar lavage fluid (BALF) and lung tissue in a dose-dependent manner ( $p < 0.01$ ). Furthermore, AD increased the levels of p-Smad2/3. AD-induced increases in collagen I and  $\alpha$ -SMA levels were reversed by loureirin B (LB). In addition, LB reduced AD-induced increased levels of the inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in both bronchoalveolar lavage fluid (BALF) and lung tissue ( $p < 0.01$ ).

**Conclusion:** These results demonstrate that LB downregulates expression of fibrosis-related proteins and suppresses AD-induced PF. The mechanism responsible for the protective effect of LB on AD-induced PF might involve inhibition of the Smad2/3 pathway. Thus, LB is a potential therapeutic agent for the management of PF.

**Keywords:** Amiodarone, Loureirin B, Pulmonary fibrosis, Smad, Inflammation

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## INTRODUCTION

Amiodarone (AD) is an effective antiarrhythmic drug that has been linked to lung damage and

can lead to fatal pulmonary fibrosis (PF) [1]. AD-induced lung injury may result from direct toxicity to lung tissues, hypersensitivity to AD, increased oxidative stress, changes in membrane

properties, activation of alveolar macrophages, and cytokine release [2]. Several studies investigating the mechanism of AD-induced PF have been reported. However, treatment for PF is quite challenging, and few treatments have been shown to inhibit the development of AD-induced PF.

*Sanguis draxonis*, commonly known as dragon's blood, is a traditional Chinese medicine with effects on promoting blood circulation and dispersing stasis, relieving pain, and stopping bleeding, and is often used to treat conditions such as blood stasis and traumatic bleeding [3]. Loureirin B (LB), the main component of *Sanguis draxonis*, has been widely used for the treatment of a variety of diseases including blood stasis, cancers, inflammatory disease, and immune disorders [4]. In a previous study, LB was reported to inhibit liver fibrosis and proliferation of hepatic stellate cells in Sprague Dawley rats. Moreover, LB has been shown to inhibit TGF- $\beta$ 1-induced fibrosis [5] and act as a PAI-1 inhibitor to improve liver fibrosis in mice [6]. These observations suggest that LB treatment is a potential therapeutic approach to liver fibrosis. However, the protective role of LB in PF has not been reported.

This study aims to clarify the protective effect of LB on AD-induced PF in mice and to discuss potential underlying mechanisms.

## EXPERIMENTAL

### Animals

Healthy male C57BL/6 mice (8–10 weeks) were purchased from Guangdong Provincial Experimental Animal Center for Medicine. Mice were housed in individually ventilated cages (Tianhuan, Shanghai, China) supplied with filtered air and free access to food and water. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committees (IACUCs) of the United States [7] and approved by the Ethics Committee of Xi'an Hospital of Traditional Chinese Medicine (approval no. 2018031).

### Animal experiments

PF was induced in mice by AD using established methods [8]. Amiodarone hydrochloride was dissolved in sterile distilled water, stored at 4°C, and warmed to 25°C before use. To establish mouse models of PF, C57BL/6 mice were separated into three groups and orally administered sterile distilled water alone

(control), 20 mg/kg body weight AD, or 40 mg/kg AD. To investigate the effect of LB on AD-induced PF, a dose of 0.25 mg/kg LB was selected. Mice were separated into four groups and received sterile distilled water, 40 mg/kg AD, 0.25 mg/kg LB, or 40 mg/kg of AD plus 0.25 mg/kg of LB. All mice were maintained for 5 weeks and then euthanized. All animals received humane care in the experiment period.

### Masson trichrome staining

For histopathological analysis, lungs were fixed by perfusion with formalin and embedded in paraffin. Sections (4- $\mu$ m) were prepared, deparaffinized, and washed, followed by staining in hematoxylin for 5–10 min. Sections were then washed in distilled water and stained with Masson trichrome for 5–10 min. Sections were treated with a mixture of 2% glacial acetic acid and 1% phosphomolybdate for 3–5 min. Finally, the sections were stained with aniline blue for 5 min and sealed with resin.

### Western analysis

Fresh lung tissue was cut into pieces, homogenized in RIPA lysis buffer, centrifuged at 4°C and 12,000 rpm, and the supernatant was collected [9]. Protein was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA) using a blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% skim milk at room temperature for 2 h followed by incubation with the appropriate primary antibodies at 4°C for 12 h. Membranes were washed with Tris-buffered saline containing 5% Tween (TBST) 3 times for 10 min each. Finally, the membranes were incubated with secondary antibodies at room temperature for 2 h. Following additional washes, bands were visualized using enhanced chemiluminescence plus (ECL) reagents (Millipore). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. All experiments were repeated at least 3 times.

### ELISA

Inflammatory cytokines in bronchoalveolar lavage fluid (BALF) and serum were detected using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, each sample was diluted in phosphate-buffered saline (PBS) and 100  $\mu$ L

was assayed. Optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific). All samples were assayed in at least triplicate.

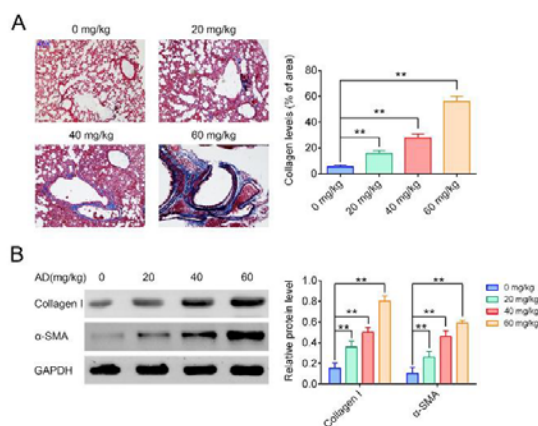
### Statistical analysis

Comparisons between two groups were performed using the paired *t*-test. Statistical analysis was performed with SPSS version 18 software (IBM, Armonk, NY, USA). Values of  $p < 0.05$  were considered statistically significant. All data are presented as mean  $\pm$  standard deviation (SD).

## RESULTS

### AD induced PF in mice

AD induced collagen deposition in mouse lung in a dose-dependent manner at the indicated concentrations, as shown by Masson's trichrome staining (Figure 1 A). In addition, western analysis showed that AD increased  $\alpha$ -SMA and collagen I levels in a dose-dependent manner at the same doses (Figure 1 B). The results indicate that AD induced PF in mice, and 60 mg/kg AD exhibited the strongest destructive effect on mouse lung. Therefore, a middle dose of 40 mg/kg AD was used in subsequent experiments.

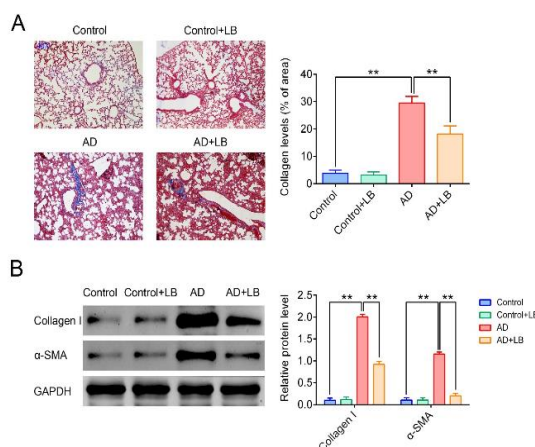


**Figure 1: Amiodarone (AD) induces pulmonary fibrosis (PF).** (A) Masson's trichrome staining of collagen in lungs of mice treated with the indicated amounts of AD (left) and quantitation (right). (B) Western analysis of  $\alpha$ -smooth muscle actin (SMA) and collagen I levels (left) and quantitation (right); \*\* $p < 0.01$

### LB alleviated AD-induced PF

As shown in Figure 2 A, compared with control, Masson's trichrome staining showed that AD induced collagen deposition, whereas 0.25 ml/kg LB reduced the collagen levels induced by AD in

mouse lung. However, LB alone showed few changes in lung tissue when compared with the control. Moreover, western analysis showed that  $\alpha$ -SMA and collagen I levels were increased in the AD group compared with control, whereas the protein levels were decreased in the AD + LB group compared with the AD group (Figure 2 B). These results show that LB alleviated AD-induced collagen deposition in mouse lung, and suggest that LB might inhibit AD-induced PF in mice.



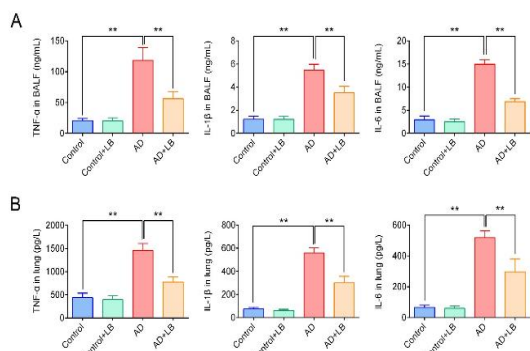
**Figure 2: Effect of loureirin B (LB) on AD-induced PF.** (A) Masson's trichrome staining of collagen in lungs of mice treated with the indicated amounts of distilled water (control), AD, and/or LB (left) and quantitation (right). (B) Western analysis of  $\alpha$ -SMA and collagen I levels (left) and quantitation (right); \*\* $p < 0.01$

### LB inhibited inflammation in mice with PF

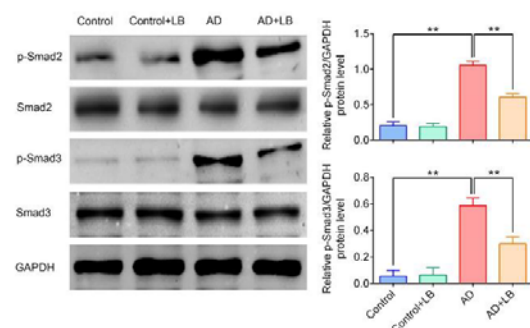
ELISA showed that AD significantly elevated the levels of inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF compared with control, whereas LB showed no obvious effects on inflammatory cytokine levels (Figure 3A). Compared with the AD group, the AD+LB group showed reduced levels of these inflammatory cytokines in BALF. Furthermore, as shown in Figure 3 B, inflammatory cytokines in serum showed the same trend as in BALF. The results indicated that LB alleviated AD-induced inflammation in mice.

### LB inhibits Smad2/3 signaling pathway

Smad2 and Smad3 might be major factors related to tissue fibrosis. As shown in Figure 4, AD significantly increased the levels of p-Smad2 and p-Smad3, whereas LB reduced these AD-induced increases. The results indicate that LB might inhibit the activation of the Smad2/3 signaling pathway induced by AD (Figure 4).



**Figure 3:** LB inhibits inflammation in mice with PF. (A) ELISA assessment of cytokines in bronchoalveolar lavage fluid (BALF) (A) and serum (B); \*\* $p < 0.01$



**Figure 4:** LB inhibits TGFβ1/Smad2/3 signaling pathway. Western analysis of Smad2, p-Smad2, Smad3, and p-Smad3 (left) and quantitation (right); \*\* $p < 0.01$

## DISCUSSION

Pulmonary fibrosis is characterized by chronic and invasive scar formation and deposition of extracellular matrix (ECM), followed by death due to impaired lung function and respiratory failure [10]. Studies have shown that increased epithelial-mesenchymal transition (EMT) and perturbation of the matrix synthesis/degradation balance are the driving factors in PF [11,12]. Numerous cytokines in the lung promote the proliferation and activation of myofibroblasts, formation of fibroblast foci, and generation and deposition of fibroblasts, eventually leading to PF [13].

The effect of drugs currently used in the treatment of PF is still unsatisfactory. Therefore, it is essential to develop new strategies for the prevention and treatment of this respiratory disease. In the present study, the traditional Chinese medicine extract of Dragon's blood, LB, inhibited AD-induced PF in mice.

LB has already been reported to contribute to improvement of the fibrotic diseases. For example, LB has been reported to inhibit liver fibrosis in Sprague Dawley rats [14] and to inhibit scar-fibroblast proliferation and suppress TGF-β1-induced fibrosis [5]. In another report, LB was shown to alleviate liver fibrosis in mice, acting as a PAI-1 inhibitor [6]. Consistent with previous reports, this study indicates that LB might inhibit AD-induced PF in mice.

Collagen deposition and inflammation are the main features of PF [15]. A recent study demonstrated that LB inhibits collagen deposition in scars [16]. Consistent with previous findings in literature [18], our Masson's trichrome staining and western analysis data show reduced expression levels of α-SMA and collagen I, demonstrating that LB indeed alleviated collagen deposition in PF mice. Three studies (including two original studies and one systematic review) indicated the anti-inflammatory role of LB [17]. In this study, the decreased levels of inflammatory cytokines TNF-α, IL-1β, and IL-6 were observed in the LB treatment group, consistent with the previous study [17], confirming the anti-inflammatory role of LB.

A variety of studies have demonstrated that the TGF-β1/Smad pathway is involved in the regulation of fibrotic disease and inflammation [18]. Smad family members, including Smad2 and Smad3, are major downstream regulators that promote TGF-β1-mediated tissue fibrosis. Several studies have shown that the TGF-β1/Smad pathway is activated in PF, and inhibition of the pathway is believed to suppress PF development. The studies cited above [19-21] indicate that downregulation of the TGF-β1/Smad pathway effectively alleviated PF, which may be a new strategy for the treatment of PF. This study showed that expression of key TGF-β1/Smad pathway members Smad2 and Smad3 was upregulated in PF mice, and suggests that LB might inhibit activation of the TGF-β1/Smad pathway via downregulation of these proteins.

Taken together, these data demonstrate that LB may inhibit AD-induced PF in mice and that the underlying mechanism might involve the TGF-β1/Smad pathway.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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