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# Inhibitory effect of L-mimosine on bleomycin-induced pulmonary fibrosis in rats: Role of eIF3a and p27

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ABSTRACT

It has also been shown that the decreased expression of eukaryotic translation initiation factor 3a (eIF3a) by L-mimosine caused cell cycle arrest. Our previous study has found that eIF3a is involved in bleomycin-induced pulmonary fibrosis. Whether the eIF3a/p27 signal pathway is involved in the inhibitory effect of L-mimosine on bleomycin-induced pulmonary fibrosis remains unknown. Pulmonary fibrosis was induced by intratracheal instillation of bleomycin (5 mg/kg) in rats. Primary pulmonary fibroblasts were cultured to investigate the proliferation by BrdU incorporation method and flow cytometry. The expression of eIF3a, p27, α-SMA, collagen I and collagen III was analyzed by qPCR and Western blot. *In vivo*, L-mimosine treatment significantly ameliorated the bleomycin-mediated histological fibrosis alterations and blocked collagen deposition concomitantly with reversing bleomycin-induced expression up-regulation of eIF3a, α-SMA, collagen I and collagen III (both mRNA and protein) and expression down- regulation of p27. *In vitro*, L-mimosine remarkably attenuated proliferation of pulmonary fibroblasts and expression of α-SMA, collagen I and collagen III induced by TGF-$\beta_1$, and this inhibitory effect of L-mimosine was accompanied by inhibiting eIF3a expression and increasing p27 expression. Knockdown of eIF3a gene expression reversed TGF-$\beta_1$-induced proliferation of fibroblasts, down-regulation of p27 expression and up-regulation of α-SMA, collagen I, and collagen III expression. These results suggest that L-mimosine inhibited the progression of bleomycin-induced pulmonary fibrosis in rats *via* the eIF3a/p27 pathway.



## 1. Introduction

Pulmonary fibrosis is a progressive and fatal disease with multiple etiologies. It is characterized by fibroproliferation, deposition of the extracellular matrix in the lung parenchyma and tissue remodeling [1,2]. The long-term survival of pulmonary fibrosis patients with current treatment regimens is poor. The mean survival is only two to three years following diagnosis of pulmonary fibrosis; therefore, novel drugs with better efficacy and tolerance are urgently needed for the treatment of pulmonary fibrosis.

Translational control plays a major role in regulating gene expression and occurs primarily at the initiation step involving multiple eukaryotic translation initiation factors (eIFs) [3]. eIF3a (also known as p170), the largest subunit of the eIF3 complex, has been suggested to play roles in regulating translation of a subset of mRNAs and in regulating cell cycle progression and cell proliferation [4,5]. Suppressing endogenous eIF3a expression has been shown to inhibit the malignant phenotype of human cancer cells while over-expression of ectopic eIF3a has been shown to promote malignant transformation of mammalian cells [6,7]. Our previous study has found that eIF3a plays an important role in bleomycin-induced pulmonary fibrosis and TGF-$\beta_1$-induced proliferation and differentiation of pulmonary fibroblasts [8]. These findings suggest that eIF3a is involved in the regulation of fibroblast proliferation in pulmonary fibrosis besides in tumorigenesis including lung cancer.

L-Mimosine {(*S*)-α-amino-β-[1-(3-hydroxy-4-oxopyridine)] propionic acid; $C_8H_{10}N_2O_4$}, a plant amino acid, acts as an iron chelator and reversibly blocks mammalian cell proliferation at the late $G_1$ phase [9]. It effectively prevents DNA synthesis by blocking the late $G_1$ phase [10], interferes with the synthesis of histone H1 kinase, specifically inhibits cyclin $D_1$ expression [11,12], and up-regulates p27 protein level [13,14]. An earlier study indicated that numbers of $G_0/G_1$ cells

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were elevated after incubation with L-mimosine in human prostate carcinoma DU145 cells [15]. It has also been shown that the decreased expression of eIF3a by L-mimosine caused elevated translation of p27 before cell cycle arrest [16]. It has also been demonstrated that regulation of protein expression such as p27 by eIF3a is involved in the function of eIF3a in cell proliferation [17].

Based on the potential and critical role of eIF3a in pathogenesis of pulmonary fibrosis and pulmonary fibroblast proliferation and differentiation, the present study aimed to assess whether L-mimosine inhibits bleomycin-induced pulmonary fibrosis in rats. In particular, we tried to explore the effect of L-mimosine on eIF3a and p27 expression, collagen accumulation, and cellular proliferation of primary cultured pulmonary fibroblasts.

## 2. Materials and methods

### 2.1. Reagents and materials

L-Mimosine (from Koa hoale seeds) was purchased from Sigma (St. Louis, MO, Cat. no.: M0253). Bleomycin was purchased from Nippon Kayaku Co. Ltd (Tokyo, Japan). Transforming growth factor-$\beta_1$ (TGF-$\beta_1$) was purchased from PeproTech (New Jersey, USA). Masson's trichrome stain kit was purchased from Nanjing KeyGEN Biotech (Nanjing, China). The BrdU cell proliferation assay kit was provided by Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM) was provided by GIBCO (New York, N.Y., USA). The primers were purchased from Shanghai Sangon Biological Engineering Co. Ltd. (Shanghai, China). The PrimeScript reverse transcription reagent Kit and SYBR® Premix Ex Taq™ were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Primary antibodies against p27 and eIF3a were purchased from Cell Signaling (Boston, MA, USA). Primary antibodies against α-SMA, collagen I, and collagen III were purchased from Abcam (Hong Kong, China), and GAPDH (sc-137179, 1:2000) was obtained from Santa Cruz (CA, USA).

### 2.2. Animals

Male Sprague–Dawley (SD) rats (aged 6–8 weeks, weighing 180–220 g) were obtained from Nanjing Qinglongshan Experimental Animal Company (certificate No: SCXK (jun) 2007-012; Nanjing, China). All experiments were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Medicine Animal Welfare Committee of Wannan Medical College, China.

### 2.3. Animal experiments

Forty eight rats were randomly divided into four groups, with twelve rats per group, as follows: 1) the control group, SD rats were anesthetized intraperitoneally with sodium pentobarbital (P3761, 30 mg/kg; Sigma) followed by intratracheal instillation of 0.9% saline; 2) the bleomycin (BLM) group, rats were anesthetized intraperitoneally with sodium pentobarbital followed by intratracheal instillation of 5 mg/kg bleomycin (Nippon Kayaku, Tokyo, Japan) in 1 mL of saline; and 3) the BLM treated with L-mimosine (25, 50 mg·(kg body mass)$^{-1}$·day$^{-1}$) group. Bleomycin was chosen as 5 mg/kg according to the previous study [8] and L-mimosine was chosen as 25 and 50 mg/kg based on our pilot study. L-Mimosine was first dissolved in Tris-buffer at pH 8.9, and then pH was adjusted to 7.2 with 1 N HCl. Before administration, this solution was freshly diluted by 0.9% physiologic saline to achieve the required concentration. L-Mimosine was administered *via* subcutaneous injection once daily from day 1 to day 28 after BLM or saline treatment (day 0) and all rats were sacrificed with exsanguination on day 29. Pulmonary fibrosis was assessed by lung histology as described in the following section [18].

### 2.4. Lung tissue histology and Masson's trichrome staining

For light microscopic investigation, right lung tissues were fixed by inflation with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 24 h and embedded in paraffin. Tissue sections (5 μm) from the apex to bottom longitudinal of the right lung were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain to enable histological evaluation of lung fibrosis. Masson's trichrome stain was used to demonstration collagen deposition, and collagen fiber is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. The procedure is according to the manufacturer's instructions (KeyGEN Biotech, Nanjing, China). All the histological assays were performed blind to the interventions.

### 2.5. Cell experiments

Primary rat pulmonary fibroblasts were prepared from the lung tissue of male 10-week-old healthy SD rats using the trypsin digestion method as described previously [19]. The cells were cultured at 37 °C under 5% $CO_2$ in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. Fibroblasts were identified by immunofluorescence staining with the antibody of Vimentin (ab8978, 1:50; Abcam, Hong Kong, China). The cells between passages 3 and 6 were used for the experiments. Two series of experiments were designed. The first series of experiments were to explore the effect of L-mimosine on proliferation of fibroblasts and the correlation with the eIF3a/p27 pathway. The cells were divided into 6 groups as follows: i) control, cells were incubated with double distilled water (TGF-$\beta_1$ solvent) for 24 h; ii) TGF-$\beta_1$, cells were incubated with TGF-$\beta_1$ (5 ng/mL) for 24 h; iii–v) +L-mimosine (1, 10, 100 μM): cells were pre-treated with L-mimosine (1, 10, 100 μM) for 1 h, and then subjected to TGF-$\beta_1$ (5 ng/mL) for 24 h; and vi) L-mimosine (100 μM): cells were pre-treated with L-mimosine (100 μM) for 1 h, and then incubated with double distilled water for 24 h. The second series of experiments were to evaluate the role of the eIF3a/p27 signal pathway in TGF-$\beta_1$-induced proliferation of rat fibroblasts. The cells were divided into 4 groups as follows: i) control, cells were incubated with double distilled water (TGF-$\beta_1$ solvent) for 24 h; ii) TGF-$\beta_1$, cells were incubated with TGF-$\beta_1$ (5 ng/mL) for 24 h; iii) +Scrambled: cells were pre-transfected with eIF3a small interfering RNA negative control for 24 h before treated with TGF-$\beta_1$ (5 ng/mL) for 24 h; and iv) +eIF3a siRNA: cells were pre-transfected with small interfering RNA against eIF3a for 24 h before treated with TGF-$\beta_1$ (5 ng/mL) for 24 h. Cell proliferation assays were performed. The expressions of collagen I, collagen III, α-SMA, and eIF3a were analyzed. The 24 h duration of TGF-$\beta_1$ was based on our pilot study.

### 2.6. Small interfering RNA transfection

Small interfering RNA (siRNA) against eIF3a gene (GenePharma CO, Shanghai, China) was generated against the following rat eIF3a sequences: sense, 5′-GGCCAAACAAGUUGAACAA-3′; and antisense, 5′-UUGUUCAAC UUGUUUGGCC-3′. Fibroblasts grown to 70% to 80% confluence were transfected with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was evaluated by eIF3a mRNA and protein expression using real-time PCR and Western blot analysis, respectively. Twenty-four hours after transfection, the cells were used for the experiments as mentioned above.