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Ligustilide Attenuates Bleomycin-induced Pulmonary Fibrosis in Mice via Nrf2 Pathway Activation

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Abstract

Idiopathic pulmonary fibrosis is a progressive interstitial lung disease without effective therapeutic strategies at present. Ligustilide (LIG) is a main bioactive component of angelica. The present study aimed to observe the effect of LIG on mice lung fibrosis at late stage of pulmonary fibrosis and to explore the underlying mechanism. After 14 days for a single BLM instillation, the mice were daily treated with LIG for 2 weeks. Then the effect of LIG on lung fibrosis was observed then. The pulmonary function measurement, Hematoxylin- eosin (H&E) and Masson's trichrome staining, Immunofluorescence and Western blot were used to evaluate the effect of LIG on pulmonary fibrosis. We investigated the underlying mechanism after transforming growth factor-β1 (TGF-β1) treatment *in vitro*. The results showed LIG attenuated BLM-induced pulmonary fibrosis and improved pulmonary function. LIG reduced fibroblast activation and reactive oxygen species (ROS) production after TGF-β1 treatment. LIG regulated the activation of nuclear factor E2-related factor 2 (Nrf2) pathways in fibroblasts after TGF-β1 exposed, which might be related to the protective effect of LIG on fibroblasts. LIG reduced pulmonary fibrosis in mice after BLM infusion, which might be related to Nrf2pathway activation. LIG might be a promising drug for the treatment of pulmonary fibrosis.

Keywords: Lung fibrosis; Bleomycin; Ligustilide; Fibroblasts; Nrf2; Oxidative stress

Introduction

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease and is characterized by the excessive deposition of extracellular matrix in the pulmonary interstitium, leading to a progressive and irreversible decline in the lung function [1-3]. The number of patients with IPF is increasing year by year at present. As a chronic interstitial lung disease, the etiology of IPF is unknown and the mean survival time was only 2-5 years after diagnosis. Although extensive exploration, the pathogenesis for IPF still remains not clear. Nintedanib and Prfenidone (PFD) have been approved for clinical treatment of pulmonary fibrosis [4,5] but they cannot completely cure the disease. Therefore, new therapeutic agents for pulmonary fibrosis are urgently needed to be explored.

Under pathological conditions, a large number of fibroblasts proliferate, migrate, activate and accumulate in the area of lung injury [6,7], accompanied by the deposition of a large number of extracellular matrix and other proteins, leading to irreversible lung fibrosis [1]. Transforming growth factor-β1 (TGF-β1) plays a significant role in the initiation of lung fibrosis by promoting fibroblasts proliferation, differentiation and extracellular matrix secretion [8,9]. Studies show that both canonical (Smad-based) and non-canonical (non-Smad-based) signalling pathways could be activated in fibrosis induced by TGF-β1 [10]. A large number of studies have proved that dysregulation of TGF-β1/Smad pathway was an important pathogenic mechanism in lung fibrosis [11] and Smad3 is one of the key downstream regulator that promote tissue fibrosis induced by TGF-β1 [12].

Emerging studies indicate that cytokines and reactive oxygen species (ROS) might be responsible for the activation of fibroblasts [13,14]. Excessive ROS generation emerges when oxidative stress induced [15]. It has been certificated that Nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important transcription factor that induces many genes such as HO-1 and NQO1 transcription thereby protects tissues from damage and neutralizes ROS production under oxidative stress [14,16]. The regulation of Nrf2 can restrain myofibroblast activation and reduce extracellular matrix deposition in b BLM-treated mice [17].

Ligustilide (LIG; 3-butylidene-4,5-dihydrophthalide; Figure 1A), the main bioactive component of the Umbelliferae family in Traditional Chinese Medicine such as *Rhizoma Ligustici Chuanxiong, Radix Angelicae Sinensis*, has been found to be related to widely pharmacological activities, such as anti-oxidant, anti-inflammatory, neuroprotective and protective effects on many diseases [18-21]. However, it is not known the effect of LIG on fibroblast activation after lung fibrosis. In this study, we observed the effects of LIG on lung fibrosis in mice after BLM treatment and further probed the possible mechanism of LIG on fibroblast activation after TGF-β1 exposed *invitro*.

Materials and Methods

Animals

Male C57BL / 6 mice (20 ± 2 g) were purchased from Hangzhou Ziyuan Laboratory Animal Technology Co., Ltd. All animals were raised in colony cages at constant temperature (23°C) and humidity

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(50%) for 12-h light/dark cycles, and they could freely gain food and water. All animal procedures were approved by the Laboratory Animal Care and Use Committee at Southeast University (20210106011) and were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs and Administration

LIG was purchased from Chengdu herbpurify, Co, Ltd (HPLC > 98%). All mice were randomly divided into six groups (n≥8 each group), the sham, BLM, BLM plus PFD (300 mg/kg), BLM plus 10 mg/kg of LIG, BLM plus 30 mg/kg of LIG, BLM plus 90 mg/kg of LIG groups. The mice were intra-tracheally administered 100 μL of bleomycin (2 mg/kg) in all groups except the sham group after anesthesia with pentobarbital sodium (1%, 50 mg/kg). Mice in the sham group were given equivalent volume of saline by the same way. 14 days after BLM infusion, mice were treated with LIG, PFD or 0.5% CMC-Na solution continuously for 14 days. Body weight of mice was measured every day.

Pulmonary Function Measurement and Sample Collection

After 14 days of continuous treatment, the mice were euthanized with pentobarbital sodium for pulmonary function tests as described previously [40]. After euthanized, mice were inserted a tracheal catheter and fastened it to the trachea followed by trachea exposed. Then, IC (inspiratory capacity, volume inspired during slow inspiration), ERV (expiratory reserve volume), FVC (forced vital capacity, volume expired during fast expiration) and TLC (total lung capacity, FRC+IC) were tested with the Forced Manoeuvres System (EMMS, Hants, UK). Three times measurements were operated each mouse. Finally, mice were sacrificed and lung sample collection was performed for further study.

Histopathological Analysis

The lung samples were removed and immediately fixed in 4% paraformaldehyde followed by cryoprotected and then sliced into 8-μm frozen sections with a freezing microtome, stained with Masson's trichrome (Biyun Tian, China) and hematoxylin-eosin (H & E, Biyun Tian, China) according to the instructions to evaluate for lung damage. The images were visualized with a microscope (EVOS FL AUTO2, Thermo, America).

Cell Culture and Treatment

The mouse lung fibroblast cell line (MLG) was bought from ScienCell. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with fetal bovine serum (10%) and penicillin-streptomycin solution at 37°C in 5% CO_2 incubator. The medium was changed every two days. To simulate fibrosis conditions *in vitro*, cells were exposed to recombinant mouse TGF-β1 (Kingsley Biotechnology, China). After 80-90% confluency, cells were treated with LIG (3, 10, 30 μM) followed by TGF-β1 administration for 24 h. Then, cells were used for various follow-up experiments.

Assay of ROS Production

MLG cells were given indicated agents for 24 h, and the intracellular ROS generation was observed by measuring the oxidative conversion of DCFH-DA (cell permeable) to fluorescent dichlorofluorescein with a commercial assay kit. Images were observed with a microscope (EVOS FL AUTO2, Thermo, America). DCFH-DA was measured at an excitation wavelength 488 nm and an emission wavelength 525 nm with a microplate reader.

Cell Proliferation Assay

Bromodeoxyuridine (BrdU, Sigma-Aldrich) assay was used to evaluate MLG cell proliferation according to the instructions. Briefly, MLG cells were cultured in 24-well plate with indicated treatment for 24 h. Then 10 μM BrdU was given to these cells followed by 4 h incubation. Next, the cells were washed with PBS and fixed with paraformaldehyde (4%). Then, the cells were washed with PBS and permeabilized with Triton X-100 (0.3%) for 30 minutes. Cells were incubated in 2N HCl for 10 minutes followed by boric acid buffer (pH 7.4) incubation at room temperature. Cells were incubated in antibody blocking solution for 1 h after PBS washed. Next, cells were incubated in antibody staining buffer with anti-BrdU primary antibody (1:1000, SantaCruz) overnight at 4°C and then Fluorescent labeled secondary antibody (1:200, invitrogen). The images were observed with a fluorescence microscope (EVOS FL AUTO2, Thermo, America).

Cell Migration Assay

To assess cell migration capacity after treatment, a 2D scratch assay was performed as described previously [41]. Specifically, MLG cells were cultured in 24-well plates in a 37°C incubator. After confluency, a straight line of moderate width was scratched using a sterile 200-μl pipette tip. The fresh medium was given to each well after cells washed twice with PBS. Simultaneously, cells were cultured under different condition, and the scratch gap images were captured at 0 h, 12 h and 24 h after gap formation. Each experiment was repeated at least three times.

Western Blot

The solution composed of RIPA buffer (Beyotime) and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) was used to gain protein samples from cells or lung tissue. SDS-polyacrylamide gel was used to separate proteins from different preparations which then transferred to a PVDF membrane. Next, the membrane was shook in skim milk (5%) for 1 h at room temperature and then incubated in primary antibody at 4°C overnight. Primary antibodies against collagen I (1:1000, BioWorld), α-SMA (1:1000, Proteintech), Nrf2 (1:1000, Proteintech), HO-1 (1:1000, Proteintech), and NQO-1 (1:1000, Affinity) were used. GAPDH (1:1000, BioWorld) was used as an internal reference protein. HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (according to what primary antibody origination was used) were used to detect the proteins. The images were observed with a fluorescence microscope (EVOS FL AUTO2, Thermo, America).

Immunofluorescence

Lung frozen sections or cells were fixed in paraformaldehyde (4%) and then incubated in a blocking solution (10% normal goat serum and 0.3% Triton X-100 in PBS) for 1 h at room temperature. Next, the lung frozen sections or cells were incubated with primary antibodies against collagen-I (1:200, BioWorld), α-SMA (1:200, Proteintech), TGF-β1 (1:300, Affinity), pSmad3 (1:300, Affinity) or HO-1 (1:200, Proteintech). Cy3-conjugated secondary antibodies (1:500, Invitrogen) and/or Alexa Fluor 488 (1:300, Invitrogen) was added to the sections or cells. The cell nuclei were labeled by Hoechst 33342 if needed. The images were observed with a fluorescence microscope (EVOS FL AUTO2, Thermo, America).

Molecular Docking Study

To evaluate whether LIG could bind with Keap1 protein, we performed in silico protein-ligand docking experiment using

Autodock vina (1.1.2) and Chimera (1.15). The Keap1 structure was found in the protein data bank (PDB ID: 3wdz), and then removed the crystal water molecules and other small molecules. The conformational ensemble for LIG to Keap1 protein was generated with the Autodock vina. The conformational search was performed with the genetic algorithm. 100 individual genetical gorithm runs were performed to generate 100 docked conformations for the conformational space of LIG exploration extensively. We make sure that the docking box size enclose the possible binding pocket and keep a fixed protein structure during molecular docking.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Comparison between two groups was analyzed using a two tailed Student's t test. Differences were served significant at $p<0.05$. All analysis was conducted using GraphPad Prism version 8.0.

Results

LIG attenuated Pulmonary Fibrosis in Mice induced by BLM

In order to ensure drug safety, toxicity of LIG was first evaluated. As shown in supplementary (Figure1B,E,H) staining demonstrated that no evident histological damages in lung were detected between the Con and LIG-treated mice. To determine whether treatment with LIG at the late stage of pulmonary fibrosis mitigates BLM-induced lung damage, mice were stimulated with BLM (2 mg/kg) followed by treatment shown in (Figure 1C). Six treatment groups were designed as follows, LIG treatment groups (10, 30 or 90 mg/kg), the PFD group, the vehicle group and the sham group. As displayed in (Figure 1D), LIG treatment after BLM infusion 2 weeks increased the survival rate of mice subjected to BLM. LIG also maintained body weight of mice compared with the vehicle group (Figure 1E). Lung function measurements showed that LIG improved the pulmonary function compared with the vehicle group (Figure 1F). Hematoxylin–eosin (H&E) staining showed that LIG reduced BLM-induced diffuse alveolar collapse and wall thickening in the lung tissue (Figure 1G). In addition, Masson's trichrome staining revealed that LIG suppressed collagen deposition in the lungs of BLM-treated mice (Figure 1H). The results proved that LIG improved survival rate and lung function of mice after BLM infusion and protected against BLM induced lung damage, implying that LIG might be a promising drug for the treatment of pulmonary fibrosis.

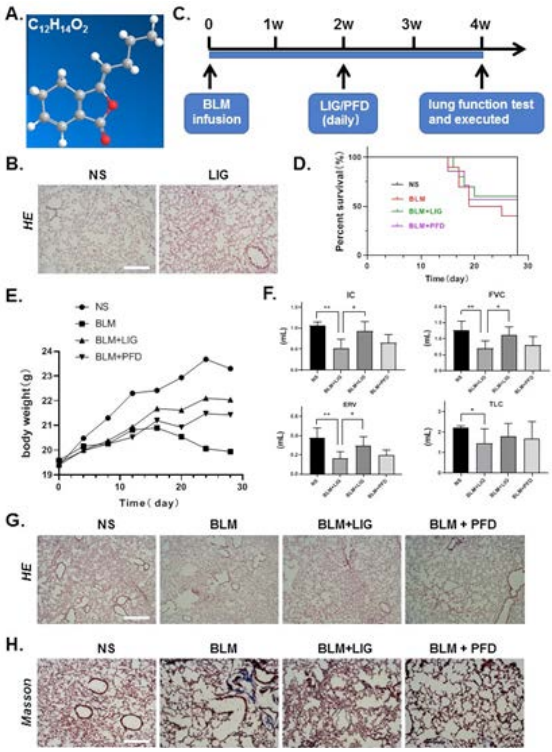

Figure 1: LIG attenuated pulmonary fibrosis in mice induced by BLM.

A: Molecular structure of LIG
B: H&E staining staining in the lung tissues of mice with BLM treatment, scale bar=100μm, n≥3
C: The experimental groups included the sham, BLM, BLM plus PFD, BLM plus LIG groups
D: The effect of LIG on mice survival rate after BLM treatment, n≥8
E: The effect of LIG on the body weight of mice subjected to BLM, n≥8
F: The effect of LIG on pulmonary function of mice subjected to BLM, n≥8, $^{**}p<0.01$
G: H&E staining in the lung tissues of mice treatment with BLM, n≥3, scale bar= 200μm
H: Masson trichrome staining in the lung tissues of mice treatment with BLM, n≥3, scale bar= 200μm

LIG reduced Extracellular Matrix deposition in BLM-treated Mice

The process of pulmonary fibrosis is accompanied by the deposition of extracellular matrix, so the effect of LIG on extracellular matrix deposition was evaluated then. As shown in (Figure 2A,B) LIG decreased α-SMA and collagen I deposition compared with the vehicle group. Western blot also showed the effect of LIG on the protein level of α-SMA and collagen I (Figure 2C-E). (Figure 2) showed that LIG reduces extracellular matrix deposition in mice induced by BLM which might be related to its effect on pulmonary fibrosis induced by BLM.

The effect of LIG on TGF-β1 Pathway in Pulmonary Fibrosis induced by BLM

TGF-β1 is key role in the process of pulmonary fibrosis by promoting fibroblasts to proliferation, differentiation and extracellular matrix secretion (7). Therefore, the effect on TGF-β1 pathway in lung of mice subjected to BLM was assessed. As shown in (Figure 3A) BLM significantly increased TGF-β1 level in mouse lung compared with the vehicle group, and LIG reduced the effect of BLM. Smad 3 is an important element in TGF-β1 pathway, and activated after phosphorylated [12,22]. LIG decreased Smad 3 phosphorylation induced by BLM (Figure 3B). These data indicated LIG could regulate TGF-β1 pathway activation in pulmonary fibrosis after BLM treatment.

LIG reduces TGF-β1 induced Fibroblast Activation

The activation of fibroblasts plays a significant role in the process of pulmonary fibrosis. To evaluate the protective effect of LIG on fibroblast activation induced by TGF-β1, we first assessed the protein level of cells after TGF-β1 exposed. Before that, we confirmed that fibroblasts incubated with LIG (3, 10 and 30 μM) for 24 h showed no cytotoxic effect on their viability (data not shown). As shown in (Figure 4A,B) LIG reduced the increased protein level of collagen I after TGF-β1 treatment. A 2D scratch assay was performed to measure cell migration capacity after treatment. LIG restrained cell migration in response to TGF-β1 as shown in (Figure 4C). Since cell proliferation is essential for fibroblast activation, we explored whether LIG treatment could inhibit fibroblast proliferation after TGF-β1 administration. Data showed decreased fibroblast proliferation in LIG-treated group (Figure 4D,E). Our data showed that LIG attenuated TGF-β1 induced fibroblast activation, indicating that the protective effect of LIG on

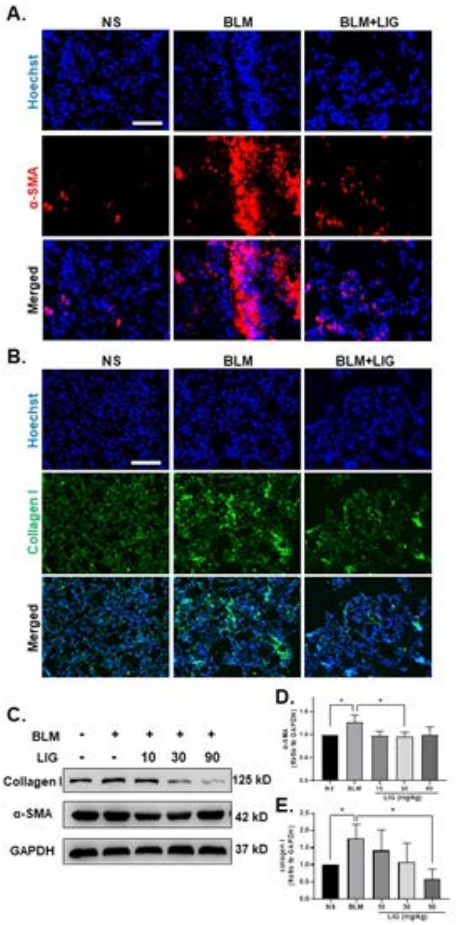

Figure 2: LIG reduced α-SMA and collagen I deposition in mice induced by BLM.
A: Immunohistochemistry staining of α-SMA in the lung tissues of BLM-treated mice, scale bar = 200μm, n≥3
B: Immunohistochemistry staining of collagen I in the lung tissues of mice BLM-treated mice, n≥3, scale bar 200μm
C-E: Western blot assay for α-SMA and collagen I, n≥3, *p<0.05

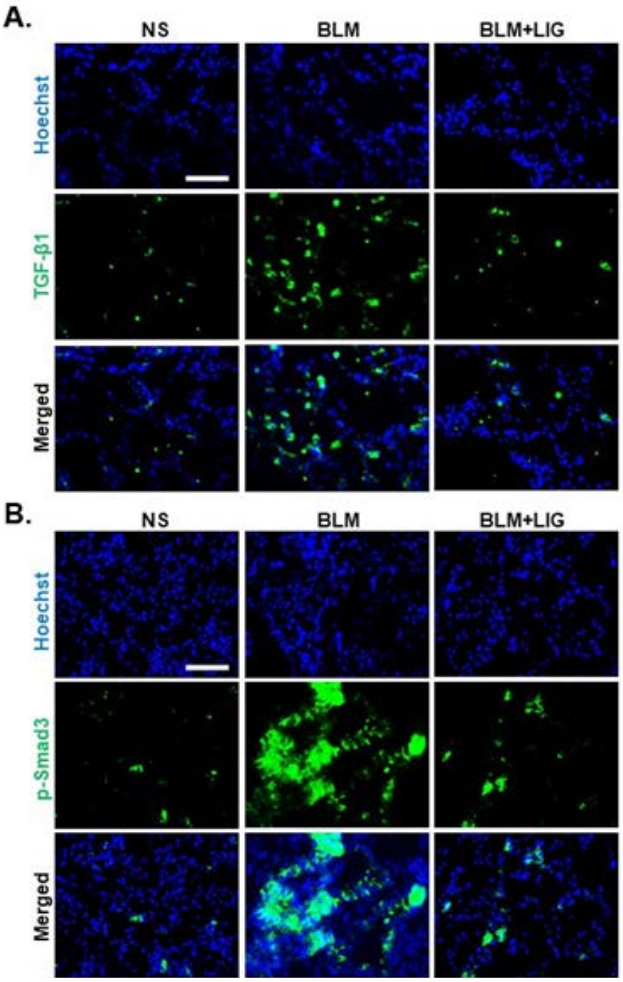

Figure 3: The effect of LIG on TGF-β1 pathway in pulmonary fibrosis induced by BLM.
A: Immunohistochemistry staining of TGF-β1 in the lung tissues of BLM-treated mice, n≥3, scale bar = 200μm
B: Immunohistochemistry staining of pSmad3 in the lung tissues of BLM-treated mice, n≥3, scale bar = 200μm

pulmonary fibrosis might be related to the inhibition of fibroblast activation after TGF-β1 exposed.

Effect of LIG on Nrf2 Pathway and ROS production in Fibroblasts

Considering that oxidative stress might be responsible for the activation of fibroblasts, we determined whether the effect of LIG on fibroblast activation involved in antioxidant effect of LIG. According to the joint analysis of online public database and literature reports, we predicted the pathway related to oxidative stress stimulated by TGF-β1 (Figure 5A). The molecular docking result showed that LIG docks into the cavity of the Keap1 protein with a reasonable fit, and the binding energy between Keap1 and LIG was -6.7 kJ/mol, indicating a strong bond between them (Figure 5B), however, the Nrf2 was not enough to bind with LIG (data not shown). Due to the Nrf2 pathway is a crucial transcription factor under oxidative stress, Nrf2 expression and its target antioxidant genes including NQO1 and HO-1 were evaluated. As showed, LIG raised the protein level of Nrf2, HO-1 and NQO1 (Figure 5C-F) after TGF-β1 exposed. The effect of LIG on ROS production was investigated then. Data showed that LIG reduced ROS production in fibroblasts subjected to TGF-β1 (Figure 5G,H). ML385, an inhibitor of Nrf2, reduced the effect of LIG on the production of ROS, suggesting that the effect of LIG on the oxidative stress might be concerned with the regulation of Nrf2 pathway.

LIG regulated Protein level of Nrf2 pathway in Mice Lung subjected to BLM

To ascertain the crucial role of Nrf2 in anti-fibrosis, the expression of Nrf2 and HO-1 was observed in the lung tissue of mice after BLM exposed then. Compared with the vehicle group, LIG increased the protein level of Nrf2 and HO-1 (Figure 6A,B). These data suggested that LIG restrained lung fibrosis via Nrf2 pathway regulation.

Discussion

In this work, we found that LIG proved survival rate and lung function, and protected against lung damage and collagen deposition after BLM infusion for 2 weeks. Additionally, LIG reduced TGF-β1 induced ROS production and fibroblast activation including cell migration, proliferation and the protein level of collagen I along with Nrf2 pathway activation (Figure 7). Thus, LIG activated Nrf2 pathway to reduce oxidative stress response, thus contributing to inhibit myofibroblast activation in pulmonary fibrosis.

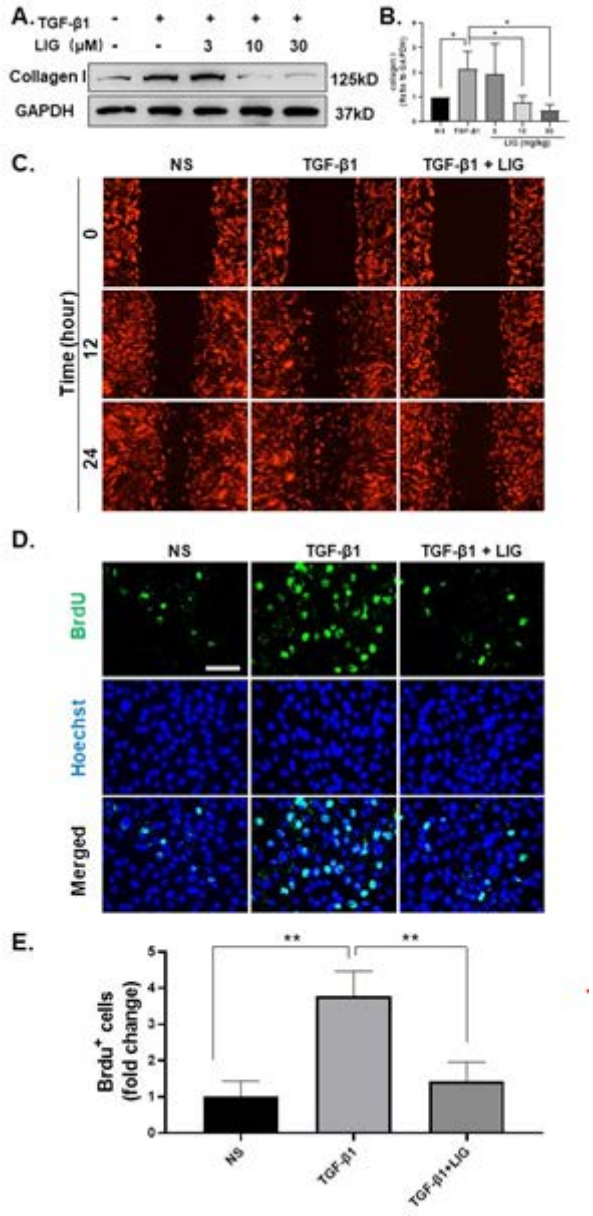

Figure 4: LIG reduces TGF-β1 induced fibroblast activation.
A-B: Western blot assay for collagen I protein level of MLG after TGF-β1 treatment for 24 h, n≥3, **p*<0.05
C: A 2D scratch assay was performed to measure cell migration capacity after TGF-β1 treatment for 24 h, n≥3
D-E: Cell proliferation was assessed for MLG with BrdU assay, n≥3, ***p*<0.01, scale bar = 200μm

Pulmonary fibrosis is characterized by the differentiation of fibroblasts to myofibroblasts followed by excessive ECM deposition [1]. Respiratory function is severely impaired after pulmonary fibrosis, manifested as dry cough and progressive dyspnea. The respiratory function of patients continues to deteriorate with the aggravation of the disease and lung injury. The incidence rate and mortality rate of idiopathic pulmonary fibrosis increase year by year, however, there is a lack of effective drugs and therapeutic strategies to treat it. Thus, it is urgent to find new drugs to treat pulmonary fibrosis.

BLM is a chemotherapy drug, one of which side effects is to lead

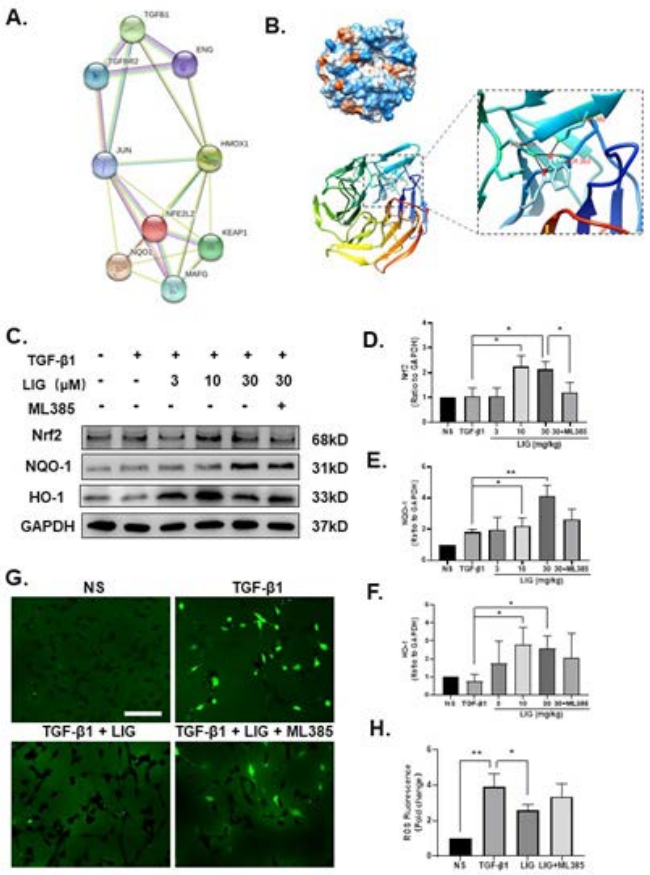

Figure 5: Effect of LIG on Nrf2 pathway and ROS production in fibroblasts .
A: The pathway related to oxidative stress stimulated by TGF-β1
B: The molecular docking result between LIG and Keap1 protein
C-F: Western blot assay for Nrf2, HO-1 and NQO-1 protein level of MLG, n≥3, **p*<0.05, ***p*<0.01
G-H: The effect of LIG on ROS production, n≥3, **p*<0.05, ***p*<0.01s, cale bar = 200μm

pulmonary fibrosis [23]. Therefore, BLM is a widely used inducer for the formation of animal models of pulmonary fibrosis. TGF-β1 is a profibrotic cytokine and obviously increased after BLM treatment, suggesting that TGF-β1 is important during the pathogenesis of pulmonary fibrosis induced by BLM [24]. TGF-β1 is produced in the lungs by various cells, including alveolar macrophages, fibroblasts and activated alveolar epithelial cells [11,25]. Studies have shown that inhibiting TGF-β1-induced fibroblast activation might attenuate lung fibrosis [26,27].

A great many reports have proved that natural bioactive substances have anti-inflammatory, anti-cancer, antioxidant and other physiological activities, and are widely distributed in many kinds of animals and plants, marine organisms and microorganisms [28]. LIG, also known as Angelica phthalide, is the main active component of the volatile oil of Angelica sinensis, an umbrella plant in China. It was reported that LIG have many pharmacological functions including anti-oxidant and anti-inflammatory, while it also plays a protective role in cardiovascular disease. For example, LIG inhibited the production of proinflammatory cytokines and inflammatory pain mediated by microglia [29]. LIG suppressed the SIRT1/NF-κB signaling pathways thereby alleviating podocyte injury [30]. LIG also attenuated intervertebral disc degeneration as well as apoptosis and the

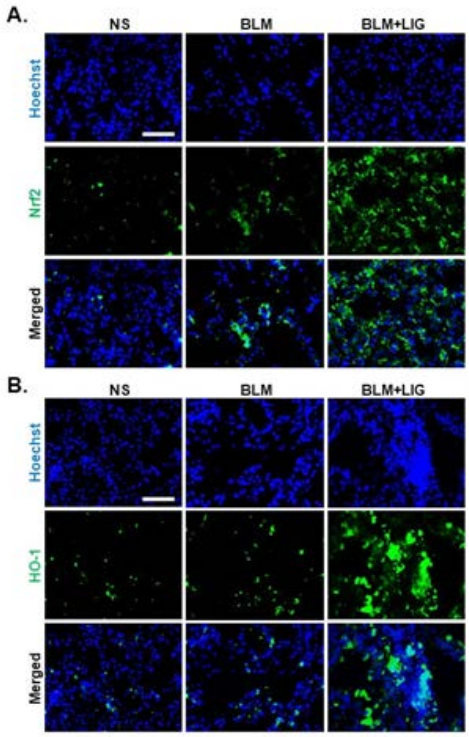

Figure 6: LIG regulated protein level of Nrf2 pathway in mice lung subjected to BLM.
A: Immunohistochemistry staining of Nrf2 in the lung tissues of mice with BLM treatment, n≥3, scale bar= 200μm
B: Immunohistochemistry staining of HO-1 in the lung tissues of mice with BLM treatment, n≥3, scale bar= 200μm

degradation of extracellular matrix in nucleus pulposus cells [18]. LIG regulated GPR30/EGFR pathway thereby promoting bone formation [31]. LIG modulated the activation of PI3K/Akt pathway and thus decreased hippocampal neuronal apoptosis induced by ischemia reperfusion [20]. LIG regulated Nrf2/HO-1 activation and the synthesis of NO in HUVECs [32]. In this work, we found that LIG activated Nrf2 pathway to reduce oxidative stress response, thus contributing to inhibit myofibroblast activation in pulmonary fibrosis.

It is worth noting that, in previous studies, most of drug treatments began at the next day after BLM infusion to evaluate their anti-fibrotic effect. However, in our study, we started drug treatment 2 weeks after BLM stimulation, that is, after the formation of pulmonary fibrosis, which still showed that the drug had the effect of alleviating fibrosis. We know that most clinical patients start treatment after the occurrence of pulmonary fibrosis confirmation by doctors, so the anti-fibrotic effect shown by LIG is more appropriate to the actual clinical situation. PFD has been approved for pulmonary fibrosis treatment, and we chose it as the positive drug. According to literature research, we attempted that the dosage of PFD is 300 mg/kg. Combined with the experimental results, we found that LIG is no worse than PFD in the effect on pulmonary fibrosis, which is mainly reflected in mouse survival rate, mouse weight, mouse lung function and so on. In addition, the dosage of LIG was 90 mg/kg, less than the dosage of PFD. Therefore, our data showed LIG is potential to become a clinical drug for pulmonary fibrosis treatment.

There's a mountain of evidence proving inflammation and oxidant-antioxidant balances disruption in the lung to be involved in pulmonary fibrosis [2,33]. In the process of lung fibrosis, the imbalance

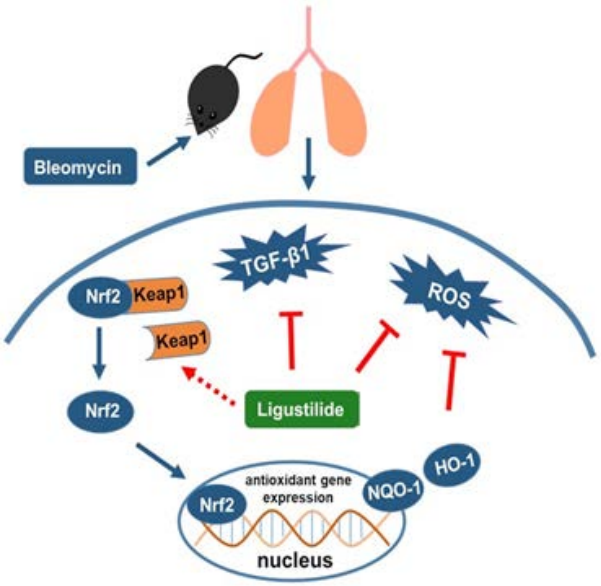

Figure 7: Schematic drawing of the effect of LIG on fibroblast activation in mice lung after BLM infusion.

of oxidant production and antioxidant defences during oxidative stress have been proved to be a significant molecular mechanism and altered levels of antioxidants in the epithelial lining fluid in lungs of IPF patients [34]. Studies showed increased oxidative stress in IPF, meanwhile, antioxidants could prevent pulmonary fibrosis induced by BLM and asbestos [35,36].

Nrf2 promotes downstream antioxidant substances (such as HO-1 and NQO-1) production, indicating a crucial role in resisting oxidative stress damage in cells and tissues [14]. Extensive data indicated that Nrf2 activation decreased the lung damage, and meanwhile HO-1 activity and expression was obviously reduced in the fibrotic lung tissue of bleomycin-treated mice [35,37], indicating Nrf2 a very important role in the pathological progress of pulmonary fibrosis. In our present study, we observed that LIG could raise the decreased protein expressions of Nrf2, HO-1 and NQO-1 in TGF-β1-treated fibroblasts and lung tissue of mice with BLM treatment, suggesting that the Nrf2-dependent antioxidant factor might play an important role in the pathogenesis of pulmonary fibrosis.

A lot of evidence points out that Nrf2 bind with kelch-like ECH associated protein 1 (Keap1) followed by degradation via protein ubiquitination in resting cells [38,39]. The molecular docking result proved LIG docks into the cavity of the Keap1 protein with a reasonable fit, indicating that LIG might increase the Keap1 degradation, and reduce the effect of protein ubiquitination on Nrf2. As a result, LIG might activate Nrf2 pathway and stand up to oxidative stress caused by BLM. However, this is only a conjecture based on molecular docking analysis. Whether it is true or not still needs to be supported by experimental data. Therefore, our data could confirm that LIG protected against oxidative stress induced by BLM stimulation via its regulation on Nrf2 pathway.

In a conclusion, our present study demonstrated that LIG could reduce the formation of pulmonary fibrosis in mice subjected to BLM. The protective effect of LIG might be related to Nrf2 pathway activation. This finding may provide a potential therapeutic treatment for pulmonary fibrosis.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

JW performed experiments of this research, analyzed the data, generated the figures and edited the manuscript. TW, QY, JY, JY, ZJ, JY and YC performed the experiments and assisted with editing. XZ and WL conceived and designed experiments of this research. JC conceived the idea, interpreted the data and directed the project. All authors read, discussed, and approved the final version of the manuscript.

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