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

Jie Chao*[1,3,4,5], Jing Wang[1], Tianqing Wang[1], Qiuchen Yu[1], Juan Yin[1], Jingyan Yong[1], Zexi Jiang[1], Jiangkai Yu[1], Yusi Cheng[1], Wei Luo[1] and Xinxin Zhang[2]

[1]Department of Physiology, School of Medicine, Southeast University, Nanjing, Jiangsu, 210009, China
[2]Department of Histology and Embryology, School of Medicine, Southeast University, Nanjing 210009, PR China
[3]

(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. e mice were intra-tracheally administered 100 μL of bleomycin (2 mg/kg) in all groups except the sham group a er 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 a er 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

A er 14 days of continuous treatment, the mice were euthanized with pentobarbital sodium for pulmonary function tests as described previously [40]. A er euthanized, mice were inserted a tracheal catheter and fastened it to the trachea followed by trachea exposed. en, 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). ree times measurements were operated each mouse. Finally, mice were sacri ced and lung sample collection was performed for further study.

Histopathological Analysis

e lung samples were removed and immediately xed 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.

T1xed96g days. Botwo

Autodock vina (1.1.2) and Chimera (1.15). e Keap1 structure was found in the protein data bank (PDB ID: 3wdz), and then removed the crystal water molecules and other small molecules. e conformational ensemble for LIG to Keap1 protein was generated with the Autodock vina. e 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 xed 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. Di erences were served signi cant 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 rst 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 brosis 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 a er 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 di use 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). e results proved that LIG improved survival rate and lung function of mice a er BLM infusion and protected against BLM induced lung damage, implying that LIG might be a promising drug for the treatment of pulmonary brosis.

LIG reduced Extracellular Matrix deposition in BLM-treated Mice

e process of pulmonary brosis is accompanied by the deposition of extracellular matrix, so the e ect 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 e ect 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 e ect on pulmonary brosis induced by BLM.

e e ect of LIG on TGF-β1 Pathway in Pulmonary Fibrosis induced by BLM

TGF-β1 is key role in the process of pulmonary brosis by promoting broblasts to proliferation, di erentiation and extracellular matrix secretion (7). erefore, the e ect on TGF-β1 pathway in lung of mice subjected to BLM was assessed. As shown in (Figure 3A) BLM signi cantly increased TGF-β1 level in mouse lung compared with the vehicle group, and LIG reduced the e ect of BLM. Smad 3 is an important element in TGF-β1 pathway, and activated a er phosphorylated [12,22]. LIG decreased Smad 3 phosphorylation induced by BLM (Figure 3B). ese data indicated LIG could regulate TGF-β1 pathway activation in pulmonary brosis a er BLM treatment.

LIG reduces TGF-β1 induced Fibroblast Activation

e activation of broblasts plays a signi cant role in the process of pulmonary brosis. To evaluate the protective e ect of LIG on broblast activation induced by TGF-β1, we rst assessed the protein level of cells a er TGF-β1 exposed. Before that, we con rmed that broblasts incubated with LIG (3, 10 and 30 μM) for 24 h showed no cytotoxic e ect on their viability (data not shown). As shown in

pulmonary brosis might be related to the inhibition of broblast activation a er TGF-β1 exposed.

E ect of LIG on Nrf2 Pathway and ROS production in Fibroblasts

Considering that oxidative stress might be responsible for the activation of broblasts, we determined whether the e ect of LIG on

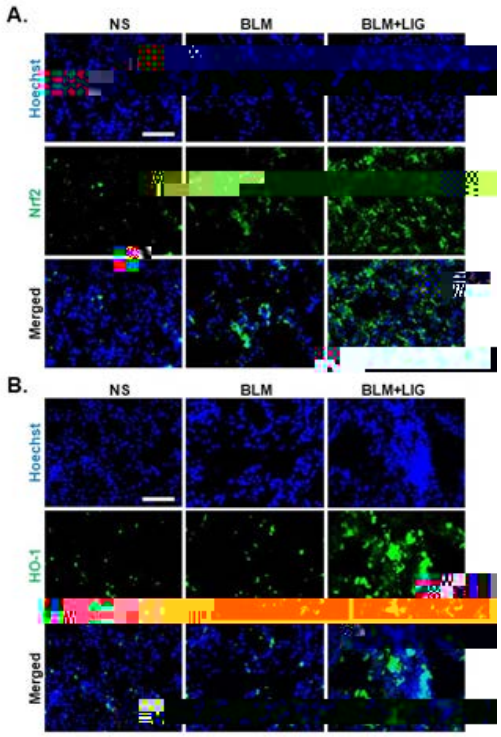

Figure 6: LIG regulated protein level of Nrf2 pathway in mice lung subjected to BLM.
A: Immunohistochemistry staining of Nrf2 in the lung tissues of mice with BLM treatment, nó3, scale bar= 200ɛm
B: Immunohistochemistry staining of HO-1 in the lung tissues of mice with BLM treatment, nó3, scale bar= 200ɛm

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 myo broblast activation in pulmonary brosis.

It is worth noting that, in previous studies, most of drug treatments began at the next day a er BLM infusion to evaluate their anti- brotic e ect. However, in our study, we started drug treatment 2 weeks a er BLM stimulation, that is, a er the formation of pulmonary brosis, which still showed that the drug had the e ect of alleviating brosis. We know that most clinical patients start treatment a er the occurrence of pulmonary brosis con rmation by doctors, so the anti- brotic e ect shown by LIG is more appropriate to the actual clinical situation. PFD has been approved for pulmonary brosis 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 e ect on pulmonary brosis, which is mainly re ected 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. erefore, our data showed LIG is potential to become a clinical drug for pulmonary brosis treatment.

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

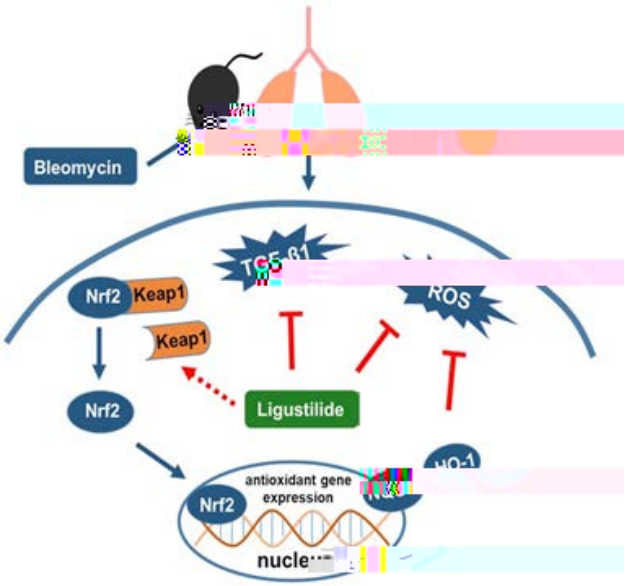

Figure 7: Schematic drawing of the e ect of LIG on ýbroblast activation in mice lung after BLM infusion.

of oxidant production and antioxidant defences during oxidative stress have been proved to be a signi cant molecular mechanism and altered levels of antioxidants in the epithelial lining uid in lungs of IPF patients [34]. Studies showed increased oxidative stress in IPF, meanwhile, antioxidants could prevent pulmonary brosis 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 brotic lung tissue of bleomycin-treated mice [35,37], indicating Nrf2 a very important role in the pathological progress of pulmonary brosis. 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 broblasts 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 brosis.

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]. e molecular docking result proved LIG docks into the cavity of the Keap1 protein with a reasonable t, indicating that LIG might increase the Keap1 degradation, and reduce the e ect 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. erefore, our data could con rm 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 brosis in mice subjected to BLM. e protective e ect of LIG might be related to Nrf2 pathway activation. is nding may provide a potential therapeutic treatment for pulmonary brosis.

Con ict of Interest

e authors declare that they have no con ict of interest.

Author Contributions

JW performed experiments of this research, analyzed the data, generated the gures 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 nal version of the manuscript.

References

1. King TE, Pardo A, Selman M (2011) Idiopathic pulmonary ýbrosis. The Lancet 378: 1949-1961.
2. Heukels P, Moor CC, Von der Thusen JH, Wijsenbeek MS, Kool M (2019) Inþammation and immunity in IPF pathogenesis and treatment. Respir Med 147: 79-91.
3. Schafer MJ, White TA, Iijima K, Haak AJ, Ligresti G, et al. (2017) Cellular senescence mediates ýbrotic pulmonary disease. Nat Commun 8: 14532.
4. Saito S, Alkhatib A, Kolls JK, Kondoh Y, Lasky JA (2019) Pharmacotherapy and adjunctive treatment for idiopathic pulmonary ýbrosis (IPF). J Thorac Dis 11: S1740-S1754.
5. Ogura T, Taniguchi H, Azuma A, Inoue Y, Kondoh Y, et al. (2015) Safety and pharmacokinetics of nintedanib and pirfenidone in idiopathic pulmonary ýbrosis. Eur Respir J 45: 1382-1392.
6. puurRe £ - ã pi ñ S1743

5.