Sinomenine attenuates pulmonary fibrosis by downregulating TGF-β1/Smad3, PI3K/Akt and NF-κB signaling pathways

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Abstract

Background

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive and irreversible interstitial lung disease. Pulmonary fibrosis (PF) has become more common among people severely infected with COVID-19, with IPF being the most common form since this virus became a global epidemic disease in 2019. At present, the etiology is unclear, the treatment methods are limited, and the prognosis is quite poor. Sinomenine (SIN) extracted from the dried stems of *Sinomenium actum*, is used in traditional Chinese medicine to treat several diseases, alleviate liver fibrosis injury, and improve airway remodeling caused by chronic asthma. However, the mechanism used by SIN to combat PF is unclear. Therefore, this work describes the effect of SIN on IPF.

Methods

PF was induced by bleomycin (BLM) in C57BL-6J mice, and then Inflammatory factors, lung histopathological changes, and TGF-β/Smad signaling pathway were evaluated. Serum-starved human embryonic lung fibroblasts (HFL-1) and A549 cells were treated with different doses of SIN. We observed the effects of SIN on HFL-1 and A549 cells, including proliferation and migration, the transformation of fibroblasts into myofibroblasts (FMT), epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) deposition and signal pathways (TGF-β/Smad, PI3K/Akt and NF-κB signal pathways). In addition, the TGF-β receptor inhibitor SB-431542 was used to evaluate not only the classical Smad pathways downstream of TGF-β, but also non-Smad pathways (PI3K/Akt and NF-κB signaling pathways) involved in the process of PF.

Results

The *in vivo* experiments showed that SIN reduced the pathological changes in the lung tissue induced by BLM, reduced the abnormal expression of inflammatory cytokines, and improved the weight and survival rate of mice induced by BLM. The *in vitro* experiments showed that SIN inhibited the migration and proliferation of HFL-1 and A549 cells by inhibiting TGF-β1/Smad3, PI3K/Akt, and NF-κB pathways, prevented the FMT of HFL-1, reversed the EMT of A549 cells, restored the balance of matrix metalloenzymes, and reduced the expression of ECM proteins.

Conclusion

SIN attenuated PF by down-regulating TGF-β/Smad3, PI3K/Akt, and NF-κB signaling pathways, being a potential effective drug in the treatment of PF.
Introduction

The appearance of the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in Wuhan in December 2019 caused more than 641 million cases of severe acute progressive respiratory diseases worldwide and more than 6.6 million deaths by December 2022[1]. Some infected people are still prone to develop interstitial pneumonia, and even life-threatening acute respiratory distress syndrome (ARDS)[2]. COVID-19 is a human pathogen with strong immune escape, transmission, and adaptability, and pulmonary fibrosis (PF) is a recognized consequence of COVID-19[3]. Idiopathic pulmonary fibrosis (IPF) is the most common form of PF, with a median survival time of 2–4 years[4]. The only feasible treatment for patients with end-stage PF is still lung transplantation due to the lack of appropriate drug treatments. The FDA recently approved two new drugs to treat IPF, such as nintedanib and pirfenidone. These drugs, including some glucocorticoids, stabilize the patient’s condition, but they cannot reverse the progress of fibrosis. At present, the drugs for treating PF have side effects on the liver, skin, myocardium, and growth and development of organism which limit their use, especially in patients with liver problems[5]. Therefore, it is of the utmost importance to find new drugs with improved therapeutic effects and fewer side effects.

PF is considered an inflammatory repair mechanism of repeated micro-injury of the alveolar epithelium, which leads to the directional chemotaxis of fibrogenic mediators, specifically activating fibroblasts to differentiate into myofibroblasts and producing extracellular matrix (ECM). The excessive accumulation of ECM destroys the structure of the normal lung tissue, affects gas exchange, and the ECM-cell signaling induces positive feedback in ECM production, which further aggravates the progress of fibrosis[6, 7].

Although the mechanism of PF is not clear at present, many hypotheses have been formulated to explain its mechanism, and among these, that of EMT of alveolar epithelial cells is one of the most substantial. Nuclear factor κB (NF-κB) is an important mediator of EMT. It promotes the transcription of various inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL), and transforming growth factor β (TGF-β), which are strongly related to the progress of IPF, especially TGF-β[8–10]. The classical pathway of TGF-β-mediated fibrosis is related to the Smad protein family signaling pathway. Once the downstream effector complex enters the nucleus, it regulates the expression of EMT-related target genes. However, also non-Smad pathways exist downstream of TGF-β[11]. PI3K/Akt pathway is the key signal node in the process of fibrosis, and it interacts with the TGF-β/Smad signal transduction pathway to alleviate the progress of PF[12, 13]. Therefore, these factors should be considered when evaluating the effect of drugs on IPF.

Sinomenine (SIN) is an alkaloid monomer extracted from the dried stems of Sinomenium actum Rehd.et wils., and is a popular traditional Chinese medicine used to treat rheumatoid arthritis and arrhythmia. Recent studies show that SIN is effective against tumor and inflammatory diseases. It inhibits the migration and invasion of breast cancer cells, prostate cancer cells, and glioblastoma cells[14–16], as well as oxidative stress, inflammation, and apoptosis to alleviate acute liver injury[17]. Moreover, SIN alleviates liver fibrosis and improves airway remodeling caused by chronic asthma by regulating TGF-
β/Smad in vitro and in vivo[18, 19]. However, the anti-fibrosis mechanism of action of SIN on PF is unclear.

This study showed that SIN alleviated the pathological changes in the lung induced by bleomycin (BLM), reduced the abnormal expression of inflammatory cytokines, and improved the weight and survival rate of mice treated with BLM. In vitro experiments showed that SIN inhibited the migration and proliferation of human embryonic lung fibroblast cell line (HFL-1) and human lung adenocarcinoma basal epithelial cell line (A549) cells by the inhibiting TGF-β1/Smad3, PI3K/Akt, and NF-κB pathways, prevented the transformation of HFL-1 into myofibroblasts (FMT), reversed the EMT of A549 cells, restored the balance of matrix metalloenzymes, and reduced the expression of ECM marker proteins. These results support the role of SIN as a potential medicine for IPF treatment.

Materials and methods

BLM-induced IPF mouse model

A total of 36 male C57BL-6J mice (8–10 weeks, 25–28 g) (SCXK(Zhe)2022-0005) were purchased from Hangzhou Qizhen Laboratory Animal Technology Co., Ltd. (Hangzhou, China), and were housed under controlled environmental conditions (22 ℃ and 12:12-hour light-dark cycle). A total of 36 mice were randomly allocated into 6 groups (6 mice per group): Control group, BLM group, B + L group (SIN 50 mg/kg), B + M group (SIN 100 mg/kg), B + H group (SIN 150 mg/kg) and SIN group (SIN 150 mg/kg). Except Control and SIN group, PF was induced with 3mg/kg bleomycin (BLM) (HY-17565; MCE, Monmouth Junction, NJ, USA) preparation solution via Intratracheal injection on the rst day. The mice were injected with SIN or 0.9% NaCl solution (control group) intraperitoneally for two weeks after day 1 of modeling. The body weight was monitored and recorded every day, and the mice were sacrificed at day 14.

Cell culture and reagents

HFL-1 and A549 were purchased from the cell bank of China Academy of Sciences (Shanghai, China). All the cells used were passaged less than 20 times. Both cell types were cultured in Ham's F12K medium (Bio-Channel, Nanjing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, GrandIsland, NY, USA), and incubated in a humid environment of 37 ℃ and 5% CO₂. After adhering to the bottom of the well, the cells in the logarithmic phase were digested with 0.25% trypsin. Then, the cells were diluted to 1×10⁵ cells/mL with a medium containing 10% FBS and seeded into a 6-well plate (1 mL/well, NEST, Wuxi, China). When the cells reached 70–80% confluence, they were divided into six groups: control group (containing 0.5% FBS medium), TGF-β1 group (10 ng/mL TGF-β1), TGF-β1 + SIN (125 µM) group, TGF-β1 + SIN (250 µM) group, TGF-β1 + SIN (500 µM) group and TGF-β1 + SIN (1000 µM) group. All the experiments were carried out in triplicate. After reaching 70% confluence, they were subjected to several analyses. Moreover, HFL-1 and A549 cells in each group were starved in serum-free F12K for 12 h, then 10 ng/mL TGF-β1 (PeproTech, Cranbury, NJ, USA) was added for 12 h to induce fibrosis, and then the cells
were treated with SIN (S235903, Selleck, Shanghai, China) for 12 h. A stock solution of SIN was prepared (solvent: water) at a concentration of 1 mM, stored at -20 °C, and diluted to the specified working solution before each experiment. The TGF-β receptor inhibitor SB-431542 (MCE, Monmouth Junction, NJ, USA) was also used to further explore the mechanism of fibrosis in the cell model, and starved cells were treated with the inhibitor 30 min before TGF-β1 treatment.

**Determination of drug toxicity and cell proliferation rate**

HFL-1 and A549 cells were seeded into 96-well plates (100 µL/well, NEST, Wuxi, China) at a density of 8,000–10,000 cells per well and cultured overnight. When the cells reached 70–80% confluence, they were treated with different concentrations of SIN for 12 h and 24 h. Cell viability was assessed by Cell counting kit – 8 (CCK-8; MCE, Monmouth Junction, NJ, USA) after the addition of 10 µL/well reagent and incubated for 1.5 h.

In addition, A549 and HFL-1 cells were seeded into 96-well plates in the same way as above, and treated with 10 ng/mL TGF-β1 for 12 h to induce EMT after the density was appropriate. A549 cells and HFL-1 cells induced by TGF-β1 were treated with different suitable concentrations of SIN (125, 250, 500, and 1000 µM) for 12 h, and cell viability was measured by CCK-8 reagent used in the same way as above.

**Wound healing assay**

HFL-1 and A549 cells were cultured in F12K supplemented with 10% FBS. After adhesion on the bottom of the well, the cells in the logarithmic phase were digested with 0.25% trypsin. A549 cells and HFL-1 cells were seeded into 6-well plates (1 mL/well, NEST, Wuxi, China) at a density of 10^5 cells per well. They were divided into four groups: control group (without drugs and inducers), TGF-β1 group, TGF-β1 + SIN (500 µM) group and TGF-β1 + SIN (1000 µM) group. Except control group, the A549 and HFL-1 cells induced by TGF-β1 for 12h were cultured in serum-free F12K medium with or without different concentrations of SIN for 6 h or 12 h. A 200 µL micropipette tip was used to generate horizontal scratches in the center of the well. At different time points, the image of scratch width in each well was obtained by optical microscope imaging and analyzed by ImageJ software. The wound healing rate was calculated as follows = (0 h wound width – 6 h/12 h wound width) / 0 h wound width.

**Detection of cytokines in bronchoalveolar lavage fluid (BALF) and serum of mice**

The mice lungs were inflated in situ with 1 mL phosphate-buffered saline (PBS) using intratracheal perfusion, and they were washed by drawing and pushing the syringe three times. The washing fluid was collected as the bronchial alveolar lavage fluid (BALF). Left lungs were flash-frozen in liquid nitrogen for molecular analysis. Right lungs were collected and fixed in formalin for 48 h before being processed to make histological slides. Blood (1 mL) was collected from the inferior vena cava using a 1 mL syringe. Then, BALF was centrifuged (1000× rpm, 5 min) to remove cell debris, and the blood was centrifuged (3000× rpm, 10 min) to obtain the serum. BALF and serum were placed into a 100 µL tube and stored at -80 °C. ELISA kits (EK0527, mouse TNF-α ELISA Kit; EK 0411, mouse IL-6 ELISA Kit; EK0398, mouse IL-2
ELISA Kit; EK0515, mouse TGF-β1 ELISA Kit; Wuhan Boster Biological Technology, Ltd., Wuhan, China) were used for testing the correspondent cytokine according to the manufacturer's instructions. The unit of the calculation result was unified as pg/mL.

**Histology and immunohistochemistry**

The left lung of the mice was fixed in 4% paraformaldehyde for 2 days, embedded in paraffin, and cut into 4 μm-thick slides. After dewaxing and gradient ethanol hydration, the paraffin sections were stained with hematoxylin-eosin (H&E) staining kit or Masson trichrome staining kit. Three slices were randomly selected from each group, and five views were randomly selected from each slice under the microscope at 200x magnification. Assessment of outcome was performed by pathologists blinded to the treatment group, and the degree of lung injury was scored according to a method previously reported. In addition, the Ashcroft score was used to evaluate the PF. As regards immunohistochemistry, 3% hydrogen peroxide solution was added dropwise to tissue sections to quench endogenous peroxidase activity. Then, the slices were immersed in citrate antigen repair solution (AR0024, Wuhan Boston Biological Technology, Ltd., Wuhan, China), and the antigen was repaired by heat-induced epitope repair method. After fully cooling, the slices were incubated at room temperature with QuickBlock™ immunostaining blocking solution (Beyotime, P0260, Shanghai, China) for 15 min to block the nonspecific binding. Subsequently, the blocking solution was removed; the primary antibody was added dropwise; and the slides were incubated at 4 °C overnight. A working solution of a streptavidin biotin complex peroxidase elite rabbit IgG kit (SA1022, Wuhan Boston Biological Technology Ltd., China) was added dropwise and incubated at room temperature for 1 h. The freshly prepared DAB working solution (AR1022, Wuhan Boston Biological Technology, Ltd., Wuhan, China) was dropped on the glass slide and the slides were counterstained with hematoxylin for 2 min. The stained tissue sections were observed under a microscope (Leica, DM3000) and the staining degree was evaluated. The following antibodies were used: mouse monoclonal antibody against E-cadherin (1:200, #14472, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal antibody against vimentin (1:200, #5741, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibody against α-SMA (1:200, #14395-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody against collagen I (1:200, ab34710, Abcam, Cambridge, UK), rabbit monoclonal antibody against phospho-Smad3 (S423/S425, 1:200, ab52903, Abcam, Cambridge, UK), and rabbit polyclonal antibody against TGF-β1 (1:1000, #21898-1-AP, Proteintech, Wuhan, China).

**Determination of hydroxyproline content**

The right lung was accurately weighed according to the instructions of the hydroxyproline test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results were expressed as μg of hydroxyproline/mg of protein.

**Western blot analysis**

Cell and mouse lung were lysed using RIPA buffer (P0013, Beyotime, Shanghai, China) containing PMSF (ST506, Beyotime, Shanghai, China), protease inhibitor, and phosphatase inhibitor (P1050, Beyotime,
Shanghai, China), and centrifuged at 12000 rpm and 4 °C. The concentration of total proteins was
detected by BCA kit (P0009, Beyotime, Shanghai, China). The protein samples were diluted with 5×
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, P0015, Beyotime, Shanghai,
China) and boiled for 5 min. An amount of 30–50 µg of the total proteins was added to each lane of the
SDS-polyacrylamide gel, then they were transferred to a PVDF membrane (Immobilon-P, Darmstadt,
Germany) and incubated with the primary antibody at 4 °C overnight, followed by incubation with HRP-
labeled secondary antibody for 1 h at room temperature. An ECL chemiluminescence detection kit
(P00185, Beyotime, Shanghai, China) was used for visualization and imaging with Tanon 5200
automatic chemiluminescence fluorescence image analysis system (Shanghai, China). The intensity of
the bands was analyzed and standardized by the internal control (GAPDH) of each sample using ImageJ
(Version 1.37c, Bethesda, MD, USA). The following antibodies were used: rabbit monoclonal antibody
against NF-κB(1:1000, #8242, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibody
against Akt(1:1000, #9272, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal antibody
against phospho-Akt(S473,1:1000, #4060, Cell Signaling Technology, Danvers, MA, USA), rabbit
monoclonal antibody against PI3K(1:1000, WL02849, WanleiBio, Shenyang, Liaoning province, China),
rabbit polyclonal antibody against phospho-PI3K (Tyr458/Tyr199, 1:1000, #4228, Cell Signaling
Technology, Danvers, MA, USA), mouse monoclonal antibody against E-Cadherin(1:1000, #14472, Cell
Signaling Technology, Danvers, MA, USA), rabbit monoclonal antibody against Vimentin(1:1000, #5741,
Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibody against α-SMA (1:1000,
#14395-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody against MMP-2(1:1000, #10373-2-
AP, Proteintech, Wuhan, China), rabbit polyclonal antibody against MMP-9 (1:1000, #10375-1-AP,
Proteintech, Wuhan, China), rabbit polyclonal antibody against TIMP1 (1:1000, ab61224, Abcam,
Cambridge, UK), rabbit polyclonal antibody against Collagen I (1:1000, ab34710, Abcam, Cambridge, UK),
rabbit monoclonal antibody against phospho-Smad3 (S423/S425, 1:1000, ab52903, Abcam, Cambridge,
UK), rabbit monoclonal antibody against Smad3(1:1000, #9523, Cell Signaling Technology, Danvers, MA,
USA), rabbit polyclonal antibody against Fibronectin (1:1000, ab2413, Abcam, Cambridge, UK), rabbit
polyclonal antibody against TGF-β1(1:1000, #21898-1-AP, Proteintech, Wuhan, China), and mouse
monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, #60004-1-Ig,
Proteintech, Wuhan, China). The secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:5000,
SA00001-2, Proteintech, Wuhan, China) or HRP-conjugated goat anti-mouse IgG (1:5000, SA00001-1,
Proteintech, Wuhan, China).

RNA isolation and quantitative real-time PCR (qRT-PCR)

At the end of treatment, total RNA was isolated from the mouse lung and HFL-1 cells by RNA-Quick
Purification Kit (ES Science Biotechnology, Shanghai, China) according to the manufacturer’s
instructions. GAPDH was used as the internal control for HFL-1 cells and mouse lung. RNA amplification
kit SYBR® Premix EX TAG™ II (Takara Biotechnology Co., Ltd., Dalian, China) was used and qRT-PCR was
performed using LightCycler® 480 II system (Roche, Alameda, CA, USA). The relative quantification of
mRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method, where Ct is the cycle threshold.
Primers were synthesized by Shanghai Sangon Bioengineering Co., Ltd. The primers used are listed in Table S1.

**Immunofluorescence by confocal microscope**

Cells were seeded in confocal Petri dishes (#801001, NEST, Wuxi, China) and incubated overnight. After EMT induction and SIN treatment, 4% paraformaldehyde (PFA, P0099, Beyotime, Shanghai, China) was added at room temperature for 15 min. Hydrogen peroxide 3% was used to block endogenous peroxidase activity. Cells were treated with Triton X-100 (P0096, Beyotime, Shanghai, China) for 15 min. Then, they were incubated with QuickBlockTM immunostaining blocking solution (P0260, Beyotime, Shanghai, China) for 15 min at room temperature to block nonspecific binding. Afterward, the primary antibody was added and incubated at 4 °C overnight, and the appropriate HRP-labeled secondary antibody was added and incubated at room temperature for 1 h. The nucleus was stained with DAPI solution (P0131, Beyotime, Shanghai, China), incubated for 15 min and the slides were sealed. The glass slide was observed under a confocal microscope (Leica Stellaris) and imaged. The following antibodies were used: rabbit monoclonal antibody against phospho-Akt(S473,1:200, #4060, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal antibody against E-Cadherin(1:200, #14472, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal antibody against Vimentin(1:200, #5741, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal antibody against α-SMA (1:200, #14395-1-AP, Proteintech, Wuhan, China), rabbit polyclonal antibody against Collagen I (1:200, ab34710, Abcam, Cambridge, UK), and rabbit monoclonal antibody against phospho-Smad3 (S423/S425, 1:200 ab52903, Abcam, Cambridge, UK). The secondary antibody was Goat Anti-Rabbit IgG(Dylight 488, Abbkine, Wuhan, China) or Goat Anti-Mouse IgG(Dylight 594, Abbkine, Wuhan, China).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (version 6.01). Student t-test was used for the comparison between two groups, and one-way analysis of variance was used for the comparison among more than two groups. Results are expressed as mean ± SEM of at least three independent experiments (n ≥ 3). A value of p < 0.05 was considered statistically significant.

**Results**

**SIN reduces BLM-induced PF in mice**

A preclinical IPF model was established by BLM to evaluate the potential anti-fibrosis effect of SIN in vivo (Figure A). Mice treated with BLM began to die on day 6. The weight loss trend in the SIN treatment group was less and the weight increased with the increase of the treatment dose at a certain range as compared with the BLM group (Fig. 1B, C), although the dose of 200 mg/kg SIN also showed certain toxic effects (Fig. S1A, B). The mortality of the BLM group on day 10 and 14 was 33–69%. In contrast, the mortality of mice treated with SIN was 12.5–37.5% (Fig. 1C). In addition, the weight of the drug control group
increased rapidly after one week compared with the model group, and the weight gain reached that of the control group. The above findings indicated that SIN reduced BLM-induced PF in mice.

**SIN attenuates lung tissue injury and fibrosis in a dose-dependent manner**

The lungs of mice showed different characteristics. The lungs of the PF model mice are dark red and lack the spongy texture, while the control mice showed pink and soft lungs. Surprisingly, SIN treatment improved the color and texture of the lungs (Fig. 2A). H&E staining showed pathological changes in the lungs, and the normal alveolar structure in the BLM group was blurred or disappeared, with incomplete alveolar shape, diffuse fibrosis, and inflammatory cell infiltration. In addition, SIN helped the lung to keep intact most of the alveolar structure and significantly reduced the fibrotic lesions. The higher the dose, the less proliferating cells (Fig. 2A). The inflammation of the lungs in the SIN plus BLM group was gradually relieved and the degree of relief was dose-dependent, although edema, inflammatory cell infiltration, and vascularization still existed in some areas, as compared with the control group (Fig. 2A). Masson staining showed that the lung in the control group had a small amount of blue staining, which represented the collagen component of the ECM of the normal lung. After 14 days, a large amount of blue staining appeared in the lung of the BLM group mice, suggesting the deposit of a large amount of collagen due to inflammation. With the increase of the SIN dose, the blue staining gradually decreased after treatment. Sirius red staining also showed the same trend as Masson (Fig. S1C). In addition, the changes in the expression of pulmonary fibrosis marker proteins such as a-SMA, collagen I, fibronectin, and connective tissue growth factor are important features of pulmonary fibrosis progress. The immunohistochemical detection of pulmonary fibrosis markers in mice showed that a-SMA, collagen I, fibronectin, and connective tissue growth factor in the lung were significantly increased by a single intratracheal administration of BLM, while the concentration of SIN in the treatment group was negatively correlated with the staining degree of fibronectin. In addition, the intraperitoneal injection of SIN did not affect the expression of a-SMA, collagen I, and fibronectin (brown) (Fig. 2A).

Hydroxyproline content indirectly quantifies collagen deposition, since this amino acid exists almost exclusively in collagen. The collagen deposition in the fibrosis focus of BLM mice increased significantly compared with that in the control mice. The continuous treatment with SIN resulted in a gradual reduction of the collagen deposition. It is worth noting that 150 mg/kg of SIN significantly inhibited the collagen deposition (Fig. 2B). The Ashcroft score and lung injury score also showed that lung inflammation and collagen deposition in the BLM group were consistent with the previous trend (Fig. 2C, D). In addition, the degree of inflammation in the lung assessed by the detection of the expression of the inflammatory factors TNF-α, IL-2, and IL-6 in the BALF revealed that BLM caused their significant increase compared with the control group. However, the release of cytokines was inhibited by SIN, suggesting the role of SIN on inflammation in BLM-induced pulmonary fibrosis (Fig. 2E-G).

**SIN alleviates BLM-induced PF, as well as neutralizes ECM deposition and EMT**
Excessive deposition of ECM is the characteristic of pulmonary fibrosis, and ECM is mainly secreted by fibroblasts and alveolar epithelial cells during pulmonary fibrosis. Under normal circumstances, the ECM in the lung is degraded by matrix metalloproteinases (MMPs), but during the formation of pulmonary fibrosis caused by BLM, ECM deposition is due to the imbalance of MMPs and tissue inhibitors of metalloproteinases (TIMPs)[22]. MMPs are a family of enzymes that degrade collagen, and TIMPs specifically inhibit MMPs activity. TIMP-1 not only inhibits MMPs activity and zymogen activation, but also promotes cell growth factor-like effect, which in turn promotes fibroblast proliferation and collagen synthesis[23]. Western blot results showed that the expressions of fibronectin, collagen I, and α-SMA in the BLM group were significantly higher than those in the control group, and the expressions of these proteins were effectively inhibited after SIN treatment (Fig. 3A, B). Western blot also showed that the expressions of MMP-9, MMP-2, and TIMP-1 in the lungs of mice in the BLM group were significantly increased, and this trend was effectively suppressed by SIN (Fig. 3C, D). This suggests that SIN effectively inhibited the abnormal ECM deposition after BLM-induced pulmonary fibrosis in mice.

The classical markers of EMT such as E-cadherin and vimentin, were assessed by immunohistochemistry to verify whether SIN was able to alleviate pulmonary fibrosis by inhibiting BLM-induced EMT [24]. Immunohistochemistry (Fig. 3E) showed the same trend. The expression of vimentin in the lung significantly increased after BLM induction, while the expression of E-cadherin significantly decreased compared with the control group. However, SIN induces a decrease in the expression of vimentin and an increase in the expression of E-cadherin. These results indicated that SIN reversed EMT in BLM-induced pulmonary fibrosis.

**SIN regulates TGF-β1/Smad3 signaling pathway**

TGF-β pathway is the key axis in the pathogenesis of PF. The quantitative analysis of TGF-β1 by qRT-PCR showed that the transcription of TGF-β1 in the BLM group was significantly increased, and it decreased after the treatment with SIN in a dose-dependent manner (Fig. 4A). In addition, the results of the assessment of TGF-β1 signaling pathway revealed that the expression of TGF-β1 in the lung was significantly increased after BLM induction, and the downstream key protein P-smad3 was significantly activated. SIN induced an inhibition in the expression of TGF-β1 and the hyperphosphorylation of Smad3 induced by BLM (Fig. 4B-D). In addition, the results of BALF and serum of mice showed that the up-regulation of serum TGF-β1 stimulated by BLM was significantly inhibited by SIN treatment (Fig. 4E, F). Similarly, the immunohistochemical analysis of the lung showed that TGF-β1 and P-Smad3 had the same trend in SIN-treated mouse lungs (Fig. 4G). These results suggested that SIN effectively inhibited BLM-induced pulmonary fibrosis in mice by regulating TGF-β1/Smad 3 signaling pathway.

**SIN inhibits the proliferation and migration of HFL-1 and A549 cells induced by TGF-β1**

SIN showed an anti-fibrotic effect in the *in vivo* PF model. Thus, *in vitro* experiments using two cell lines were performed to further explore the anti-fibrotic mechanism. Epithelial cells have the ability to respond
to changes in the microenvironment through EMT. EMT is also the driving force of normal repair, promoting the emergence of myofibroblasts that secrete collagen in IPF. Therefore, the down-regulation of epithelial markers, up-regulation of mesenchymal markers, and ECM deposition are observed during EMT[24].

TGF-β1 is considered a pathogenic cytokine during EMT[25]; thus, in this study, HFL-1 and A549 cells were treated with TGF-β1 to induce EMT \textit{in vitro}. HFL-1 and A549 cells were treated with SIN at different concentrations, and the results revealed that 62.5–1000 µM SIN did not induce evident cell death within 24 h (Fig. 5A, B). Therefore, the dose of 125–1000 µM was selected for all cell experiments. The proliferation of HFL-1 and A549 cells was promoted when treated with TGF-β1, but the proliferation of HFL-1 and A549 cells was inhibited by TGF-β1 combined with SIN in a dose-dependent manner (Fig. 5C, D). The cytoskeleton structure during EMT is remodeled and the cell migration ability is enhanced. Thus, the wound scratch healing was assessed to clarify that the inhibition of SIN on TGF-β1-induced cell proliferation was related to the EMT process. The results showed that the migration of HFL-1 and A549 cells treated with TGF-β1 was increased, but it was inhibited by the treatment of SIN combined with TGF-β1, with a migration inhibition effect that increased in a dose-dependent manner (Fig. 5E-H). These results suggested that SIN inhibited the proliferation and migration of HFL-1 cells induced by TGF-β1.

**SIN prevents TGF-β1-induced fibroblasts transformation into FMT and improves ECM**

The mRNA expression of α-SMA, collagen I, and fibronectin significantly increased in the TGF-β1 group, and decreased after the treatment with SIN in a dose-dependence manner (Fig. 6A). The protein expression of α-SMA in HFL-1 cells increased after TGF-β1 treatment, but it was inhibited after SIN treatment (Fig. 6B). In addition, the expression of α-SMA, collagen I, vimentin, and fibronectin in the TGF-β1 group was significantly higher than that in the control group, and the expression of the above proteins was effectively inhibited by SIN (Fig. 6C, D).

The protein expression of MMP-9 and TIMP-1 in HFL-1 cells induced by TGF-β1 was significantly increased compared with that in the control group, but their expression was down-regulated by SIN (Fig. 6E-G). In a word, the above results suggested that SIN prevented TGF-β1-induced FMT and reduced ECM deposition.

**SIN reverses ECM and EMT induced by TGF-β1 in A549 cells**

The anti-fibrotic effect of SIN on A549 cells was confirmed by the ability of SIN to reduce the expression of α-SMA, fibronectin, and collagen I that were increased by TGF-β1 (Fig. 7A, B). In addition, the protein expression of MMP-9 and TIMP-1 in A549 cells induced by TGF-β1 was significantly increased compared with that in the control group, while their expression was also decreased by SIN (Fig. 7C, D). Moreover, the expression of E-cadherin decreased in TGF-β1 group compared with that in the control group, while the
expression of vimentin increased, while the treatment with SIN combined with TGF-β1 significantly increased the expression of E-cadherin and decreased the expression of vimentin (Fig. 7A, B, E). Finally, the trend after the treatment with SB-431542 (10 mM) combined with TGF-β1 was basically the same as that after the treatment with SIN combined with TGF-β1 (Fig. 7E).

Therefore, the above results suggested that SIN significantly reduced the expression of mesenchymal proteins (α-SMA and vimentin) to prevent FMT in HFL-1 cells, increased the expression of E-cadherin and decrease the expression of vimentin in A549 cells to reverse EMT, and reduced fibrotic proteins (α-SMA, fibronectin and collagen I) in HFL-1 and A549 cells to improve ECM deposition and restore the balance of MMP-9 and TIMP-1 (Fig. 6, 7), indicating that SIN inhibited the process of fibrosis in vitro.

**Effect of SIN on TGF-β1/Smad3, PI3K/Akt and NF-κB pathways in HFL-1 cells induced by TGF-β1**

The phosphorylation level of Smad3, PI3K/Akt, and the protein expression of NF-κB in HFL-1 cells treated with TGF-β1 alone or in combination with SIN were evaluated to explore the regulatory effect of SIN on the signaling pathway in the HFL-1 cells. The results showed that SIN decreased the increase of phosphorylated Smad3, PI3K/Akt, and NF-κB in TGF-β1-induced cells without changing the overall levels of Smad3 and PI3K/Akt (Fig. 8A-E). These results suggested that SIN inhibited TGF-β1-induced fibrosis in HFL-1 cells by inhibiting Smad3, PI3K/Akt, and NF-κB signaling pathways.

**SIN plays an anti-fibrotic role by regulating TGF-β1-induced abnormal activation of Smad pathway and non-Smad downstream pathway.**

The TGF-β receptor inhibitor SB-431542 (10 mM) was added to HFL-1 cells for 30 min before the treatment with TGF-β to further confirm whether Smad3, PI3K/Akt, and NF-κB signal transduction pathways are regulated by TGF-β1. The treatment with SIN (1000 µM) for 12 hours significantly reduced the protein expression of α-SMA, collagen I, and fibronectin induced by TGF-β1, and the degree of inhibition was similar to that induced by SB-431542 combined with TGF-β1 (Fig. 8F, G). Furthermore, SIN combined with TGF-β1 inhibited the expression of MMP-9 and TIMP-1 to some extent compared with TGF-β1, and the degree of inhibition was similar to that of SB-431542 combined with TGF-β1 (Fig. 8F, G). In addition, the expression of collagen I and α-SMA in HFL-1 cells induced by TGF-β1 showed that SIN reversed the fibrosis markers induced by TGF-β1, which was consistent with the above trend (Fig. 8I). Finally, the expression of the core functional proteins P-Smad3 and P-AKT of the above-mentioned pathways were evaluated and the results revealed that SIN decreased the expression of the proteins mentioned above by different degrees, which was similar to the trend obtained by the treatment with SB-431542 combined with TGF-β1 treatment group (Fig. 8F, H, S1E, S1F). These results suggested that SIN played an anti-fibrotic role by regulating the abnormal activation of TGF-β1-induced Smad downstream pathway (TGF-β1/Smad3) and non-Smad downstream pathway (PI3K/Akt and NF-κB).

**Discussion**
IPF is a progressive disease, with most of the patients worsening within 2–3 years after diagnosis, with a mortality rate of 50%[26]. The outbreak of COVID-19 resulted in the occurrence of complications including IPF in critically ill patients, making it one of the main problems that need to be solved and effectively treated[27]. IPF is a progressive disease caused by the continuous injury of the alveolar epithelium, which leads to the continuous activation of repair mechanisms, the uncontrolled proliferation and differentiation of fibroblasts into myofibroblasts, and then excessive proliferation, EMT and ECM production as well as collagen deposition in the affected organs[28]. In the initial stage of IPF, a variety of proinflammatory factors (such as TNF-α, IL-6, IL-1, and TGF-β) and matrix metalloenzymes secreted by fibroblasts participate in the chemotaxis and proliferation of inflammatory cells and the mediation of intercellular interaction, promoting the further progress of inflammatory repair reaction[29]. NF-κB is one of the classical molecules involved in inflammation, usually activated to protect it from pathogens, but its abnormal activation is usually the cause of chronic inflammation.

SIN has anti-rheumatic and pharmacological effects, as demonstrated already in the 70s[30]. At present, SIN has been approved by China Food and Drug Administration (CFDA) for the treatment of rheumatoid arthritis, but its mechanism of action against other diseases has not been fully clarified. Previous studies reported that SIN has anti-inflammatory effects in adjuvant arthritis rats, and it regulates T cells and Th17 cells in intestinal-associated lymphoid tissues[29, 31]. In addition, it inhibits EMT by down-regulating the expression of TGF-β1/Smad3, thus alleviating airway remodeling and renal fibrosis[32, 33]. This study mainly observed the changes in cell proliferation and migration and the expression of specific proteins through in vitro experiments combined with in vivo experiments to confirm the therapeutic effect of SIN on IPF. In addition, our results showed that the anti-fibrotic effect of SIN was related to the regulation of TGF-β1/Smad3, PI3K/Akt, and NF-κB signaling pathways.

Fibroblasts are vital in the formation of IPF structure and in the maintenance of the lung tissue function. The continuous proliferation of fibroblasts combined with alveolar epithelial cells interaction due to the stimulation of cytokines promotes fibrosis [34, 35]. In the process of alveolar epithelial damage and abnormal repair, excessive collagen deposition occurs in the tissue, and fibroblasts are transformed into myofibroblasts, with α-SMA being the main sign of the transformation from fibroblasts to myofibroblasts[36]. Our results showed that SIN inhibited the proliferation and migration of HFL-1 cells in a dose-dependent manner. MMPs are a group of enzymes mainly responsible for the degradation of ECM, but their activity increases during the pathogenesis of PF and the repair or remodeling of inflammatory tissues[34, 37]. TIMPs, which inhibit MMP cleavage activity, are abundant in lung parenchyma of IPF patients and animals with induced PF, which contributes to the failure of degradation of collagen and other ECM components [38]. This study showed that SIN inhibited the abnormal expression of TIMP-1 and MMP-2/9 caused by pulmonary fibrosis and improved the physiological homeostasis of ECM. Therefore, our hypothesis was that SIN restored the imbalance of MMPs/TIMPs ratio caused by PF, delaying its further development.

In addition, SIN treatment significantly reduced the EMT of BLM-induced PF mice. EMT is a key step in the process of PF. Lung epithelial cells are common targets of injury, the driving force of normal repair,
and the key factor of fibrotic lung disease. An important feature of epithelial cells is that they have the ability to respond to microenvironment signals through EMT. EMT regulation consists of a series of key steps to produce pro-inflammatory signals that cause cell damage. EMT is not the transformation from alveolar epithelial cells to fibroblasts, but the ability to reversibly acquire mesenchymal characteristics and enhance mesenchymal crosstalk[6, 39]. In these highly regulated repair pathways, repeated injuries are superimposed with persistent inflammation and hypoxia, which leads to excessive ECM deposition in activated fibroblasts, which in turn destroys normal lung structure and affects gas exchange[7]. Once this positive feedback mechanism is formed, PF continues to progress[40]. Our experiments in vitro and in vivo showed that SIN inhibited the increase of collagen I, fibronectin, and α-SMA protein expression induced by BLM. Furthermore, TNF-α, IL-1β, IL-6, and TGF-β1 in BALF of mice significantly decreased after SIN treatment, as well as TGF-β1 in the serum. The expression of NF-κB protein also significantly decreased in a dose-dependent manner in the SIN treatment group, suggesting that the potential mechanism to inhibit the inflammatory response might be the down-regulation of NF-κB expression and the inhibition of the downstream release of inflammatory cytokines. Interestingly, SIN reversed the expression of E-cadherin and vimentin in A549 cells treated with TGF-β1, and also reduced the expression of vimentin and α-SMA in HFL-1 cells treated with TGF-β1 to inhibit EMT. Our research results showed for the first time that SIN alleviated the damage in the structure of the lung, collagen fiber deposition, and inflammatory cell accumulation induced by BLM, significantly inhibited the release of inflammatory cytokines in the lung, reversed EMT, and improved PF.

Next, the molecular mechanism used by SIN to alleviate PF was investigated. TGF-β/Smad is recognized as a key signaling pathway in the process of fibrosis[41, 42]. Our experiment showed that BLM led to a significant increase in the expression of TGF-β1 and P-Smad3 proteins in the lung, and SIN selectively reduced the accumulation of TGF-β1 and P-Smad3. The cell fibrosis model induced by TGF-β1 is widely used in vitro to explore the anti-fibrotic effect and mechanism of a specific drug[43, 44]. TGF-β1 induced the proliferation of HFL-1 cells in a time-dependent manner, while SIN significantly reduced the cell proliferation induced by TGF-β1 in a dose-dependent manner. Many studies reported that TGF-β/Smad and PI3K/Akt signaling pathways are involved in regulating the formation of PF[45, 46]. Based on the fact that the increased TGF-β1 production in BLM-induced IPF was effectively inhibited by SIN, the anti-fibrotic effect of SIN might be due to the inhibition of Smad and PI3K/Akt signaling pathways. TGF-β1/Smad3 and PI3K/Akt signaling pathways were activated by TGF-β1, and the expression of phosphorylated proteins in Smad3 and PI3K/Akt signaling pathways was significantly inhibited by SIN. This suggested that SIN blocked the TGF-β1/Smad3 and PI3K/Akt signaling pathways. The effect of SIN on the downstream target of TGF-β1 was confirmed using SB-431542 to explore its mechanism. Indeed, the expression of fibronectin, α-SMA, type I collagen, and MMP-related proteins in HFL-1 cells pretreated with SB-431542 was inhibited compared with their expression in the TGF-β1 group, and the same trend was observed in the SIN treatment group, but the degree of inhibition was not complete. NF-κB, P-smad3, and P-AKT were significantly decreased in the inhibitor group, and the slight difference from the treatment group might be due to the interaction and relationship between signaling pathways. The up-regulation of collagen and α-SMA expression in the TGF-β1 group indicated that fibroblasts were transformed into
myofibroblasts, while the expression of the same proteins in the SIN and SB-431542 group was inhibited, indicating that SIN inhibited the differentiation and collagen deposition of HFL-1 cells. These results suggested that SIN also blocked the non-Smad signaling pathways downstream of TGF-β1, including PI3K/AKT and NF-κB pathways, during the treatment of PF.

Overall, our results demonstrated that SIN inhibited TGF-β1/Smad3, PI3K/Akt, and NF-κB pathways to produce its therapeutic effects on PF (Figure. 9). However, considering the shortcomings of the experimental design and the problems to be solved in the future, further studies should be performed to assess whether SIN treatment on TGF-β1-induced cells mediates other mechanisms, such as the expression of autophagy-related proteins downstream of Akt.

**Conclusion**

SIN is a natural monomer used in traditional Chinese medicine that might become an effective drug for treating PF. Indeed, our evidence revealed that SIN might represent a more effective way in the clinical treatment of the consequences of SARS-COV-2 PF, providing ideas for the treatment of IPF with traditional Chinese medicine.

**Abbreviations**

IPF: Idiopathic pulmonary fibrosis; PF: Pulmonary fibrosis; SIN: Sinomenine; BLM: bleomycin; HFL-1: human embryonic lung fibroblasts; FMT: transformation of fibroblasts into myofibroblasts; EMT: epithelial-mesenchymal transition; ECM: extracellular matrix; TGF-β: Transforming growth factor; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; NF-κB: Nuclear factor κB; SARS-CoV-2: the severe acute respiratory syndrome coronavirus type 2; ARDS: acute respiratory distress syndrome; FDA: Food and Drug Administration; TNF-α: tumor necrosis factor α; IL: interleukin; FBS: fetal bovine serum; BALF: bronchoalveolar lavage fluid; ELISA: Enzyme-linked immunosorbent assay; H&E: Haemotoxylin and eosin stain; DAB: 3,3′-Diaminobenzidine; PMSF: Phenylmethanesulfonyl fluoride; BCA: Bicinchoninic acid; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; ECL: Electrochemiluminescence;

**Declarations**

**Author Contributions:** F.Y., M.X., L.D., designed and performed in vitro studies, F.Y., M.X., L.D., designed, performed and analysed in vivo experiments. F.Y., M.X. and L.D. provided guidance on data processing and writing. X.S., Y.S., Y.J., contributed to cell culture and histopathological analysis. F.Y., T.Z., C.Z. and G.Y. contributed to the study design, supervision of the study, draft and review of the manuscript. All authors had full access to the data. All authors have read and agreed to the published version of the manuscript.
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**Availability of data and materials:** All data generated or analyzed in this study are included in this article. Other data that are relevant to this article are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate:**

All animal experiments were approved by Shaoxing People's Hospital Ethics Committee and performed according to the guidelines from the National Institutes of Health. All efforts were made to minimize suffering.

**Consent for publication:** Not applicable.

**Acknowledgments:** Not applicable.

**Competing interests:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**References**


Figures
Figure 1

SIN reduces BLM-induced PF in mice. (A) C57BL-6 J mice were treated with BLM or 0.9% NaCl solution as BLM (control) at a dose of 3 mg/kg; BLM-induced mice were treated with an intraperitoneal injection of SIN (50, 100 and 150mg/kg) or vehicle once a day for 2 weeks from the first day after intratracheal instillation of the drug (vehicle). Lungs were collected at designated time points for H&E staining, Masson staining, BALF, hydroxyproline, and other indicators. (B, C) Body weight of mice monitored every day during the treatment, and assessment of the survival rate in each group. n = 6 mice per group. # p<0.05, compared with control; * p<0.05, compared with BLM; one-way analysis of variance.
Figure 2

SIN attenuates lung tissue injury and fibrosis in a dose-dependent manner. (A) Morphological changes of mouse lung: pink represents normal lung tissue and dark red represents fibrotic lung tissue. Representative micrographs (200x magnification) of paraffin-embedded lung tissue sections stained with H&E and Masson trichrome. Effect of SIN on the expression of collagen I, fibronectin, and α-SMA proteins in the lung of mice after BLM-induced PF on day 14 (200x magnification). (B) Analysis and quantification
of the content of hydroxyproline in the lung of different groups. (C) PF score. (D) Lung injury severity score. (E-G) Concentrations of TNF-α, IL-2, and IL-6 in BALF detected by ELISA kit. n = 6 mice per group. Scale bar: 50 µm. # p<0.05, ## p<0.01, ### p<0.001, compared with control; * p<0.05, ** p<0.01, *** p<0.001, and ns, not significant, compared with BLM; one-way analysis of variance.

Figure 3

SIN alleviates BLM-induced PF, as well as neutralizes ECM deposition and EMT. (A) Western blot image showing the expression of collagen I, fibronectin, and α-SMA in the lung. (B) The expression of collagen I,
fibronectin, and a-SMA in Figure A was normalized to the expression of GAPDH (n = 3). (C) Western blot image showing the expression of MMP-9, MMP-2, and TIMP-1 in the lung. GAPDH was used as the loading control. (D) The expression of MMP-9, MMP-2, and TIMP-1 in Figure C was normalized to the expression of GAPDH (n = 3). (E) Effect of SIN on the expression of E-cadherin and vimentin in the lung of BLM-induced PF mice on day 14 by immunohistochemistry (200x magnification). n = 6 mice per group. Scale bar: 50 µm. # p <0.05, ### p<0.001, compared with control; * p<0.05, ** p<0.01, *** p<0.001, and ns, not significant, compared with BLM; one-way analysis of variance.

Figure 4

SIN regulates TGF-β1/Smad3 signaling pathway. (A) Relative expression of TGF-β1 mRNA in the lung of mice from each group measured by qRT-PCR. (B) Western blot image showing the expression of TGF-β/Smad signaling pathway-related protein P-Smad 3 in the lung. (C-D) The expression of TGF-β1, P-Smad3, and Smad3 in Figure B was normalized to the expression of GAPDH (n = 3). (E) The concentration of TGF-β1 in BALF. (F) The concentration of TGF-β1 in serum. (G) Effect of SIN on the expression of TGF-
β1 and P-Smad3 in the lung of BLM-induced PF mice on day 14 by immunohistochemistry (200x magnification). n = 6 mice per group. Scale bar: 50 µm. # p<0.05, ### p<0.001, compared with control; * p<0.05, ** p<0.01, *** p<0.001, and ns, not significant, compared with BLM; one-way analysis of variance.

Figure 5

SIN inhibits the proliferation and migration of HFL-1 and A549 cells induced by TGF-β1. (A, B) Toxicity of different concentrations of SIN on HFL-1 and A549 cells by CCK-8. (C, D) Effect of different concentrations of SIN (125, 250, 500, and 1000 µM) on the proliferation of different cells after TGF-β1 treatment. (E) Migration and migration width of HFL-1 cells treated with SIN (500 and 1000 µM)
Figure 6

SIN prevents TGF-β1-induced fibroblasts transformation into FMT and improves ECM. (A) mRNA expression of α-SMA, fibronectin, and collagen detected by qRT-PCR. (B) HFL-1 cells treated with TGF-β1 were then treated with different concentrations of SIN (500 and 1000 μM), and the representative image...
of α-SMA protein by immunofluorescence. α-SMA: green; DAPI: blue staining in the nuclei. (C) Expression of α-SMA, collagen I, vimentin, and fibronectin in HFL1 cells treated with SIN by western blot (125, 250, 500, and 1000 μM). (D) The expression of different proteins in Figure C was normalized to GAPDH expression. (E) Expression of MMP-9 and TIMP1 by western blot. (F, G) The expression of different proteins in Figure E was normalized to GAPDH expression. n = 3. Results are shown as mean ± SEM. Statistical analysis was performed by one-way analysis of variance. # p<0.05, ## p<0.01, ### p<0.001, compared with control. * p<0.05, *** p<0.001 and ns, not significant, compared with TGF-β1.
Figure 7

SIN reverses ECM and EMT induced by TGF-β1 in A549 cells. (A) Expression of α-SMA, fibronectin, collagen I, E-cadherin, and vimentin in A549 cells treated with SIN (125, 250, 500, and 1000 μM) by western blot. (B) The expression of different proteins in Figure A was normalized to GAPDH expression. (C) Expression of MMP-9 and TIMP1 in A549 cells by western blot. (D) The expression of different proteins in Figure C was normalized to GAPDH expression. (E) A549 cells were treated with SB431542 as control, and representative images of E-cadherin and vimentin by immunofluorescence in each group. Scale bar: 20 μm. n = 3. Results are shown as mean ± SEM. Statistical analysis was performed by one-way analysis of variance. # p<0.05, ## p<0.01, compared with control. * p<0.05, *** p<0.001 and ns, not significant, compared with TGF-β1.
Figure 8

Effect of SIN on TGF-β1/Smad3, PI3K/AKT and NF-κB pathways in HFL-1 cells induced by TGF-β1. (A) Expression of NF-κB, Smad3, and PI3K/AKT signaling pathway analyzed by western blot. (B-E) The expression of the different proteins in Figure A was normalized to GAPDH expression (n = 3). (F) Expression of fibronectin, α-SMA, collagen I, MMP-9, and TIMP-1 in HFL-1 cells analyzed by western blot in the following four groups. Control group treated with nothing, TGF-β1 group treated with TGF-β1 (10 ng/ml) for 12 hours, the treatment group was treated with SIN (1000 μM) for 12 hours after TGF-β1, and
the TGF-β receptor inhibitor group was not treated with SIN after SB-431542. (G, H) The expression of different proteins in Figure F was normalized to GAPDH expression (n = 3). (I) Representative images showing the co-localization of collagen I (green) and α-SMA (red), and the nuclei stained by DAPI (blue). n = 3. Scale bar: 5 μm. Results are shown as mean ± SEM. Statistical analysis was performed by one-way analysis of variance. # p<0.05, ## p<0.01, ### p<0.001, compared with control. * p<0.05, ** p<0.01, *** p<0.001, compared with TGF-β1.

Figure 9

Summary of the effect of SIN on pulmonary fibrosis. SIN exerts anti-fibrotic effects through regulating TGF-β1/Smad3 PI3K/AKT and NF-κB pathways negatively, and inhibiting epithelial-mesenchymal transition and fibroblast proliferation, reducing collagen deposition, and alleviating the inflammatory response.

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