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Research Paper

Anlotinib attenuated bleomycin-induced pulmonary fibrosis via the TGF-β1 signalling pathway

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Keywords
anlotinib; idiopathic pulmonary fibrosis; inflammation; oxidative stress; TGF-β signalling pathway

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Abstract

Objectives Anlotinib hydrochloride (AL3818) is a novel multitarget tyrosine kinase inhibitor which has the same targets as nintedanib, an effective drug has been approved for the treatment of idiopathic pulmonary fibrosis. Here, we examined whether anlotinib could also attenuate bleomycin-induced pulmonary fibrosis in mice and explored the antifibrosis mechanism.

Methods We have evaluated the effect of anlotinib on bleomycin-induced pulmonary fibrosis in mice. Inflammatory cytokines in alveolar lavage fluid including IL-1β, IL-4, IL-6 and TNF-α were determined by ELISA. Biomarkers of oxidative stress were measured by corresponding kit. Histopathologic examination was analysed by H&E staining and immunohistochemistry. In vitro, we investigated whether anlotinib inhibited TGFβ/Smad3 and non-Smad pathways by luciferase assay or Western blotting. We also evaluated whether anlotinib inhibited TGF-β1-induced epithelial–mesenchymal transition (EMT) and promoted myofibroblast apoptosis in order to explore the possible molecular mechanism.

Key findings The results indicated that anlotinib treatment remarkably attenuated inflammation, oxidative stress and pulmonary fibrosis in mouse lungs. Anlotinib could inhibit the TGF-β1 signalling pathway. Additionally, anlotinib not only profoundly inhibited TGF-β1-induced EMT in alveolar epithelial cells, but also simultaneously reduced the proliferation and promoted the apoptosis in fibroblasts.

Conclusions In summary, the results suggest that anlotinib-mediated suppression of pulmonary fibrosis is related to the inhibition of TGF-β1 signalling pathway.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease caused by multiple causes, mostly in the elder and confined to the lung. The clinical manifestations are progressive respiratory aggravation and decreased lung function, which can lead to respiratory failure and even death. IPF is a clinically heterogeneous disease but its prognosis is overall poor, with a median survival of 3–4 years.[1] Currently, only pirfenidone and nintedanib were approved therapeutics for IPF, but they have limited efficacy,[2] so it is urgently needed to identify new potential therapeutic agents for IPF patients.

The pathogenesis of IPF is not definitively identified but thought to involve excessive inflammation, oxidative stress and chronic or repetitive microinjuries of the alveolar epithelium as triggers of the disease.[3] Certainly, a multitude of profibrotic mediators and signalling pathways are involved in the pathogenesis of pulmonary fibrosis, of which TGF-β is a particularly potent cytokine which can

induce a large number of fibroblasts proliferating and differentiating into myofibroblasts, which show resistance to apoptosis and accumulate at the sites of active fibrosis in fibroblastic foci, leading to excessive production and deposition of extracellular matrix (ECM).[4,5] During the progression of pulmonary fibrosis, epithelial–mesenchymal transition (EMT) also contributes to the expansion of myofibroblasts causing the development and sustainment of the fibrotic process.[6] Therefore, inhibiting the proliferation and activation of lung fibroblasts, promoting the apoptosis of myofibroblasts, and blocking the EMT process are fatal for the treatment of pulmonary fibrosis.

Anlotinib (1-[[[4-(4-fluoro-2-methyl-1H-indol-5-yloxy)-6-methoxyquinolin-7-Yl] oxy] methyl]cyclopropanamine dihydrochloride) (Figure 1) is a multitarget small molecule tyrosine kinase inhibitor that has been approved for the treatment of advanced non-small-cell lung cancer (NSCLC).[7] Its targets are VEGFR, FGFR, PDGFR and c-Kit.[7] At the same time, recent studies suggested that anlotinib has shown a promising antitumor activity against hepatocellular carcinoma (HCC) via inducing HCC cells apoptosis and inhibiting proliferation by suppressing ERK1/2 and Akt pathways.[8] We hypothesize that in the pulmonary fibrosis, anlotinib may also have the activity of inhibiting the proliferation of pulmonary fibroblasts and promoting apoptosis of fibroblasts. More importantly, anlotinib has similar targets with nintedanib, and the IC50 of anlotinib to VEGFR and FGFR is much lower than nintedanib,[9] which indicated anlotinib may have an effect on IPF to some extent. However, no studies have reported the efficacy and pharmacological properties of anlotinib in the treatment of IPF. Here, we firstly explored the therapeutic effect of anlotinib on IPF and its possible mechanisms. The results of this study showed that anlotinib could attenuate bleomycin-induced pulmonary fibrosis through suppressing TGF-β signalling pathway, inhibit EMT and promote myofibroblasts apoptosis in vitro. This will provide a theoretical basis for the clinical development and application of anlotinib for the treatment of pulmonary fibrosis.

Materials and Methods

Antibodies and reagents

The primary antibodies used in the study include p-Smad3, Smad3, p-ERK1/2, ERK1/2, p-Akt, Akt, E-cadherin, vimentin, ZEB1, caspase-3, cleaved caspase-3, PARP and cleaved PARP all purchased from Cell Signaling Technology (Danvers, MA, USA); the antibodies to α-SMA, fibronectin, collagen I, GAPDH and β-tubulin were purchased from Affinity Biosciences (Cincinnati, OH, USA). Human TGF-β1-mammalian was purchased from PeproTech (Rocky Hill, CT, USA). Anlotinib hydrochloride was manufactured by YU ANG Biotech Co., Ltd. (Shanghai, China), purity greater than 99%.

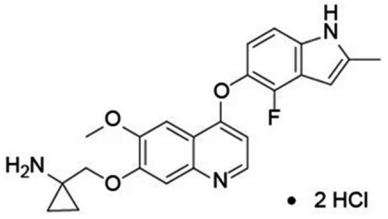

Figure 1 Molecular structure of Anlotinib Dihydrochloride.

Animals

Male C57BL/6 mice of about 6–8 weeks were purchased from Beijing Academy of Military Sciences (Beijing, China). Mice were housed in a room at a temperature of 20–25 °C and a humidity between 40% and 70%, with 12-h light/dark cycle. Mice were free to eat and drink. Mice were randomly divided into normal saline group, bleomycin group and anlotinib group, with 6 mice in each group. All experimental protocols were approved by the Animal Experiment Committee of Tianjin International Joint Academy of Biomedicine (approval no. SYXK(JIN)2017-0003). All efforts were made to minimize suffering.

Cell culture and treatments

All cells were obtained from ATCC (Manassas, VA, USA). CAGA-NIH3T3 cells were cultured in DMEM (contain PS; Solarbio, Beijing, China), A549 were cultured in RPMI medium 1640 (contain PS; Solarbio), and HFL1 cell lines were cultured in F12K medium (Gibco, Grand island, NY, USA), both supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel) at 37 °C with 5% CO_2. After 24 h of serum starvation, 5 ng/ml TGF-β1 was supplemented to induce lung fibroblast activation and EMT. In most experiments, cells were maintained in media supplemented with TGF-β1 with or without anlotinib hydrochloride (1 μM) for 24 h to evaluate the effect of anlotinib on cell proliferation, apoptosis and EMT. When studying the effects of anlotinib on TGF-β1/Smad3, TGFβ1/MAPK (ERK1/2 and P38), PI3K/Akt and other signalling pathways, the cells were preincubated for 2 h with anlotinib before treatment with exogenous TGF-β1.

Luciferase assay

The CAGA-NIH3T3 cells stably transfected with the TGF-β1/Smad3 signalling pathway reporter gene plasmid were cultured in a 96-well plate. When the confluence of cells reached 80%, the serum was removed and the cells were

starved for 24 h. Then, cells were treated with 5 ng/ml TGF-β1 with or without anlotinib (concentration of 8, 4, 2, 1, 0.8, 0.4, 0.2, 0.1 μM) for 18 h. Luciferase activity was tested using the Glomax Multi+ Detection System.

Quantitative real-time PCR

Total RNA was isolated from cells by the guanidinium isothiocyanate acidic phenol chloroform method using TRIzol (Invitrogen Corp., Carlsbad, CA, USA). RNA was reverse-transcribed using the FastKing gDNA Dispelling RT SuperMix (TIANGEN Biotech, Beijing, China). Real-time qPCR analysis was run on the Roche system using SYBR Green PCR Master Mix (Roche, Basel, Switzerland). Each measurement was repeated in triplicate and normalized to the corresponding GAPDH content values. The primers optimized for real-time PCR assays are listed in Table 1.

Western blotting

Cells were lysed in RIPA (Beyotime Biotechnology, Shanghai, China), and protein concentrations were determined using the BCA Protein Assay Kit (Beyotime Biotechnology). Equal amounts of proteins (50 μg) were resolved by SDS-PAGE, and the proteins were transferred to a PVDF membrane and incubated with the appropriate antibodies. The protein bands were visualized using an enhanced chemiluminescence system (Affinity Biosciences, Cincinnati, OH, USA). β-Tubulin and GAPDH were used as loading control.

Immunofluorescence staining

The A549 cells were fixed in 4% paraformaldehyde, stained with specific primary antibodies, including those against E-cadherin and vimentin, at 4 °C overnight and then incubated with FITC-conjugated anti-rabbit IgG (Solarbio). The nuclei were stained with DAPI. Representative micrographs were observed using a confocal laser scanning microscope (Leica, Wetzlar, Germany).

Cell apoptosis analysis

The impact of anlotinib on cell apoptosis was determined by immunoblot analysis on related proapoptotic gene (cleaved caspase-3) and further evaluated by flow cytometry. Briefly, Mlg cells were seeded into a 6-well plate and treated with TGF-β1 or sorafenib for 24 h. Thereafter, after washing with precooling PBS, the cells were fixed with 70% ethanol for 24 h. Finally, quantitative analysis of cell apoptosis was carried out by BD FACSCalibur flow cytometer (BD Biosciences, New York, NY, USA) staining with an Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology). The data were analysed using FlowJo 7.6.1 software.

BLM-induced pulmonary fibrosis model

According to the sequence of events in bleomycin-induced pulmonary fibrosis described by Antje Moeller *et al*.,[10] after administration of bleomycin, there is the onset of an acute inflammatory response lasting up to 8 days, and the fibrogenic changes occur after day 8. So we have established bleomycin-induced early inflammation model and bleomycin-induced pulmonary fibrosis model testing the anlotinib intervention both towards injury and inflammation and the potential antifibrotic activity. At day 0, C57BL/6 mice were intratracheally injected with 2 mg/kg BLM (Nippon Kayaku Co. Ltd, Tokyo, Japan) after anaesthesia with an intraperitoneal injection of 10% chloral hydrate (5 ml/kg). Saline group received normal saline by the same procedure. In the bleomycin-induced early inflammation model, mice received normal saline or anlotinib by gavage once per day at a dose of 3 mg/kg body weight from day 1 to day 7 after bleomycin administration. At day 8, the mice were sacrificed, and their alveolar lavage fluid and lung tissues were collected to analyse the levels of inflammatory factors and biomarkers of oxidative stress. In the bleomycin-induced pulmonary fibrosis model, normal saline or anlotinib was applied intragastrically from day 7 to day 13. At day 14, the forced vital capacity (FVC) of mice was recorded by Animal Lung Function Testing instrument, and the lung tissues were taken for the determination of hydroxyproline and histopathology.

Histological and immunohistochemical analyses

The lung tissues were fixed in 4% paraformaldehyde, dehydrated in a graded alcohol series and embedded in paraffin

Table 1 Primer sequences for real-time PCR

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (3′–5′)
Human α-SMA	CTATGAGGGCTATGCCTTGCC	GCTCAGCAGTAGTAACGAAGGA
Human fibronectin	CGGTGGCTGTCAGTCAAAG	AAACCTCGGCTTCCTCCATAA
Human E-cadherin	CACGGTAACCGATCAGAATG	ACCTCCATCACAGAGGTTCC
Human vimentin	TGCCGTTGAAGCTGCTAACTA	CCAGAGGGAGTGAATCCAGATTA
Human GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

blocks. Five-micron-thick sections were then stained with haematoxylin and eosin (H&E; Solarbio) for routine examination. The histologic severity for fibrosis was quantified by Ashcroft scoring system.[11] In brief, the degree of fibrosis ranges from grade 0 (normal lung) to grade 8 (fibrous obliteration). The average score of each microscopic field in the entire lung slide was used as the fibrosis score.

For immunohistochemistry assays, paraffin-embedded lung tissue sections were deparaffinised and antigen-retrieved. The following experimental steps were performed according to the instructions of UltraSensitive™ s-p immunohistochemical staining kit (MAIXIN.BIO, Fuzhou, China). Sections were stained with DAB solution (MAIXIN.BIO) and counterstained using haematoxylin. Eight images per slide and six slides per treatment group were quantified by Image Pro Plus 6.0 for integral optical density (IOD) and the positive area (Area). The mean optical density (MOD = IOD/Area) was calculated to evaluate the expression of α-SMA and collagen I.

Hydroxyproline determination

The right lung tissues of the mice in bleomycin-induced pulmonary fibrosis model were isolated for determining the Hyp levels according to a modified method described by Dong Y *et al.*[12]

Pulmonary function test

After being anaesthetized, the mice were fixed to the operating table and kept in a supine position. When the trachea is exposed, the intubation is inserted into the trachea and fixed with cotton thread. The forced lung capacity (FVC) of mice was measured after the mice were transferred to the body description platform.

ELISA for the detection of inflammatory factors

The levels of IL-1β, IL-4, IL-6 and IFN-γ in BALF of bleomycin-induced inflammation model groups were determined by enzyme-linked immunosorbent assay (ELISA) kit. Strictly follow the instructions of the ELISA kit (BioLegend, San Diego, CA, USA).

Measurement of oxidative stress

The lung homogenates were centrifuged at 4 °C, and the supernatant was retained for testing. The levels of superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and malondialdehyde (MDA) in lung tissue were measured according to the instructions of detection kit (Solarbio).

Statistics

All statistical analyses were performed using GraphPad prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as means ± SEM. All statistical comparisons were analysed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test to identify significant differences between groups. $P < 0.05$ was considered to be statistically significant.

Results

Anlotinib attenuates bleomycin-induced pulmonary fibrosis in mice

We have evaluated the effect of anlotinib on bleomycin-induced pulmonary fibrosis in mice. The histological changes at 14 days are shown in Figure 2a. In the saline group, there was no obvious inflammatory reaction and fibrosis in the lung tissue of mice, and the lung tissue structure was normal. After bleomycin injected, not only the airway wall was significantly thickened, but also the alveolar structure was disordered. Meanwhile, the lung tissue of mice showed obvious alveolar inflammation, and some alveoli appeared realistic changes and fibrosis. In addition, the mice with BLM injection showed higher Ashcroft score than in control group. After anlotinib intervention, the above lesions were alleviated, and the Ashcroft score was significantly reduced (Figure 2b). We also observed that the content of hydroxyproline in the left lung of mice in bleomycin group was significantly higher than that in saline group, as shown in Figure 2c. In anlotinib-treated group, the hydroxyproline content decreased, indicating that anlotinib inhibited ECM deposition. We also evaluated the effect of anlotinib on the forced vital capacity in bleomycin-induced pulmonary fibrosis mice. The results showed that anlotinib increased the lung capacity of mice (Figure 2d).

α-SMA was a generally acknowledged biomarker of myofibroblast activation, and collagen I was an important indicative factor of ECM deposition in pulmonary mesenchyme. The expression of α-SMA and collagen I was observed in the alveolar and interstitial space of the lung by immunohistochemistry on day 14. The results are illustrated in Figure 3; in normal lung tissue (NaCl group), immunostaining could be evidently observed in tracheal and vascular wall, and interstitial area was exceedingly weakly positive. In fibrotic lung tissues (BLM group), α-SMA and collagen I were highly expressed in the interstitial as seen by the large positive immunolocalization area in lung sections. Evidently, the expression of α-SMA and collagen I was reduced when treated by anlotinib compared with BLM group.

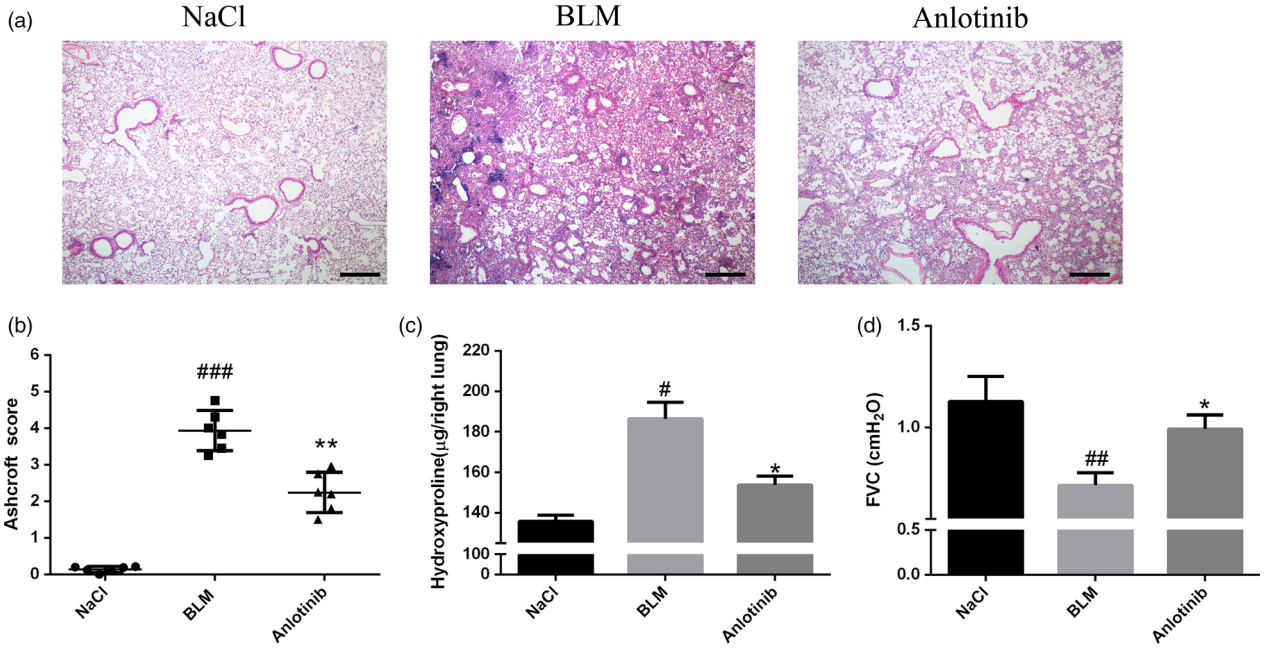

Figure 2 Anlotinib improves BLM-induced pulmonary fibrosis in mice. (a) The representative photographs of the effects of anlotinib against BLM-induced pathological changes (HE stain. Bars, 100 μm). At day 14, interstitial fibrosis was evident in mouse's lung from the BLM-treated group, compared with NaCl group. The pathological changes were obviously relieved in the lungs of the anlotinib-treated group. (b) Degree of lung fibrosis was quantified by Ashcroft score system. (c) The hydroxyproline content of the right lung of mice. (d) The forced vital capacity of the mice. (Values are expressed as mean ± SEM and $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs BLM-treated group.)

Anlotinib suppresses pulmonary inflammation and oxidative stress in a mouse model of bleomycin-induced lung injury

Injecting bleomycin into the trachea of mice can cause early lung damage. During this process, excessive free radicals are produced, leading to inflammatory reactions, accompanied by the activation of inflammatory cells and release of inflammatory factors. A large number of inflammatory cells infiltrate into the pulmonary interstitium or alveolar, and are activated to release excessive oxygen radicals, which react with surrounding biomacromolecular substances (such as nucleic acid, protein, lipid) for oxidation. We successfully established a bleomycin-induced early inflammatory model in mice to assess the ability of anlotinib to inhibit inflammatory responses and improve oxidative stress. As shown in Figure 4a–4d, compared with the normal saline group, the content of inflammatory factors IL-1β, IL-4, IL-6 and TNF-α in alveolar lavage fluid increased significantly after the injection of bleomycin, while the content decreased significantly after the administration of anlotinib. Additionally, we also observed that MDA and ROS levels were significantly elevated in the lung tissue homogenate of the bleomycin group, in which SOD and T-AOC levels were decreased. In contrast, after anlotinib administration, the levels of MDA and ROS were significantly reduced, while the levels of SOD and T-AOC were increased (Figure 4e–4g). Therefore, anlotinib can inhibit bleomycin-induced inflammatory response and enhance antioxidant capacity.

Anlotinib antagonizes TGF-β1-Smad and non-Smad signalling in vitro

Transforming growth factor-β1 (TGF-β1) is considered to be the most critical fibrogenic factor involved in the development of IPF, and its function depends on the signal transduction and regulation of Smad and non-Smad.[13,14] Smad3 is the main activation protein of TGF-β/Smad signalling pathway, regulating a series of gene expression, and promotes fibroblast differentiation and proliferation.[13] We used the constructed CAGA-NIH3T3 cell line stably transfected with the TGF-β1/Smad3 signalling pathway reporter plasmid to detect the effect of anlotinib hydrochloride on TGF-β1/Smad3 signalling pathway. As shown in Figure 5a, luciferases assay showed that anlotinib inhibited the TGFβ1/Smad3 signalling pathway in a dose-dependent manner. According to the results of Luciferases assay, 1 μM was selected as

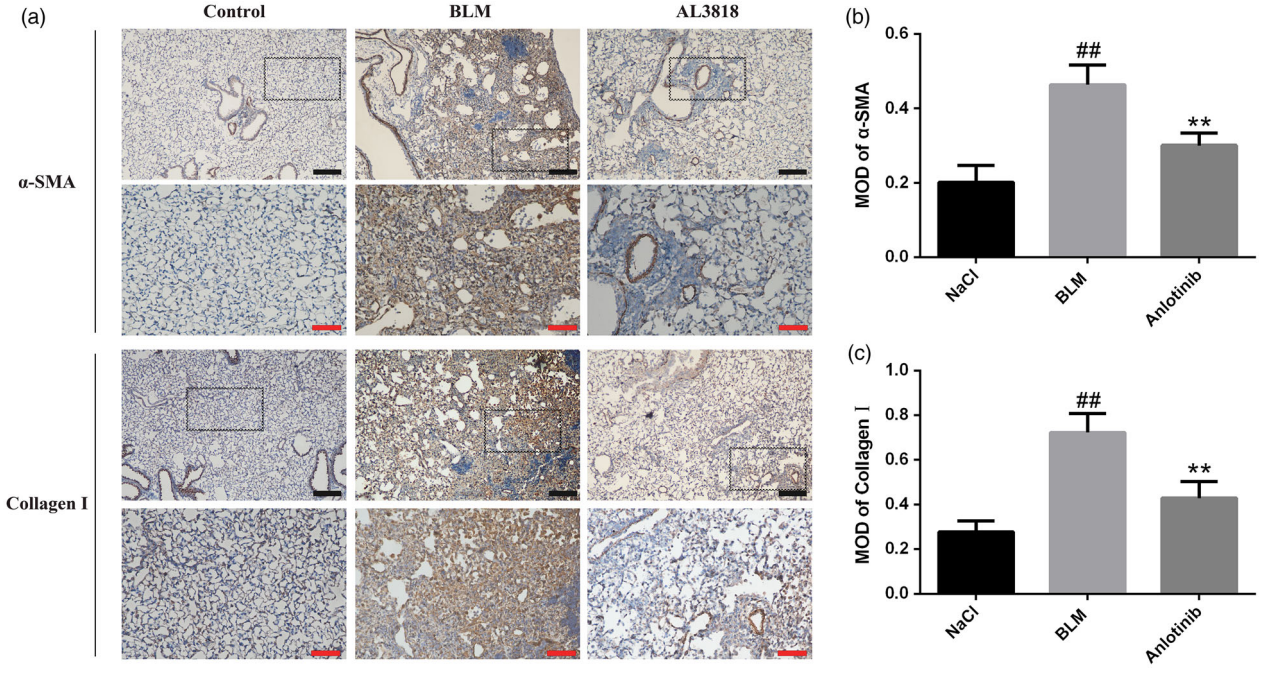

Figure 3 (a) Immunohistochemistry for α-SMA and collagen I (Brown area) in bleomycin-induced pulmonary fibrosis in mice on day 14. Representative images of lung sections from each group are shown. Dashed boxes on top panels indicate region shown at higher magnification in bottom panels. Magnification = ×200 (top panels), ×400 (bottom panels). (b) The mean optical density (MOD) of positive areas was quantified by Image Pro Plus 6.0 to evaluate the levels of α-SMA and collagen I expression. (Data were shown as mean ± SEM and $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs BLM-treated group.)

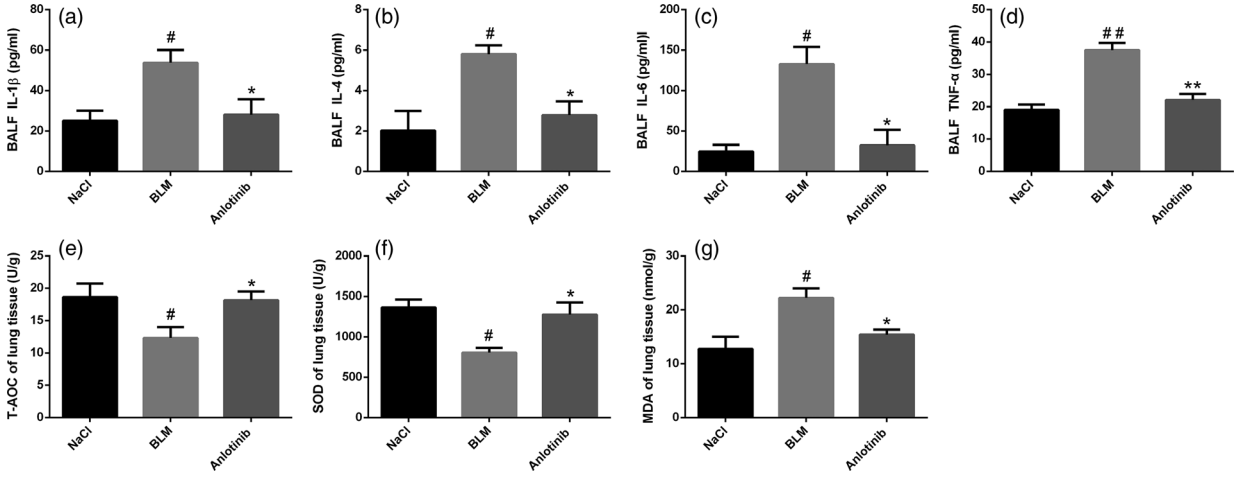

Figure 4 (a–d) Effects of anlotinib on inflammatory mediators in BALF. Cytokines in BALF were determined using ELISA. (e–g) Effects of anlotinib oxidative stress induced by BLM. (a–g) The experiments were performed three technical replicates and two biological repeats. (Data were shown as mean ± SEM and $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs BLM-treated group.)

the best effective concentration for subsequent experiments. Further, we observed that anlotinib hydrochloride significantly inhibited TGF-β1-induced phosphorylation of Smad3 protein, as shown in Figure 5b.

In addition to the TGF-β/Smad signalling pathway, non-Smad pathways, including the MAPK pathway and the PI3K/AKT pathway are also involved in the formation of pulmonary fibrosis.[15,16] Therefore, we examined whether

anlotinib can inhibit TGF-β1-induced phosphorylation of p38, ERK1/2 and AKT. The results showed that anlotinib inhibited TGF-β1-induced phosphorylation of p38, ERK1/2 and AKT in lung fibroblasts (Figure 5c). In conclusion, anlotinib may exert antipulmonary fibrosis by blocking TGF-β1-mediated Smad and non-Smad signalling pathways.

Anlotinib counteracts TGF-β1-induced EMT in A549 cells

Epithelial–mesenchymal transition is a process in which epithelial cells are morphologically transformed into a mesenchymal cell phenotype. This process is closely related to the development of pulmonary fibrosis and is one of the important sources of myofibroblasts.[17] When EMT occurs, the phenotype of the cells changes, and the expression of E-cadherin, which make the epithelial cells tight, is reduced. The expression of E-cadherin, which is tightly linked to the epithelial cells, is decreased, and the expression of vimentin in the interstitial cell type is increased.

In pulmonary fibrosis, the key fibrogenic factor TGF-β1 induces the occurrence of EMT. Based on this mechanism, we evaluated the effects of anlotinib on TGF-β1-induced EMT in A549 cells. As shown in Figure 6a and 6b, real-time PCR indicated that the transcription level of E-cadherin was decreased after TGF-β1 stimulation, while the transcription level of vimentin was increased, which was consistent with the results of Western blot (Figure 6c). Anlotinib treatment could increase the expression of E-cadherin, decrease the expression of vimentin and significantly reduce the expression of transcription factor ZEB1. We further confirmed by immunofluorescence that anlotinib increased E-cadherin expression and inhibited TGF-β1-induced vimentin expression (Figure 6d). Therefore, anlotinib can attenuate pulmonary fibrosis by inhibiting TGF-β1-induced EMT.

Anlotinib inhibits cell activation and induces progressive apoptosis in human lung fibroblasts

In pulmonary fibrosis, fibroblasts proliferate and differentiate into myofibroblasts, which characteristically express α-SMA and produce excessive ECM, accelerating the progression of pulmonary fibrosis. Therefore, inhibiting the activation of fibroblasts, or removing activated fibroblasts, which leads to reduced ECM deposition, is an effective strategy for the treatment of pulmonary fibrosis. We observed that anlotinib inhibited TGF-β1-induced expression of the myofibroblast activation marker α-SMA and the extracellular matrix-associated protein fibronectin (Figure 7a–7c), indicating that anlotinib can stifle the activation and proliferation of fibroblasts.

Although inhibiting the proliferation of fibroblasts/myofibroblasts and reducing the secretion and deposition of ECM can alleviate the progression of pulmonary fibrosis to a certain extent, fibroblasts/myofibroblasts have strong proliferation and anti-apoptotic ability, so it is very difficult to completely inhibit their proliferation. Therefore, effectively

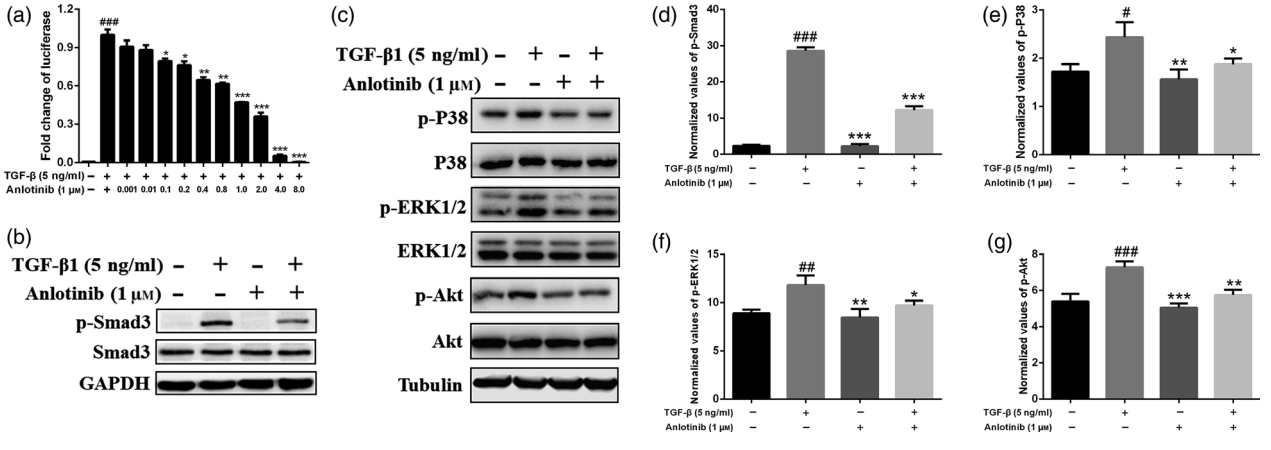

Figure 5 Anlotinib antagonizes TGF-β1-mediated Smad and non-Smad signalling. (a) The CAGA-NIH3T3 were cells treated with anlotinib (8, 4, 2, 1, 0.8, 0.4, 0.2, 0.1 μM) and 5 ng/ml TGF-β1 for 18 h. Luciferase activity was tested using the Glomax Multi+ Detection system. (b and d) HFL1 cells were treated with anlotinib(1 μM) for 2 h, then stimulated with 5 ng/ml TGF-β1 for 30 min and assayed by Western blot. The bar graph showed p-Smad3 expression after normalization with Smad3 expression. GAPDH was used as a loading control. (c) Assessing the phosphorylation of p38, Akt and ERK1/2 under the same condition. (e–g) Quantification of p-p38, p-Akt and p-ERK1/2 expression is achieved using densitometric values, respectively, normalized to p38, Akt and ERK1/2 levels. Tubulin was used as a loading control. (a) The experiments were performed three technical replicates and two biological repeats. (b and c) The experiments were performed three times. (Data were shown as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs TGF-β1 group (untreated).)

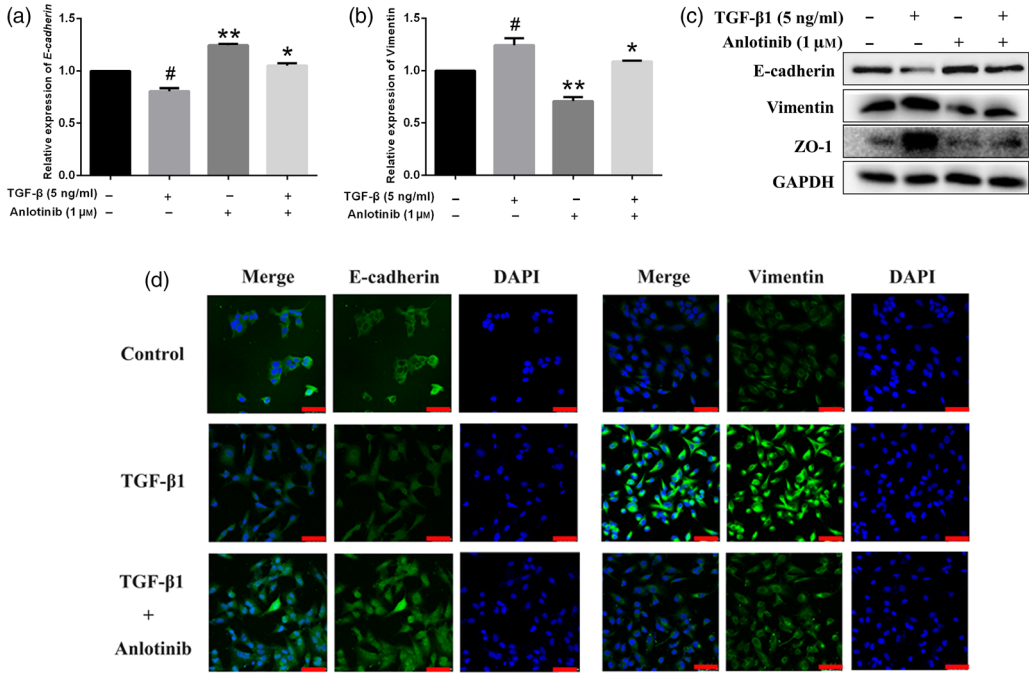

Figure 6 Anlotinib counteracts TGF-β1-induced EMT in A549 cells. (a and b) A549 type II alveolar cell line was treated with anlotinib (1 μM) and TGF-β1 (5 ng/ml) for 24 h. Real-time PCR analyses the mRNA transcription level of E-cadherin and vimentin. GAPDH was used as a loading control. (c) Under the same processing conditions, Western blot analyses of E-cadherin, TCF8/ZEB1 and vimentin expression in A549 cells. GAPDH was used as a loading control. (d) A549 cells were grown on coverslips and treated with TGF-β1 (5 ng/ml) and anlotinib (1 μM) for 24 h. Cells were fixed and stained with vimentin and E-cadherin antibodies followed by incubation with fluorescently tagged secondary antibodies. DAPI was used to stain nuclei. Vimentin (green), E-cadherin (green) and nuclei (blue) were visualized by confocal fluorescence microscopy (400×). (a, b and d) The experiments were performed two technical replicates and three biological repeats. (c) The experiments were performed three times. (Data were shown as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs TGF-β1 group (untreated).)

promoting the apoptosis of fibroblasts/myofibroblasts and fundamentally eliminating fibroblasts/myofibroblasts are of great value in the treatment of pulmonary fibrosis. As shown in Figure 7c, treatment with anlotinib has produced the cleaved forms of caspase-3 and PARP, which are considered reliable markers of apoptosis. More importantly, Figure 7c showed no changes in cleaved caspase-3 and cleaved PARP with the treatment of anlotinib alone compared with the control group, which indicated that anlotinib had no effect on the activity of normal lung fibroblasts, but only promoted the apoptosis of activated fibroblasts under pathological conditions. We further used flow cytometry to analyse the apoptosis of fibroblasts, and the results showed that the apoptosis of fibroblasts was inhibited after TGF-β1 exposure. With the treatment of anlotinib, the percentage of apoptosis cells was significantly increased (Figure 7d). Taken together, these data suggested anlotinib can not only inhibit the proliferation and activation of fibroblasts, but also promote the apoptosis of lung fibroblasts.

Discussion

Idiopathic pulmonary fibrosis is a chronic progressive interstitial lung disease of unknown origin which leads rapidly to death. Currently, BLM-induced pulmonary fibrosis is the most classic model for exploring the pathogenesis of IPF and testing the effectiveness of new antifibrotic drugs.[18] Recent studies have shown the mechanism of bleomycin-induced pulmonary fibrosis: ANXA2(membrane-associated protein A2) is a specific bleomycin target, and bleomycin binding with ANXA2 blocks TFEB (transcription factor EB)induced autophagic flux and further promotes apoptosis of epithelial cells, leading to pulmonary fibrosis.[19] In short, BLM-induced fibrosis animal model mimics the clinicopathological features of IPF, which is an effective model to test the effects of novel pharmacological agents. In this study, we established two models: bleomycin-induced inflammatory model and pulmonary fibrosis model to evaluate the antifibrotic effects of anlotinib. First, in inflammatory

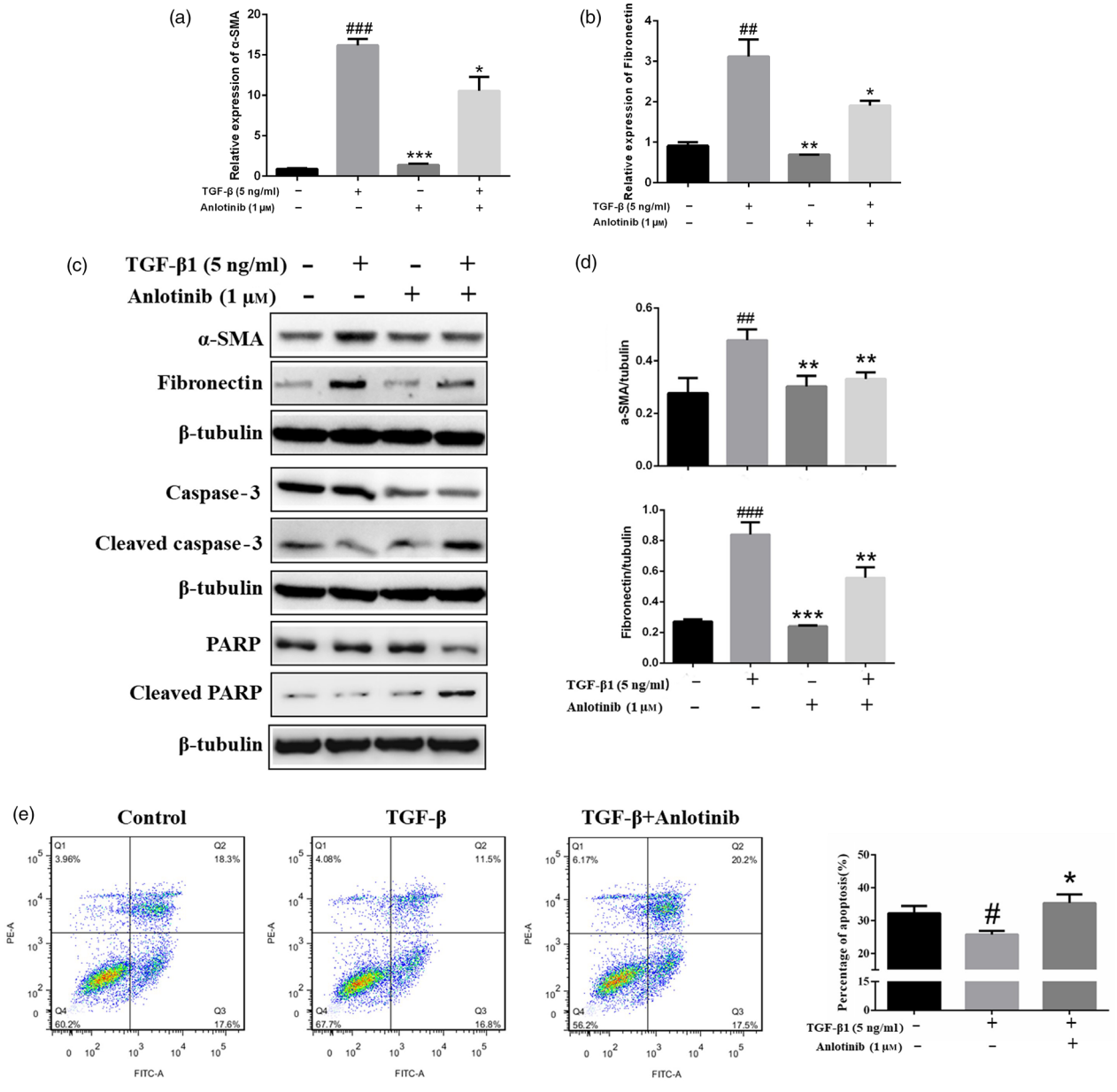

Figure 7 Anlotinib inhibits lung fibroblast excitation and promotes apoptosis. HFL1 cells were treated with anlotinib (1 μM) and the presence or absence of TGF-β1 (5 ng/ml) for 24 h. (a and b) Real-time qPCR on related genes, including α-SMA and fibronectin. (c) Cell proliferation and apoptosis were measured by Western blot. Quantification of α-SMA and fibronectin expression is achieved using densitometric values as shown in (d). β-Tubulin was used as a loading control. (e) Quantitative analysis of cell apoptosis was carried out by flow cytometry. Identified by flow cytometry, cells were divided into four sections: Q1: Annexin V-FITC− PI+ was representative of mechanical error; Q2: Annexin V-FITC+ PI+ was representative of late apoptosis or necrosis cells; Q3: Annexin V-FITC− PI− was representative of living cells; and Q4: Annexin V-FITC+ PI− was representative of early apoptosis cells. (a, b and e) The experiments were performed two technical replicates and three biological repeats. (c) The experiments were performed three times. (Data were shown as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs control group,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs TGF-β1 group (untreated).)

models, we found that anlotinib significantly reduced inflammatory cytokines such as IL-1β, IL-4, IL-6 and TNF-α, which have been demonstrated that were enhanced levels in the lungs of patients with IPF.[20–22] In addition, anlotinib not only reduced lipid peroxidation levels, but also enhanced SOD activity and total antioxidant capacity in mouse lung tissue. It is suggested that anlotinib may inhibit early lung injury through

suppressing inflammatory response and oxidative stress. Subsequently, in the bleomycin-induced pulmonary fibrosis model, anlotinib significantly normalized the alveolar structure and reduced the area of pulmonary fibrosis, as demonstrated by histopathological sections. In addition, anlotinib can also reduce hydroxyproline content, which means that collagen secretion and ECM deposition are reduced. Similarly, it has been proved in immunohistochemical sections. We have also observed that it can improve lung function in mice with pulmonary fibrosis. In the bleomycin-induced pulmonary fibrosis model, anlotinib showed a favourable antifibrotic effect, which laid a foundation for us to study the pharmacological mechanism intensively.

Although the pathogenesis of pulmonary fibrosis is not clear at present, there is evidence that cytokines play a key role in the development of pulmonary fibrosis. They regulate each other and restrict each other, forming a complex network and participating in the process of pulmonary fibrosis.[23] TGFβ1 is currently the most potent profibrogenic cytokines and is recognized as a key factor involved in the development of pulmonary fibrosis. In patients with IPF, TGF-β1 is mainly secreted by pulmonary macrophages, alveolar epithelial cells, which can induce EMT in alveolar epithelial cells, stimulate fibroblast proliferation and activation, and promote myofibroblast formation and ECM deposition.[24] It mainly induces pulmonary fibrosis through the TGF-β1/Smad3 signalling pathway, so we have tested whether anlotinib can exert antifibrotic effects by blocking the TGFβ1/Smad3 signalling pathway. As we expected, anlotinib can significantly inhibit TGF-β1/Smad3 signalling pathway.

In addition to relying on the Smad pathway, TGF-β1 can also trigger pulmonary fibrosis by activating the non-Smad signalling pathway (ERK1/2, P38MAPK and PI3K/Akt). Among them, ERK1/2 and P38 belong to the classical pathway of MAPK signalling pathway. Different growth factors and cytokines are the major activators of the ERK1/2 signalling pathway, such as TGF-β, FGF and PDGF. ERK1/2 signalling pathway plays an important role in the proliferation and apoptosis of pulmonary fibroblasts.[25] If the signal pathway activity is limited, fibroblasts proliferation and ECM deposition can be suppressed. As an important member of the MAPK family, the p38 signalling pathway regulates inflammatory reaction and wound healing was regulated by controlling the activation of transcription factors and the synthesis of inflammatory factors.[26] Simultaneously, Xia H and colleagues have found that PI3K and Akt were abnormally activated in the fibroblastic foci of human IPF tissue.[27] In addition, the PI3K/AKT signalling pathway interacts with the p38MAPK signalling pathway, which plays a complementary role in the development of pulmonary fibrosis.[28] We have observed that anlotinib could inhibit Smad3, ERK1/2, p38 and AKT signalling pathways. Therefore, anlotinib may be inhibited early events in TGF-β1 Signalling, such as inhibiting phosphorylation of the TGF-β receptor. In addition, tyrosine kinase receptors such as VEGFR, PDGFR and FGFR can also activate MAPK and PI3K/AKT signalling pathways.[29] In this way, anlotinib, a tyrosine kinase receptor inhibitor may also inhibit the MAPK and PI3K/AKT signalling pathways by blocking the tyrosine kinase receptor in pulmonary fibrosis. It is worth considering that there are substantial evidences from preclinical studies that some tyrosine kinases inhibitor, such as nintedanib, sorafenib, thalidomide and sunitinib, can also strongly inhibit the TGF-β1/Smad3 signalling pathway,[30–33] suggesting that there is a special connection between the tyrosine kinase pathway and the TGF-β1 signalling pathway. Further research is underway to elucidate how specific tyrosine kinases interact with the TGF-β1 signalling pathway, contributing to the pathogenic processes in lung fibrosis, which may elucidate the pathogenesis of pulmonary fibrosis and confirm additional targets for therapeutic interventions.

In addition, we observed that anlotinib inhibits TGF-β-induced proliferation of NIH3T3 fibroblasts and reduces ECM deposition. Although this can alleviate the progression of pulmonary fibrosis to some extent, it is very difficult to completely inhibit the proliferation of fibroblasts due to their strong proliferation capacity. And the key effector cells, myofibroblasts, cannot be fundamentally removed, which has a strong anti-apoptotic effect and can induce apoptosis of alveolar epithelial cells, thereby accelerating the progression of pulmonary fibrosis.[34] So, it is of great value in the treatment of pulmonary fibrosis to effectively inhibit the source of myofibroblasts or promote the apoptosis of myofibroblasts. There are studies indicate that when pulmonary fibrosis occurs, myofibroblasts, in addition to the proliferation and differentiation of fibroblasts originating in the lungs and the circulating fibroblasts derived from bone marrow, can also be transformed from alveolar epithelial cells by EMT.[6] We observed that anlotinib suppresses the transformation of A549 into a mesenchymal phenotype, which may be related to the inhibition of TGF-β1/Smad, MAPK and PI3K/Akt signalling pathways involved in the regulation of EMT. Besides, we also tested whether anlotinib could promote apoptosis of myofibroblasts and found that it could block the cell cycle of myofibroblasts and promote its apoptosis. Kim and colleagues demonstrated that the activation of the PI3K/AKT pathway by TGF-β1 was, at least in part, necessary for myofibroblast growth and anti-apoptotic effects.[35]

Therefore, we believe that anlotinib facilitates the apoptosis of myofibroblasts mainly by inhibiting the activation of AKT induced by TGF-β1. Besides anlotinib, several tyrosine kinase inhibitors originally used to treat tumours have shown to reduce pulmonary fibrosis in animal models, for example nintedanib, imatinib, gefitinib and sorafenib, all exerting antifibrotic effects by suppressing the activation of TGF-β signalling.[30,36,37] However, only nintedanib has a positive efficacy and safety, and benefit patients with IPF in clinical trials. Thus, it can be seen that challenges and hopes may coexist in the course of clinical translation. In view of the IC50 of anlotinib to VEGFR and FGFR is much lower than nintedanib, we believe that our findings will be promising for the consideration of anlotinib as an antifibrotic drug. However, the bioavailability and side effects of anlotinib and nintedanib could also affect the efficacy of the drugs in vivo, so it is necessary to further compare the treatment effects of the two drugs at the same experiment condition to determine whether anlotinib is safe and effective agent for the treatment of pulmonary fibrosis.

Conclusions

In summary, we demonstrated that anlotinib not only ameliorated BLM-induced pulmonary fibrosis in mice, but also suppressed the profibrogenic activity of TGF-β1 signalling in vitro. Therefore, anlotinib may be a potential agent for the treatment of IPF.

Declarations

Conflicts of interest

The Authors declare no conflict of interest.

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Author contributions

X.H.L. conceived and designed the experiments; H.R., Z.W.L. and S.S.Z. performed the experiments, analysed the data and wrote the manuscript; S.S.L. and L.Z. performed experiments and collected data; K.H., S.Y.G., W.H.G., X.W.L. and K.Y.H. performed experiments; and H.G.Z. and C.Y. administered and supervised the experimental work. All authors read and approved the final manuscript.

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