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Zanubrutinib Attenuated Bleomycin-Induced Pulmonary Fibrosis by Inhibiting the TGF- β 1 Signaling Pathway

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and fatal interstitial lung disease with high mortality and limited treatment. So far, the only drugs approved for the treatment of IPF are Nintedanib and Pirfenidone. Zanubrutinib, a BTK small molecule inhibitor, is approved for the treatment of mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL). In this study, we explored the potential effect and mechanisms of zanubrutinib on pulmonary fibrosis in vivo and in vitro.

Methods: In the in vivo experiments, different doses of zanubrutinib were administered in a mouse model of bleomycin-induced pulmonary fibrosis, and pathological manifestations and lung function indexes were evaluated. The in vitro experiments were used a TGF- β 1-treated fibroblast model to evaluate the effect of zanubrutinib on the activation and autophagy phenotype of fibroblasts and explored the underlying signaling pathways mechanism.

Results: In vivo experiments proved that zanubrutinib effectively attenuated bleomycin (BLM)-induced pulmonary fibrosis in mice. In vitro mechanism study indicated that zanubrutinib could suppress collagen deposition, myofibroblast activation by inhibiting the TGF- β 1/Smad pathway and induce autophagy through the TGF- β 1/mTOR pathway.

Conclusions: Zanubrutinib could alleviate bleomycin- induced lung fibrosis in mice by inhibiting the TGF- β 1 signaling pathway.

Key Words: Pulmonary fibrosis; Zanubrutinib; TGF- β signaling pathway; BTK; myofibroblasts; Autophagy

1. Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and fibrotic interstitial lung disease characterized by fibrosis and inflammation in the lung interstitium and surrounding airspace [1]. Patients with idiopathic pulmonary fibrosis generally use high-resolution chest CT (HRCT) as the diagnostic gold standard and the most obvious pathological manifestations are diffuse or patchy shadows of the lung, as well as bronchial traction and dilation [2]. The median survival time of IPF patients is only

2.41 years and the incidence of IPF is increasing year by year[3]. The specific pathogenesis of IPF has not been clarified so far [4]. It is generally believed that regenerative repair and scar repair are out of balance, and eventually lead to pulmonary fibrosis [5, 6]. Currently, there are only two drugs approved for the treatment of IPF, Nintedanib and Pirfenidone, and they cannot significantly delay the survival of patients. There is an urgent need to continue to develop effective therapeutic drugs.

Transforming growth factor- β 1 (TGF- β 1) was a core regulator in the development of pulmonary fibrosis [7]. Active TGF- β 1 may induce myofibroblast activation, migration and proliferation [8]. Phosphorylated smad2 and smad3 as the downstream of TGF- β are motivated by TGF- β 1 to regulate fibroblast activation and extracellular matrix (ECM) deposition [9]. Autophagy is an important process involving in cell growth, survival, development and death [10]. mTOR signal is the core regulatory signal pathway of autophagy, inhibiting mTOR signal can induce the formation of autophagy [11]. mTOR signal is mediated by many upstream proteins, TGF- β 1 can activate mTOR signal [12]. The overexpression of ECM proteins is a fatal factor in pathogenesis of pulmonary fibrosis, and its levels could be down-regulated by autophagy [13]. Therefore, reducing the activation of fibroblasts and extracellular matrix deposition induced by TGF- β 1 and increasing the level of autophagy in fibroblasts are important means to treat fibrosis.

Zanubrutinib (ZB, BGB-3111) is an FDA-approved irreversible inhibitor of Bruton's tyrosine kinase (BTK). BTK is a key kinase in the B cell receptor (BCR)

signal transduction pathway, involved in the proliferation, differentiation and apoptosis of B cells [14]. ZB is a second-generation BTK inhibitor with increased specificity to BTK. The role of this greater selectivity toward BTK aims to increase potency of the drug and reduce off-target effects [15]. ZB is clinically used for the treatment of Mantle cell lymphoma (MCL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) [16]. It has been reported that Ibrutinib, the first-generation small molecule inhibitor of BTK, can aggravate fibrosis [17]. However, another study reported that the expression of BTK in B cells is increasing in IPF patients [18]. Therefore, the relationship between BTK and pulmonary fibrosis is controversial. Our study evaluated the role of second-generation BTK inhibitor ZB in bleomycin-induced pulmonary fibrosis in mice, and further explored the mechanism of ZB in pulmonary fibrosis *in vivo and in vitro*.

2. Materials and methods

2.1. Animal Model of Pulmonary Fibrosis

Male C57BL/6 mice (6- to 8-week old; 20–25 g body weight) were bought from Charles River Laboratories (Beijing, China). All animal care and experimental procedures complied with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (Permit No. SYXK 2014-0003). Mice were housed under controlled temperature (22–26°C), humidity (60 ± 2%) and a 12 h light–dark cycle. During the whole experiment, the mice were free access to food and water.

The modeling method of the animal model of pulmonary fibrosis is according to the

procedures previously described[19]. In briefly, 42 mice were randomly divided into 6 groups: control group, BLM group, BLM + Nintedanib group (100 mg/kg), BLM + Zanubrutinib group (5 mg/kg), BLM + Zanubrutinib group (10 mg/kg), BLM + Zanubrutinib group (20 mg/kg). Nintedanib was used as the positive control. For control group saline was injected intratracheally. Intratracheal injection of 2.5 U/kg BLM (Hanhui Pharmaceuticals CO., LTD, China) in other groups. Zanubrutinib and nintedanib were given by gavage once a day from the seventh day of BLM injury. The mice were sacrificed under anesthesia 14 days after the BLM injection to evaluate the degree of pulmonary fibrosis.

2.2. Cell Culture

NIH3T3 and the mouse lung fibroblast (Mlg) cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were cultured in DMEM (Solarbio, Beijing, China) containing 10% FBS (Gibico, Carlsbad, CA, USA) and 1% penicillin–streptomycin (Gibico, Carlsbad, CA, USA).

2.3. Western Blotting

All proteins were extracted from cells and lung tissues as reported previously[20]. The proteins were separated by the polypropylene gel according to the molecular weight and transferred to polyvinylidene difluoride (PVDF) membrane (Roche, Switzerland).

After incubating with 5% skimmed milk at room temperature for 30 minutes, incubate the primary antibody overnight at 4 degrees. Then, the membranes were incubated with HRP-conjugated secondary antibodies (Abcam, UK) for 2 h at room temperature

and tested with chemiluminescence reagent (Affinity, USA). The following primary antibodies (Table 1) were used to detect the protein expression levels.

Table 1. The list of primary antibodies.

Antibody	Company and Item No.	Antibody	Company and Item No.
GAPDH	Affinity, AF7021	p-Smad3 (Ser425)	Affinity, AF3362
β -tubulin	Affinity, T0023	Smad3	Affinity, AF6362
α -SMA	Affinity, AF1032	p-Smad2(S465+Ser467)	CST, 18338S
Collagen I	Proteintech, 66761-1-Ig	Smad2	Affinity, AF6449
Fibronectin	Proteintech, 15613-1-AP	P62	Affinity, AF5384
S6RP	CST, 5G10	mTOR	CST, 2983
p-S6RP(S235/236)	CST, 4858T	p-mTOR(S2448)	Affinity, AF3308
P-ULK1(Ser757)	CST, 14202T	p-p70 S6K(Thr289/412)	Affinity, AF3228
ULK1	CST,8054T	p70 S6K	Affinity, AF6226

2.4. Real-Time Quantitative PCR

Total RNA was extracted from cells using trizol (Thermo Scientific Inc, Waltham, MA, USA) according to the manufacturer's instructions. The generation of cDNA from the total RNA was performed using the Reverse SYBR Select Master Mix kit (Tiangen, Beijing, China), followed by fluorescence quantitative real-time PCR (Yeasen, Shanghai, China). The list of gene primers was as follow.

Table 2. The list of gene primers.

Gene (Mouse)	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Gene accession number
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α -SMA	CTGGTGATGATGCTCCCA	GCCCATTCCTCAACCATTACTCC	NM_007392.2
Collagen I	CCAAGAAGACATCCCTGAAGTCA	TGCACGTCATCGCACACA	NM_007742.4
Fibronectin	AAGGATGGAGTGATAGCAACCC	TCTGCTTGAAATCTGGTGTGC	NM_001276413.1
GAPDH	TGGATTTGGACGCATTGGTC	TTTGCACTGGTACGTGTTGAT	NM_001289726.1

2.5. MTT Assay

The MTT assay can be used to detect the toxicity of Zanutrutinib to cells. NIH3T3 cells were seeded in 96-well plates. After incubation of TGF- β 1 (5 ng/mL) with/without Zanutrutinib (12.5-1600nM) for 24 h, 20 μ L MTT (0.5 mg/mL) was added. After four hours, use a pipette to remove the supernatant, add 120ul of DMSO to dissolve the precipitated crystals. Then, the OD540 nm was measured to reflect the relative number of cell.

2.6. Wound-Healing Assays

Spread NIH3T3 and Mlg cells in a six-well plate for Wound-Healing Assays. Use a 200ul pipette tip to draw a line in the middle of each hole of the six-well plate. After washing with phosphate buffer, add TGF- β 1 (5 ng/mL) or Zanutrutinib (100 nM, 200 nM, 400 nM) and take images at 0h, 6h, 12h, 24h to observe cell migration by using a light microscope (Nikon, Tokyo, Japan).

2.7. Immunofluorescence

This experiment used the NIH3T3 cell line. All the steps are the same as previously reported[21]. For microtubule-associated protein 1 light chain 3 (LC3)-B plasmid transfection, according to the supplier's instructions (SinoBiological, Beijing, China), 1.5 mg GFP-LC3 and 2 mg Cherry-GFP-LC3 plasmids incubate with PEI in

serum-free Opti for 20 minutes at room temperature respectively. Then they were transfected into NIH3T3 cells. After adding TGF- β 1 (5 ng/mL) or Zanubrutinib (100 nM, 200 nM, 400 nM) for 24 hours, fix the cells and stain the nucleus with DAPI. The fluorescent was examined with the confocal microscope (Nikon, Japan).

2.8. Immunohistochemistry

Use the UltraSensitiveTM SP (Mouse/Rabbit) IHC Kit and DAB Kit (Maxim, Fuzhou, China) according to the instructions to detect the expression of the measured makers . Randomly select three fields of view for each slice for positive area statistics.

2.9. Hydroxyproline content determination

Hydroxyproline was tested according to the methods as described previously [22]. Adjust the PH to 7 after dissolving the three lung lobes on the right of the mice with hydrochloric acid. The results were calculated as μg hydroxyproline per right weight using hydroxyproline standards (Sigma, USA).

2.10. Histological examination

The left lung of the mice is filled with 10% formaldehyde and embedded in paraffin to prepare tissue sections with a thickness of 5 μM . Tissue sections for H&E and Masson staining (Solarbio, Shanghai, China). Images were collected using a fluorescence microscope (Nikon, Japan). Pulmonary fibrosis area was evaluated as described previously [23].

2.11. Evaluation of Pulmonary Function

Pulmonary function analysis of mice using Anires2005 system (Biolab, Beijing, China). The system will automatically measure lung function parameters including

forced vital capacity (FVC), dynamic compliance (C_{dyn}), expiratory resistance (R_e) and inspiratory resistance (R_i).

2.12. Statistical Analysis

GraphPad Prism 6.0 was used for Statistical analysis. Differences between experimental and control group were assessed by Student's t test. Significant differences among multiple groups were detected by one-way ANOVA. Significance was described as follows: # represent the difference between control/NaCl and TGF- β 1/BLM-treated group, and * represents the difference between the TGF- β 1/BLM-treated and the ZB -treated group:

* p < 0.05, ** p < 0.01, *** p < 0.001; # p < 0.05, ## p < 0.01, ### p < 0.001

3. Results

3.1. ZB alleviates BLM-induced pulmonary fibrosis in mice.

In order to evaluate the anti-fibrotic effect of ZB and explore the mechanism, we verified the anti-fibrotic effect of ZB in BLM-induced animal model of pulmonary fibrosis. From day 7 to day 14 after BLM injury, ZB was administered per day and Nintedanib acted as a positive control (**Figure 1A**). Compared with the model group, the hydroxyproline content and fibrosis area in the administration group were significantly reduced, and the highest dose was almost equal to the positive drug (**Figure 1B-C**). H&E and Masson staining of lung tissue pathological sections also showed that ZB can improve the deposition of collagen in lung tissues and restored lung structure damage caused by BLM (**Figure 1D**). The change of lung function is also an important indicator for evaluating pulmonary fibrosis. Our experimental

results showed that ZB can alleviate the decline of forced vital capacity (FVC) and dynamic compliance (Cdyn), and decreased inspiratory resistance (Ri) and expiratory resistance (Re) (**Figure 1E**).

Figure 1. ZB attenuates pulmonary fibrosis and improves pulmonary function in BLM-treated mice. (A) Dosing regimen in BLM-induced fibrosis model. (B-C) Hydroxyproline content of lung tissues in each group and statistics of lung fibrosis area in each group. (D) H&E and Masson staining of lung tissues in each group (Scale: 50μM). (E) Lung function parameters, include forced vital capacity (FVC), dynamic compliance (Cdyn), inspiratory resistance (Ri) and expiratory resistance (Re). Data was noted as the means ± SD, n = 5. # represent the difference between NaCl and BLM-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between BLM-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P <

0.001, ****P < 0.0001.

3.2. ZB alleviates the migration and activation of fibroblasts.

TGF- β 1-induced fibroblast activation and myofibroblast differentiation, thereby increasing the production of ECM, is an important process in the pathogenesis of IPF[24]. Within 0-1600 nM, ZB has no obvious toxicity to NIH3T3 cells and has an inhibitory effect on cell proliferation caused by TGF- β 1 (**Figure 2A-B**). ZB can reduce the migration of NIH3T3 and Mlg cells (**Figure 2C-D**). ZB attenuated the TGF- β 1-induced expression of Fibronectin, Collagen I and α -SMA in NIH3T3 and Mlg cells (**Figure 3A-B**). Quantitative real-time PCR results also showed that ZB can attenuated the TGF- β 1-induced mRNA expression of Fibronectin, Collagen I and α -SMA in NIH3T3 and Mlg cells (**Figure 3C-D**). In order to further verify that ZB can alleviate fibroblast activation and myofibroblast differentiation, we performed α -SMA immunofluorescence results to further prove that ZB can reduce the expression of α -SMA (**Figure 3E-F**). Finally, our experimental results show that ZB can attenuates TGF- β 1-induced myofibroblast differentiation, ECM deposition and the migration of fibroblasts *in vitro*.

Figure 2. ZB attenuates the migration of fibroblasts. (A) MTT assays of NIH3T3 cells treated with ZB (12.5-1600 nM). (B) MTT assays of NIH3T3 cells treated with ZB (12.5-1600 nM) and were exposed to TGF-β1 (5 ng/mL). (C-D) Wound healing assays of NIH3T3 cells (C) and Mlg cells (D) at 0, 6, 12 and 24 h (Scale: 50μM). Data was noted as the means ± SD, n=3. # represent the difference between control and TGF-β1-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between TGF-β1-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 3. ZB attenuates TGF-β1-induced myofibroblast differentiation and ECM deposition in vitro. (A-B) Western blot analysis of the protein levels of α-SMA, Collagen I, Fibronectin in NIH3T3 cells (A) and Mlg cells (B). β-tubulin was used as an internal reference in densitometric analysis. (C-D) NIH3T3 cells (C) and Mlg cells (D) were exposed to TGF- β 1 (5 ng/mL) and/or ZB (100 nM, 200 nM, 400nM) for 24 h, and quantitative real-time PCR was used to detect the mRNA levels ofα-SMA, Collagen I and Fibronectin. (E-F) Using immunofluorescence to detect fibrosis marker a-SMA in NIH3T3 cells (E) and Mlg cells (F) respectively (Scale: 50μM).

Data was noted as the means $\pm$ SD, n=3. # represent the difference between control and TGF- β 1-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between TGF- β 1-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.3. ZB down-regulates TGF- β 1/Smad signal pathway in fibroblasts.

TGF- β 1 is the most important driving factor of pulmonary fibrosis, and the activation of TGF- β 1/Smad signaling pathway will promote the processing of fibrosis [25]. ZB can reduce the expression level of p-smad2 and p-Smad3 induced by TGF- β 1 in NIH3T3 and Mlg cells (**Figure 4A-B**). Immunofluorescence results also showed that ZB can reduce the expression level of p-Smad3 induced by TGF- β 1 in NIH3T3 and Mlg cells (**Figure 4C-D**).

Figure 4. ZB down-regulates TGF-β1/Smad signal pathway in fibroblasts. (A-B)

NIH3T3 cells (A) and Mlg cells (B) were treated with TGF-β1 (5 ng/mL) and/or ZB (100 nM, 200 nM, 400 nM) for 30 min, and Western blot was used to detect Smad3, Smad2, and p-Smad2, p-Smad3 expression levels. GAPDH was used as an internal reference in densitometric analysis. (C-D) Using immunofluorescence to detect TGF-β1/Smad signal activation marker p-Smad3 in NIH3T3 cells (C) and Mlg cells (D) respectively (Scale: 50μM). Data was noted as the means ± SD, n=3. # represent the difference between control and TGF-β1-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between TGF-β1-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.4. ZB promotes autophagy by inhibiting the TGF- β 1/mTOR signaling pathway in fibroblasts.

Autophagy is a physiological process that maintains cell homeostasis and is also closely related to pulmonary fibrosis. In pulmonary fibrosis, autophagy triggered by TGF- β 1 can enhance the apoptosis resistance of fibroblasts, induces epithelial-mesenchymal transition to promote the processing of fibrosis [26]. ZB can induce autophagy and reduce the expression of P62 in NIH3T3 and Mlg cells (**Figure 5A-B**). Autophagy inhibitor chloroquine (CQ) can inhibit autophagy and increase the expression level of P62, but ZB can alleviate the inhibitory effect of chloroquine in NIH3T3 and Mlg cells (**Figure 5C-D**). The LC3B-GFP and LC3B-mCherry-GFP plasmids were transferred into the NIH3T3 cells to evaluate the effect of ZB on autophagic flux. Experimental results show that ZB can significantly increase the number of autophagosomes (GFP+) and autophagolysosomes (Cherry+GFP-) induced by TGF- β 1 (**Figure 5E-F**). The formation of autophagy is regulated by many protein signals, among which mTOR protein is the core regulator [27]. Experimental results show that in NIH3T3 and Mlg cells, TGF- β 1 can activate mTOR and downstream signaling proteins, and ZB can significantly inhibit the expression of p-mTOR, p-ULK1, p-p70 S6K and pS6RP induced by TGF- β 1 (**Figure 6A-B**). All in all, ZB can induce autophagy and increase the number of autophagolysosomes by inhibiting the TGF- β 1/mTOR signaling pathway.

Figure 5. ZB can induce autophagy and increase the number of autophagolysosomes in fibroblasts. (A-B) NIH3T3 cells (A) and Mlg cells (B) were treated with TGF- β 1 (5 ng/mL) and/or ZB (100 nM, 200 nM, 400 nM) for 24 h, and Western blot was used to detect P62, LC3 expression levels. (C-D) NIH3T3 cells (C) and Mlg cells (D) were treated with Chloroquine (CQ, autophagy inhibitor, 5 ng/mL)

and/or ZB (100 nM, 200 nM, 400 nM) for 24 h, and Western blot was used to detect P62, LC3 expression levels. GAPDH was used as an internal reference in densitometric analysis. (E-F) 1.5 mg GFP-LC3 plasmids (E) and 2 mg Cherry-GFP-LC3 plasmids (F) were transfected into NIH3T3 cells. Immunofluorescence was used to detect the number of autophagolysosomes in NIH3T3 cells (Scale: 50μM). Data was noted as the means $\pm$ SD, n=3. # represent the difference between control and TGF-β1-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between TGF-β1-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6. ZB down-regulates TGF-β1/mTOR signal pathway. (A-B) NIH3T3 cells (A) and Mlg cells (B) were exposed to TGF-β1 (5 ng/mL) and/or ZB (100 nM, 200 nM, 400 nM) for 12 h, and Western blot was used to detect proteins expression

levels of TGF- β 1/mTOR signal pathway including mTOR, ULK1, p70 S6K, S6RP and their phosphorylation expression levels. β -tubulin was used as an internal reference in densitometric analysis. Data was noted as the means $\pm$ SD, n=3. # represent the difference between control and TGF- β 1-treated group, ##P<0.01, ###P<0.001, ####P<0.0001. * represent the difference between TGF- β 1-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.5. ZB inhibits ECM deposition and induces autophagy via regulating TGF- β 1 signaling pathway *in vivo*.

In order to better prove the anti-fibrosis efficacy of ZB, we performed immunohistochemistry, Western Blot and Quantitative real-time PCR to detect α -SMA and extracellular matrix (ECM) deposition marker Fibronectin, Collagen I in lung tissues. The results showed that ZB can reduce the protein and mRNA expression levels of Fibronectin, Collagen I and α -SMA *in vivo* (**Figure 7A-C**). The results of immunohistochemistry showed that the expression levels of P62 and p-Smad3 in the administration group were significantly reduced compared with the model group (**Figure 7C**). The Western Blot results of the lung tissues of each group showed that ZB can reduce the phosphorylation level of the protein on the TGF- β 1/Smad and TGF- β 1/mTOR pathway including p-Smad3, p-Smad2, p-mTOR and p-S6RP (**Figure 7D**). At the same time, Western Blot results showed that ZB reduced the expression of autophagy marker P62 (**Figure 7D**). In conclusion, ZB could attenuate BLM-induced pulmonary fibrosis via regulating TGF- β 1 signaling pathway *in vivo*.

Figure 7. ZB attenuates ECM deposition and induces autophagy via regulating TGF- β 1 signaling pathway *in vivo*. (A) Western Blot analysis of Fibronectin, Collagen I, α -SMA expression levels in the lung tissues of each group. (B) Quantitative real-time PCR analysis of Fibronectin, Collagen I, α -SMA mRNA expression levels in the lung tissues of each group. (C) Immunohistochemical pictures of Fibronectin, Collagen I, α -SMA, p-Smad3 and P62(Scale: 50 μ M). (D) Western Blot analysis of Smad signal pathway proteins and autophagy protein including p-Smad3, Smad3, p-Smad2, Smad2, P62, p-mTOR, mTOR, p-S6RP and S6RP. GAPDH was used as an internal reference in densitometric analysis. Data was noted as the means $\pm$

SD, n=5. # represent the difference between NaCl and BLM-treated group, ##P<0.01, ###P< 0.001, ####P< 0.0001. * represent the difference between BLM-treated and ZB-treated group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

4. Discussion

IPF is a disease of unknown cause, characterized by diffuse alveolitis and alveolar structural disorders, which eventually lead to pulmonary interstitial fibrosis [2]. IPF has an insidious onset, with more than 200 pathogenic factors, and an average survival period of 2.41 years [3]. The mortality rate is higher than that of most cancers [28]. At present, the only drugs approved by the FDA for the treatment of IPF are Nintedanib and Pirfenidone, but they can only delay the decline of lung function and cannot reverse the progression of the disease. Therefore, there is an urgent need to develop better drugs [29]. In this study, the experimental results showed that ZB can alleviate bleomycin-induced lung fibrosis in mice.

ZB is a small molecule inhibitor of BTK. ZB forms a covalent bond with the cysteine residue in the active site of BTK, thereby inhibiting BTK activity. BTK is a signaling molecule in the B cell antigen receptor (BCR) and cytokine receptor pathways. In B cells, BTK signal transduction activates the pathways necessary for B cell proliferation, transportation, chemotaxis and adhesion. In non-clinical studies, ZB inhibited the proliferation of malignant B cells and reduced tumor growth [30]. FDA approves ZB for the treatment of adult mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL). Studies have reported that ZB can also treat Waldenström macroglobulinemia (WM) [31].

TGF- β 1 is an important regulatory factor for the occurrence of fibrotic diseases. It can regulate fibroblasts transform into myofibroblasts, and induce collagen synthesis by interacting with the downstream Smads [32]. The formation process of pulmonary fibrosis involves the classic TGF- β 1/Smads signaling pathway and the non-classical TGF- β signaling pathway. The TGF- β 1 signaling pathway is involved in the occurrence and development of pulmonary fibrosis [33]. Our experimental results showed that ZB can inhibit the activation of TGF- β 1/Smad pathway caused by TGF- β 1 to alleviate fibrosis. Recent studies have also shown that mTOR-dependent autophagy plays an important role in the development of pulmonary fibrosis. Increased mTOR activity in IPF fibroblasts. TGF- β 1 stimulation inhibits autophagy flux in fibroblasts, our results showed ZB can induce autophagy [34].

It has been reported that Ibrutinib, the first-generation small molecule inhibitor of BTK, can aggravate fibrosis by aggravating inflammation [17]. Compared with our study, Ibrutinib (10 mg/kg) was administered by oral gavage daily since the beginning till sacrifice and ZB was given by gavage once a day from the seventh day after BLM injury. The fibrosis process caused by bleomycin includes the early inflammatory phase (days 2-7) and the late fibrotic phase (days 7-14) [35]. At the same time, ibrutinib could increase the apoptosis of bleomycin-induced epithelial cell, but cannot increase the differentiation of myofibroblasts *in vitro* and also has off-target effects. Other studies have shown that ibrutinib can reduce the expression of activated NF- κ B and NLRP3 inflammasomes and reduce inflammation by targeting BTK [36]. Therefore, the relationship between BTK and inflammation, fibrosis is controversial

and needs further research.

Conclusions

Our study found that ZB attenuated BLM-induced pulmonary fibrosis in mice, inhibited myofibroblast migration and activation by suppressing the TGF- β 1/Smad signaling, and induced autophagy via regulating TGF- β 1/mTOR pathways. Although the specific target of ZB to treat pulmonary fibrosis has not yet been elucidated, ZB may be used as a potential drug candidate for the treatment of pulmonary fibrosis.

Abbreviation

IPF: Idiopathic pulmonary fibrosis; MCL: mantle cell lymphoma; CLL: chronic lymphocytic leukemia; SLL: small lymphocytic lymphoma; BLM: Bleomycin; mTOR: mammalian target of rapamycin; TGF- β 1: transforming growth factor- β 1; ECM: extracellular matrix; ZB: Zanubrutinib; BTK: Bruton's tyrosine kinase; BCR: B cell receptor; Mlg: Mouse lung fibroblasts cell; NIH-3T3: Mouse embryonic fibroblasts cell line; FBS: Fetal bovine serum; DMEM: Dulbecco's modified eagle medium; PVDF: polyvinylidene difluoride; H&E: Hematoxylin–eosin; FVC: Forced vital capacity; Cydn: Dynamic compliance; Re: Expiratory resistance; Ri: Inspiratory resistance; LC3: Microtubule-associated protein 1 light chain 3; GFP-LC3B: Green fluorescent protein-microtubule-associated protein 1 light chain 3B; mCherry-GFP-LC3B: mCherry fluorescent protein-Green fluorescent protein-microtubule-associated protein 1 light chain 3B; ULK-1: Protein kinase ULK1/Autophagy-related protein 1; p70 S6K: p70 S6 Kinase; S6RP: S6 ribosomal protein; α -SMA: α -smooth muscle actin; CQ: Chloroquine; WM: Waldenström

macroglobulinemia; NF- κ B: Nuclear factor- κ B; NLRP3: NOD-like receptor 3.

Declarations

Ethics approval and consent to participate

All animal care and experimental procedures complied with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (project code: SYXK 2019-0001, date of approval: 1 November 2019).

Consent for publication

Not applicable.

Availability of data and materials

Data available on request from the authors.

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Competing Interests

The authors declare that they have no conflict of interest.

Authors' contributions

Conceived and designed the study: H.Z. and C.Y.; cell experiments: X.L. and Y.W.; animal experiments: J.G. and J.L.; collection and assembly of data: S.L. and Y.C.; data

analysis and interpretation: R.L. and X.L. All authors have read and agreed to the published version of the manuscript.

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