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SB216763, a selective small molecule inhibitor of glycogen synthase kinase-3, improves bleomycin-induced pulmonary fibrosis *via* activating autophagy

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Abstract An excessive accumulation of extracellular matrix composed by insoluble collagen is the core pathogenic change of fibroproliferative diseases including pulmonary fibrosis. We recently found that autophagy, a self-catabolic process that maintains intracellular homeostasis, participates critically in the regulation of collagen degradation in the fibrotic tissues. Here we report that treatment of mice with SB216763, a potent and selective inhibitor of glycogen synthase kinase-3 (GSK3), significantly decreased the bleomycin (BLM)-induced acute inflammation, attenuated pulmonary fibrosis, improved the lung function and increased animal survival through activating autophagy. Indeed, we found that treatment of mice or cultured cells with SB216763 restored the autophagy *in vivo* and *in vitro* through increasing the expression of autophagy related proteins and decreasing the physical interaction of Bcl-2 and Beclin-1. Additionally, SB216763 stimulated the binding of Bcl-2 and GSK3, which reduces the interaction of Bcl-2 and Beclin-1. We conclude that the GSK3 inhibitor SB216763 improves the BLM-induced pulmonary fibrosis through stimulating autophagy core complex to restore the activity of autophagy in fibrotic lung tissue. Our work suggests that using a moderate autophagic agonist, such as SB216763, is a promising

Abbreviations: Bcl-2, B-cell lymphoma 2; BLM, bleomycin; ECM, extracellular matrix; GSK3, glycogen synthase kinase-3; IL-17A, interleukin-17A; LAMP-1, lysosome-associated membrane protein 1; LC3, light chain 3; TGF-1, transforming growth factor 1

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therapeutic strategy for the treatment of devastating fibroproliferative diseases such as idiopathic pulmonary fibrosis.

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1. Introduction

Tissue fibrosis is a core structure change and underlying mechanism for a variety of incurable chronic lung diseases. It is characterized by an excessive accumulation of extracellular matrix (ECM) leading to stiffening and/or scarring of the involved tissue, which destructs the normal lung architecture, affects lung functions, and causes failure of lung organ^{1,2}. Because no convincing or effective therapeutics are available for the treatment of pulmonary fibrosis, it has a mortality rate that exceeds that of many cancers³. The parenchymal collagen, the major insoluble fibrous protein, is the main substance of the ECM, and is deposited in the fibrotic lung disease⁴. There are various signal pathways that regulate the expression of collagen and induce the development of pulmonary fibrosis, including stimulating the transforming growth factor 1 (TGF-1) signal pathway⁵. Moreover, the attenuation of collagen degradation is also observed increasingly in the pulmonary fibrosis^{6,7}.

Autophagy, a self-catabolic process that maintains intracellular homeostasis and determines cell fates under stress, has been recently found to degrade the collagen and resolute the pulmonary fibrosis in tissue repairing process^{6,7}. Indeed, autophagy has been recognized as a key mechanism for cellular homeostasis and survival in a variety of lung diseases⁸. The autophagy core complex, comprised of Bcl-2, Beclin-1, Vps34, Vps15, Atg14, and Ambra1, plays a crucial role in autophagy activation process⁹. Bcl-2 binds to Beclin-1 by BH3 domain to protect the activation of autophagy under quiescent conditions. When cells are stimulated by a variety of stimuli, Bcl-2 is isolated from Beclin-1 and the autophagic activity is enhanced in the cells⁹. Therefore, the integration of Bcl-2 and Beclin-1 is a switcher of autophagy activation.

Glycogen synthase kinase-3 (GSK3) is a serine-threonine kinase, and it is involved in energy metabolism, neuronal cell development, and body pattern formation. GSK3 regulates these cellular activities through regulating the integration of signaling or functional proteins. GSK3 binds to tau and phosphorylates it to regulate negatively its ability of stabilizing microtubule structure¹⁰. And GSK3 promotes the expression of p53, a tumor suppressor gene, through binding to p53, and induces the cell apoptosis in the mitochondria¹¹. Moreover, GSK3 up-regulates the activity of autophagy through integrating acetyltransferase TIP60 and stimulating the protein kinase ULK1, which is required for activating autophagy¹². And our previous work reported that SB216763, the potent and selective GSK3 inhibitor, increases the expression of Bcl-2¹³. It suggests that SB216763 may affect the activity of autophagy by regulating the integration of Bcl-2 and Beclin-1, which may be due to its capacity of inhibiting GSK3. Thus SB216763 may attenuate the pulmonary fibrosis through regaining the activity of autophagy.

In this study, we document that the GSK3 inhibitor SB216763 attenuates tissue injury, inflammation, pulmonary fibrosis, and lung dysfunction by restoring the autophagic activity in acutely injured lungs. Treatment of mice with SB216763 activates the autophagic core complex and promotes the degradation of collagen in the BLM-induced fibrotic lungs through interrupting the Bcl-2

and Beclin-1 interaction. SB216763 attenuates the activity of GSK3 and enhances the integration of GSK3 and Bcl-2, which restrains the binding of Bcl-2 and Beclin-1. Our studies suggest that using a moderate autophagic agonists such as GSK3 inhibitor, is a promising therapeutic strategy for the treatment of fibroproliferative diseases such as idiopathic pulmonary fibrosis.

2. Materials and methods

2.1. Reagents

BLM was purchased from Nippon Kayaku (Tokyo, Japan). SB216763 was obtained from Sigma (St. Louis, MO). Alexa Fluor 488 and 647 Abs were obtained from Invitrogen. Mouse IgG and anti-mouse Bcl-2, Beclin-1, Vps34, p-Bcl-2 (S70), p62, Light chain 3 (LC3) III, lysosome-associated membrane protein 1 (LAMP-1), and β -actin antibodies were obtained from Cell Signaling Technology (Danvers, MA). Other materials were purchased from commercial sources.

2.2. Preparation of pulmonary fibrosis

Male C57BL/6J mice (17 ± 1 g, 6–8 weeks) were obtained from Vital River Laboratory Animal Technology. The mice were anesthetized with 50 mg/kg pentobarbital ip (Merck). Using an insulin syringe, 50 mL of LPS-free saline, clinical grade BLM (3.0 U/kg) was injected directly into the trachea as previously described¹⁴. The SB216763 (20 mg/kg)¹⁵ was injected ip in saline on day 14, 17, and 21 after BLM instillation. Mice were sacrificed by excessive doses of anesthesia for the lungs on day 28 after BLM instillation. The lungs were excised and were fixed or frozen for morphological evaluation or measurement of hydroxyproline content.

All animal protocols were conformed to the Guidelines for the Care and Use of Laboratory Animals prepared and approved by the Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

2.3. Lung functions measurement

Twenty eight days after bleomycin injured, mice were anesthetized with 50 mg/kg ip pentobarbital and placed on flexivent system (flexivent, SCIREQ Inc., Montreal, Canada). Mice were mechanically ventilated with tidal volume 10 mL/kg and respiratory rate of 150 breaths/min. Lung function parameters were calculated by the measurements of total lung capacity (deep inflation of the mice lungs to a pressure of 30 cm H₂O followed by a breath hold for a few seconds), Snapshot (single-frequency forced oscillation waveform with the frequency at 150 breaths/min), Quickprime-3 (multi-frequency forced oscillation waveforms), and pressure–volume loops (slow stepwise inflation to TLC and deflation back to FRC). All perturbations were performed until three acceptable measurements with coefficient of determination (COD) ≥ 0.9 were recorded in each individual subject.

2.4. Histomorphology

The lungs were rapidly excised, fixed with 4% paraformaldehyde, and embedded in paraffin for histopathological examination as previously described¹⁴. Lung tissue sections (5 mm thick) were prepared and stained with H&E, or Masson's trichrome. The grade of pulmonary inflammation and fibrosis was blindly assessed by a professional pathologist. The average integrated OD (IOD) of collagen deposition from 10 randomly chosen regions per tissue sample at a magnification of 200× was determined using Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD).

2.5. Measurement of lung hydroxyproline

Collagen deposition was determined by measuring the total hydroxyproline content of the lungs according to the revised Reddy GK method¹⁴. In brief, the lungs were hydrolyzed with 2.5 M NaOH at 120 °C, 0.1 kPa for 40 min. After neutralization with hydrochloric acid, the hydrolyzation products were diluted with distilled water. The hydroxyproline content of the hydrolyzation products was assessed calorimetrically at 550 nm with *p*-dimethylaminobenzaldehyde. The results were represented as micrograms per lung.

2.6. Plasmids construction

Mouse GSK3 was generated by PCR with appropriate primer plasmids and subcloned in frame to the pcDNA6-HA expression plasmid. Mouse Beclin-1 was generated by PCR and subcloned in frame to the pFLAG-CMV-2 expression plasmid. Myc-tagged Bcl-2 plasmid was a gift from Dr. W. Stratford May, Jr. (University of Florida Shands Cancer Center).

2.7. Cell culture

The mouse type II alveolar epithelial cells (MLE-12) were cultured in DMEM-Ham's Nutrient Mixture F12 (1:1; Hyclone) supplemented with 2% FBS and L-glutamine (full-nutrient medium). The cells were passaged every 2 days.

2.8. Semi-quantitative RT-PCR

The expression of Bcl-2 mRNA was analyzed as described previously¹⁶. Total RNA was isolated from MLE-12 cells using the TRIzol kit (Invitrogen) according to the manufacturer's instructions. Next, the RNA was reverse-transcribed and amplified. PCR was performed using a Mycycler thermal cycler, and the amplified products were analyzed by agarose gel. The following specific primer sequences were used: 5'-ACGGCTGCACGAGT-CACAC-3' and 5'-GGCAGGCGGGAGGTCTT-3' (mouse collagen I); and 5'-TAACCAACTGGGACGATATG-3' and 5'-AAACAGGGACAGCACAGCCT-3' (mouse β -actin). All values obtained were normalized to the values obtained for β -actin.

2.9. Immunoprecipitation and Western blotting

Cells were washed three times with phosphate-buffered saline, harvested, and lysed in co-immunoprecipitation buffer that has been described before¹⁷. Total cell lysates (5 mg protein) were subjected to immunoprecipitation with appropriate antibodies, as indicated, overnight at 4 °C with gentle agitation, followed by incubation with protein A/G Plus-agarose for 24 h at 4 °C. The

immunocomplex was washed three times and then mixed with 2× SDS sample buffer and boiled for 5 min. For Western blotting, coprecipitates or whole cell extracts were resolved by SDS-PAGE and blotted on PVDF membranes (Millipore). The membranes were immunoblotted with the indicated antibodies and developed using an ECL detection system (Amersham Bioscience).

2.10. Confocal assay

Standard protocols for immunofluorescence microscopy were used as described previously¹⁷. The lung tissue (0.5 μ m thick) on the glass slide was prepared and stained with indicated primary Abs overnight at 4 °C. The sections were washed twice, incubated with fluorochrome-labeled secondary Abs (1:200) for 30 min, and washed three times after staining. Images were obtained with a Leica SP2 confocal microscope and analyzed with Leica confocal software.

2.11. Statistics

Data are represented as the mean \pm SE. Statistical analyses were performed using Student's *t*-test for two-group comparisons. The survival rates were analyzed using the Kaplan–Meier method. The *P* value was set at 0.05. All statistics were analyzed using SPSS 17.0 software.

3. Results

3.1. SB216763 attenuates BLM-induced pulmonary fibrosis through accelerating the degradation of collagen

We recently reported that IL-17A inhibits the GSK3-induced degradation of Bcl-2 and promotes the expression of Bcl-2 to attenuate the activity of autophagy¹³. Moreover, blockade of IL-17A activity restores the autophagic flux to suppress pulmonary fibrotic after BLM challenged⁷. And SB216763, the GSK3 inhibitor, also revealed the capacity of suspending Bcl-2 degradation¹³. Hence, we further determined the regulatory effect of SB216763 in BLM-induced pulmonary fibrotic. However, we found that SB216763 reversed BLM-established pulmonary fibrosis, as demonstrated by the reduced acute inflammation, deposition of collagen (Fig. 1A and B) and the hydroxyproline content (Fig. 1C) in the damaged lung. These factors also contributed to the increased survival of BLM challenged mice (Fig. 1D). But SB216763 did not reduce the collagen mRNA expression after BLM challenged (Fig. 1E). These data suggest that SB216763 reduces the deposition of collagen in the BLM treatment lung through accelerating the degradation of collagen.

3.2. SB216763 reverses the BLM-induced reduction in pulmonary function

Subsequently, we investigated the physiological function of the lung tissue through detecting the pulmonary function in the BLM treatment mouse. We found that SB216763 increased inspiratory capacity (IC), compliance of the respiratory system (Crs) and static compliance (Cst) after BLM-challenged (Fig. 2A, D and E). Moreover, SB216763 decreased the respiratory resistance (Rrs), elastic resistance (Ers) and static elastance (Est) while the BLM increased them (Fig. 2B, C and F). These data indicate that SB216763 reverses the BLM-induced reduction in pulmonary function.

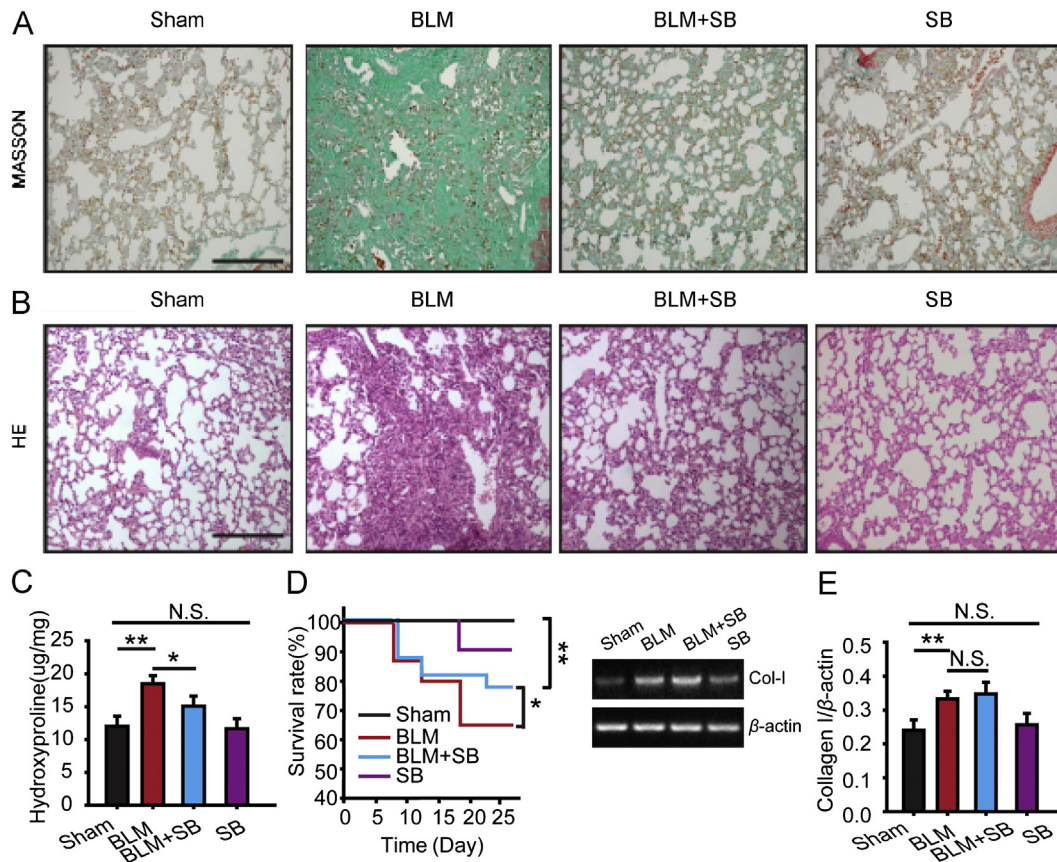


Figure 1 SB216763 attenuates BLM-induced pulmonary fibrosis through accelerating the degradation of collagen. (A–C) SB216763 promotes the resolution of the BLM-induced pulmonary inflammation and fibrosis as indicated by Masson's trichrome staining of lung sections (A), H&E staining of the lung sections (B) and hydroxyproline content (C), scale bar: 75 μ m. Data are represented as the mean \pm SE ($n=7$ /group/experiment). (D) SB216763 treatment increased the survival rates of BLM-treated mice. (E) SB216763 treatment does not affect the mRNA of collagen after BLM challenge. Data are presented as the mean \pm SE of four independent assays. * $P<0.05$; ** $P<0.01$; N.S., non-significant.

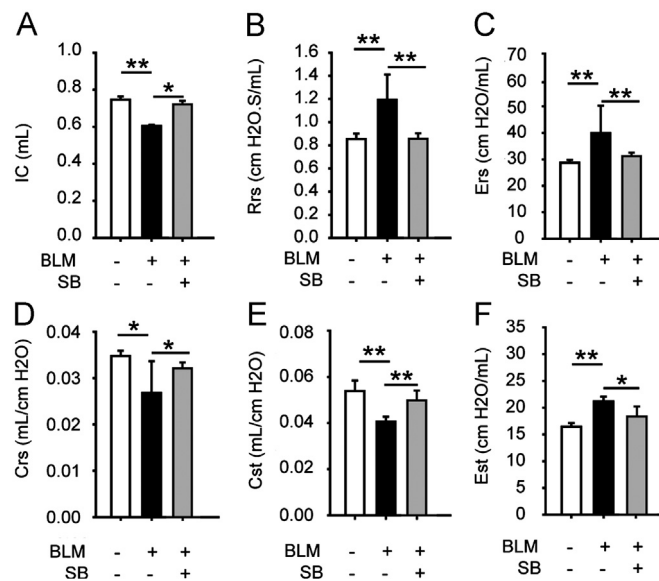


Figure 2 SB216763 reverses the BLM-induced reduction in pulmonary function. (A–F) The parameters of lung function. SB216763 significantly reduces the BLM-induced IC, Rrs, Ers, Crs, Cst, and Est. Data were presented as mean \pm SE ($n=5$ /group/experiment). * $P<0.05$; ** $P<0.01$.

3.3. SB216763 stimulates the activity of autophagy through attenuating the interaction of Bcl-2 and Beclin-1

Autophagy, a self-catabolic process that maintains intracellular homeostasis and determines cell fates under stress, has been recently found to be involved in the regulation of tissue repairing process and fibrosis⁶. Our previous work pointed out that neutralizing IL-17A, a negative regulator of autophagy, promotes the degradation of collagen through activation of autophagy⁷. We therefore examined if SB216763 augments the activity of autophagy to degrade collagen in the MLE-12 cells. We found that SB216763 increased the ratio of LC3-II/LC3-I and p-Bcl-2/Bcl-2, and increased the expression of Beclin-1 and Vps34 in the lung epithelial cells (Fig. 3A). Because Bcl-2 binds to Beclin-1, the key component of autophagy core complex, to inhibit the activity of autophagy, we further determined the regulatory effect of SB216763

on the interaction of Bcl-2 and Beclin-1. The immunoprecipitation assay revealed that stimulation of lung epithelial cells with SB216763 reduced the integration of Bcl-2 and Beclin-1 (Fig. 3B). At the meanwhile, SB216763 increased the integration of Bcl-2 and GSK3 (Fig. 3C). However, SB216763 did not effect the activity of JNK-1, a stimulator of autophagy through weakening the interaction of Bcl-2 and Beclin-1 (Fig. 3D). These data indicate that SB216763 can stimulate the activity of autophagy through attenuating the interaction of Bcl-2 and Beclin-1.

3.4. SB216763 restores autophagic activity in the lung tissue of BLM-treated mouse

Subsequently, we examined the regulatory effect of SB216763 on the activity of autophagy in the BLM treatment lung. We found

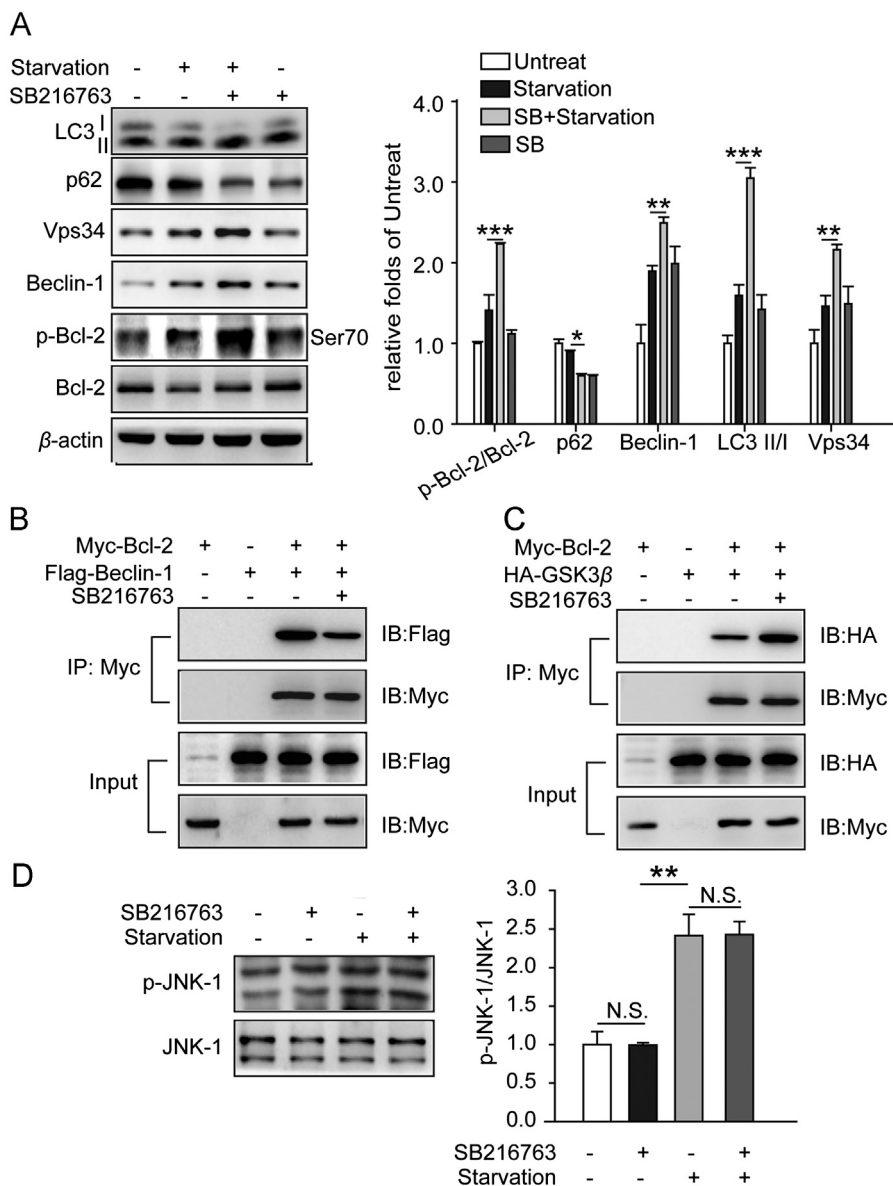


Figure 3 SB216763 stimulates the activity of autophagy through attenuating the integration of Bcl-2 and Beclin-1. (A) SB216763 enhances the expression of autophagy-associated proteins. (B) SB216763 reduces the integration of Beclin-1 and Bcl-2. (C) SB216763 enhances the integration of GSK3 and Bcl-2. (D) SB216763 does not attenuate phosphorylation of JNK-1 when the cells are starved. Data are presented as the mean \pm SE of four independent assays. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., non-significant.

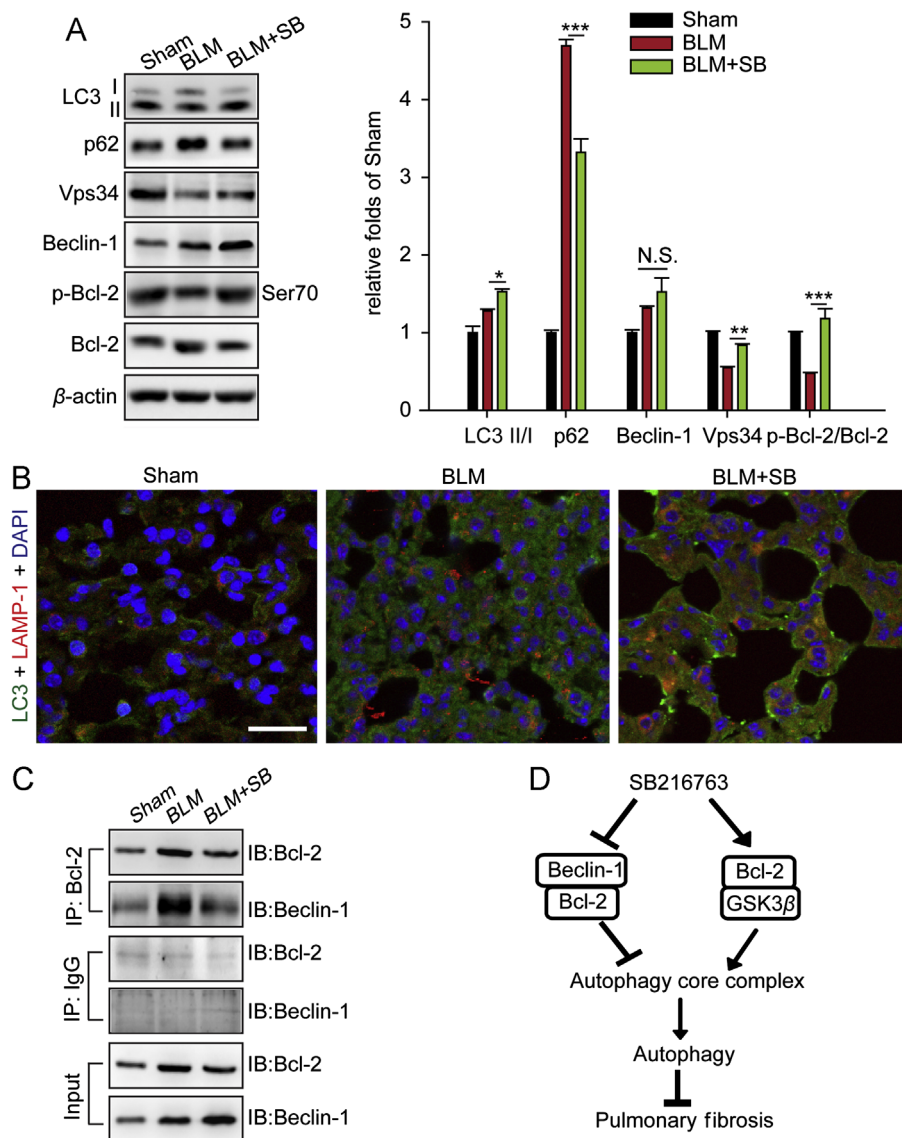


Figure 4 SB216763 restores autophagic activity in the lung tissue of BLM-treated mouse. (A) SB216763 enhances the expression of autophagy-associated proteins in the lung of BLM-treated mouse. (B) SB216763 enhances the numbers of autophagolysosomes in the lung. The autophagolysosomes were evaluated by the co-expression of LC3 and LAMP-1 staining, green for LC3 and red for LAMP-1, scale bar: 7.5 μm. The images are representatives of three independent experiments ($n=6$ /group/experiment). (C) SB216763 reduces the integration of Beclin-1 and Bcl-2 in the BLM-treated mouse. Data are presented as the mean \pm SE of four independent assays. (D) Schematic diagram of the mechanism of SB216763-mediated attenuation of pulmonary fibrosis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., non-significant.

that SB216763 increased autophagy activation significantly, as indicated by the increased ratio of LC3-II/LC3-I in fibrotic tissues (Fig. 4A). Moreover, SB216763 further decreased the expression of p62 in fibrotic tissues (Fig. 4A). We subsequently examined the expression of Beclin-1, Vps34, and the ratio of p-Bcl-2/Bcl-2, because the autophagic core complex regulates the activity of autophagy and is required for autophagosome formation in the autophagy process¹⁸. The expression of Beclin-1 was increased, but the expression of Vps34 and the ratio of p-Bcl-2/Bcl-2 were decreased in the fibrotic lung tissues from the BLM-treated mice. However, the expression of Beclin-1 and Vps34, as well as the ratio of p-Bcl-2/Bcl-2, was increased in the fibrotic lung tissues from the SB216763-treated animals (Fig. 4A). And SB216763 enhanced the co-expression of lysosome-associated membrane

protein 1 (LAMP-1) and the autolysosomal marker LC3¹⁹ in fibrotic lung tissues (Fig. 4B), which indicated that SB216763 promoted the formation of autolysosomes. Moreover, the immunoprecipitation assay revealed that the treatment of SB216763 reduced the integration of Bcl-2 and Beclin-1 (Fig. 4C). Taken together, these data indicate that SB216763 can induce an effective autophagy in the fibrotic lung tissue.

4. Discussion

SB216763 has been found in the regulation of tumorigenesis and neurodegenerative disease because of the potent function about selective inhibiting glycogen synthase kinase-3. It is reported that

the activity of GSK3 is hyperactive in the patients with osteosarcoma specimens and suppression of GSK3 by SB216763 resulted in apoptosis of osteosarcoma cells²⁰. SB216763 could reverse the hippocampus-dependent learning deficits and rescue adult hippocampal neurogenesis at multiple stages in *Fmr1 KO* mice through inhibiting the activity of GSK3²¹. However, the exact relationship of SB216763 and fibroproliferative diseases is rarely reported, except that Gurrieri et al.¹⁵ pointed out that SB216763 prevented lung inflammation and the subsequent fibrosis when co-administered with BLM. But they neither detect the changes of pulmonary function after SB216763 treatment nor involve the SB216763 regulatory effect on autophagy in the lung fibrosis.

A number of studies have demonstrated that the activity of autophagy attenuates the development and progression of pulmonary fibrosis through stimulating the collagen degradation^{6,7}. And Kim et al.²² also indicated that the autophagy promotes intracellular degradation of type I collagen induced by TGF-1 in the kidney fibrosis. Our current studies indicate that SB216763 stimulates the autophagy *in vivo* and *in vitro* through regulating the expression of autophagy relate proteins (such as Bcl-2, p-Bcl-2, Beclin-1, Vps34, LC3, and p62) and the integration of Bcl-2 and Beclin-1 (Figs. 3 and 4). This finding is consistent with that SB216763 promotes the expression of LC3-II and aggregation of punctate dots containing LC3 when the rats are attacked by ischemic brain injury²³. However, SB216763 increases the protein level of Atg5 and mTOR and decreases the activity of autophagy in the chronic myeloid leukemia cells with imatinib mesylate resistance²⁴, which suggested that inhibiting GSK3 by SB216763, which induces a state of the growth factor deprivation, has different regulatory effects on the activation of autophagy in the fibrosis and tumorigenesis.

The autophagy core complex plays a crucial role in autophagy activation process⁹. Bcl-2 binds to Beclin-1 by BH3 domain to protect the activation of autophagy under quiescent conditions. Our previous studies indicate that IL-17A-PI3K-GSK3-Bcl-2 signaling pathway participates in the attenuation of autophagic activity in lung epithelial cells¹³. Indeed, we found the GSK3 inhibitor SB216763 increased the integration of GSK3 and Bcl-2 and decreased the integration of Beclin-1 and Bcl-2 (Fig. 3B and C), which means that the SB216763 stimulated the autophagy through attenuation of the integration of Beclin-1 and Bcl-2. Wei and his colleagues found that JNK1 promotes Bcl-2 dissociation from Beclin-1 and autophagy activation through phosphorylating Bcl-2²⁵. However, we found SB216763 did not stimulate JNK1 in the MLE-12 cells at the present work (Fig. 3D). In spite of this observation, we did not deny that SB216763 may activate autophagy through regulating the activity of some protein kinases, because selectivity of SB216763 is not enough special like knockdown of gene.

5. Conclusions

In summary, our current study shows that SB216763, a GSK3 inhibitor, attenuates BLM-induced lung fibrosis through augmenting the Bcl-2 dissociation from Beclin-1 and activating autophagy subsequently (Fig. 4D). SB216763 inhibits the integration of Bcl-2 and Beclin-1 while it aggregates the binding of Bcl-2 and GSK3. The free Beclin-1 stimulates the autophagy core complex and autophagy, the primary pathway for degradation of collagen and the clearance of injured tissue debris. Our findings revealed a

novel molecular mechanism of SB216763 in the regulation of autophagy in lung epithelial cells and lung fibrosis tissue. Our work indicates that blocking GSK3 activity to stimulate autophagy is a promising therapeutic strategy for the prevention and treatment of fibroproliferative diseases such as pulmonary fibrosis.

Acknowledgments

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