

RESEARCH ARTICLE



Baicalein attenuates bleomycin-induced lung fibroblast senescence and lung fibrosis through restoration of Sirt3 expression

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ABSTRACT

Context: Fibroblast senescence was reported to contribute to the pathological development of idiopathic pulmonary fibrosis (IPF), and baicalein is reported to attenuate IPF.

Objective: This study explores whether baicalein attenuates lung fibrosis by regulating lung fibroblast senescence.

Materials and methods: Institute of Cancer Research (ICR) mice were randomly assigned to control, bleomycin (BLM), baicalein and BLM + baicalein groups. Lung fibrosis was established by a single intratracheal dose of BLM (3 mg/kg). The baicalein group received baicalein orally (100 mg/kg/day). Sirtuin 3 (Sirt3) siRNA (50 µg) was injected through the tail vein once a week for 2 weeks to explore its effect on the anti-pulmonary fibrosis of baicalein.

Results: BLM-treated mice exhibited obvious lung fibrosis and fibroblast senescence by showing increased levels of collagen deposition (27.29% vs. 4.14%), hydroxyproline (208.05 vs. 40.16 ng/mg), collagen I (25.18 vs. 9.15 µg/mg), p53, p21, p16, MCP-1, PAI-1, TNF-α, MMP-10 and MMP-12 in lung tissues, which were attenuated by baicalein. Baicalein also mitigated BLM-mediated activation of TGF-β1/Smad signalling pathway. Baicalein restored the BLM-induced downregulation of Sirt3 expression in lung tissues and silencing of Sirt3 abolished the inhibitory role of baicalein against BLM-induced lung fibrosis, fibroblast senescence and activation of TGF-β1/Smad signalling pathway.

Conclusions: Baicalein preserved the BLM-induced downregulation of lung Sirt3 expression, and thus the suppression of TGF-β1/Smad signalling pathway and lung fibrosis, which might provide an experimental basis for treatment of IPF.

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Introduction

Idiopathic pulmonary fibrosis (IPF), the most common form of idiopathic interstitial pneumonia, causes progressive pulmonary fibrosis, which is characterized by repeated epithelial cell damage, destruction of alveolar structure and pro-fibrotic mediator-induced extracellular matrix (ECM) deposition by myofibroblasts. The occurrence and factors associated with IPF include genetic susceptibility and other risk factors, such as bacterial or viral infection, smoking and environmental pollution (Cao et al. 2021). IPF has a high mortality rate, with the average survival time being 2–4 years from diagnosis (Wolters et al. 2014). Currently, only pirfenidone and nintedanib are approved by the Food and Drug Administration (FDA) (Ma et al. 2022) and recommended in the recent ATS/ERS/JRS/ALAT clinical practice guideline for the treatment of IPF (Raghu et al. 2022). Although the clinical trials with pirfenidone and nintedanib in IPF were

reported to reduce the decline in FVC compared to placebo and the antifibrotic therapies are associated with improved survival in patients with IPF, this therapy could not completely stop the deterioration of lung function over time (Ma et al. 2022; Takehara et al. 2022). Therefore, identifying effective therapeutic methods for IPF is urgent.

The formation of fibrotic foci in IPF is mainly due to excessive ECM protein deposition in the alveolar space, with activated myofibroblasts being the main producers of pulmonary ECM (Du S-F et al. 2019; Blokland et al. 2021; Chen T et al. 2021; Wang Q et al. 2021; Zhang et al. 2021; Röhrich et al. 2022; Wu et al. 2022). Wu et al. (2022) demonstrated that the expression of checkpoint kinases 1 and 2 (CHK1/2) was markedly increased in the lungs, remodelled pulmonary arteries and isolated fibroblasts from IPF patients and animal models. CHK1/2 inhibition could interfere with TGF-β1-mediated fibroblast activation, attenuating fibrosis and pulmonary vascular remodelling. Zhang

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et al. (2021) indicated that N-methyladenosine modification expression was upregulated in a bleomycin (BLM)-induced pulmonary fibrosis mouse model, fibroblast-to-myofibroblast (FMT)-derived myofibroblasts and IPF patient lung samples. N⁶-methyladenosine (m⁶A) modification contributes to IPF-induced pulmonary fibrosis by regulating FMT. Of note, an increasing number of studies highlight the critical role of fibroblast senescence in the pathological process of IPF, as well as the existence of an increased and persistent number of senescent fibroblasts in lung tissues with IPF (Schafer et al. 2017). Senolytic drugs, including quercetin and dasatinib plus quercetin have been reported to attenuate IPF-induced pulmonary fibrosis and dysfunction in mice by selectively eliminating senescent fibroblasts and/or myofibroblasts (Hecker et al. 2014; Alvarez et al. 2017; Schafer et al. 2017; Hohmann et al. 2019), providing a potential therapeutic method for IPF.

Baicalein (5,6,7-trihydroxyflavone) is a major phenolic flavonoid extracted from the root of *Scutellaria baicalensis* Georgi (Lamiaceae) and widely used to treat multiple diseases, such as Alzheimer's disease, Parkinson's disease (Li Y et al. 2017), neuro-inflammation (Rui et al. 2020), ischemia-reperfusion-induced brain injury (Yang et al. 2019), hyperuricaemia (Chen Y et al. 2021), sepsis-induced liver injury (Liu A et al. 2015), avian pathogenic *Escherichia coli*-induced acute lung injury, pulmonary arterial hypertension (Shi et al. 2018) and pulmonary fibrosis (Gao et al. 2013; Sun X et al. 2020), due to its anti-inflammatory, antioxidant and anti-apoptosis effects. Gao et al. (2013) reported that baicalein mitigated lung fibrosis in a rat model of IPF by suppressing miRNA (miR)-21 and TGF- β /Smad signalling. Sun et al. (2020) showed that baicalein attenuated TGF- β 1-mediated collagen production in lung fibroblasts by inhibiting the expression of connective tissue growth factor.

In the present study, a mouse model of BLM-induced IPF was used to explore the protective effects of baicalein. Baicalein could inhibit BLM-induced lung fibroblast senescence and pulmonary fibrosis by suppressing the TGF- β 1/Smad signalling pathway. It was also found that baicalein markedly increased sir-tuin 3 (Sirt3) expression in the lung tissue of BLM-treated mice, which may contribute to the inhibitory effects of baicalein against BLM-induced lung fibroblast senescence and pulmonary fibrosis.

Materials and methods

Animals and drug administration

Institute of Cancer Research (ICR) mice (male; 8 weeks old) were provided by Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and provided with free access to food and water in a controlled temperature of 23–25 °C. All animal protocols were approved by the Ethics Committee of the Experimental Animals of Shanghai Seventh People's Hospital (approval no. 2020-AR-053). Mice were anesthetized using sodium pentobarbital (i.p., 30 mg/kg; MilliporeSigma, Burlington, MA) and then subjected to a single intratracheal dose of BLM (3 mg/kg; Selleck Chemicals, Houston, TX) diluted in 50 μ L sterile saline. Mice that had been intratracheally administrated 50 μ L sterile saline served as the control. In the baicalein group, mice received baicalein orally (100 mg/kg/day; MilliporeSigma, Burlington, MA); the dose was based on a previous study, which showed that baicalein (p.o. 100 mg/kg/day) markedly mitigated BLM-induced lung fibrosis (Gao et al. 2013).

Experimental groups and drug treatment

The first experiment was performed to explore the effect of baicalein on BLM-mediated pulmonary fibrosis. Mice were randomly divided into four groups: (i) control group ($n=7$), mice were intratracheally administrated sterile saline; (ii) BLM group ($n=7$), mice were intratracheally administrated BLM; (iii) baicalein group ($n=7$), mice were administrated saline intratracheally and baicalein orally; and (iv) BLM + baicalein group ($n=7$), mice were administrated BLM intratracheally and baicalein orally. Mice were sacrificed after 2 weeks of BLM and baicalein administration.

The second experiment aimed to examine whether Sirt3 siRNA could abolish the protective role of baicalein against BLM-induced pulmonary fibrosis. Mice were randomly divided into six groups: (i) control group ($n=7$); (ii) BLM group ($n=7$); (iii) BLM + baicalein group ($n=7$); (iv) Sirt3 siRNA group ($n=7$); (v) BLM + Sirt3 siRNA group ($n=7$); and (vi) BLM + baicalein + Sirt3 siRNA group ($n=7$). Following drug administration, mice in the control siRNA group were injected with 50 μ g control siRNA and those in the Sirt3 siRNA group were injected 50 μ g Sirt3 siRNA through the tail vein once a week for 2 weeks.

Sirt3 siRNA administration

In vivo-jetPEITM was used for control and Sirt3 siRNA administration, according to the manufacturer's instructions. Briefly, control or Sirt3 siRNA (50 μ g) was dissolved in a 100- μ L mixture of equal volumes of in vivo-jetPEITM and 10% glucose, and then injected into the tail vein once a week for 2 weeks. The Sirt3 siRNAs were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and the sequences were as follows: Sirt3 siRNA sense, 5'-GUCUGAAGCAGUACAGAAAtt-3' and antisense, 5'-UUUCUGUACUGCUUCAGACaa-3' (Srivastava et al. 2018); control siRNA sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3' antisense (Tang et al. 2018).

Masson's trichrome staining

The left lower pulmonary tissues were fixed in paraformaldehyde and then dehydrated in graded alcohol and embedded in paraffin. Paraffin-embedded sections (5 μ m) were subjected to Masson's trichrome staining (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) to measure the fibrotic areas, according to the manufacturer's instructions (Du S-F et al. 2019). Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD) was used to quantify the fibrotic area by manually examining the blue area (Du S-F et al. 2019). The researcher examining the fibrotic area was blinded to group allocation.

Measurement of hydroxyproline and collagen I content

Cold PBS containing proteinase inhibitor cocktail (Sigma-Aldrich; Merck KgaA, Darmstadt, Germany) was used to homogenize pulmonary tissues, and hydroxyproline content (Winching, Nanjing, China) and collagen I (R&D Systems, Inc., Minneapolis, MN) were examined according to the manufacturer's instructions.

Isolation of mouse lung fibroblasts

Mouse pulmonary tissues were cut into pieces and then digested for 90 min at 37°C with gentle shaking. Dulbecco's modified Eagle's medium-prepared digestion solution contained collagenase type III (0.1 U/mL, Worthington, Lakewood, NJ), trypsin (0.125%, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA) and DNase I (0.1 U/mL, Thermo Fisher Scientific, Inc., Waltham, MA). Following filtration, cells were collected for subsequent experiments (Sun X et al. 2015).

Reverse transcription-quantitative PCR (RT-qPCR)

TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) was used to extract total RNA from lung fibroblasts and pulmonary tissues, which was then reverse-transcribed to cDNA using superscript reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA) with oligodeoxythymidine for mRNAs. A CFX Connect real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) was used to perform qPCR with the following primer sequences: Sirt3 (accession no. NM_001127351.1) forward, 5'-AGCAACCTTC AGCAGTATGACATCC-3' and reverse, 5'-TTTCACAACGCCAGTACAGACAGG-3'; monocyte chemoattractant protein-1 (MCP-1; accession no. NM_011333) forward, 5'-CCACTCACCTGCTGCTACTCATTC-3' and reverse, 5'-GTTCACCTGTCACACTGGTCACTCC-3'; TNF-α (accession no. NM_001278601.1) forward, 5'-CACCACGCTCTTCTGTC TACTGAAC-3' and reverse, 5'-TGACGGCAGAGAGGA GGTGAC-3'; plasminogen activator inhibitor-1 (PAI-1; accession no. NM_008871.2) forward, 5'-TCAATGACT GGGTGGAAGGCATAC-3' and reverse, 5'-AGATGTTGGTG AGGCGGAGAG-3' MMP-10 (accession no. NM_019471.3) forward, 5'-GCCTACCAATCTGCTCAGCGTATC-3' and reverse, 5'-TGAAGCCACCAACATCAGGAACAC-3' reverse; and MMP-12 (accession no. NM_001320076.1) forward, 5'-TCAATGACTGGGTGGAAAGGCATAC-3' and reverse, 5'-AGATGTTGGTGAGGCGGAGAG-3'. The 20 μL reaction solution contained 5 μL diluted cDNA, 0.5 μM paired primer, 10 μL SYBRGreen mix (Aidlab Biotechnologies Co., Ltd., Beijing, China) and 4.9 μL DEPC water. The annealing temperature and amplification were set at 60°C and 40 cycles, respectively. The comparative C_q method ($2^{-\Delta\Delta C_q}$) was performed to measure the relative gene expression (Du JK et al. 2016). mRNA levels were normalized to those of β-actin.

Western blotting

Cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland) was used to lyse lung fibroblasts and pulmonary tissues. Following determination of protein concentrations using BCA assays, the isolated protein was separated using 10% SDS-PAGE and then transferred to PVDF membranes. Following blocking with non-fat dry milk dissolved in TBS for 2 h at room temperature, the membranes were incubated with BSA-diluted primary antibodies against Sirt3 (1:1000; cat. no. sc-365175; Santa Cruz Biotechnology, Inc., Dallas, TX), p16 (1:1000, cat. no. sc-56330; Santa Cruz Biotechnology, Inc., Dallas, TX), Smad4 (1:1000; cat. no. sc-7966; Santa Cruz Biotechnology, Inc., Dallas, TX), p-Smad2 (p-Smad2; 1:1000; Cell Signaling Technology, Inc., Danvers, MA), p-Smad3 (1:1000; Cell Signaling Technology, Inc., Danvers, MA), p53 (1:1000; cat. no. 10422-

1-AP; ProteinTech Group, Inc., Rosemont, IL), p21 (1:1000; cat. no. 27296-1-AP; ProteinTech Group, Inc., Rosemont, IL), αSMA (1:1000, cat. no. sc-8432; Santa Cruz Biotechnology, Inc., Dallas, TX), fibronectin (1:1000, cat. no. sc-8422; Santa Cruz Biotechnology, Inc., Dallas, TX), Smad2 (1:1000; cat. no. sc-393312 Santa Cruz Biotechnology, Inc., Dallas, TX), Smad3 (1:1000; cat. no. sc-101154; Santa Cruz Biotechnology, Inc., Dallas, TX) or β-actin (1:1000; cat. no. sc-81178; Santa Cruz Biotechnology, Inc., Dallas, TX) at 4°C overnight. Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:1000) for 2 h at room temperature. An Enhanced Chemiluminescence Western Blotting Detection system (Santa Cruz Biotechnology, Inc., Dallas, TX) and a GeneGnome HR scanner (Syngene Europe, Frederick, MD) were used to visualize the bands.

Statistical analysis

The data are presented as the mean ± SEM. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL). Statistical comparisons between two groups were determined using a two-tailed Student's *t*-test. One- or two-way ANOVA with a Bonferroni's *post hoc* test was performed for comparisons among multiple groups. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Baicalein mitigates BLM-induced lung fibrosis in the mouse model

The impact of baicalein on BLM-induced lung fibrosis was first explored. As demonstrated in Figure 1(A,B), Masson's trichrome staining revealed considerable collagen deposition in the pulmonary interstitium, with collagen deposition primarily observed in the alveolar walls along with epithelial thickening and cellular infiltrates. BLM also increased the expression of fibronectin and α-SMA in the protein level of lung tissue (Fig. S1A). Baicalein could significantly mitigate BLM-induced collagen deposition, structural damage and elevated expression of fibronectin and α-SMA in the lung tissue, as evidenced by a decrease in the amount of collagen deposition, relatively normal pulmonary architecture and low expression of fibronectin and α-SMA (Figure 1(A,B), Fig. S1A).

Next, the amount of hydroxyproline and collagen I was measured to quantify the amount of collagen deposited in the lungs. As shown in Figure 1(C,D), the amount of hydroxyproline and collagen I was significantly increased in BLM-treated mice and baicalein could reverse the BLM-induced increase in the content of hydroxyproline and collagen I, indicating that baicalein mitigates BLM-induced lung fibrosis.

Baicalein mitigates BLM-induced lung fibroblast senescence in the mouse model

Next, the effects of baicalein on BLM-induced cell senescence in the lung tissue were explored. As shown in Figure 2(A), the protein levels of senescence effectors, including p53, p21 and p16, were significantly elevated in the lung tissue of BLM-treated mice. The ratio of the SA-β-gal-positive senescent cells was also increased in isolated lung fibroblast of BLM-treated mice (Fig. S1B and C). However, the increased expression of p53, p21 and p16 and the elevated ratio of SA-β-gal-positive senescent cells

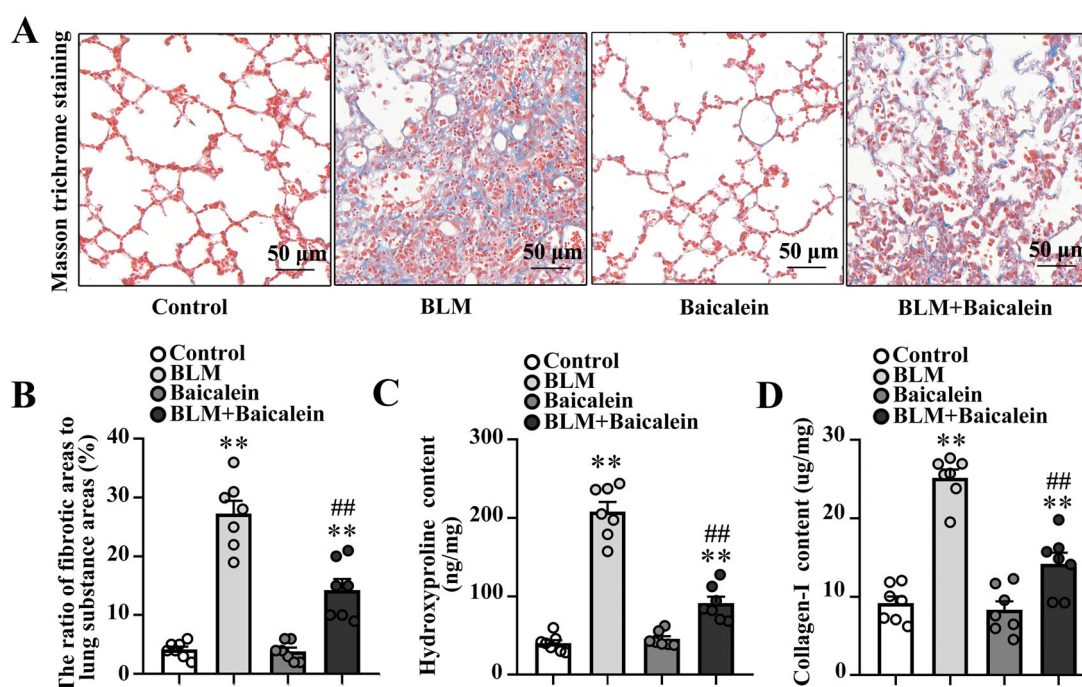


Figure 1. Baicalein mitigates BLM-induced lung fibrosis. (A, B) Masson's trichrome staining was performed to measure collagen deposition in pulmonary tissues in control, BLM, baicalein and BLM + baicalein groups. Representative images (A) and changes in the ratio of collagen-deposited areas to lung substance areas (a morphometric measure of pulmonary fibrosis) (B). Hydroxyproline (C) and collagen I (D) contents in pulmonary tissues were examined by ELISA in control, BLM, baicalein and BLM + baicalein groups. Data are presented as the mean \pm SEM ($n = 7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. BLM: bleomycin.

were markedly reversed by baicalein treatment. In addition, the lung tissue in BLM-treated mice demonstrated markedly increased transcript levels of proinflammatory and profibrotic senescence-associated secretory phenotype (SASP) factors, including MCP-1, PAI-1, TNF- α , MMP-10 and MMP-12, which were partly attenuated by baicalein treatment (Figure 2(B)).

To further clarify the direct effect of baicalein on lung fibroblast senescence in BLM-treated mice, the lung fibroblasts in BLM-treated mice exhibited an obvious increase in the protein levels of p53, p21 and p16, as well as the transcript levels of proinflammatory and profibrotic SASP factors, and this was reversed by baicalein treatment (Figure 2(C,D)), indicating that baicalein mitigated BLM-induced fibroblast senescence.

Baicalein ameliorates BLM-induced activation of TGF- β 1/Smad signalling in lung tissues

The TGF- β 1/Smad pathway has been widely reported to play a crucial role in tissue fibrosis (Tseliou et al. 2014; Tran et al. 2019; Yao et al. 2019; Chale-Dzul et al. 2020; Hussein et al. 2020; Li X-F et al. 2020; Du J-K et al. 2021). As shown in Figure 3(A,B), the transcript levels of TGF- β 1 and protein levels of p-Smad2, p-Smad3 and Smad4 were increased in the lung tissue of BLM-treated mice compared with the control, suggesting that BLM activates TGF- β 1-Smad signalling in the lung. It was also found that baicalein could suppress the BLM-induced TGF- β 1 production, p-Smad2, p-Smad3 and Smad4 expression in the lung.

Baicalein restores Sirt3 expression in the lung tissues of BLM-treated mice

A previous study indicated that the downregulation of Sirt3 contributes to aging-associated tissue fibrosis by blocking TGF- β

expression (Sundaresan et al. 2015). As shown in Figure 4(A,B), BLM treatment resulted in an obvious decrease in the mRNA and protein expression levels of Sirt3 in the lungs. Baicalein could prevent the BLM-induced decrease in the lung tissue expression of Sirt3.

Sirt3 siRNA abrogates the protective role of baicalein against BLM-induced pulmonary fibrosis

Next, the impact of Sirt3 siRNA on the protective role of baicalein against BLM-induced pulmonary fibrosis was explored. As shown in Figure 5(A), Sirt3 siRNA resulted in an $\sim 80\%$ decrease in Sirt3 expression in the lung tissues, and Sirt3 knockdown blocked the protective effects of baicalein against BLM-induced pulmonary fibrosis, as evidenced by the decreased collagen deposition (Figure 5(B)), and the levels of hydroxyproline (Figure 5(C)), collagen I (Figure 5(D)), α SMA and fibronectin (Fig. S2A and B). These results suggested that Sirt3 siRNA could abolish the protective role of baicalein against BLM-induced pulmonary fibrosis.

Sirt3 siRNA abolishes the protective role of baicalein against BLM-induced lung fibroblast senescence and activation of TGF- β 1/Smad signalling in lung tissues

Next, the impact of Sirt3 siRNA on the beneficial role of baicalein against BLM-induced lung fibroblast senescence was explored. As shown in Figure 6, Fig. S2C and D, Sirt3 siRNA blocked the protective effects of baicalein against BLM-induced senescence and the alteration of the levels of proinflammatory and profibrotic SASP factors in the lung tissue and isolated lung fibroblasts, as evidenced by the decreased protein levels of p53, p21, p16 and the ratio of the SA- β -gal-positive senescent cells in

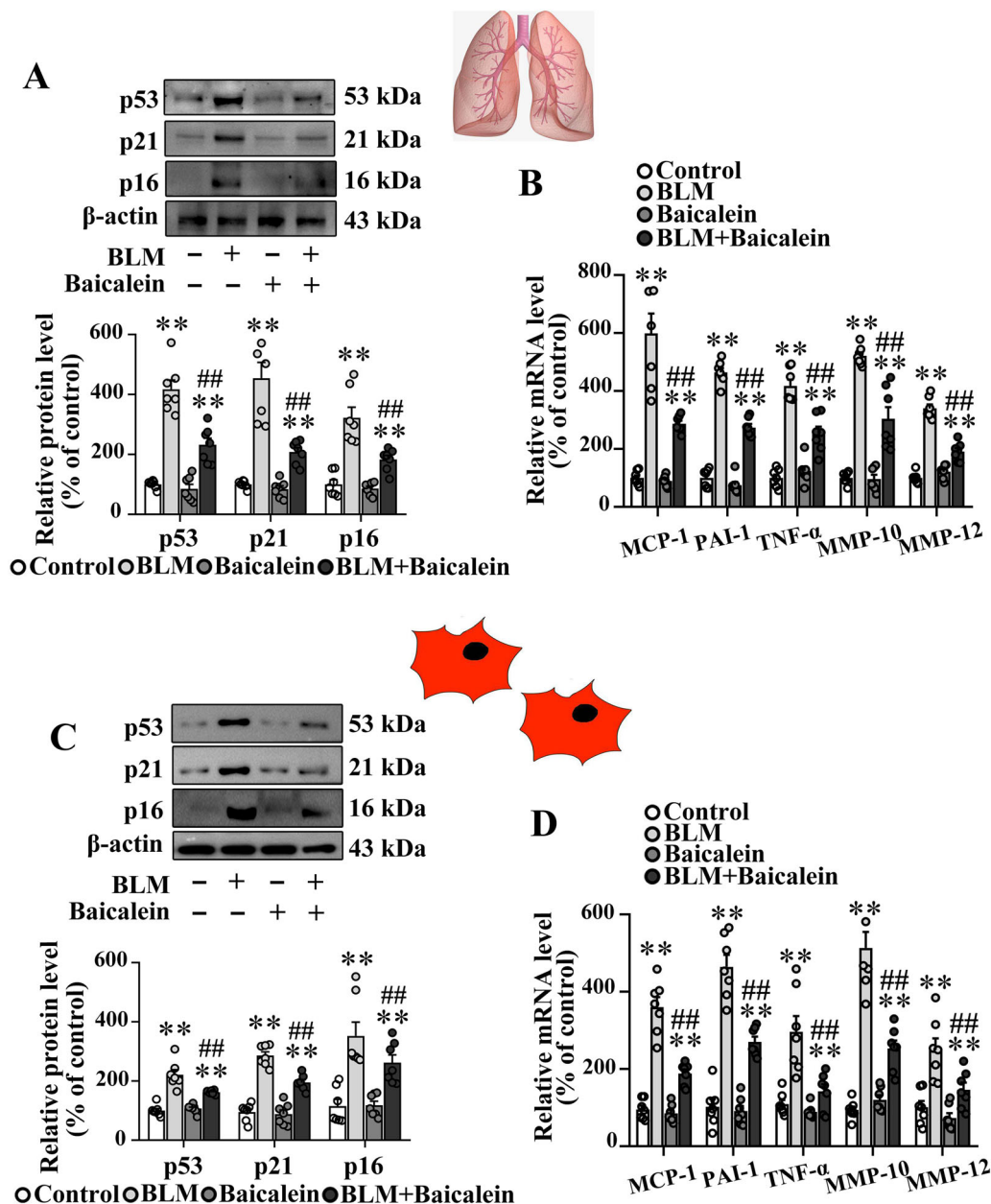


Figure 2. Baicalein mitigates BLM-induced senescence in lung tissues and isolated lung fibroblasts. (A, B) Protein levels of p53, p21 and p16 and the mRNA levels of SASP factors, including MCP-1, PAI-1, TNF- α , MMP-10 and MMP-12 in lung tissues were measured in control, BLM, baicalein and BLM + baicalein groups. (C, D) Protein levels of p53, p21 and p16 and the mRNA levels of MCP-1, PAI-1, TNF- α , MMP-10 and MMP-12 in isolated lung fibroblasts were measured using western blotting and RT-qPCR, respectively. Representative protein bands were presented on the top of the histograms (A, C). Data are presented as the mean \pm SEM ($n = 7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. SASP: senescence-associated secretory phenotype; MCP-1: monocyte chemoattractant protein-1; PAI-1: plasminogen activator inhibitor-1; TNF- α : tumour necrosis factor- α ; MMP: matrix metalloproteinase; RT-qPCR: reverse transcription-quantitative PCR; BLM: bleomycin.

isolated lung fibroblast, as well as the transcript levels of proinflammatory and profibrotic SASP factors.

We then explored the impact of Sirt3 siRNA on the role of baicalein against BLM-induced activation of TGF- β 1-Smad pathway. Results showed that Sirt3 siRNA blocked the inhibitory effect of baicalein against BLM-induced activation of TGF- β 1/Smad signalling, as shown by increased transcript levels of TGF- β 1 (Figure 7(A)) and protein levels of p-Smad2, p-Smad3 and Smad4 (Figure 7(B,C)) in the lung tissue.

Discussion

IPF is a chronic, progressive, fibrosing interstitial lung disease that affects hundreds of thousands of people worldwide, reducing

their quality of life and leading to death from respiratory failure within years of diagnosis. Herein, BLM administration was found to result in significant pulmonary fibrosis and inflammation, and baicalein was found to play a positive role in preventing BLM-induced fibrosis and inflammatory responses in a mouse model. Our findings suggested that the dysregulation of Sirt3-mediated pulmonary fibroblast senescence contributed to BLM-induced lung fibrosis, and Sirt3 knockdown blocked the protective role of baicalein in preventing BLM-induced fibrosis.

Baicalein is a common plant flavonoid with a wide range of beneficial pharmacological properties, including anti-inflammatory (Teng et al. 2020; D'Amico et al. 2021), antioxidative (D'Amico et al. 2019), anti-apoptotic (Liu C et al. 2010; Li X-x et al. 2012; Lin M et al. 2014; Hung et al. 2016; Wang M et al.

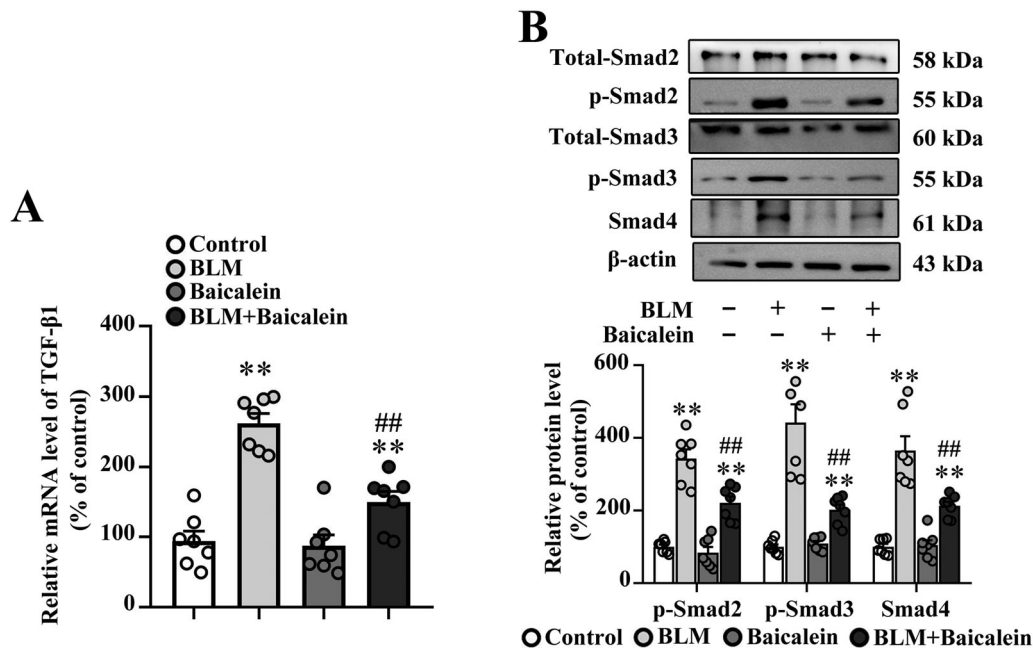


Figure 3. Baicalein mitigates BLM-induced TGF-β1/Smad signalling in lung tissue. (A) The mRNA expression levels of TGF-β1 in pulmonary tissues were examined using RT-qPCR. (B) The protein expression levels of p-Smad2, p-Smad3 and Smad4 in pulmonary tissues were examined using western blotting. Representative immunoblots and the corresponding histograms are presented. Data are presented as the mean ± SEM ($n = 7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. BLM: bleomycin; TGF-β1: transforming growth factor-β1; Smad: mothers against decapentaplegic homolog; RT-qPCR: reverse transcription-quantitative PCR.

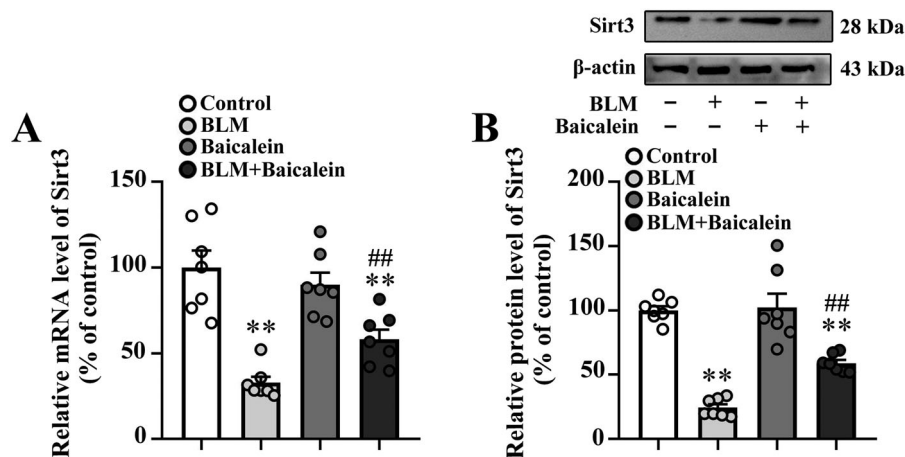


Figure 4. Baicalein prevents BLM-induced downregulation of Sirt3 in lung tissues. RT-qPCR and western blotting analysis were performed to measure the (A) mRNA and (B) protein Sirt3 expression and in the lung tissues of control, BLM, baicalein and BLM + baicalein groups. Representative immunoblots of Sirt3 and the corresponding histograms are presented. Data are presented as the mean ± SEM ($n = 7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. p-: phosphorylated. Sirt3: sirtuin 3; BLM: bleomycin.

2020), antitumorigenic (Li J et al. 2019) and pro-immunoregulatory functions (Shi et al. 2018). In addition, previous studies suggested that baicalein has antifibrotic potential in several types of tissues, including the lung (Gao et al. 2013), kidney (Hu et al. 2017), liver (Sun H et al. 2010) and heart (Kong et al. 2010). Baicalein has been reported to reverse BLM-induced lung fibrosis in rats, which was partly achieved through the inhibition of TGF-β/Smad signalling. Consistent with these findings, it was also found that baicalein could attenuate BLM-induced pulmonary fibrosis in mice. The protective role of baicalein against BLM-induced pulmonary fibrosis was partly independent in inhibiting TGF-β/Smad signalling, as shown by the improved lung architecture, decreased collagen deposition and amounts of hydroxyproline and type I collagen, as well as TGF-β1 production and phosphorylation of Smad2, Smad3 and Smad4 expression in the lung.

Cell senescence refers to a relatively stable state in which cells irreversibly leave the cell cycle and lose their proliferative ability under the action of signal transduction. In recent decades, cell senescence has attracted widespread attention as it increases the morbidity of fibroproliferative pulmonary diseases in elderly individuals (Parimon et al. 2021). Recent studies have demonstrated that epithelial progenitor cell dysfunction and cellular senescence, including epithelial and fibroblast senescence, were associated with the pathological development of IPF (Demaria et al. 2014; Lehmann et al. 2017). Hohmann et al. (2019) found that quercetin could attenuate BLM-induced lung fibrosis and injury by inhibiting fibroblast senescence and enhancing FasL- or TRAIL-induced apoptosis. Of note, Cui et al. (2018) demonstrated that baicalein could mitigate TGF-β1-mediated lung FMT differentiation through the inhibition of miR-21 expression. In the present study, it was found that baicalein reversed

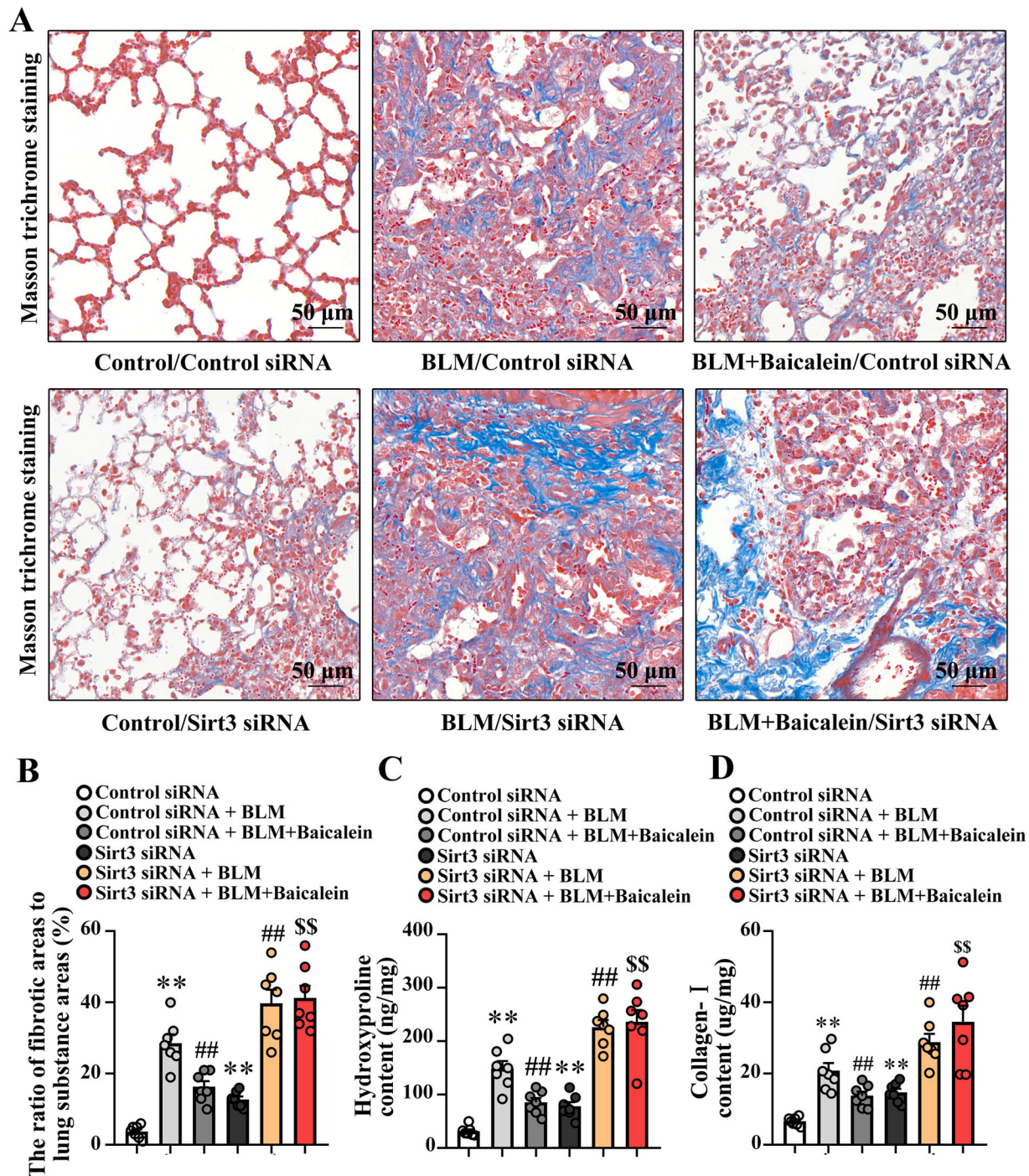


Figure 5. Silencing of Sirt3 abolishes the protective effect of baicalein against BLM-induced lung fibrosis. Masson's trichrome staining was performed to measure collagen deposition in pulmonary tissues. (A, B) Representative images and the ratio of fibrotic areas to the total lung area. (C) Hydroxyproline and (D) collagen I content in pulmonary tissues was examined using ELISA. Data are presented as the mean \pm SEM ($n=7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. \$\$ $p < 0.01$ vs. BLM + baicalein. Sirt3: sirtuin 3; BLM: bleomycin.

BLM-induced lung fibroblast senescence and increased the transcript levels of proinflammatory and profibrotic SASP factors in mice. However, whether baicalein enhances lung fibroblast apoptosis in BLM-treated mice and regulates the expression of miR-21 is worthy of further study.

Sirt3 dysregulation was reported to be involved in the pathological process of lung fibrosis. Clinically, it was demonstrated that Sirt3 was absent within fibrotic areas, as compared with adjacent areas within the same tissue in the scleroderma and IPF specimens (Sosulski et al. 2017). It was also reported that Sirt3

was deficient in the alveolar epithelial cells of IPF patients (Cheresh et al. 2021); these findings indicated that Sirt3 may have therapeutic potential in the management of lung fibrosis. In animal models of pulmonary fibrosis, the expression of Sirt3 was significantly decreased in the lung tissue of Ad-TGF- β 1-treated mice (Sosulski et al. 2017). Sirt3 overexpression attenuated asbestos-mediated lung fibrosis and the beneficial effect of Sirt3 overexpression was associated with decreased lung mtDNA damage and Mo-AM recruitment (Jacobs et al. 2008). Consistent with the study, Sosulski et al. (2017) found that Sirt3-deficient mice

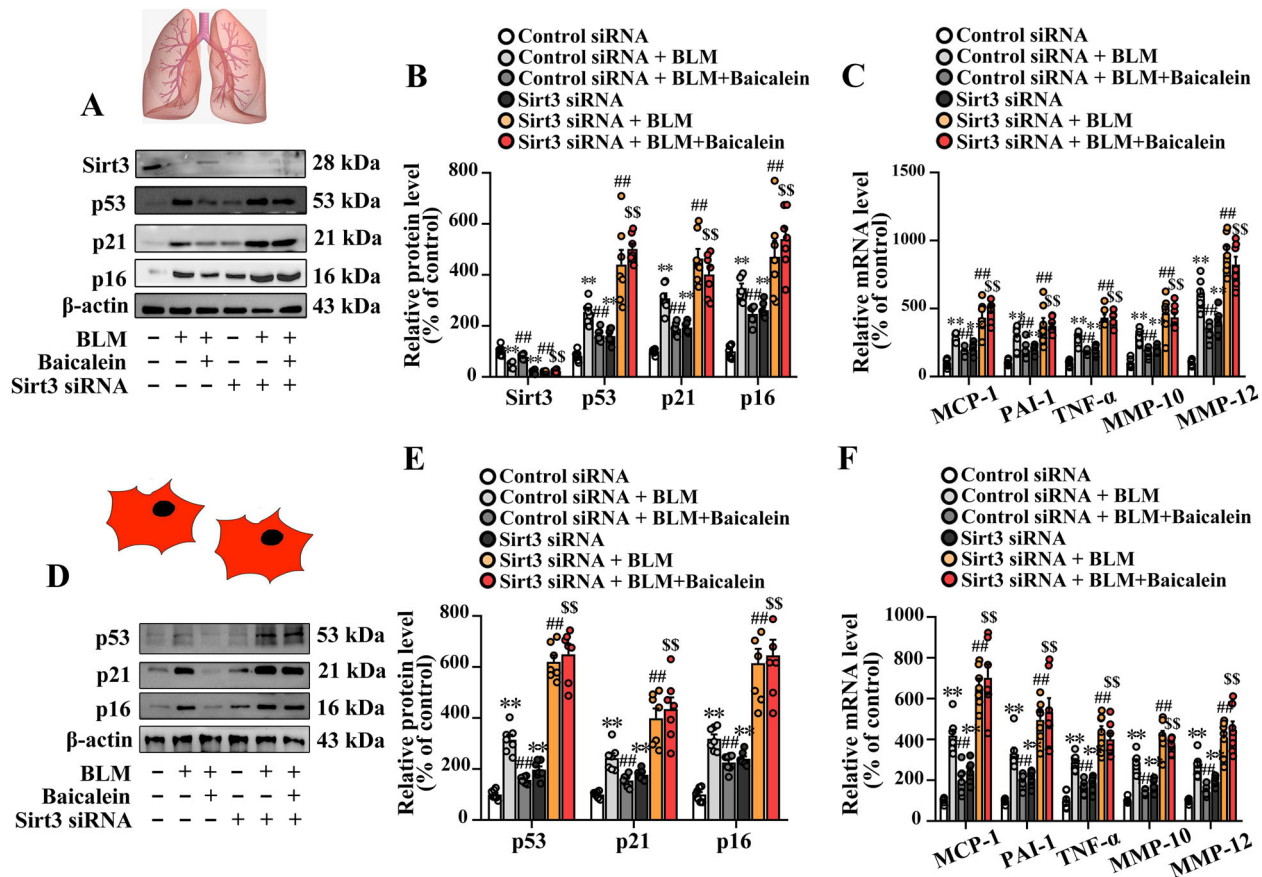


Figure 6. Sirt3 silencing abolishes the protective effect of baicalein against BLM-induced senescence in lung tissue and isolated lung fibroblasts. (A, B, D and E) The protein expression levels of Sirt3, p53, p21 and p16 in pulmonary tissues were measured using western blotting. (C, F) The mRNA levels of MCP-1, PAI-1, TNF- α , MMP-10 and MMP-12 were measured using RT-qPCR. Data are presented as the mean \pm SEM ($n=7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM + baicalein. Sirt3: siRNA; BLM: bleomycin; MCP-1: monocyte chemoattractant protein-1; PAI-1: plasminogen activator inhibitor-1; TNF- α : tumour necrosis factor- α ; MMP: matrix metalloproteinase; RT-qPCR: reverse transcription-quantitative PCR.

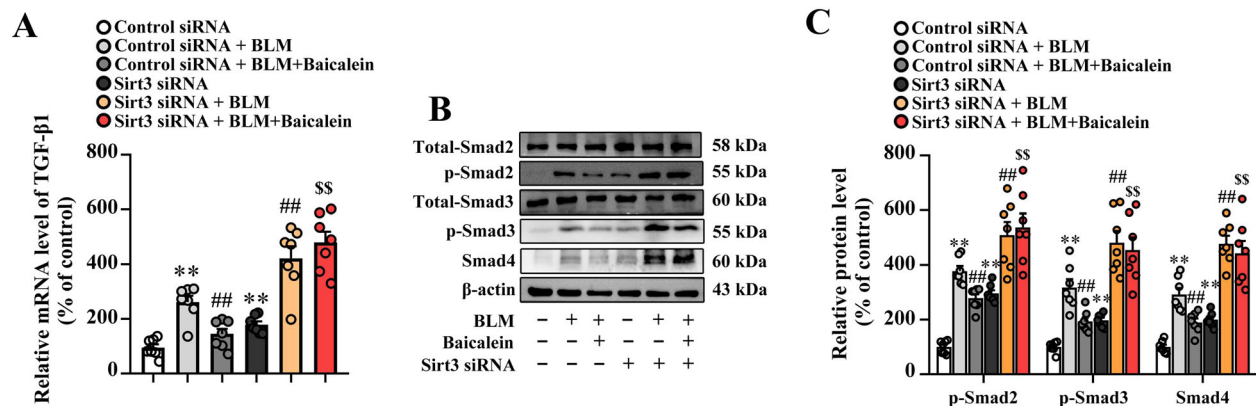


Figure 7. Sirt3 knockdown abolishes the protective effects of baicalein on BLM-induced TGF- β 1/Smad signalling in the lung tissue. (A) The mRNA expression levels of TGF- β 1 in pulmonary tissues were examined using RT-qPCR. (B) The protein expression levels of p-Smad2, p-Smad3 and Smad4 in pulmonary tissues were examined using western blotting. Data are presented as the mean \pm SEM ($n=7$). ** $p < 0.01$ vs. control. ## $p < 0.01$ vs. BLM. \$\$\$ $p < 0.01$ vs. BLM + baicalein. Sirt3: siRNA; BLM: bleomycin; TGF- β 1: transforming growth factor- β 1; Smad: mothers against decapentaplegic homology; RT-qPCR: reverse transcription-quantitative PCR.

were susceptible to lung fibrosis, and Sirt3 overexpression mitigated TGF- β 1-induced FMT differentiation. The role of Sirt3 in lung fibrosis and FMT differentiation was associated with Smad3 (Schafer et al. 2017). Nevertheless, it was also found that Sirt3 expression was significantly decreased in the lung tissues of BLM-treated mice and Sirt3 downregulation contributed to the pathological process of BLM-mediated pulmonary fibrosis.

Located in the mitochondrial matrix, Sirt3 is a member of the Sirtuin family. It was reported to deacetylate and activate

mitochondrial forkhead box class O 3a, which then regulated defective mitochondrial clearance through transcriptional regulation of autophagy-related genes (Jacobs et al. 2008). In addition, Im et al. (2015) showed that autophagy was deficient in lung fibroblasts and in the fibrotic lungs of IPF patients. A limitation of the present study was that it did not examine mitochondrial function and autophagy. In addition, no *in vitro* experiments were performed in the present study, which would have improved its relevance for IPF and/or senescence. Inhibition

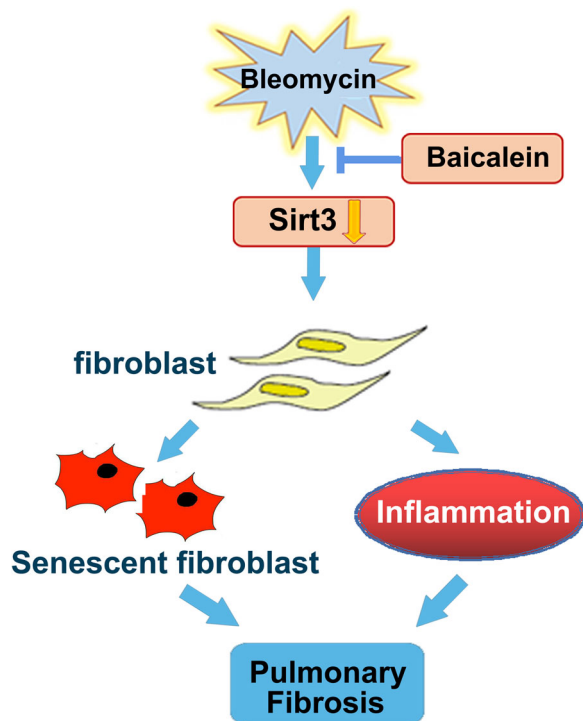


Figure 8. Schematic diagram of the mechanism through which baicalein attenuates BLM-induced pulmonary fibrosis through upregulating Sirt3 expression. BLM suppressed Sirt3 expression, and Sirt3 dysregulation contributed to pulmonary fibroblast senescence and inflammation, resulting in pulmonary fibrosis, which was markedly mitigated by baicalein administration. BLM: bleomycin; Sirt3: sirtuin 3.

experiments with baicalein using primary human IPF fibroblasts should be performed, and the robustness of senescence markers can be improved by performing senescence-associated- β -galactosidase staining and measuring SASP factors, in addition to gene expression, using ELISA.

Conclusions

Baicalein inhibited the BLM-mediated activation of TGF- β 1/Smad and lung fibroblast senescence, which was in parallel with the protective roles of baicalein against BLM-mediated lung fibrosis. Furthermore, baicalein preserved the BLM-induced downregulation of lung Sirt3 expression, and thus the suppression of TGF- β 1/Smad signalling pathway and lung fibrosis, which might provide an experimental basis for treatment of IPF (Figure 8).

Disclosure statement

All authors declare no conflict of interest.

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