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ORIGINAL ARTICLE

Pulmonary

Lanatoside C protects mice against bleomycin-induced pulmonary fibrosis through suppression of fibroblast proliferation and differentiation

Yunjuan Nie[1] | Dan Zhang[2] | Zhewu Jin[1] | Boyu Li[1] | Xue Wang[1] | Huilian Che[1] | Yaqian You[1] | Xiaohang Qian[1] | Yang Zhang[3] | Peng Zhao[1] | Gaoshang Chai[1]

[1]Department of Basic Medicine, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu, China

[2]Department of Laboratory Medicine, Research Center for Cancer Precision Medicine, Bengbu Medical College, Bengbu, Anhui Province, China

[3]Department of Orthopedic, Lu'an Fourth People's Hospital, Lu'an, Anhui, China

Correspondence
Peng Zhao, Gaoshang Chai, Department of Basic Medicine, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu, China.
Emails: zhaopeng336@jiangnan.edu.cn (PZ); chaigaoshang@jiangnan.edu.cn (GC)

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Summary

It has been established that lanatoside C, a FDA-approved cardiac glycoside, reduces proliferation of cancer cell lines. The proliferation of fibroblasts is critical to the pathogenesis of pulmonary fibrosis (PF), a progressive and fatal fibrotic lung disease lacking effective treatment. In this study we have investigated the impact of lanatoside C on a bleomycin (BLM)-induced mouse model of PF and through the evaluation of fibroblast proliferation and activation in vitro. We evaluated explanted lung tissue by histological staining, western blot analysis, qRT-PCR and survival analysis, demonstrating that lanatoside C was able to protect mice against BLM-induced pulmonary fibrosis. The proliferation of cultured pulmonary fibroblasts isolated from BLM-induced PF mice was suppressed by lanatoside C, as hypothesized, through the induction of cell apoptosis and cell cycle arrest at the G2/M phase. The Akt signalling pathway was involved in this process. Interestingly, the production of α-SMA, fibronectin, and collagen I and III in response to TGF-β1 in healthy mouse fibroblasts was suppressed following lanatoside C administration by inhibition of TGF-β1/Smad signalling. In addition, TGF-β1-induced migration in lung fibroblasts was also impeded after lanatoside C treatment. Together, our data revealed that lanatoside C alleviated BLM-induced pulmonary fibrosis in mice via attenuation of growth and differentiation of fibroblasts, suggesting that it has potential as a candidate therapy for PF patients.

KEYWORDS

Akt, differentiation, lanatoside C, proliferation, pulmonary fibrosis, TGF-β1

1 | INTRODUCTION

Pulmonary fibrosis (PF) is a progressive and fatal lung disease in which median survival time following diagnosis is 2-5 years.[1] Despite previous extensive studies, the pathogenesis of PF is still poorly understood and effective therapeutic strategies remain ambiguous.[2]

It is now universally acknowledged that widely distributed fibroblasts in the structures of the lungs perform a critical role during the fibrotic process.[3] Fibroblasts isolated from lungs of pulmonary fibrosis patients have been shown to display marked proliferation

Nie, Zhang and Jin equally contributed to this paper.
Zhao is the first corresponding author.

when cultured in specific stimulatory conditions.[4,5] Both cell cycle arrest and enhanced apoptosis have been identified as central mechanisms for controlling cell growth and proliferation in various cancer cell lines.[6,7] Interestingly, those two key components of proliferation have also been identified as being adaptive for fibroblasts.[8] Thus, suppression of proliferation of fibroblasts via the regulation of apoptosis and cell cycle progression is a pivotal therapeutic direction for pulmonary fibrosis.[9]

Additionally, pulmonary fibroblasts are capable of differentiation into myofibroblasts in response to profibrotic cytokines such as TGF-β1 during the process of wound healing leading to the development of pulmonary fibrosis and resulting in overproduction of extracellular matrix (ECM) including alpha smooth muscle actin (α-SMA), fibronectin and various collagen types.[10] Therefore, differentiation of fibroblasts is a second key process that occurs during the course of fibrosis.[11] We hypothesize that control of the activation, proliferation and migration of fibroblasts could attenuate fibrogenesis in pulmonary fibrosis.[12,13]

Lanatoside C, a cardiac glycoside approved by the US Food and Drug Administration (FDA), is widely used in the treatment of heart failure and cardiac arrhythmia.[14] Recent studies have reported that its therapeutic effect on cancer occurs via induction of apoptosis and suppression of cellular proliferation.[15,16] Characteristics of pulmonary fibrosis exhibit a degree of overlap with cancer during the process of fibrosis. Fibroblasts in PF are capable of proliferation and invasion of the alveolar basement membrane. This observation has prompted researchers to speculate about the possibility of oncology drugs being effective in the prevention and treatment of BLM-induced pulmonary fibrosis.[17,18]

To verify this hypothesis, we assessed the impact of lanatoside C in a BLM-induced mouse model of pulmonary fibrosis.[19] We found that treatment with lanatoside C protected mice against pulmonary

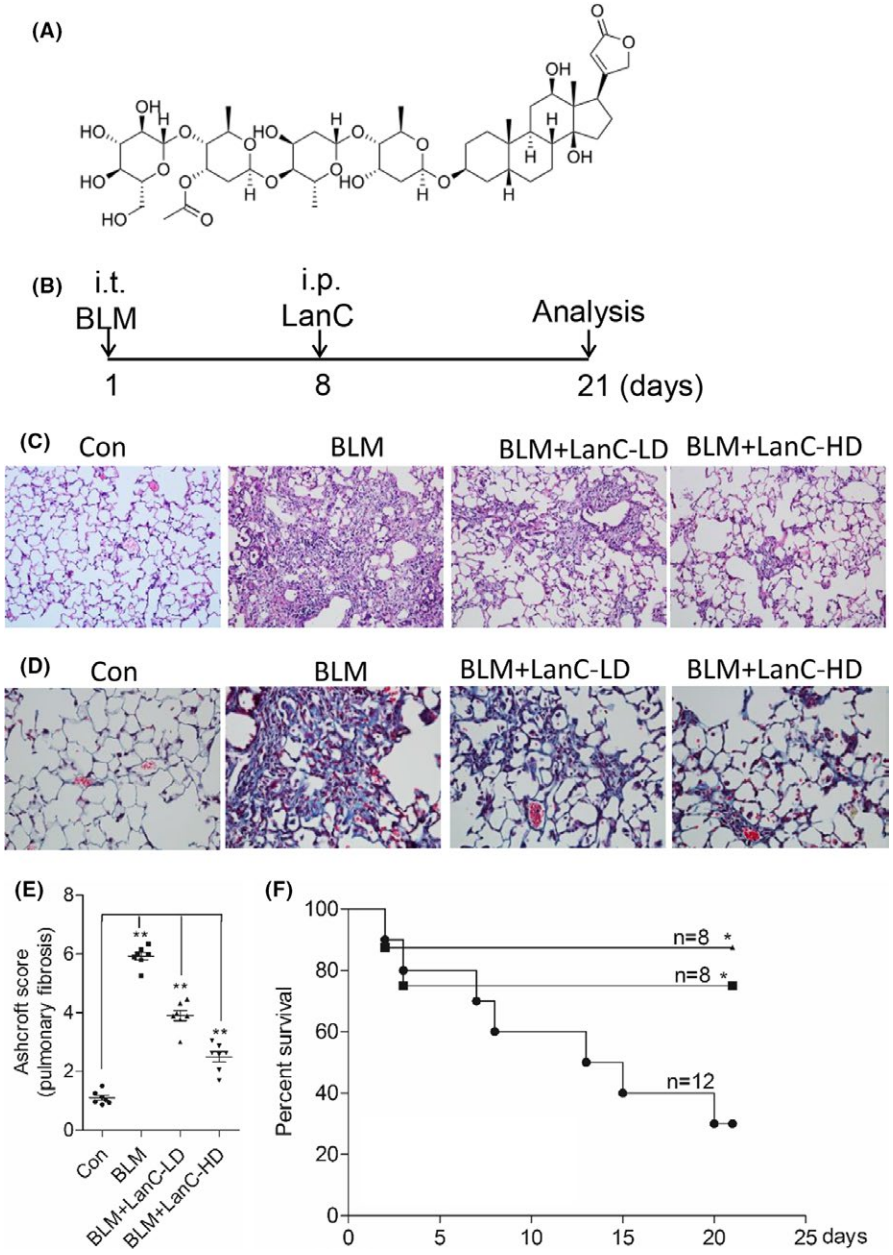

FIGURE 1 Lanatoside C attenuated pathological changes and reduced mortality in bleomycin-induced pulmonary fibrosis of mice. A, Chemical structure of lanatoside C (LanC). B, Schematic timeline shows the process of establishing bleomycin (BLM) model and LanC administration. C57BL/6 mice were i.t. injected with saline or BLM (1.4 U/kg) at day 1, followed by i.p. injection of LanC at the indicated dosage at day 8. Lungs were collected for analysis of fibrosis 21 d post challenge. C, Representative results for H&E, images taken under ×200 magnification. D, Masson's trichrome staining, ×400. E, Assessment of pulmonary fibrosis by Ashcroft score. F, Survival rate of mice after bleomycin challenge (1.5 U/kg body weight) with or without treatment by various concentrations of lanatoside C. Data represents means ± SEM using at least 6–8 mice in each group (*P < 0.05, **P < 0.01 and ***P < 0.001). ●— BLM; ■— BLM + LanC-LD; ▲— BLM + LanC-HD

fibrosis in a dose-dependent manner and reduced fibroblast proliferation, differentiation and migration in lung tissue. To explore the mechanism of this suppression, we quantified the relative expression of relevant genes and found that lanatoside C downregulated Akt and FoxO1 phosphorylation, thus modifying the expression of the downstream apoptosis-associated genes Bcl2 and cell cycle arrest genes cyclin D1 and cyclin E. In addition, the TGF-β1/Smad signalling pathway was also downregulated after lanatoside C administration. These observations support the notion that lanatoside C could be a potential treatment for pulmonary fibrosis.

2 | RESULTS

2.1 | Lanatoside C administration ameliorated BLM-induced pulmonary fibrosis

The chemical structure of lanatoside C, which is widely used to treat arrhythmias and heart failure, is shown in Figure 1A. It is currently considered to have a therapeutic effect on cancer. However, whether it could be an effective therapy for pulmonary fibrosis remains unclear. To determine the effect of lanatoside C on pulmonary fibrosis, we established a murine model of PF by intratracheal injection of BLM at day 1, intraperitoneal treatment with lanatoside C at day 8 then harvest of samples for analysis at day 21 (Figure 1B).

Histologic analysis demonstrated that fibroblasts and myofibroblasts aggregated and infiltrated a large number of normal alveolar structures, with infiltration of inflammatory cells 21 days after injection of bleomycin, while conversely, a considerable attenuation of pulmonary fibrosis was observed in a dose-dependent manner in the lanatoside C-treated mice (Figure 1C). Collagen deposition in lung sections was evaluated using Masson's trichrome staining. The pulmonary interstitium from lanatoside C-treated mice exhibited reduced deposition of blue stain compared with BLM-induced mice, suggesting that lanatoside C reduced collagen accumulation (Figure 1D). In particular, the severity of pulmonary fibrosis was much reduced as manifested by lower Ashcroft scores (Figure 1E). In addition, the survival assay revealed lanatoside C reduced long-term effects of high dosage BLM-induced lung fibrosis (Figure 1F). Collectively, these data indicate that lanatoside C protected against BLM-induced pulmonary fibrosis.

To further assess the effects of lanatoside C on pulmonary fibrosis, we evaluated the expression of α-smooth muscle actin (α-SMA) by western blot analysis, and fibronectin, collagen I and collagen Ш by RT-qPCR in whole-lung samples. In support of our hypothesis, the expression of α-SMA, fibronectin, and collagens I and Ш increased in the BLM-induced group compared with saline-treated control mice. Notably, lanatoside C-treated mice exhibited a marked reduction in these fibrotic markers in a dose-dependent manner (Figure 2A-E). To confirm these observations, we examined the concentration of

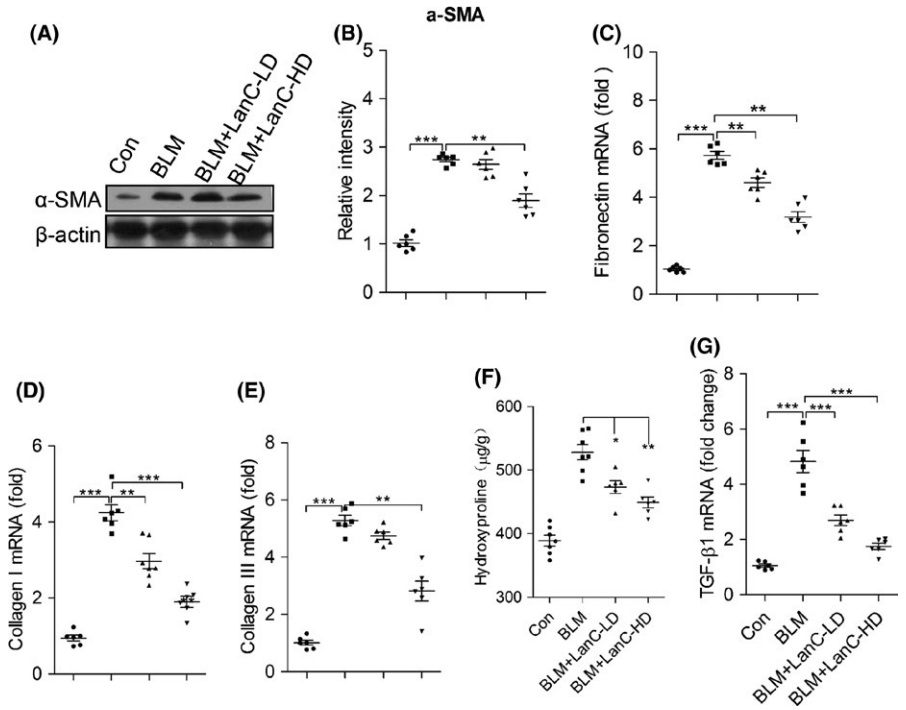

FIGURE 2 Administration of lanatoside C attenuated lung fibrosis after bleomycin (BLM) induction. Mice were given saline or bleomycin (1.4 U/kg body weight) by intratracheal instillation with or without treatment with various concentrations of lanatoside C by intraperitoneal injection in accordance with the defined protocol. Lungs were harvested for analysis of fibrosis. A, αSMA protein expression was measured by western blotting. B, quantitative analysis. C,D,E, mRNA levels of fibronectin, collagen I and collagen Ш assessed by RT-PCR analysis. F, Hydroxyproline content was determined using a commercial kit. G, mRNA levels of TGF-β1 assessed by RT-PCR analysis. Six to 8 mice were included in each study group. Data shown are means ± SEM (*P < 0.05, **P < 0.01 and ***P < 0.001) with n = 6–8 per group. ● Con; ■ BLM; ▲ BLM + LanC-LD; ▼ BLM + LanC-HD

hydroxyproline in the lungs. The results revealed that administration of lanatoside C caused only low levels of hydroxyproline to be detected after BLM induction in a dose-dependent manner (Figure 2F).

Together, these data demonstrate that administration of lanatoside C protected mice against BLM-induced pulmonary fibrosis. TGF-β1 is a key cytokine for PF progression, we examined its expression and found lanatoside C reduced the production of TGF-β1 significantly (Figure 2G).

2.2 | Lanatoside C suppressed proliferation of pulmonary fibroblasts by induction of cell apoptosis and cell cycle arrest

It has been established that the proliferation of fibroblasts is among the major pathophysiological mechanisms that cause pulmonary fibrosis.[12] The impact of lanatoside C on the viability and proliferation of pulmonary fibroblasts was assessed using an MTT assay. Cultured cells isolated from the lungs of BLM-induced mice were treated with 0-10 μmol/L doses of lanatoside C for 24 and 48 hours. The results revealed that it inhibited cellular growth at concentrations of 3 μmol/L and greater (Figure 3A).

Since cell cycle progression and apoptosis are decisive factors in cell proliferation, we investigated the phase distribution of the cell cycle and apoptosis in lanatoside C-treated pulmonary fibroblasts. Cell cycle progression was measured after treatment of cells with varying concentrations of lanatoside C for 24 hours. As shown in Figure 3B,C, the proportion of cells in the G2/M phase increased considerably in cells treated with both 3 and 10 μmol/L lanatoside C compared with the vehicle group. The results suggest that lanatoside C may inhibit cell cycle progression at the G2/M phase.

To investigate whether the anti-proliferation activity was associated with a cellular apoptotic response, pulmonary fibroblasts were treated with 3 and 10 μmol/L lanatoside C for 24 and 48 hours and then were stained with Annexin V-FITC/PI. Flow cytometry data (Figure 3D,E) indicate that lanatoside C treatment significantly increased the proportion of apoptotic cells at 24 and 48 hours. These data suggest that apoptosis was also implicated in the lanatoside C-mediated proliferation response.

2.3 | Lanatoside C downregulated Akt signalling pathways in pulmonary fibroblasts

The Akt signalling pathway is pivotal for cell growth and proliferation by activation of downstream effectors in both physiological and pathological conditions.[20,21] To elucidate the underlying mechanisms of lanatoside C treatment in the regulation of cellular apoptosis and cell cycle progression, pulmonary fibroblasts were treated with 3 μmol/L lanatoside C for 24 hours to examine the protein expression of the important signalling molecule Akt and its downstream targets FoxO1 and Bcl2, by western blot analysis. As shown in Figure 4A,B, lanatoside C significantly reduced phosphorylation of Akt and FoxO1, and downregulated the anti-apoptotic protein Bcl2 accordingly. Cyclin D1 and cyclin E are both key genes required for cell cycle progression and are regulated by Akt.[22-24] To investigate the mechanism by which lanatoside C blocked cell cycle progression, the expression of cyclin D1 and cyclin E was determined by RT-qPCR. As shown in Figure 4C,D, the mRNA levels of cyclin D1 and E were dramatically up-regulated after lanatoside C administration. Together, these data indicate that the anti-proliferative effects of lanatoside C on pulmonary fibroblasts may by mediated by the inhibition of the Akt signalling pathway.

2.4 | Lanatoside C suppressed TGF-β1 induced differentiation of pulmonary fibroblasts

Fibroblast differentiation is a pivotal step in the pulmonary fibrotic process, in which myofibroblasts are derived from fibroblasts in the presence of TGF-β1, which secrete extracellular matrix proteins causing progression of the disease. We thus evaluated the effect of lanatoside C on the differentiation of pulmonary fibroblasts. Cells were incubated with TGF-β1 (10 ng/mL) and lanatoside C (1 μmol/L) for 0, 24 and 48 hours. Cells induced with TGF-β1 alone acted as a control. The protein levels of α-SMA were downregulated in TGF-β1-induced pulmonary fibroblasts after lanatoside C treatment, as detected by western blot analysis (Figure 5A,B), indicating that administration of lanatoside C may affect the differentiation of fibroblasts. Based on the observations above, we conducted further analysis of the effect of lanatoside C on pulmonary fibroblast differentiation. We observed that lanatoside C administration significantly inhibited mRNA expression of fibronectin, collagen I and collagen Ш after TGF-β1 treatment for 24 and 48 hours, as determined by RT-qPCR (Figure 5C-E).

The results above suggest that lanatoside C suppressed the differentiation of fibroblasts.

2.5 | Lanatoside C attenuated fibroblast differentiation by suppression of TGF-β1/Smad signalling and impeded TGF-β1-mediated cell migration

To elucidate the underlying mechanism of fibroblast differentiation, we examined the Smad signalling pathway activity, pivotal for fibroblast differentiation after TGF-β1 stimulation. As shown in Figure 6A,B,C, TGF-β1 stimulation for 8 hours significantly increased levels of p-Smad2 and p-Smad3, while lanatoside C treatment significantly reduced this phosphorylation activity, resulting in attenuated Smad signal activation. These data suggest that lanatoside C suppressed the differentiation of fibroblasts by inhibition of TGF-β1/Smad signalling.

TGF-β1 is well known for its potent chemotactic effects. In consideration of the migration capacity of fibroblasts and the important role this plays in the development of pulmonary fibrosis, we examined the effect of lanatoside C on cell migration in response to TGF-β1 using a wound healing assay. TGF-β1-dependent fibroblast migration was notably reduced after lanatoside C treatment in a dose-dependent manner (Figure 6D,E).

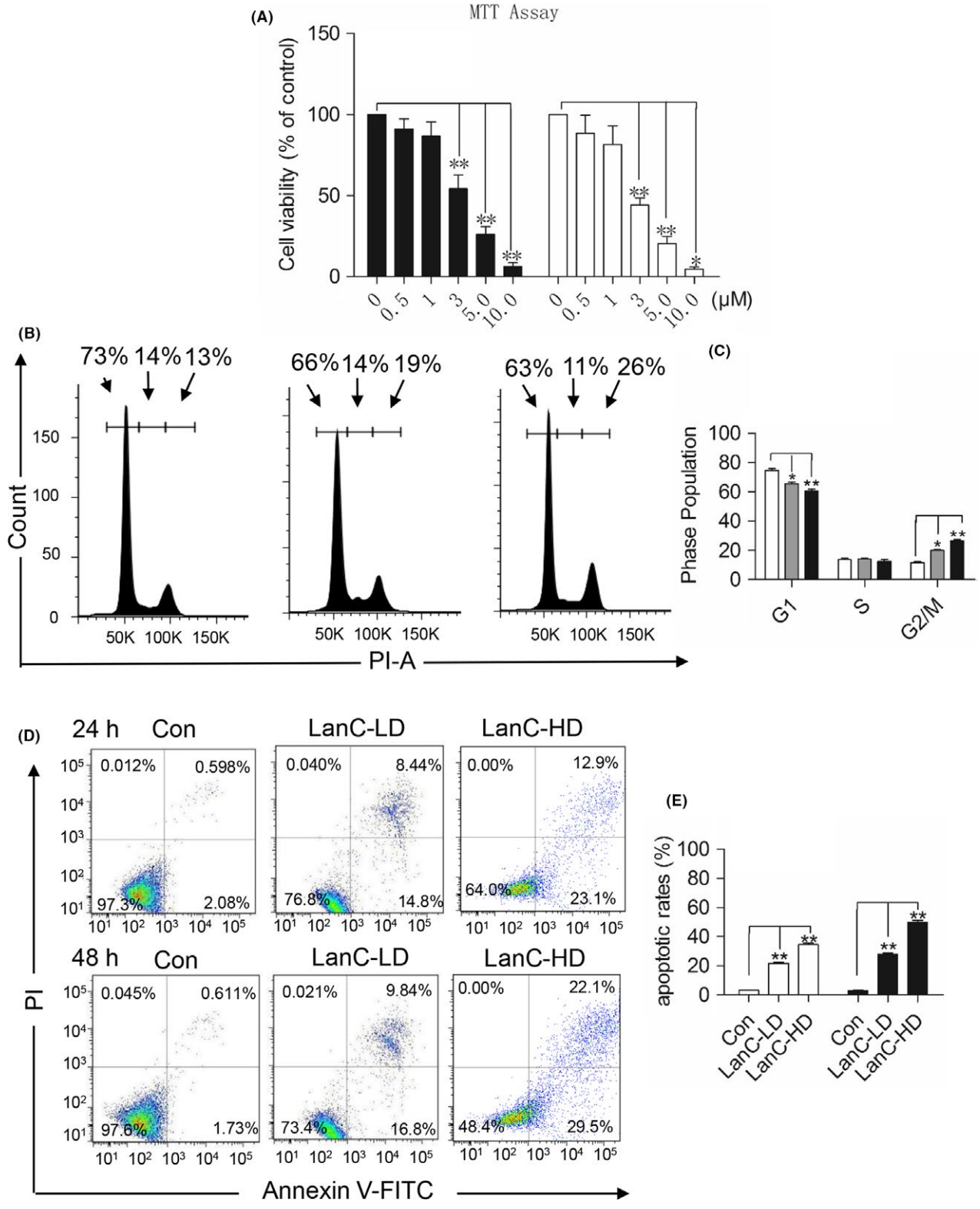

FIGURE 3 Lanatoside C reduced proliferation by induction of G2/M phase cell cycle arrest and apoptosis in pulmonary fibroblasts. Pulmonary fibroblasts were isolated from bleomycin (BLM)-treated mice. A, Cells were treated with 0–10 μmol/L lanatoside C for 24 or 48 h. The impact of lanatoside C on pulmonary fibroblast viability was assessed using an MTT assay. ■ 24 h; □ 48 h. B, Cells were treated with 3 or 10 μmol/L lanatoside C for 24 h then collected and analyzed by flow cytometry. C, Analysis of cell cycle distribution. □ DMSO; ▒ LanC-LD; ■ LanC-HD. D, Cell apoptosis after treatment with lanatoside C (3 or 10 μmol/L) or control for 24 and 48 h, analyzed by Annexin V-FITC/PI double staining by flow cytometry. E, The percentage of apoptotic cells was calculated as the apoptosis rate. □ 24 h; ■ 48 h. Data represents means ± SEM based on at least three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

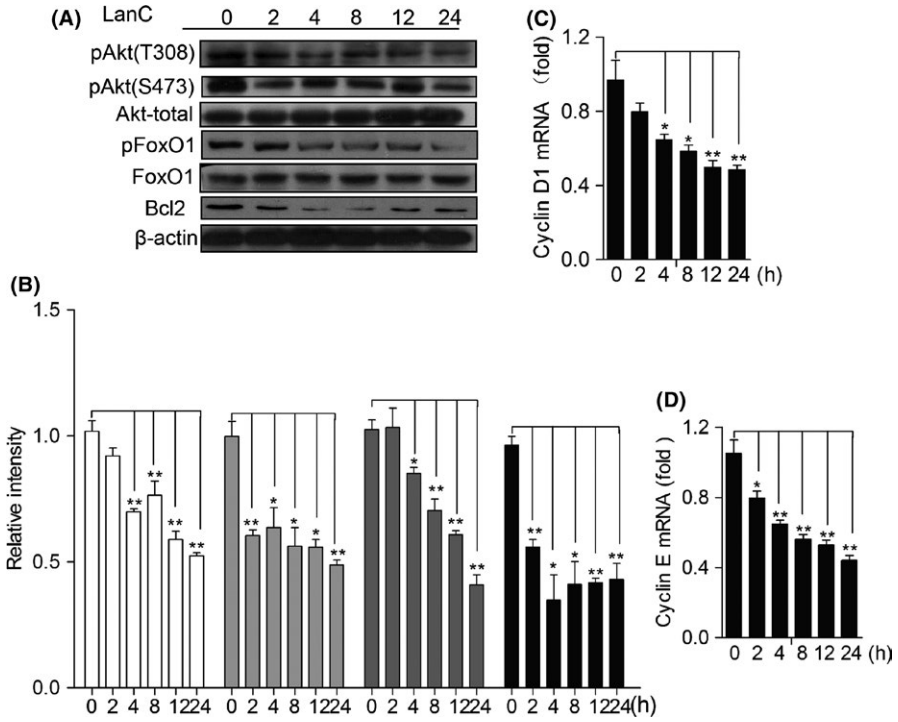

FIGURE 4 Akt signalling pathway was involved in lanatoside C-suppressed proliferation of pulmonary fibroblasts. Pulmonary fibroblasts were isolated from bleomycin (BLM)-treated mice, then dosed with lanatoside C (3 μmol/L) or vehicle (1‰ DMSO) for 0, 2, 4, 8, 12 or 24 h. A, Protein expression of phosphor-Akt, total-Akt, phosphor-FoxO1, FoxO1 and Bcl2 was quantified by western blot analysis. B, Quantification of phosphorylation of Akt, FoxO1 and expression of Bcl2 using Image J software. □ pAKT(ser318); ▒ pAkt(ser473); ▒ pFoxO1; ■ Bcl2. C,D, mRNA levels of cyclin D1 and cyclin E measured by qRT-PCR. mRNA levels of target genes were first normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) then converted to a percentage of the control. Quantitative data shown above are means ± SEM of three independent experiments. (*P < 0.05, **P < 0.01 and ***P < 0.001)

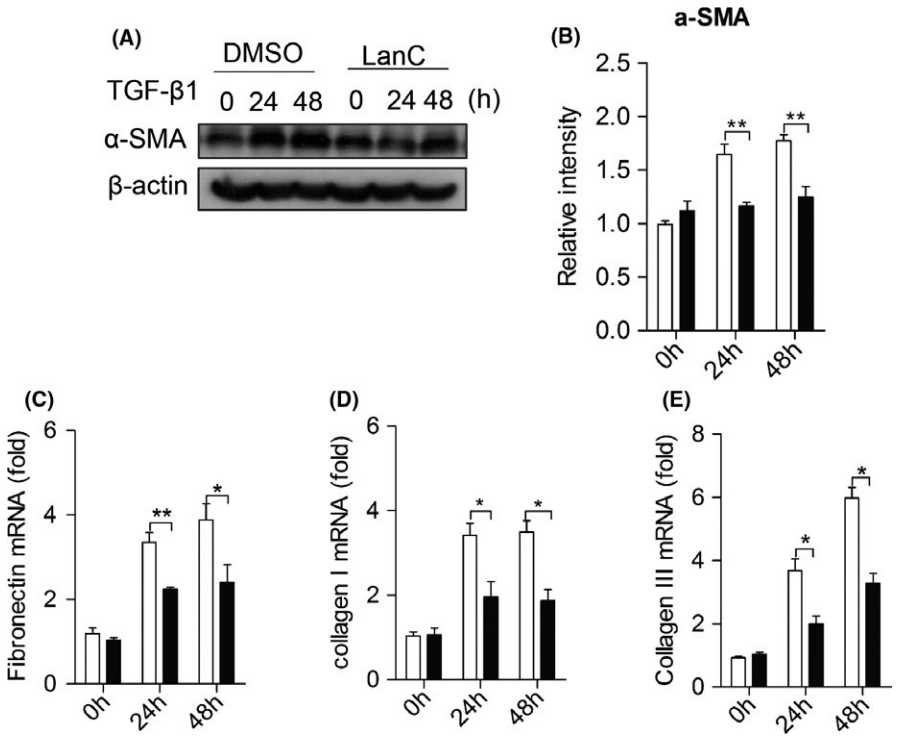

FIGURE 5 Lanatoside C treatment inhibited pulmonary fibroblast differentiation. Pulmonary fibroblasts were treated with TGF-β1 (10 ng/mL) + 1‰ DMSO or TGF-β1 (10 ng/mL) + lanatoside C (1 μmol/L) for 0, 24 or 48 h. A, Protein levels of α-SMA were quantified by western blot analysis. B, The quantification of α-SMA by Image J software. C,D,E, mRNA levels of fibronectin, collagen I and collagen III quantified by qRT-PCR and normalized to GAPDH. Quantitative data shown above are means ± SEM of three independent experiments. (*P < 0.05, **P < 0.01 and ***P < 0.001). □ DMSO + TGF-β1; ■ LanC + TGF-β1

2.6 | Lanatoside C treatment inhibited the phosphorylation of AKT, FOXO1 and Smad2/3 in vivo

We next investigated whether AKT, FOXO1 and Smad2/3 phosphorylation signalling pathway was involved in fibrotic process in vivo. As shown in Figure 7A-E, the phosphorylation of these signalling molecules in lung tissues of BLM-stimulated mice was significantly downregulated after lanatoside C treatment. These results further demonstrated that AKT, FOXO1 or Smad2/3 were involved in the protective roles of lanatoside C against BLM-induced pulmonary fibrosis.

3 | DISCUSSION

Pulmonary fibrosis is a devastating fibrotic disease with treatments that are of limited effectiveness. A key feature of PF is excessive deposition of ECM driven by fibroblast proliferation, differentiation and increased migration.[1,12] Previous studies have established that therapies targeting fibroblast proliferation and activation exhibit the most effective inhibition of PF development.[25] However, therapeutic options affecting fibroblasts are also limited and require evaluation.

Lanatoside C was initially approved as a cardiac glycoside for the efficient treatment of arrhythmias and heart failure. However, recent studies demonstrating involvement in anti-cancer functions and mechanistic studies have focused principally on its anti-proliferation, apoptosis-promoting and migration-suppressing properties.[15,26] It is well-established that the overproduction of excessive quantities of ECM proteins and the formation of fibrous scars in pulmonary fibrosis are driven mainly by pulmonary fibroblast proliferation and their differentiation into myofibroblasts.[27,28] In the pathological conditions of PF, activated fibroblasts resist apoptosis and exhibit excessive proliferation and differentiation.[29] If lanatoside C can perform an anti-proliferative role in fibroblasts, which share some overlapping characteristics with cancer cell lines,[17] it might affect the progression of pulmonary fibrosis. In order to explore this hypothesis, we thus adopted a bleomycin-induced mouse model of pulmonary fibrosis to evaluate the impact of lanatoside C on the disease. As hypothesized, our data demonstrate that BLM caused fibroblast proliferation and increased ECM production, including collagen. Treatments with lanatoside C significantly reversed these pathological injuries in a dose-dependent manner and promoted the survival of mice treated with high doses of BLM. To further confirm these results, we measured the concentrations of components of the ECM. The mRNA expression of α-SMA, fibronectin and collagens I and III declined, and hydroxyproline content decreased after administration of lanatoside C. These results clearly demonstrate the impact of lanatoside C on the suppression of PF.

Inflammatory responses are a critical mechanism for PF progression. BLM causes damage to the alveolar epithelial cells, leading to an interstitial inflammatory response within the first week after its administration.[19,30] However, we found there were few significant changes in BLM-induced inflammation after lanatoside C administration (Figure S1A-D) and that the effect of lanatoside C in regulating the development of BLM-induced PF is not through suppressing the inflammatory response. Fibroblasts are the principal effector cells in PF and extensive evidence suggests that pulmonary fibrosis is driven by proliferation, expansion and activation of fibroblasts, resulting in the production of a myofibroblast phenotype which secretes large quantities of ECM proteins.[10,31] To explore how lanatoside C could impact PF, we first cultured pulmonary fibroblasts then measured cell proliferation. Interestingly, fibroblasts treated with lanatoside C displayed reduced proliferation, increased apoptosis and disturbed cell cycle progression. These results indicate that lanatoside C administration can protect mice from BLM-induced pulmonary fibrosis and inhibit fibroblast proliferation.

Consequently, we explored the possible mechanisms of action of lanatoside C. The Phosphatidylinositol 3-kinase (PI3K)/Akt pathway, a major intracellular signalling pathway, can modulate cell proliferation, cell cycle progression and apoptosis under physiological and pathological conditions.[32,33] One important downstream target protein is FoxO1, which can be deactivated through phosphorylation at its three putative phosphorylation sites for Akt. Akt/FoxO1 has been shown to be involved in regulating cyclin D1 and cyclin E expression during cell cycle and controlling anti-apoptotic Bcl-2 levels.[34,35] Our data demonstrated that the phosphorylation of both Akt and FoxO1 was downregulated after lanatoside C treatment, indicating that reduced Akt activity contributed to enhanced FoxO1 activity. Accordingly, the expression of Bcl-2, cyclin D1 and cyclin E were significantly reduced. Together, these data indicate that the impact of lanatoside C on the proliferation and apoptosis of fibroblasts might be through the inactivation of the Akt signalling pathway, which has been identified as a pivotal molecule in the development of pulmonary fibrosis.[36-38]

Fibroblast differentiation is an additional critical aetiology in the development of pulmonary fibrosis. Accompanying changes in the microenvironment include the overproduction of a key factor, TGF-β1, resulting in the activation of fibroblasts into myofibroblasts and production of abundant levels of ECM in the interstitial tissues of the lungs, leading to irreversible damage to lung architecture and pulmonary fibrosis.[10,39] Our results revealed that lanatoside C treatment was capable of reducing the expression of α-SMA, fibronectin and collagens I and III in response to TGF-β1, indicating a suppression role for lanatoside C during the differentiation process of fibroblasts into myofibroblasts. Previous studies have established that TGF-β1 can induce Smad2 and Smad3 phosphorylation, which directly contributes to fibroblast differentiation.[40,41] The foregoing observations prompted us to examine the effect of lanatoside C on the TGF-β1/Smad signalling pathway. Indeed, Smad2 and Smad3 were phosphorylated following TGF-β1 stimulation in pulmonary fibroblasts and lanatoside C treatment inhibited this TGF-β1-induced phosphorylation. Interestingly, we observed that treatment with lanatoside C inhibited TGF-β1-induced wound closure, implying that lanatoside C was also able to regulate scar formation in PF through mediation of fibroblast migration.

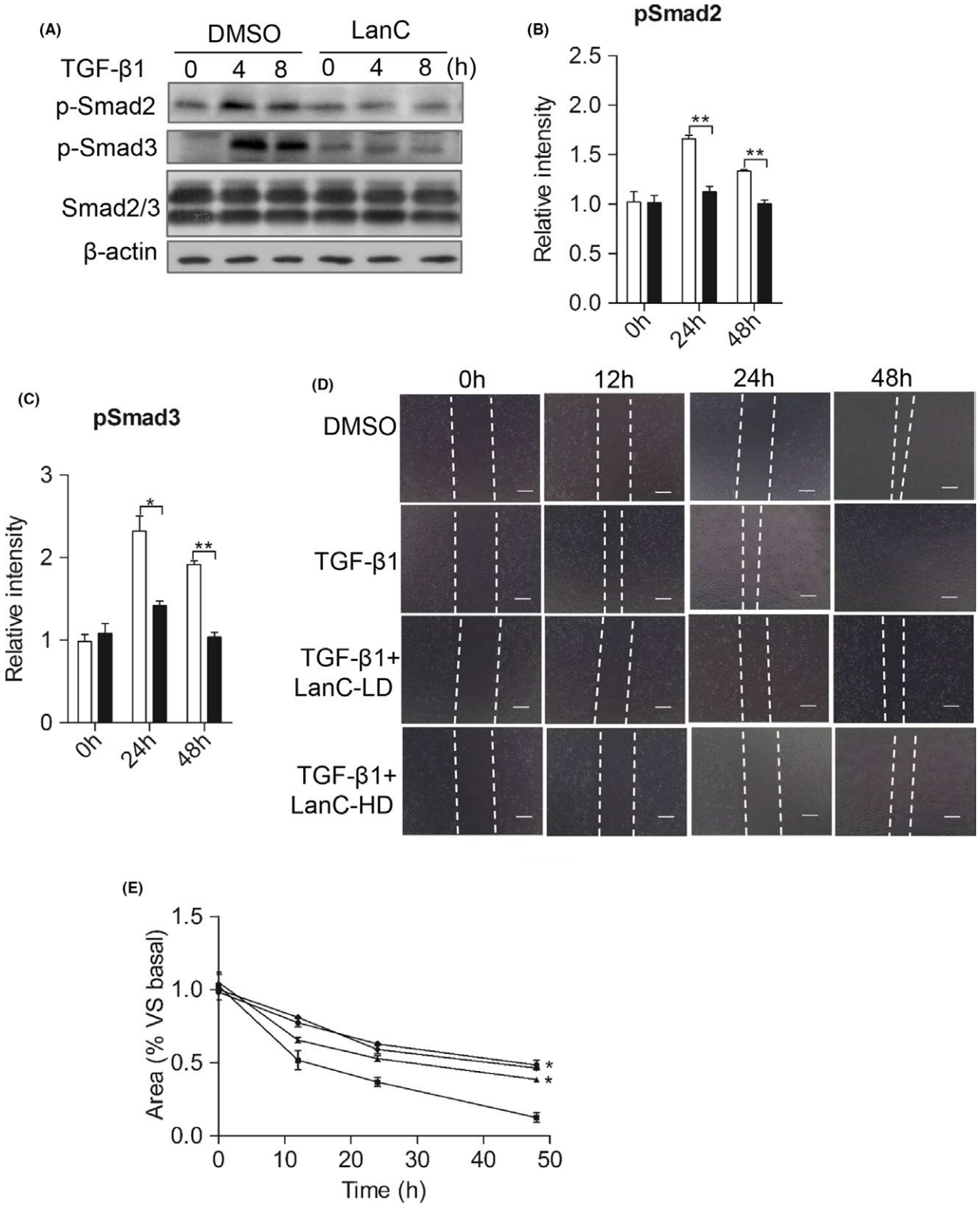

FIGURE 6 Lanatoside C treatment antagonized TGF-β1 signalling in pulmonary fibroblasts. A, Pulmonary fibroblasts were treated with TGF-β1 (10 ng/mL) + 1‰ DMSO or TGF-β1 (10 ng/mL) + lanatoside C (1 μmol/L) for 0, 4 or 8 h. Protein expression of phosphor-smad2, phosphor-smad3 and smad2/3 was quantified by western blot analysis. B,C, Quantification of phosphorylation of smad2 and smad3 by Image J software. □ DMSO + TGF-β; ■ LanC + TGF-β. D, Wound closure treated with or without lanatoside C (3 or 10 μmol/L) for 1 h then stimulated with or without TGF-β1 (10 ng/mL) for 48 h. Micrographs were produced 0, 12, 24 or 48 h after scratching. Scale bars = 300 μm. E, Analysis of wound closure area. Quantitative data shown above are means ± SEM of three independent experiments. (*P < 0.05, **P < 0.01 and ***P < 0.001). E, ●– DMSO; ■– TGF-β1; ▲– TGF-β1 + LanC-LD; ◆– TGF-β1 + LanC-HD

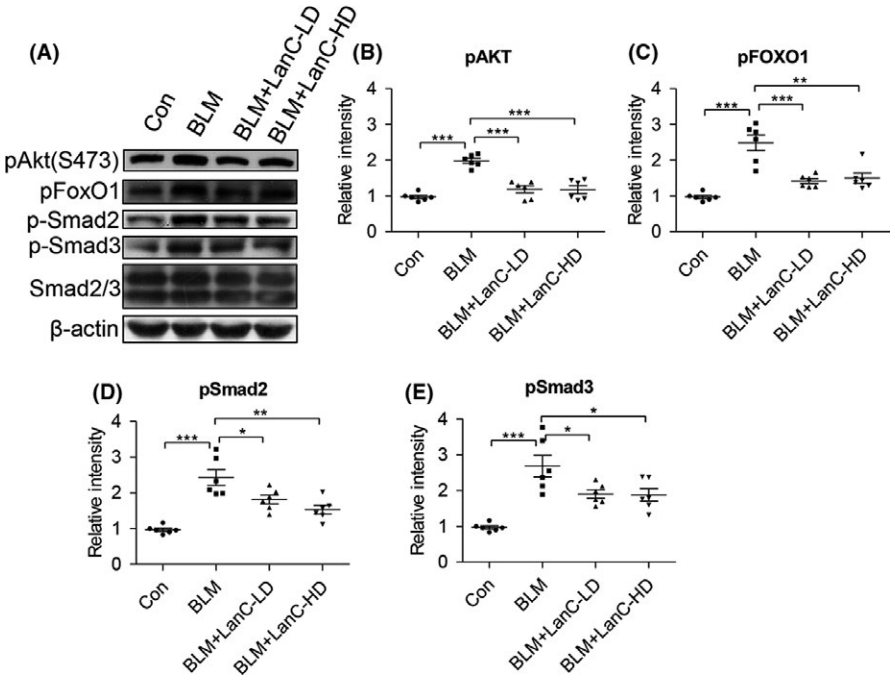

FIGURE 7 The involvement of Akt, FoxO1 and Smad2/3 phosphorylation in the effect of lanatoside C on lung tissues of bleomycin (BLM)-stimulated mice. A, Representative images of the phosphorylation and expression levels of Akt, FoxO1 and Smad2/3 in four indicated groups using western blots. β-Actin was used as a loading control. Statistical analysis of differences in the phosphorylation level of (B) Akt, (C) FoxO1, (D) Smad2 and (E) Smad3. Values represent means ± SEM, n = 5–8, $^{**}P < 0.01$, $^{***}P < 0.001$

Collectively, our results demonstrated that lanatoside C treatment protected mice from BLM-induced pulmonary fibrosis. Mechanistic experiments revealed that lanatoside C performed this therapeutic function against pulmonary fibrosis through suppression of fibroblast proliferation and differentiation through Akt/FoxO1 and TGF-β1/Smad signalling, respectively. We further investigated whether lanatoside C treatment affect AKT, FOXO1 or Smad2/3 phosphorylation in lung tissues, and the results showed the phosphorylation of these signalling molecules in lung tissues of BLM-stimulated mice was significantly downregulated after lanatoside C treatment, demonstrating that Akt, FoxO1 or Smad2/3 were involved in the protective roles of lanatoside C against BLM-induced pulmonary fibrosis.

Pulmonary fibrosis is a major health concern that requires the development of effective therapies. Our evidence supports the potential application of lanatoside C as a novel therapeutic strategy for pulmonary fibrosis.

4 | METHODS

4.1 | Mice

Six to eight week old male C57BL/6 mice were ordered from Nanjing Model Animal Research Center of Nanjing University of China (license No. SCXK (Su) 2017-0052) and housed in separate pathogen-free conditions at the Animal Center of Jiangnan University. The mouse study was approved by the Institutional Animal Care and Use Committee at Jiangnan University, Jiangsu, China.

4.2 | Reagents

Lanatoside C (CAS no.: 17575-22-3) was purchased from MedChemExpress (MCE, China) and was dissolved in DMSO (Sigma. Louis, MO, USA) for the in vitro study or in the solvent H_2O : ethanol : polyoxyethylene hydrogenated castor oil at a ratio of 8:1:1 for the in vivo study. TGF-β1 was purchased from PeproTech, USA. Antibodies against Akt, phospho-Akt (Thr308), phospho-Akt (Ser473), FoxO1, phospho-FoxO1 (Ser319), Bcl2, phospho-smad2, phospho-smad3, smad2/3 and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

4.3 | Bleomycin induced pulmonary fibrosis and lanatoside C administration

Mice were injected with 5 mg/kg pentobarbital intraperitoneally for general anaesthesia. They were randomly allocated to four groups: (a) Saline was delivered to the mice by intratracheal instillation at day 1 which were treated with solvent by intraperitoneal injection at day 8 as the control group; (b) BLM (BioTang, 1.4 U/kg for fibrotic analysis) in saline was given to the mice by intratracheal instillation at day 1 which were then treated with solvent by intraperitoneal injection at day 8; (c) BLM in solvent was given to the mice by intratracheal instillation at day 1 which were then treated with 4 mg/kg of lanatoside C dissolved in solvent by intraperitoneal injection at day 8; (d) BLM in solvent was given to the mice by intratracheal instillation at day 1 which were then treated with 8 mg/kg of lanatoside C dissolved in solvent by intraperitoneal injection at day 8. After 21 consecutive days, samples were harvested for histological analysis, protein expression by western blotting, mRNA levels by real-time RT-PCR and collagen content by hydroxyproline assay (kit from NanJing Jiancheng Bioengineering Institute, Nanjing, China).

A separate set of experiments was conducted for survival analysis. Mice were treated with 1.5 U/kg BLM at day 1 then treated with different concentrations of lanatoside C at day 8. Survival rates were observed for 21 days.

4.4 | Lung histology and hydroxyproline content

After treatment with BLM with or without lanatoside C, the mice were killed after 21 days. The lungs were cannulated, inflated with 1 mL 4% paraformaldehyde under constant pressure, removed from the animal then placed in fresh 4% paraformaldehyde for 24 hours at room temperature. The lung tissues were then embedded in paraffin, sliced (4 μm) then stained with hematoxylin and eosin (H&E) or Masson's trichrome (kit from NanJing Jiancheng Bioengineering Institute). Visual grading of pulmonary fibrosis was performed using the Ashcroft score. Briefly, each field of the entire tissue was visually graded from 0 (normal lung) to 8 (total fibrotic obliteration of the field). The mean value of the grades was then used as the visual fibrotic score.[42] A hydroxyproline assay was performed to estimate lung collagen deposition. All five lung lobes were harvested from each animal in a separate set of experiments, weighed and subjected to collagen estimation in accordance with the manufacturer's instructions of the commercial kit obtained from NanJing Jiancheng Bioengineering Institute.

4.5 | Culture and treatment of primary mouse lung fibroblasts

Mouse lung fibroblasts were obtained using a protocol as previously reported,[9] with modification. Six to 8 weeks-old mice were euthanized and the whole lungs were collected and cut into small pieces, minced, and then subjected to collagenase type IV and DNase I digestion (Sigma, St Louis, MO, USA) then placed in DMEM with 100 IU/mL penicillin-streptomycin and 5% foetal bovine serum (FBS) for 30 minutes. After centrifugation, pellets containing tissue fragments and cells were washed and cultured at 37°C and 5% CO_2 for 7–14 days in DMEM supplemented with 10% FBS and penicillin/streptomycin. Tissue fragments were discarded and the cells used for analysis. For fibroblast proliferation, BLM was instilled intratracheally into the mice 14 days prior to isolation of the fibroblasts, the period at which pathologic fibrosis changes had formed within the lungs.[43] The cells were treated with various doses of lanatoside C for a specified duration, with 1‰ DMSO used as a control. For fibroblast differentiation, cells were co-cultured with recombinant TGF-β1 with or without lanatoside C for various durations for protein, RNA and protein expression analysis.

4.6 | Cell viability measurement (MTT assay)

Lung fibroblasts were seeded into 96-well plates (10^4/well in 100 μL media) and then incubated with various concentrations of lanatoside C (0, 0.5, 1.0, 3.0, 5.0 or 10 μmol/L) for 24 or 48 hours. Control group cells were incubated with 1‰ DMSO for the same duration. Cell debris was removed by washing with PBS, then 100 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours. Absorbance values (OD) at 490 nm were measured using a microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA, USA) and cell viability of each well calculated by reference to a blank and control using the formula: cell viability % = ODcon - ODtest/ODcon - ODblank. Each test condition was assayed in triplicate.

4.7 | Cell cycle measurement

Pulmonary fibroblasts were seeded at 5×10^5 cells per well into 6-well plates for 24 hours at 37°C (5% CO_2). Culture medium was replaced with fresh medium supplemented with lanatoside C (0, 3 or 10 μmol/L) or 1‰ DMSO as a control for 24 hours. Cells were then harvested, washed with cold PBS and fixed in cold 70% ethanol at 4°C overnight. Fixed cells were then treated with 10 μg/mL RNAse at 37°C for 30 minutes in the dark and stained with 50 μg/mL PI for 5 minutes. Each cell population was analyzed for cell cycle by flow cytometry (LSRFortessa X-20; BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo 7.6 software.

4.8 | Apoptosis assay

Pulmonary fibroblasts were seeded into 6-well plates and treated with 1‰ DMSO (control) or treated with lanatoside C (3 or 10 μmol/L) for 0, 24 or 48 hours. Cells were harvested, washed with cold PBS twice and re-suspended in 500 μL Annexin-binding buffer. Subsequently, cells were incubated with Annexin V-FITC and PI (BB-4101-50T; BestBio, Shanghai, China) then analyzed by flow cytometry (LSRFortess X-20; BD Biosciences). Data were analyzed by FlowJo 7.6 software (Treestar, Ashland, OR, USA).

4.9 | Real-time PCR analysis

Total RNA was harvested from mouse lung tissues and pulmonary fibroblasts using a TissueLyser system (Qiagen, Germantown, MD, USA). cDNA was prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and amplified by real-time PCR with Fast SYBR Green master mix with primer sets for : αSMA (forward, 5′-GACGCTGAAGTATCCGATAGAACACG-3′, reverse, 5′-CACCATCTCCAGAGTCCAGCACAAT-3′), fibronectin (forward, 5′-TCTGGGAAATGGAAAAGGGGAATGG3′, reverse, 5′-CACTGAAGCAGGTTTCCTCGGTTGT-3′), collagen I forward, (5′-TGCCGTGACCTCAAGATGTG-3′, reverse, 5′-CACAAGCGTGCTGTAGGTGA3′), collagen III (forward, 5′-ACGTAGATGAATTGGGATGCAG-3′, reverse, 5′-GGGTTGGGGCAGTCTAGTC-3′), Cyclin D1(forward, 5′-GGGGACAACTCTTAAGTCTCAC-3′, reverse, 5′-CCAATAAAAGACCAATCTCTC-3′), Cyclin E (forward, 5′-GAGCTTGAATACCCTAGGACTG-3′, reverse, 5′-CGTCTCTCTGTGGAGCTTATAGA C-3′), GAPDH (forward, 5′-TGCGACTTCAACAGCAACTC-3′, reverse, 5′-CTTGCTCAGTGTCCTTGCTG-3′).

Relative gene expression was calculated using the ΔΔCt method and normalized by GAPDH expression.

4.10 | Western-blot analysis

Protein lysates were obtained using RIPA buffer with 1 mmol/L PMSF (phenylmethanesulfonyl fluoride). Protein concentration

was quantified using a bicinchoninic acid (BCA) assay. The proteins (10–20 μg) in each sample were separated using 10%–12% SDS/PAGE at 200 V for 1 hour then transferred onto PVDF membranes at 80–100 V for 1 hour, blocked in TBS containing 5% nonfat skimmed milk and incubated with an appropriate primary antibody overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody for 1 hour at room temperature, followed by supersensitive ECL solution (Boster Biological Technology, Pleasanton, CA, USA) then visualized on an Amersham Imager 600 (GE Healthcare Life Sciences, Fairfield, CT, USA) chemiluminescent detector. Intensity was quantified using Image J software.

4.11 | Wound healing assay

Pulmonary fibroblasts were seeded (5×10^5 cells/well) in 12-well plates and incubated for 24 hours at 37°C in 5% CO_2 until 90% confluent. The adherent cell layer was scratched with a sterile 200 μL pipette tip. The cells were washed with cold PBS, co-cultured with recombinant TGF-β1 with lanatoside C (0, 3 or 10 μmol/L) or 1‰ DMSO as a control for a further 24 hours or 48 hours. Images of the wounded area were created at 0, 24 and 48 hours to evaluate fibroblast migration at precisely the same microscopic cross point by light microscopy (RX51, Olympus Optical Co., Tokyo, Japan). Wound healing images were analyzed using Image J software (NIH). Wound coverage rate was measured and normalized using control wells.

4.12 | Statistical analysis

Data were expressed as means ± SEM of experiments which were performed at least in triplicate. All statistical analyses for single comparisons were by student's *t*-test, differences between groups calculated using a one way ANOVA multiple comparison test using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). For all analyses, results were considered statistically significant at $P < 0.05$.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Yunjuan Nie and Dan Zhang designed the experiments; Yunjuan Nie, Dan Zhang, Zhewu Jin, Boyu Li, Xue Wang, Huilian Che, Xiaohang Qian, Yang Zhang and Yaqian You performed the experiments; Peng Zhao analyzed the data; Peng Zhao and Gaoshang Chai prepared the manuscript.

ORCID

Yunjuan Nie https://orcid.org/0000-0002-6167-4258

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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