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Benzylisoquinoline alkaloids inhibit lung fibroblast activation mainly via inhibiting TGF-β1/Smads and ERK1/2 pathway proteins

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ABSTRACT

Backgrounds: Liensinine (Lien), Neferine (Nef), Isoliensinine (Iso) and Tetrandrine (Tet), benzylisoquinoline alkaloids (BIAs), have been shown inhibitory effects on pulmonary fibrosis (PF) through anti-inflammatory, anti-oxidative activities, inhibition of cytokines and NF-κB. Effects of other similar BIAs, Dauricine (Dau), Papaverine (Pap) and lotusine (Lot), on PF remain unclear. Here, we explored the effects of five bisbenzylisoquinoline (Lien, Nef, Iso, Tet and Dau) and two monobenzylisoquinoline (Pap, Lot) alkaloids on normal and PF fibroblasts.

Methods: Primary normal and PF lung fibroblasts were cultured and treated with these alkaloids. Proliferation, activation, migration and apoptosis changes were detected by MTT, wound healing assay, flow cytometry. Protein level was analyzed by Western blot.

Results: All BIAs inhibited proliferation of normal and PF lung fibroblasts induced by TGF-β. α-SMA protein level in normal and PF lung fibroblasts decreased after Lien, Nef, Iso, Tet and Dau treatment. Pap and Lot had no influence on α-SMA expression. Dau showed the strongest inhibitory effects on proliferation and activation among alkaloids. The migration rates of normal and PF lung fibroblasts were inhibited by Lien, Nef, Iso, and Dau. Lien, Nef, Iso and Dau significantly promoted apoptosis, while Tet had no effect on apoptosis. Pap and Lot had no influence on activation, migration and apoptosis. Dau significantly inhibited Smad3/4 and p-ERK1/2 protein overexpression induced by TGF-β1.

Conclusions: Bisbenzylisoquinoline alkaloids had stronger effects on inhibiting lung fibroblasts than monobenzylisoquinoline alkaloids. Dau expressed the strongest inhibitory effects, which may be related to its inhibition of TGF-β1/Smad3/4 and p-ERK1/2 pathway proteins.

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1. Introduction

Fibroblasts are the predominant secretory cells of extracellular matrix (ECM) proteins in the lung and are also key mediators of normal and pathological lung remodeling. After stimulation, fibroblasts proliferate and differentiate into myofibroblasts which are characterized by α-smooth muscle actin (α-SMA) expression. Among those stimulus factors, transforming growth factor-β1 (TGF-β1) is considered as one of the strongest pro-fibrotic factors [1,2]. The Smads are a family of intracellular signaling molecules that act downstream of receptors for the TGF-β family of ligands. Accumulating evidence suggests that TGF-β/Smads signaling is a mediator of pro-fibrotic effects and promotes myofibroblast differentiation [3,4]. Myofibroblasts have the stronger ability to migrate and secrete collagen, ECM proteins which promote the development of fibrosis [5,6,7]. Thus the accumulation of myofibroblasts in fibroblastic foci is a pivotal feature of pulmonary fibrosis (PF). Therefore, inhibition of the fibroblast proliferation, activation, migration and promoting apoptosis may contribute to the treatment of PF.

Benzylisoquinoline alkaloids (BIAs) are among the most structurally diverse and show a myriad of pharmacological activities such as the treatment of antitumor, arrhythmia, platelet aggregation and the inhibitory of PF. Liensinine (Lien), Neferine (Nef), Isoliensine (Iso), Tetrandrine (Tet), Dauricine (Dau), Papaverine (Pap) and Lotusine (Lot) are seven different structural BIAs, shown in Fig. 1. Lien, Nef, Iso, Tet and Dau are five bisbenzylisoquinoline alkaloids which have two molecules of benzylisoquinoline structure connected by ether bond. While the structure of Pap and Lot just has one molecule of benzylisoquinoline. Previous studies have documented that Nef, Iso and Tet have extensive pharmacological effects on bleomycin- or amiodarone-induced PF model probably due to its properties of increasing superoxide dismutase, SP-D inhibition and restoring increased $CD4^{+}CD25^{+}$ Tregs, alleviating bleomycin-induced increase of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-6 and endothelin-1 in plasma or in tissue [8,9,10,11,12]. Dau is also one of the bisbenzylisoquinoline alkaloids and previous studies have shown that Dau has been commonly used for the treatment of cardiac arrhythmia and a number of immune disorders such as chronic sinusitis, chronic bronchitis, pharyngitis, rheumatism and diarrhea, antitumor and inhibit the effect of potassium ion outflow [13,14,15,16,17]. Pap as one of cyclic nucleotide phosphodiesterases (PDE) has shown pharmacological action on neurological disease and the spasmolytic effect [18,19,20,21]. Lot pretreatment has exhibited potential cardioprotective activity against DOX-induced oxidative stress by increasing the intracellular antioxidant defense [22]. It is unknown whether Dau, Pap and Lot also have effects on PF.

Thus, the present investigation was to explore the effects of these five bisbenzylisoquinoline alkaloids (Lien, Nef, Iso, Tet and Dau) and two monobenzylisoquinoline alkaloids (Pap and Lot) on proliferation, activation, migration, apoptosis of normal and PF lung fibroblasts, and compared the different effects among those alkaloids, and further investigated the mechanism of Dau on lung fibroblasts.

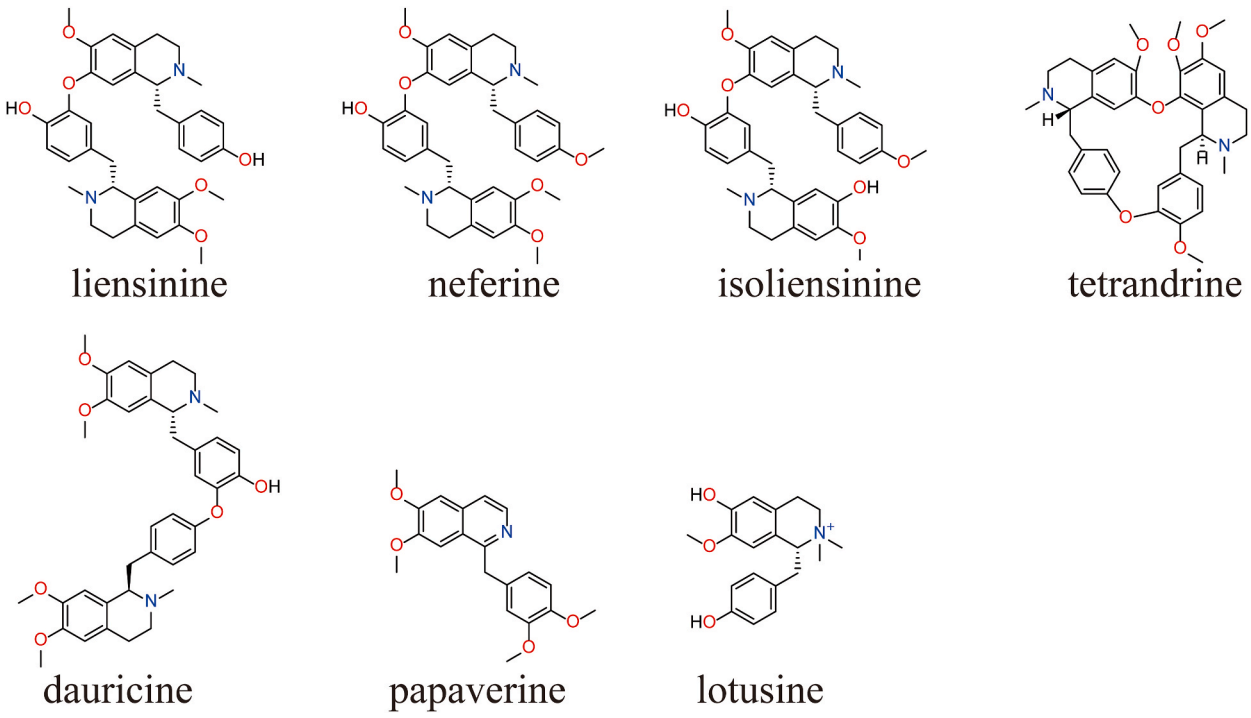

Fig. 1. Structures of seven benzylisoquinoline alkaloids Liensinine (Lien), Neferine (Nef), Isoliensinine (Iso), Tetrandrine (Tet), Dauricine (Dau), Papaverine (Pap) and lotusine (Lot).

2. Materials and methods

2.1. Materials

Lien (FW = 610.74, purity 98% by HPLC), Nef (FW = 625, purity 98% by HPLC), Iso (FW = 610.74, purity 98% by HPLC), Tet (FW = 622.76, purity 98% by HPLC), Dau (FW = 624.77, purity 98% by HPLC), Pap HCL (FW = 375.85, purity 98% by HPLC), and Lot (FW = 314, purity 98% by HPLC) was obtained from the Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology. Bleomycin was obtained from Nippon Kayaku (Tokyo, Japan). Recombinant human TGF-β1 (PeproTech, USA), anti-Smad2, anti-Smad3, anti-Smad4, anti-Smad7, anti-SMA, and anti-GAPDH were purchased from Proteintech (Chicago, USA). Anti-Erk1/2, anti-phospho-Erk1/2 and anti-caspase-3 were purchased from Cell Signaling Technology (California, USA). HRP-conjugated secondary goat anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Proteintech (Chicago, USA). Annexin V FITC and Apoptosis Detection Kit I were purchased from Becton Dickinson (San Diego, USA). DMEM, fetal bovine serum (FBS) and 0.25% trypsin-EDTA were purchased from Gibco (Rockville, MD, USA). Penicillin, streptomycin that used to fight bacterial infections and trypsin that used to digest and pass cells were from Invitrogen (Carlsbad, USA). MTT that used to measure cell viability and proliferation was from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) for easy detection of horseradish peroxidase (HRP) on immunoblots was from EngreenBiosytem (Beijing, China).

2.2. Lung fibroblasts isolation and culture

Adult male Sprague-Dawley rats weighing 180–200 g were purchased from the Department of Experimental Animals of Tongji Medical College (SCXX 2019-0009). All animal experiments were approved by the Ethics Committee of Laboratory Animals of Tongji Medical College of Huazhong University of Science and Technology. PF rats were induced by intratracheal bleomycin and evaluated by H&E and Masson staining (Xiao et al., 2005). Lung fibroblasts were isolated from normal rats and bleomycin-induced PF modal rats. Briefly, the cells were dissociated using 0.25% trypsin-EDTA at 37 °C for 40 min, and the suspension was centrifuged and then cultured in DMEM with 15% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 U/mL) at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell proliferation assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured for 24 h under normal culture medium and condition. Then, Lien, Nef, Iso, Tet, Dau, Pap and Lot were added at different concentrations of 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μM with 10 ng/ml TGF-β1. After 48 h of incubation, 10 μL MTT (5 mg/ml) was added into medium for further 4h culture. The blue dye formed was dissolved in 120 μL DMSO. The absorbance was recorded at 490 nm using a Microplate Reader (TECAN, Germany). All experiments were repeated at least four times using different batches of cells.

2.4. Wound healing assay

Cells were seeded into 12-well plates at a density of 1×10^4 cells per well and cultured in 10% FBS-containing DMEM at 37 °C overnight. Then we drew an artificial homogenous wound in the center of the culture well using a sterile 10 μL pipette tip and washed the culture well three times by sterile phosphate buffered saline (PBS). Then cells were treated with different concentrations of Nef, Lien, Iso, Tet, Dau, Pap and Lot in the presence and absence of TGF-β1 (10 ng/ml). Images of cells migrating into the wound were captured at 0, and 12 h using an inverted microscope (100x). Images were obtained for analysis using ImagePro Plus software.

2.5. Flow cytometric quantification of apoptosis

Cells were cultured in six-well plates under a humidified 5% CO_2 atmosphere at 37 °C to over 80% confluence and then treated with Lien, Nef, Iso, Tet, Dau, Pap, and Lot (3 μmol/L) in the presence of TGF-β1 (10 ng/ml) for 48 h before analysis. Then the cells were removed the culture medium and washed with PBS. Each culture well was added 1 ml 0.25% trypin and then the cells were harvested into a 5 ml culture tube. The cells were resuspended in 1x Binding Buffer at a concentration of 1×10^5 cells/ml and incubated with 5 μL FITC Annexin V and 5 μL PI for 15 min at RT (25 °C) in the dark. Finally, the cells were analyzed by flow cytometry (BD Biosciences, USA) within 1 h.

2.6. Western blot

Western blot analysis was performed with antibodies directed against Smad2, Smad3, Smad4, Smad7, Erk1/2, p- Erk1/2, and α-SMA to detect the expression of these proteins. Control, TGF-β1-treated and TGF-β1 with alkaloids-treated cells were lysed with RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The protein concentration of total cell lysates was determined using bovine serum albumin (Roche, Switzerland) as a standard. Protein samples of different groups (30 μg each) were separated electrophoretically on SDS polyacrylamide gels (10% gel for Smad2, Smad3, Smad4, Smad7, Erk1/2, p- Erk1/2 and α-SMA) at 80 V for 40 min and 100 V for Separation Gel. Then proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, USA) at 200 mA for 2 h. The membranes were blocked with 5% skimmed milk for 1 h at room temperature and then were incubated with

specific primary antibodies (1:2000 dilution for α-SMA, GAPDH and β-actin; 1:1000 for Smad2, Smad3, Smad4, Smad7, Erk1/2, p-Erk1/2) overnight at 4 °C. After wash, the membranes were incubated for 1 h at room temperature with HRP-conjugated secondary goat anti-rabbit IgG or anti-mouse IgG secondary antibody (dilution 1:5000). The bands were visualized using ECL. The results were quantified by densitometry using Image J software.

2.7. Data and statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Statistical analysis was performed using student *t*-test in two groups. One-way analysis of variance (ANOVA) following Tukey's multiple comparisons test was performed in multiple groups. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of seven BIAs on lung fibroblast proliferation

Lung fibroblasts proliferation is one of key characters in the pathophysiology of PF. After treatment with TGF-β1 (10 ng/ml) for 48 h, normal and PF lung fibroblasts proliferation were significantly increased (0.121 ± 0.006, 0.114 ± 0.003 vs 0.152 ± 0.003, 0.140 ± 0.005). As shown in Fig. 2, five bisbenzylisoquinoline alkaloids (Lien, Nef, Iso, Tet and Dau) inhibited lung fibroblasts proliferation induced by TGF-β1 in a concentration-dependent manner, and the maximum effect among them had no significant difference in normal (Fig. 2A) and PF (Fig. 2B) lung fibroblast. Pap inhibited fibroblasts proliferation at the concentration of 30 and 100 μmol/L, and the maximum inhibitory effect was lower than five dibenzylisoquinoline alkaloids. Lot (100 μmol/L) inhibited fibroblasts proliferation, but the maximum inhibitory effect was the lowest. Table 1 showed the IC_{50} value for normal and PF lung fibroblasts. The Dau IC_{50} value for normal and PF lung fibroblasts at 48 h was 7.72 ± 0.99 μmol/L and 9.35 ± 1.94 μmol/L which were lower than other alkaloids.

3.2. Effect of seven BIAs on α-SMA protein overexpression

α-SMA overexpression is a marker of myofibroblasts which produce ECM. Normal and PF lung fibroblasts were treated with TGF-β1 (10 ng/ml) and TGF-β1 with alkaloids (0.3, 3 and 30 μmol/L) for 48 h, and then total proteins were collected for Western blot. As shown in Fig. 3, the level of α-SMA was highly increased by TGF-β1 compared with control. For normal lung fibroblasts (Fig. 3B), Lien, Iso and Dau (3 μmol/L) significantly reduced the overexpression of α-SMA induced by TGF-β1. At the concentration of 30 μmol/L (Fig. 3C), Lien, Nef, Iso, Tet and Dau reduced the overexpression of α-SMA. For PF lung fibroblasts, Lien, Nef, Iso, and Dau inhibited the α-SMA overexpression at the concentration of 30 μmol/L (Fig. 3F). The two monobenzylisoquinoline alkaloids (Pap, Lot) and Tet had no significant effect on α-SMA overexpression induced by TGF-β1. Among them, Dau showed the strongest inhibitory effect on TGF-β1-induced α-SMA overexpression in normal and PF lung fibroblasts (Fig. 3C and F).

3.3. Effects of seven BIAs on the migration of lung fibroblasts

Wound healing assay was evaluated to analyze the effects of seven BIAs on the migration of normal and PF lung fibroblasts. According to the above experimental data, cells were treated with TGF-β1 (10 ng/ml) and TGF-β1 with alkaloids (3 μmol/L) for 12 h. As shown in Fig. 4, after TGF-β1 treatment, the migration rates of both normal (Fig. 4A) and PF lung fibroblasts (Fig. 4B) were enhanced. For normal lung fibroblasts (Fig. 4A and C), Lien, Nef, Iso and Dau reduced the migration rate at 3 μmol/L after 12 h (0.113 ± 0.012 , 0.08 ± 0.026 , 0.07 ± 0.006 and 0.085 ± 0.026 vs 0.208 ± 0.02396). For PF lung fibroblasts (Fig. 4B and D), Lien, Nef, Iso and Dau reduced the migration rate at 12 h, and Tet at 12 h reduced the migration rate significantly, while two monobenzylisoquinoline

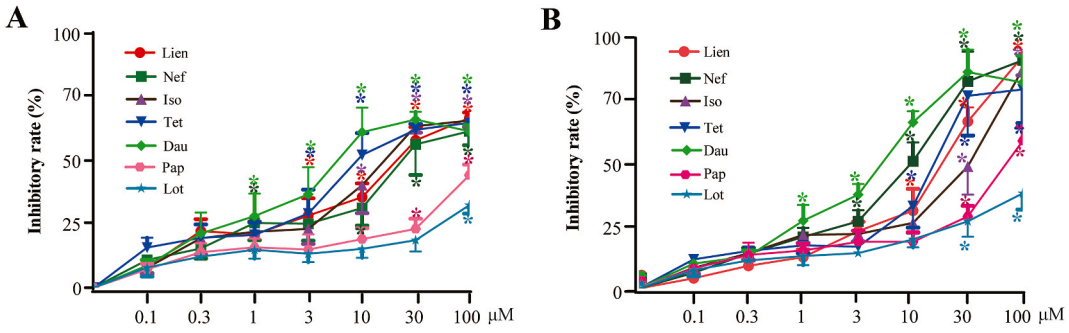

Fig. 2. Inhibitory effects of seven benzylisoquinoline alkaloids Lien, Nef, Iso, Tet, Dau, Pap and Lot on proliferation induced by TGF-β1 in normal (A) and PF (B) lung fibroblast in rats. Data are expressed as mean ± SEM, n = 6. *$P < 0.05$ by two-tailed Student's *t*-test.

Table 1
The IC_{50} value of seven benzylisoquinoline alkaloids in normal and PF lung fibroblasts.

Drugs	IC_{50} (μmol/L)	
	Normal lung fibroblasts	PF lung fibroblasts
Lien	17.02 ± 3.21	14.24 ± 3.28
Nef	10.50 ± 1.01	12.49 ± 1.65
Iso	18.90 ± 4.50	11.98 ± 2.15
Tet	13.15 ± 2.26	9.78 ± 1.46
Dau	7.72 ± 0.99	9.35 ± 1.94
Pap	64.05 ± 7.82	103.10 ± 18.21
Lot	118.00 ± 15.32	112.00 ± 3.36

Mean ± SEM, n = 5. Lien, Liensinine; Nef, Neferine; Iso, Isoliensinine; Tet, Tetrandrine; Dau, Dauricine; Pap, Papaverine; Lot, Lotusine.

alkaloids (Pap, Lot) had no significant influence on lung fibroblast migration rates (Fig. 4C and D).

3.4. Effects of seven BIAs on apoptosis of lung fibroblasts

Lung fibroblasts (normal and PF) were treated with TGF-β1 (10 ng/ml) and the indicated concentration of seven alkaloids (3 μmol/L) for 48 h. As shown in Fig. 5, TGF-β1 had no influence on apoptosis compared with control. Lien, Nef, Iso and Dau (3 μmol/L) significantly increased the apoptosis rates of normal (Fig. 5A and 5B) and PF lung fibroblasts (Fig. 5C and 5D). Tet, Pap and Lot (3 μmol/L) had no influence on apoptosis of lung fibroblasts.

3.5. Effects of Dau on TGF-β1/Smads and ERK pathway proteins in lung fibroblasts

In normal and PF lung fibroblast, the level of Smad2 (vs control: 1.03 ± 0.067 vs 0.793 ± 0.052, $P < 0.05$) (Fig. 6A and E), Smad3 (vs control: 0.400 ± 0.107 vs 0.300 ± 0.080, $P < 0.05$) (Fig. 6B and F) and Samd4 (vs control: 0.274 ± 0.019 vs 0.173 ± 0.027, $P < 0.05$) (Fig. 6C and G) were significantly up-regulated by TGF-β1, but there was no significant change about the level of Smad7 (Fig. 6D and H). After treated with Dau for 48 h, the level of Smad3 (0.312 ± 0.091 vs 0.400 ± 0.107, $P < 0.05$) (Fig. 6B and F) and Smad4 (0.1979 ± 0.0249 vs 0.274 ± 0.019, $P < 0.05$) (Fig. 6C and G) were down-regulated, but the level of Smad2 had no change (Fig. 6A and E).

Western blot (Fig. 7) revealed that TGF-β1 significantly increased p-ERK1/2 protein expression and did not affect total ERK1/2 protein expression in normal (Fig. 7A) and PF lung fibroblast (Fig. 7B). While Dau (0.3, 3, 30 μmol/L) significantly decreased p-ERK1/2 protein overexpression induced by TGF-β1 in normal (Fig. 7A) and PF lung fibroblast (Fig. 7B).

4. Discussion

In the present study, we explored the effects of seven different structures of BIAs on normal and PF lung fibroblasts, and the mechanism of Dau in inhibition of lung fibroblast activation. We found that TGF-β1 (10 ng/ml) significantly promoted cell proliferation, increased the expression of α-SMA, enhanced the ability of migration, up-regulated Smad2/3/4 and p-ERK protein expressions in normal and PF lung fibroblasts. Lien, Nef, Iso, Tet and Dau inhibited lung fibroblasts proliferation induced by TGF-β1 in a concentration-dependent manner, but the maximum effect had no significant difference. However, Pap and Lot showed the inhibition effect on proliferation at a higher concentration, and the maximum effect was lower. Lien, Nef, Iso and Dau decreased the overexpression of α-SMA induced by TGF-β1. Tet only inhibited the α-SMA overexpression in PF lung fibroblasts. The migration rates were decreased by Lien, Nef，Iso，Tet and Dau. The apoptosis rates were increased by Lien, Nef，Iso and Dau, not Tet. Pap and Lot had no significant effects on the expression of α-SMA, migration and apoptosis. Furthermore, we found that Dau showed stronger effects on inhibiting proliferation and activation compared with Lien, Nef, Iso and Tet. However, further research on the structure-activity relationships among those benzylisoquinoline is needed. Then we explored the mechanism of Dau that has been shown the strongest effects in inhibition of lung fibroblast activation. The results showed that Dau significantly inhibited the overexpression of Smad3/4 and p-ERK1/2 induced by TGF-β1.

BIAs have a diverse structure and pharmaceutical activities [23]. Previous studies have found that Nef, Iso and Tet expressed the inhibitory effects on bleomycin- and silicon-induced PF in mice [9,24]. In other fibrotic disease, these alkaloids also showed the inhibitory effects, such liver fibrosis, renal fibrosis and myocardial fibrosis [25,26,27,28]. The effects of Lien and Dau, similar structure with Nef, Iso and Tet, on fibrosis were few reported. As our results showed, these five bisbenzylisoquinoline alkaloids all showed inhibitory effects on TGF-β1-stimulated lung fibroblasts and Dau showed the higher inhibitory effect. But monobenzylisoquinoline alkaloids Pap and Lot seem to have fewer effects on TGF-β1-stimulated lung fibroblasts.

It is known that increased activity and proliferation of resident fibroblasts are important to fibrosis in all tissues. Their abilities to migration, ECM and collagen synthesis, and cytokines secretion were highly increased, once the fibroblasts were activated and differentiated into myofibroblasts. The role of myofibroblasts in PF has been known for the influence on tissue contractility or compliance, modulating signal transduction, synthesis and secretion of ECM component [29,30]. Furthermore, some studies have

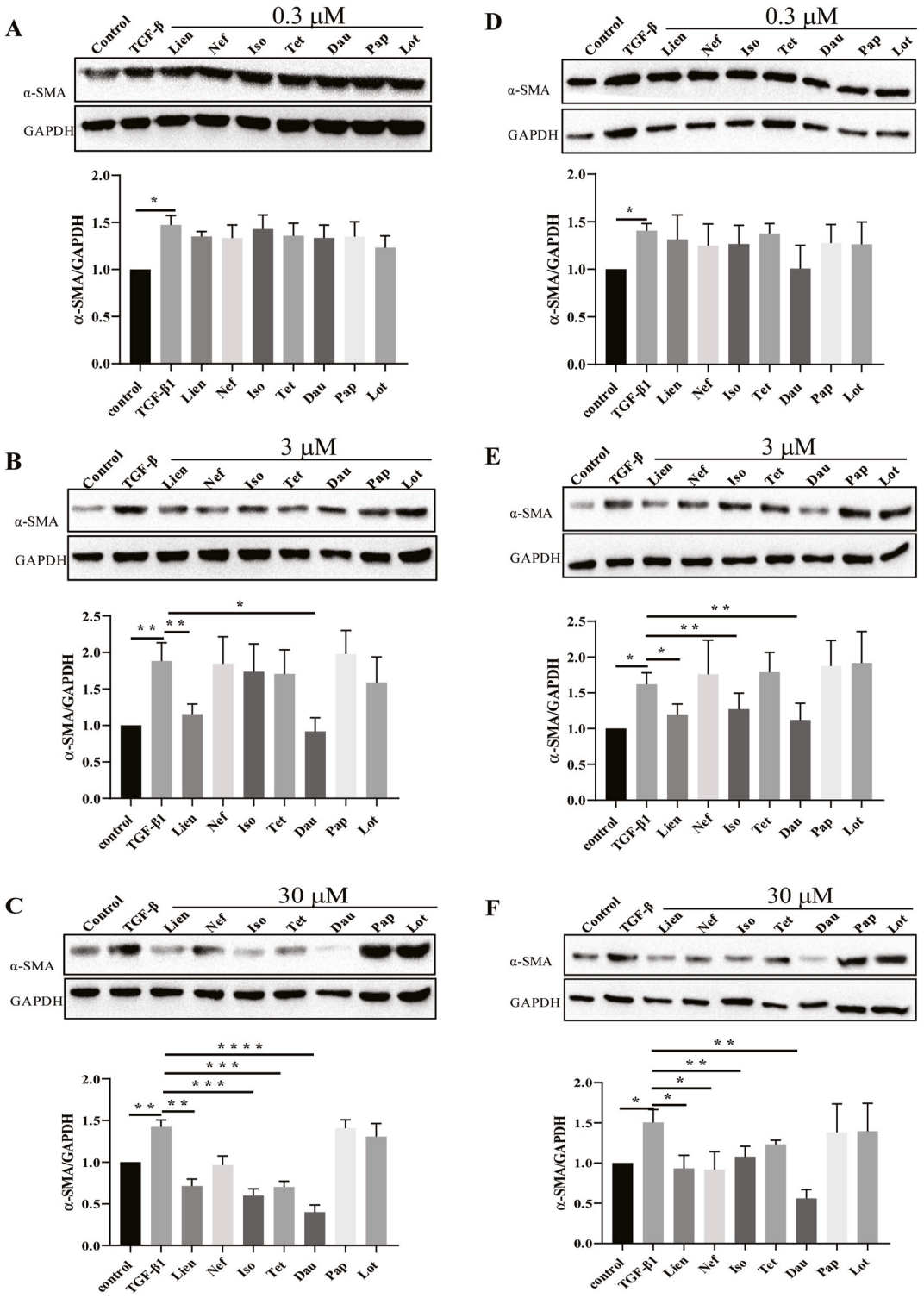

Fig. 3. Effects of five bisbenzylisoquinoline alkaloids (Lien, Nef, Iso, Tet and Dau) and two monobenzylisoquinoline alkaloids (Pap and Lot) on α-SMA overexpression induced by TGF-β1 (10 ng/ml) in normal and PF lung fibroblasts in rats. Ã C showed the changes of α-SMA protein in normal lung fibroblasts in rats. D ~ F showed the changes of α-SMA protein in PF lung fibroblasts in rats. Values are mean ± SEM, n = 5. *$P < 0.05$, **p < 0.01, ***p < 0.001, ****p < 0.0001, *vs* control or TGF-β1 by two-tailed Student's *t*-test.

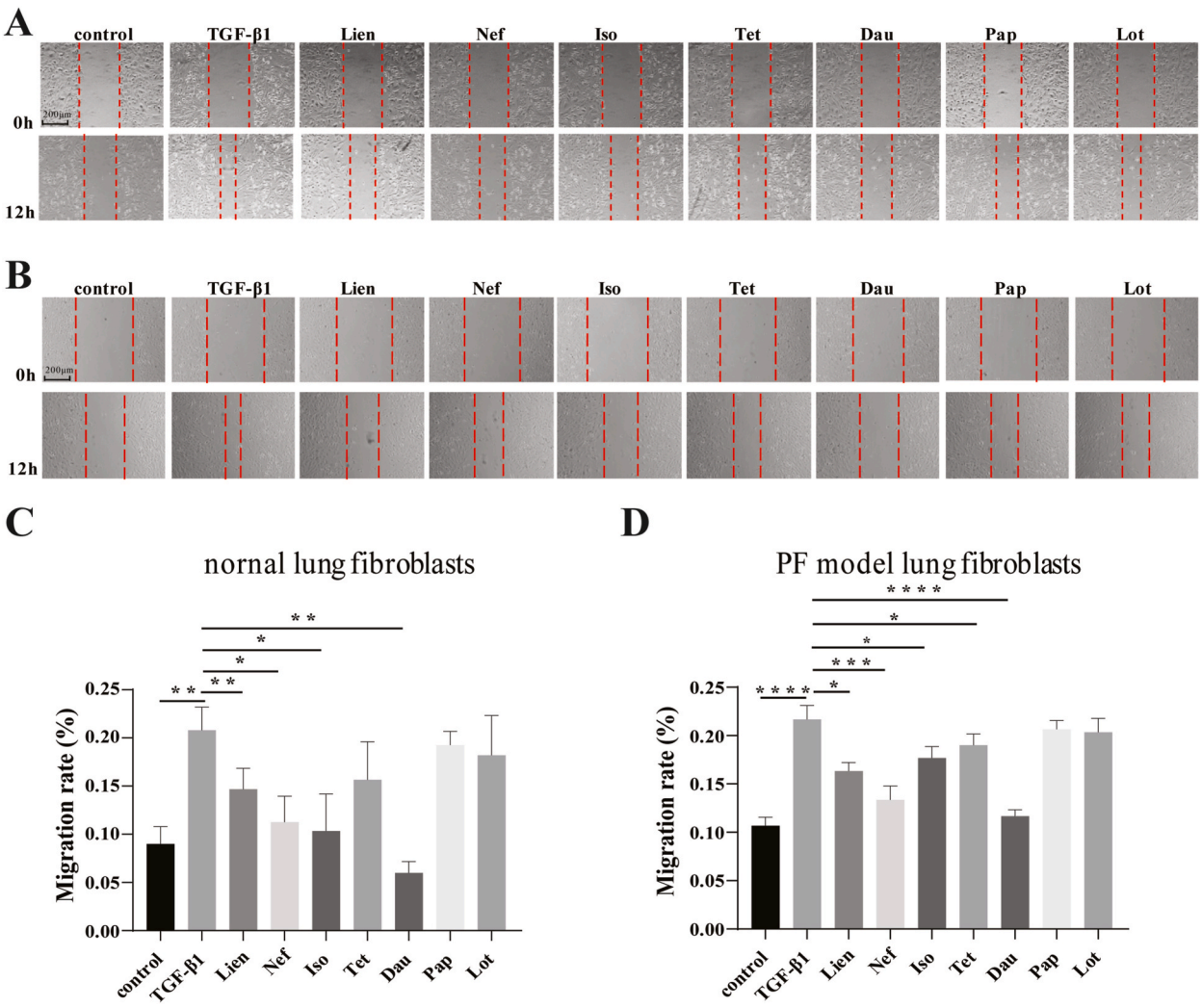

Fig. 4. Effects of five bisbenzylisoquinoline alkaloids (Lien, Nef, Iso, Tet and Dau) and two monobenzylisoquinoline alkaloids (Pap and Lot) on changes of migration induced by TGF-β1 (10 ng/ml) in normal (A, C) and PF (B, D) lung fibroblasts in rats. Values are mean ± SEM, n = 5. *$P <$ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, *vs* control or TGF-β1 by two-tailed Student's *t*-test.

found that there were plenty of myofibroblasts in areas of high ECM expression and those myofibroblasts would secret more ECM components [5]. During the pathological PF, much cytokines were related to this process, and studies have proven that TGF-β is a key factor to promote lung fibroblast differentiation [31]. In vitro and vivo, TGF-β1 mediated differentiation of lung fibroblasts and intracellular signaling events [32]. Numerous studies showed that the gene and protein level of TGF-β1 were up-regulated in the patients and animals of PF [33,34]. While inhibiting the TGF-β1 signaling by transient gene transfer could significantly prevents bleomycin-induced PF in mice [35]. TGF-β1 not only regulates the expression of α-SMA, but also plays an important role in the regulation of human pulmonary fibroblasts differentiation. Some studies demonstrated that the expression of α-SMA reduced after removal of TGF-β1 for human lung fibroblasts [36], and it has been known that the fibroblast responses to TGF-β1 may be dependent on their source of origin [37]. TGF-β1 exerts their signaling effects by binding mainly to cell surface receptors (type I, type II, type III). Type I and type II receptors are necessary for TGF-β1 signaling which is initiated when the ligand induces assembly of a heteromeric complex of type I and type II receptors. The RI kinase is closely related to recognize and phosphorylate members of the intracellular receptor-regulated Smads. The Smad branch of signaling mediators includes receptor-activated Smads (R-Smads), a common mediator Smad (co-Smad), and inhibitory Smads (I-Smads). Five different R-Smads (Smad1, Smad2, Smad3, Smad5, and Smad8) are directly phosphorylated by the type I receptors. Phosphorylated R-Smads form heteromeric complexes with Smad4 (a common co-Smad). Smad6 and Smad7 are I-Smads which limit the activities of these pathways [38]. Studies have shown that Smad3 related to the bleomycin-induced PF. In the Smad3-deficient mice, the procollagen I and CTGF mRNA induction were significantly reduced in TGF-β1-induced cells and bleomycin-induced PF mice [39,40]. By inhibiting the activin-like kinase 5 (ALK5), a type I receptor of TGF-β, the Smad2/3 nuclear translocation was attenuated, and fibroblast proliferation and type I collagen secretion and deposition

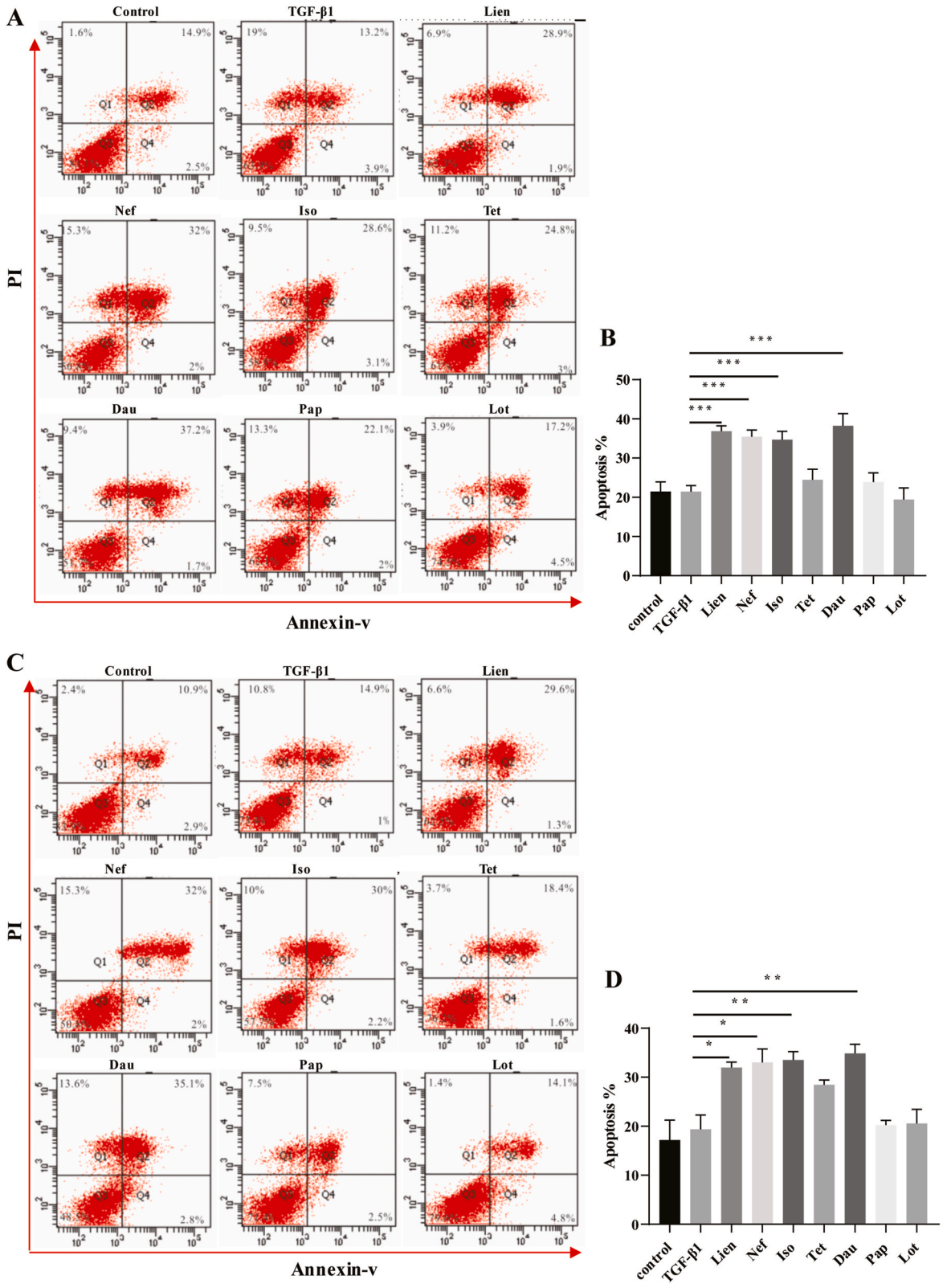

Fig. 5. Effects of five bisbenzylisoquinoline alkaloids (Lien, Nef, Iso, Tet and Dau) and two monobenzlisoquinoline alkaloids (Pap and Lot) on changes of apoptosis induced by TGF-β1 (10 ng/ml) in normal (A, B) and PF (C, D) lung fibroblasts in rats. Values are mean ± SEM, n = 5. *$P < 0.05$, **p < 0.01, ***p < 0.001, *vs* control or TGF-β1 by two-tailed Student's *t*-test.

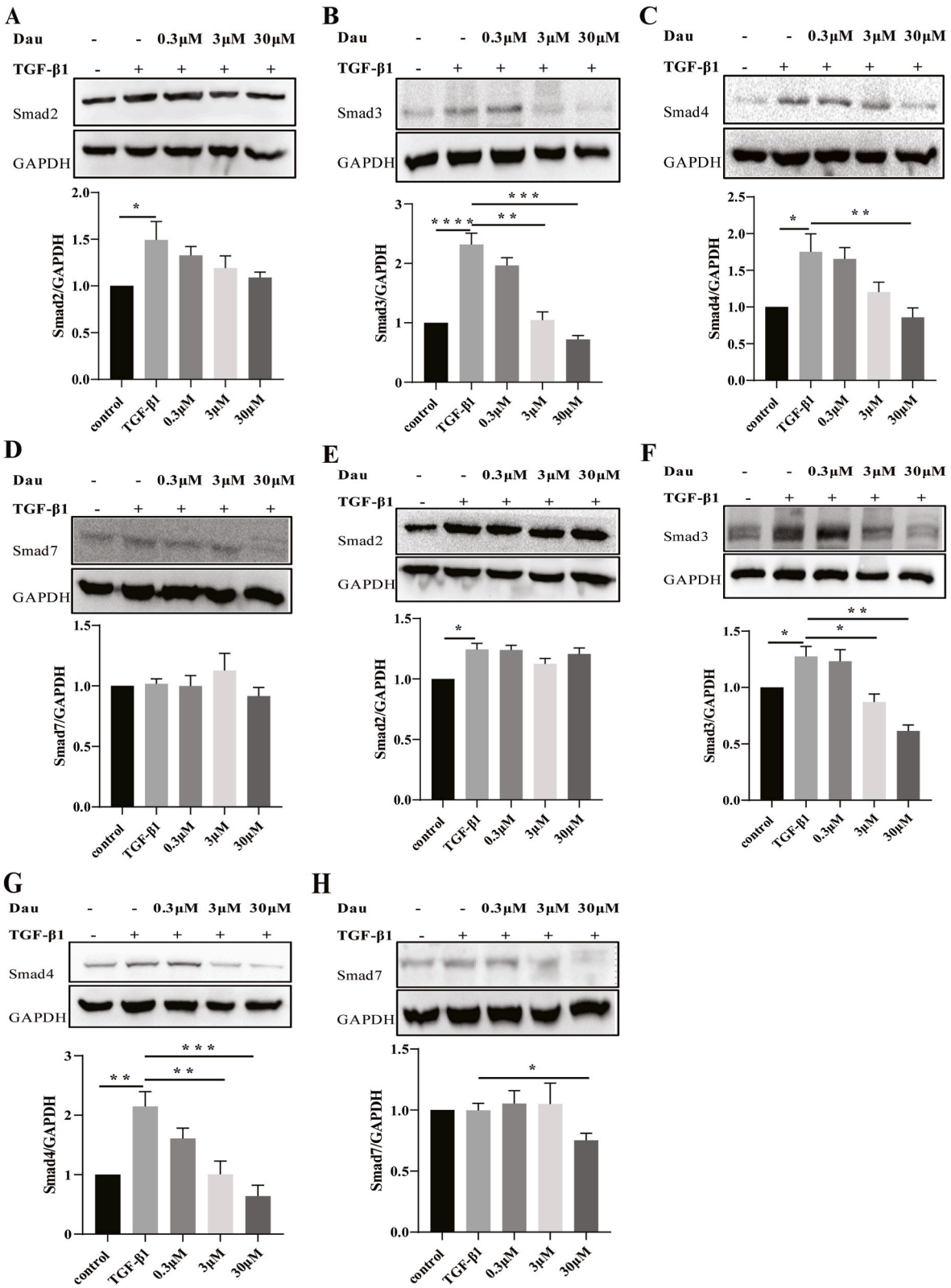

Fig. 6. Effects of Dau on TGF-β1/Smads signal pathway protein in lung fibroblast in rats. Ã D showed the changes of Smads protein in normal lung fibroblast in rats; E ~ H showed the change of Smads protein in PF lung fibroblast in rats. Values are mean ± SEM, n = 5. *$P < 0.05$, **p < 0.01, ***p < 0.001, ****p < 0.0001 *vs* control or TGF-β1 by two-tailed Student's *t*-test.

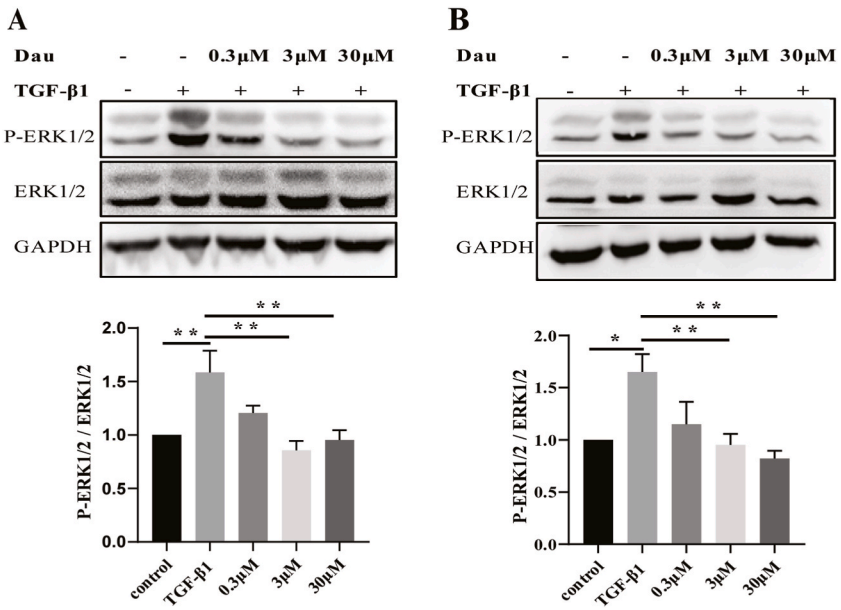

Fig. 7. Effects of Dau on TGF-β1/ERK1/2 signal pathway in normal (A) and PF (B) lung fibroblasts. Values are mean ± SEM, n = 5. *$P < 0.05$, **p < 0.01 *vs* control or TGF-β1 by two-tailed Student's *t*-test.

were decreased [41]. In our study, in normal and PF lung fibroblasts, the protein levels of Smad2/3/4 were up-regulated by TGF-β1. But this situation was reversed by Dau. Dau decreased the protein of Smad3 and Smad4, while had no influence on the Smad2 up-regulated by TGF-β1, suggesting that Dau blocked TGF-β1/Smads signal pathway via inhibition of Smad3 and Smad4.

Mitogen-activated protein kinase (MAPK) signaling pathway was also activated by TGF-β1 [42]. Studies showed that in both human umbilical vein endothelial cells and fibroblasts, TGF-β1 increased the activation of the Smads as well as MAPK and Akt [43]. As shown in Fig. 7, we found the protein level of p-ERK1/2 was highly increased in TGF-β1 treated-normal and PF lung fibroblasts, while Dau inhibited their overexpression. These data demonstrated that Dau also blocked TGF-β1/MAPK signal pathway via inhibition of p-ERK1/2.

In current study, we compared effects of 7 BIAs on normal and PF lung fibroblasts whose proliferation, activation, migration and apoptosis are well known to contribute to PF development. Our data *in vitro* showed promising findings. However, we did not observe the effects and mechanisms of these BIAs on PF *in vivo*. These limitations should be addressed in the further study.

5. Conclusion

In summary, our current data provide the first comprehensive evidence of a preliminary structure-activity relationship for BIAs. Five bisbenzylisoquinoline alkaloids Lien, Nef, Iso, Tet and Dau showed the stronger effects on inhibiting proliferation, activation, migration and promoting apoptosis of lung fibroblasts in PF than these effects of two monobenzylisoquinoline alkaloids Pap and Lot. Among them, Dau showed the strongest inhibitory effects by inhibition of TGF-β1/Smad3/4 and p-ERK1/2 pathway proteins.

Author contribution statement

Hui-Li Ren: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jia-Hua Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jun-Hua Xiao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16849.

References

[1] V. Sontake, Y. Wang, R.K. Kasam, D. Sinner, G.B. Reddy, A.P. Naren, F.X. McCormack, E.S. White, A.G. Jegga, S.K. Madala, Hsp90 regulation of fibroblast activation in pulmonary fibrosis, JCI Insight 2 (4) (2017), e91454, https://doi.org/10.1172/jci.insight.91454.

[2] M. Kimura, N. Hashimoto, M. Kusunose, D. Aoyama, K. Sakamoto, S. Miyazaki, A. Ando, N. Omote, K. Imaizumi, T. Kawabe, Y. Hasegawa, Exogenous induction of unphosphorylated PTEN reduces TGFbeta-induced extracellular matrix expressions in lung fibroblasts, Wound Repair Regen. 25 (1) (2017) 86–97, https://doi.org/10.1111/wrr.12506.

[3] C.C. Chung, R.C. Hsu, Y.H. Kao, J.P. Liou, Y.Y. Lu, Y.J. Chen, Androgen attenuates cardiac fibroblasts activations through modulations of transforming growth factor-beta and angiotensin II signaling, Int. J. Cardiol. 176 (2) (2014) 386–393, https://doi.org/10.1016/j.ijcard.2014.07.077.

[4] W.J. Piotrowski, J. Kiszalkiewicz, D. Pastuszak-Lewandoska, A. Antczak, P. Gorski, M. Migdalska-Sek, W. Gorski, K. Czarnecka, E. Nawrot, D. Domanska, E. Brzezianska-Lasota, TGF-Beta and SMADs mRNA expression in pulmonary sarcoidosis, Adv. Exp. Med. Biol. 852 (2015) 59–69, https://doi.org/10.1007/5584_2014_106.

[5] B. Hinz, S.H. Phan, V.J. Thannickal, A. Galli, M.L. Bochaton-Piallat, G. Gabbiani, The myofibroblast: one function, multiple origins, Am. J. Pathol. 170 (6) (2007) 1807–1816, https://doi.org/10.2353/ajpath.2007.070112.

[6] G. Gabbiani, The myofibroblast in wound healing and fibrocontractive diseases, J. Pathol. 200 (4) (2003) 500–503, https://doi.org/10.1002/path.1427.

[7] B. Hinz, S.H. Phan, V.J. Thannickal, M. Prunotto, A. Desmouliere, J. Varga, O. De Wever, M. Mareel, G. Gabbiani, Recent developments in myofibroblast biology: paradigms for connective tissue remodeling, Am. J. Pathol. 180 (4) (2012) 1340–1355, https://doi.org/10.1016/j.ajpath.2012.02.004.

[8] C.H. Niu, Y. Wang, J.D. Liu, J.L. Wang, J.H. Xiao, Protective effects of neferine on amiodarone-induced pulmonary fibrosis in mice, Eur. J. Pharmacol. 714 (1–3) (2013) 112–119, https://doi.org/10.1016/j.ejphar.2013.06.004.

[9] J.H. Xiao, J.H. Zhang, H.L. Chen, X.L. Feng, J.L. Wang, Inhibitory effects of isoliensinine on bleomycin-induced pulmonary fibrosis in mice, Planta Med. 71 (3) (2005) 225–230, https://doi.org/10.1055/s-2005-837821.

[10] R.H. Reist, R.D. Dey, J.P. Durham, Y. Rojanasakul, V. Castranova, Inhibition of proliferative activity of pulmonary fibroblasts by tetrandrine, Toxicol. Appl. Pharmacol. 122 (1) (1993) 70–76, https://doi.org/10.1006/taap.1993.1173.

[11] H. Jin, [A study on the inhibition effect of tetrandrine on the pingyangmycinum induced pulmonary fibrosis], Zhonghua Jiehe He Huxi Zazhi 14 (6) (1991) 359–361, 78.

[12] P.K. Mukherjee, K. Saha, R. Balasubramanian, M. Pal, B.P. Saha, Studies on psychopharmacological effects of Nelumbo nucifera Gaertn. rhizome extract, J. Ethnopharmacol. 54 (2–3) (1996) 63–67, https://doi.org/10.1016/s0378-8741(96)01455-9.

[13] J. Zhao, Y. Lian, C. Lu, L. Jing, H. Yuan, S. Peng, Inhibitory effects of a bisbenzylisoquinline alkaloid dauricine on HERG potassium channels, J. Ethnopharmacol. 141 (2) (2012) 685–691, https://doi.org/10.1016/j.jep.2011.08.054.

[14] J. Wang, Y. Li, X.B. Zu, M.F. Chen, L. Qi, Dauricine can inhibit the activity of proliferation of urinary tract tumor cells, Asian Pac. J. Tropical Med. 5 (12) (2012) 973–976, https://doi.org/10.1016/S1995-7645(12)60185-0.

[15] Z. Yang, C. Li, X. Wang, C. Zhai, Z. Yi, L. Wang, B. Liu, B. Du, H. Wu, X. Guo, M. Liu, D. Li, J. Luo, Dauricine induces apoptosis, inhibits proliferation and invasion through inhibiting NF-kappaB signaling pathway in colon cancer cells, J. Cell. Physiol. 225 (1) (2010) 266–275, https://doi.org/10.1002/jcp.22261.

[16] Y.H. Li, P.L. Gong, Neuroprotective effect of dauricine in cortical neuron culture exposed to hypoxia and hypoglycemia: involvement of correcting perturbed calcium homeostasis, Can. J. Physiol. Pharmacol. 85 (6) (2007) 621–627, https://doi.org/10.1139/y07-056.

[17] X.Y. Yang, S.Q. Jiang, L. Zhang, Q.N. Liu, P.L. Gong, Inhibitory effect of dauricine on inflammatory process following focal cerebral ischemia/reperfusion in rats, Am. J. Chin. Med. 35 (3) (2007) 477–486, https://doi.org/10.1142/S0192415X07004990.

[18] J.S. Rodefer, S.K. Saland, S.J. Eckrich, Selective phosphodiesterase inhibitors improve performance on the ED/ID cognitive task in rats, Neuropharmacology 62 (3) (2012) 1182–1190, https://doi.org/10.1016/j.neuropharm.2011.08.008.

[19] C.J. Schmidt, D.S. Chapin, J. Cianfrogna, M.L. Corman, M. Hajos, J.F. Harms, W.E. Hoffman, L.A. Lebel, S.A. McCarthy, F.R. Nelson, C. Proulx-LaFrance, M. J. Majchrzak, A.D. Ramirez, K. Schmidt, P.A. Seymour, J.A. Siuciak, F.D. Tingley 3rd, R.D. Williams, P.R. Verhoest, F.S. Menniti, Preclinical characterization of selective phosphodiesterase 10A inhibitors: a new therapeutic approach to the treatment of schizophrenia, J. Pharmacol. Exp. Therapeut. 325 (2) (2008) 681–690, https://doi.org/10.1124/jpet.107.132910.

[20] A. Becker, G. Grecksch, Phosphodiesterase inhibitors–are they potential neuroleptic drugs? Behav. Brain Res. 186 (2) (2008) 155–160, https://doi.org/10.1016/j.bbr.2007.08.002.

[21] M.I. Ragone, M. Sella, P. Conforti, M.G. Volonte, A.E. Consolini, The spasmolytic effect of Aloysia citriodora, Palau (South American cedron) is partially due to its vitexin but not isovitexin on rat duodenums, J. Ethnopharmacol. 113 (2) (2007) 258–266, https://doi.org/10.1016/j.jep.2007.06.003.

[22] R. Harishkumar, C.I. Selvaraj, Lotusine, an alkaloid from Nelumbo nucifera (Gaertn.), attenuates doxorubicin-induced toxicity in embryonically derived H9c2 cells, In Vitro Cell. Dev. Biol. Anim. 56 (5) (2020) 367–377, https://doi.org/10.1007/s11626-020-00466-1.

[23] Y. Yamada, F. Sato, Tyrosine phosphorylation and protein degradation control the transcriptional activity of WRKY involved in benzylisoquinoline alkaloid biosynthesis, Sci. Rep. 6 (2016), 31988, https://doi.org/10.1038/srep31988.

[24] L. Zhao, X. Wang, Q. Chang, J. Xu, Y. Huang, Q. Guo, S. Zhang, W. Wang, X. Chen, J. Wang, Neferine, a bisbenzylisoquinline alkaloid attenuates bleomycin-induced pulmonary fibrosis, Eur. J. Pharmacol. 627 (1–3) (2010) 304–312, https://doi.org/10.1016/j.ejphar.2009.11.007.

[25] M.S. Chen, J.H. Zhang, J.L. Wang, L. Gao, X.X. Chen, J.H. Xiao, Anti-fibrotic effects of neferine on carbon tetrachloride-induced hepatic fibrosis in mice, Am. J. Chin. Med. 43 (2) (2015) 231–240, https://doi.org/10.1142/S0192415X15500159.

[26] G. Teng, D. Svystonyuk, H.E. Mewhort, J.D. Turnbull, D.D. Belke, H.J. Duff, P.W. Fedak, Tetrandrine reverses human cardiac myofibroblast activation and myocardial fibrosis, Am. J. Physiol. Heart Circ. Physiol. 308 (12) (2015) H1564–H1574, https://doi.org/10.1152/ajpheart.00126.2015.

[27] J.L. Nortier, J.L. Vanherweghem, Renal interstitial fibrosis and urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi), Toxicology 181–182 (2002) 577–580, https://doi.org/10.1016/s0300-483x(02)00486-9.

[28] I.M. Westra, D. Oosterhuis, G.M. Groothuis, P. Olinga, Precision-cut liver slices as a model for the early onset of liver fibrosis to test antifibrotic drugs, Toxicol. Appl. Pharmacol. 274 (2) (2014) 328–338, https://doi.org/10.1016/j.taap.2013.11.017.

[29] B. Hinz, G. Gabbiani, C. Chaponnier, The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo, J. Cell Biol. 157 (4) (2002) 657–663, https://doi.org/10.1083/jcb.200201049.

[30] J. Wang, R. Zohar, C.A. McCulloch, Multiple roles of alpha-smooth muscle actin in mechanotransduction, Exp. Cell Res. 312 (3) (2006) 205–214, https://doi.org/10.1016/j.yexcr.2005.11.004.
[31] P.J. Wolters, H.R. Collard, K.D. Jones, Pathogenesis of idiopathic pulmonary fibrosis, Annu. Rev. Pathol. 9 (2014) 157–179, https://doi.org/10.1146/annurev-pathol-012513-104706.
[32] R.A. Evans, Y.C. Tian, R. Steadman, A.O. Phillips, TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins, Exp. Cell Res. 282 (2) (2003) 90–100, https://doi.org/10.1016/s0014-4827(02)00015-0.
[33] H.Y. Zhang, M. Gharaee-Kermani, K. Zhang, S. Karmiol, S.H. Phan, Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis, Am. J. Pathol. 148 (2) (1996) 527–537.
[34] A.H. Limper, T.V. Colby, M.S. Sanders, S. Asakura, P.C. Roche, R.A. DeRemee, Immunohistochemical localization of transforming growth factor-beta 1 in the nonnecrotizing granulomas of pulmonary sarcoidosis, Am. J. Respir. Crit. Care Med. 149 (1) (1994) 197–204, https://doi.org/10.1164/ajrccm.149.1.8111583.
[35] A. Nakao, M. Fujii, R. Matsumura, K. Kumano, Y. Saito, K. Miyazono, I. Iwamoto, Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice, J. Clin. Invest. 104 (1) (1999) 5–11, https://doi.org/10.1172/JCI6094.
[36] M. Miljkovic-Lolic, R. Silbergleit, G. Fiskum, R.E. Rosenthal, Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity, Brain Res. 971 (1) (2003) 90–94, https://doi.org/10.1016/s0006-8993(03)02364-3.
[37] G. Westergren-Thorsson, B. Sarnstrand, L.A. Fransson, A. Malmstrom, TGF-beta enhances the production of hyaluronan in human lung but not in skin fibroblasts, Exp. Cell Res. 186 (1) (1990) 192–195, https://doi.org/10.1016/0014-4827(90)90227-2.
[38] K. Kahata, M.S. Dadras, A. Moustakas, TGF-Beta family signaling in epithelial differentiation and epithelial-mesenchymal transition, Cold Spring Harbor Perspect. Biol. 10 (1) (2018), https://doi.org/10.1101/cshperspect.a022194.
[39] P. Bonniaud, M. Kolb, T. Galt, J. Robertson, C. Robbins, M. Stampfli, C. Lavery, P.J. Margetts, A.B. Roberts, J. Gauldie, Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis, J. Immunol. 173 (3) (2004) 2099–2108, https://doi.org/10.4049/jimmunol.173.3.2099.
[40] J. Zhao, W. Shi, Y.L. Wang, H. Chen, P. Bringas Jr., M.B. Datto, J.P. Frederick, X.F. Wang, D. Warburton, Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice, Am. J. Physiol. Lung Cell Mol. Physiol. 282 (3) (2002) L585–L593, https://doi.org/10.1152/ajplung.00151.2001.
[41] H. Higashiyama, D. Yoshimoto, T. Kaise, S. Matsubara, M. Fujiwara, H. Kikkawa, S. Asano, M. Kinoshita, Inhibition of activin receptor-like kinase 5 attenuates bleomycin-induced pulmonary fibrosis, Exp. Mol. Pathol. 83 (1) (2007) 39–46, https://doi.org/10.1016/j.yexmp.2006.12.003.
[42] A. Gunaratne, E. Chan, T.H. El-Chabib, D. Carter, G.M. Di Guglielmo, aPKC alters the TGFbeta response in NSCLC cells through both Smad-dependent and Smad-independent pathways, J. Cell Sci. 128 (3) (2015) 487–498, https://doi.org/10.1242/jcs.155440.
[43] Q.Q. Wu, Y. Xiao, X.H. Jiang, Y. Yuan, Z. Yang, W. Chang, Z.Y. Bian, Q.Z. Tang, Evodiamine attenuates TGF-beta1-induced fibroblast activation and endothelial to mesenchymal transition, Mol. Cell. Biochem. 430 (1–2) (2017) 81–90, https://doi.org/10.1007/s11010-017-2956-6.