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(5*R*)-5-hydroxytriptolide (LLDT-8) protects against bleomycin-induced lung fibrosis in mice[1]

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Key words

(5*R*)-5-hydroxytriptolide; bleomycin; lung fibrosis; cytokines

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Abstract

Aim: To study the protective effects of a triptolide-derived, novel compound, (5*R*)-5-hydroxytriptolide (LLDT-8), on bleomycin-induced lung fibrosis. **Methods:** C57BL/6 mice received an intratracheal injection of bleomycin and were then treated with LLDT-8 (0.5, 1, 2 mg/kg, ip) once daily for 7 or 14 consecutive days. The body weight loss and lung index augmentation was observed; the inflammatory response including differential cells counts of neutrophils, macrophages, and lymphocytes in the bronchoalveolar lavage fluid (BALF), superoxide dismutase (SOD), and malondialdehyde (MDA) level in the lung homogenates was detected, and the fibrosis extent was evaluated by hydroxyproline content and histopathological changes in the lungs. In addition, the pro-inflammatory and pro-fibrotic cytokines, tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and transforming growth factor-α (TGF-α) production in the lungs were measured. **Results:** LLDT-8 alleviated the body weight loss and lung index increase caused by bleomycin, reduced neutrophils and lymphocytes in the BALF, promoted SOD activity, decreased MDA production, and inhibited the hydroxyproline level and the amelioration of lung tissue histological damage. Moreover, LLDT-8 suppressed TNF-α, IL-4, and TGF-β production in the lung homogenates. **Conclusion:** LLDT-8 showed protective effects against bleomycin-induced lung fibrosis, and the results suggested the potential role of LLDT-8 in the treatment of this disease.

Introduction

Idiopathic pulmonary fibrosis is a chronic, progressive form of interstitial lung disease, associated with an extremely poor prognosis for survival in most patients. Most patients die of progressive respiratory failure within 3–8 years of the onset of symptoms. Considerable experimental evidence implicated both increased collagen production and reduced degradation, leading to an irreversible distortion of normal tissue architecture and loss of function[1–3]. However, the pathogenesis of idiopathic pulmonary fibrosis still remains unknown; early lung inflammatory response and subsequent fibrotic changes are well appreciated in the time course of this disease, and until now, there has been no satisfactory treatment for this disease. Bleomycin, a mixture of glycopeptides derived from *Streptomyces verticillus*, is a potent chemotherapeutic agent used for the treatment of lymphomas, head and neck cancers, and various tumors. Moreover, bleomycin is also known to produce lung injury and fibrosis in humans as well as in experimental animals[4].

The Chinese traditional herb *Tripterygium wilfordii* Hook F and its extracts have been widely used in the treatment of autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus[5–8]. Triptolide is identified as the most active component accounting for the immunosuppressive effects of *Tripterygium wilfordii* Hook F[9]. However, the strong toxicity of triptolide limits its application to a great extent[10]. Recently, (5*R*)-5-hydroxytriptolide (LLDT-8), a new compound derived from triptolide, was synthesized and showed similar immunosuppressive activity to that of triptolide, but its toxicity was greatly reduced *in vitro* and *in vivo*. *In vitro*, LLDT-8 significantly inhibited

mitogen-induced T and B cell proliferation, and mixed lymphocyte reaction, inflammatory, and Th1 type cytokines release[11]. *In vivo*, LLDT-8 suppressed the bovine type II collagen-induced arthritis in DBA/1 mice[12] and adjuvant-induced arthritis in Wistar rat (our unpublished observations). LLDT-8 prevented graft-versus-host disease and prolonged allogeneic cardiac transplantation survival in mice[13,14]. LLDT-8 attenuated the concanavalin A-induced liver hepatitis[15] and prolonged mice survival in the MRL-lpr/lpr murine model of systemic lupus erythematosus (our unpublished observa-tions).

The purpose of the present study was to extend our findings of LLDT-8 to an *in vivo* mouse lung fibrosis model for further exploration of its anti-inflammatory and antifibrosis action.

Materials and methods

Animals Female C57BL/6 mice (6–8 weeks old, 20–22 g) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). The animals were housed in specific pathogen-free conditions. All mice were allowed to acclimatize in our facility for 1 week before any experiments were started. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Shanghai Institute of Materia Medica (Shanghai, China).

Test compound LLDT-8 was synthesized from triptolide that was separated from *Tripterygium wilfordii* Hook F. LLDT-8 is a white, amorphous powder with 99% purity by reverse phase, high-performance liquid chromatography. The stock solution of LLDT-8 (5 mg/mL) was prepared in 2-methyl-1,3-propanediol, stored at 4 °C, and diluted to a desired concentration with saline (10 mL/kg, ip). The final concentration of 2-methyl-1,3-propanediol in the dosing solution was 4%.

Experimental model of bleomycin-induced lung fibrosis Lung fibrosis was induced as described by previous studies with minor modifications[4,16]. Briefly, after the body weight was recorded, the mice were anesthetized via intraperitoneal injection of 30 mg/kg pentobarbital sodium. A midline incision was made in the neck, and the trachea was exposed by blunt dissection. Bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan), was dissolved in 0.1 mL saline and injected into the animals' lungs via the 0.25 mL syringes at a dose of 7.5 mg/kg body weight. The normal control received an equal volume of sterile saline. After bleomycin or saline were injected into the trachea, the animal operating plate was erected and shaken to facilitate distribution of the solution throughout the lungs. The day of bleomycin injection was considered d 0 and the weight of the animals was recorded every 3–4 d.

Group assignment and drug administration The mice were randomly assigned to 5 body weight-matched groups: normal control, bleomycin, and the bleomycin with LLDT-8 treatment groups (LLDT-8 at 2 mg/kg, 1 mg/kg and 0.5 mg/kg). In most of the experiments, each group consisted of 8 mice, except the bronchoalveolar lavage assay, and 3 animals per group were analyzed. LLDT-8 was daily administered to the mice by ip from d 1. The normal and bleomycin control group were daily injected with vehicle solution by ip.

Bronchoalveolar lavage analysis On d 7 after bleomycin treatment, the mice were sacrificed. The thorax was opened by a median incision and the trachea was cannulated with a plastic catheter attached to a 2 mL syringe. Bronchoalveolar lavage was performed in 4 mL sterile saline with gentle massaging of the lungs. The bronchoalveolar lavage fluid were collected and centrifuged at 150×*g* for 10 min at 4 °C. The total number of cells in the lavage fluid was counted with trypan blue staining. The cell subsets were counted with Giemsa staining by examining 200 cells per animal. The cell numbers of macrophages, neutrophils, and lymphocytes in the lavage fluid was calculated according to their respective percentages in the total cells.

Lung tissue preparation and biochemical assay One hour after LLDT-8 or vehicle administration on d 7 or 14, the mice were sacrificed by bleeding. The lungs were removed and weighed, washed twice with cold saline, and then each lung was divided into 2 parts: the right one was fixed in 10% formalin solution for histological examination and the left one was prepared for biochemical assay and cytokine detection.

The lung samples were prepared as 10% homogenate in 0.9% saline by homogenizer on ice according to their respective weight. Then the homogenate was centrifuged, and the supernatant was collected and diluted. The assay of superoxide dismutase (SOD), malondialdehyde, and hydroxyproline levels followed the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological examination of fibrosis The lung samples were washed and fixed in buffered 10% formalin solution. After embedded in paraffin, 5 μm sections were stained with hematoxylin-eosin (HE), and examined by 2 pathologists who were blinded to the experiment.

Measurement of tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and transforming growth factor-β (TGF-β) The lung homogenate (10%) was centrifuged at

10 600×*g* for 30 min at 4 °C to remove debris, and the supernatants were assayed for TNF-α, IL-4, and TGF-β concentrations. The levels of TNF-α and IL-4 were determined using sandwich ELISA kits from PharMingen (San Diego, CA, USA) following the manufacturer's instructions. The TGF-β level was determined using a Mv1Lu cell proliferation assay[17]. Briefly, the Mv1Lu cells (2×10^4/well) were cultured in the presence of diluted acidified samples or recombinant TGF-β (R&D Systems, Minneapolis, MN, USA) in 96-well plates for 24 h at 37 °C in an incubator with 5% CO_2. The cells were pulsed with 0.5 mCi of [^{3}H]-thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was then counted using a Beta Scintillation Counter (MicroBeta Trilux, Perkin-Elmer Life Sciences, Boston, MA, USA). The concentration of TGF-β in the lung homogenate was calculated according to the rTGF-β inhibitory standard curve. The anti-TGF-β 1,2,3 antibody (Genzyme, Framingham, MA, USA) was used to confirm the specific inhibition by TGF-β in the Mv1Lu cells. The sample was acidified to pH 2 with 1 mol/L HCl for 30 min on ice and then neutralized with 1 mol/L NaOH.

Statistical analysis Data were expressed as mean±SEM or mean±SD where indicated. Statistical differences were analyzed according to the analysis of variance, followed by post-hoc multiple comparison tests (LSD). $P<0.05$ was considered to be significant.

Results

LLDT-8 attenuated bleomycin-induced lung injury Compared with the normal control, the body weight in the bleomycin-treated animals decreased gradually and reached the lowest level at d 7 after bleomycin injection, and then tended to recover. LLDT-8 displayed protective effects on the loss of body weight (Figure 1). Moreover, LLDT-8 was well tolerated in the bleomycin-treated mice, showing no change in mobility, skin hair, and respiration throughout the experiment.

In contrast to the loss of body weight, the weight of the lungs increased obviously in the bleomycin-treated mice, resulting in augmentation of the lung index (lung weight versus body weight). LLDT-8 treatment dose-dependently inhibited the increase of the lung index as compared with that of the vehicle-treated bleomycin group on d 7 and 14 (Figure 2).

The lungs were examined histologically on d 14 after bleomycin injection. Data are shown in Figure 3. The lung architecture appeared intact in the normal control group. In the bleomycin control group, there were multifocal diffuse changes consisting of some combinations of thickened

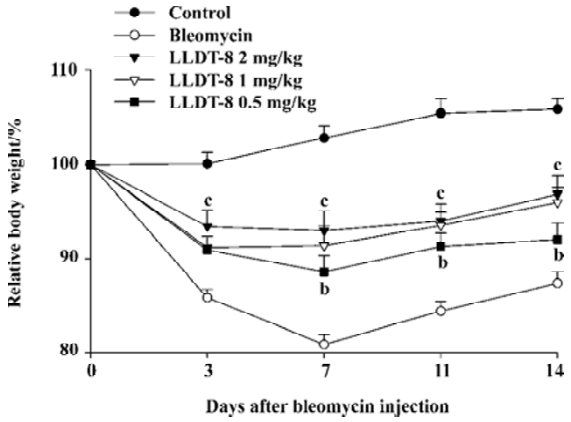

Figure 1. LLDT-8 prevented body weight loss after bleomycin exposure. C57BL/6 mice were randomly assigned in body weight-matched groups, and the body weight on d 0 (bleomycin injection) was taken as 100%. The relative body weight was calculated as the ratio to that on d 0. Data are expressed as mean±SEM (n=8). Three independent experiments were performed that gave similar results. $^bP<0.05$, $^cP<0.01$ *vs* bleomycin group.

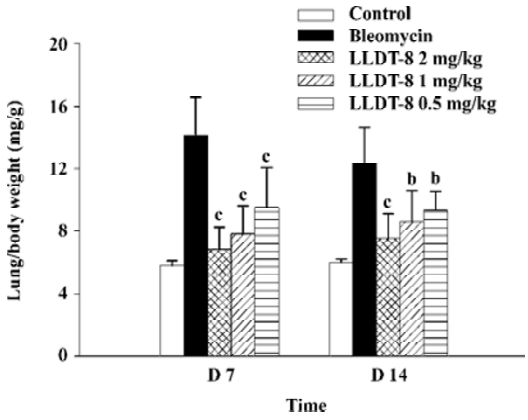

Figure 2. LLDT-8 inhibited the increase of the lung index in bleomycin-treated mice. Mice were sacrificed on d 7 and 14 after bleomycin exposure, and the lung index was calculated as a ratio of the lung weight (mg) to body weight (g) of each mouse. Data are expressed as mean±SD (n=8). Three independent experiments were performed that gave similar results. $^bP<0.05$, $^cP<0.01$ *vs* bleomycin group.

alveolar septa, interstitial hyperplasia, intra-alveolar fibrosis with myofibroblasts, occasional foci of dense fibrosis, increased alveolar macrophages, and some infiltration of inflammatory cells. However, in the LLDT-8-treated mice, there was a marked decrease in inflammation and fibrosis. No obvious fibrotic focal was observed in the 2 mg/kg LLDT-8-treated group.

LLDT-8 suppressed lung inflammatory cell expansion induced by bleomycin treatment The number of total cells

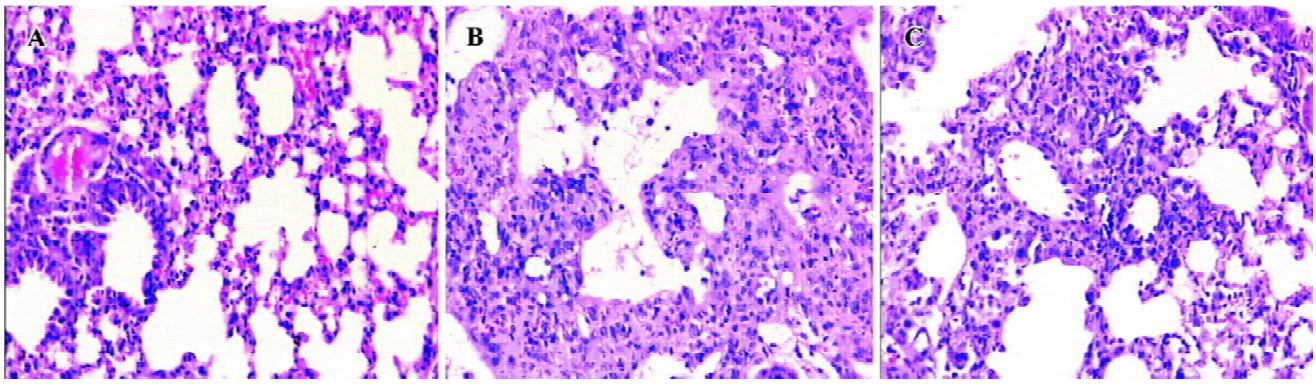

Figure 3. Histological examination of lung sections on d 14 after bleomycin treatment. Lungs were harvested and fixed in buffered 10% formalin solution. Sections were prepared and stained with HE (original magnification 40×). (A) normal mice; (B) vehicle-treated mice with lung injury. The marked thickening alveolar septa, interstitial hyperplasia, and infiltration of inflammatory cells were observed. (C) LLDT-8 (2 $mg \cdot kg^{-1} \cdot d^{-1}$)-treated mice with lung injury; a remarkable amelioration of inflammation and fibrosis was observed.

and the subsets including macrophages, lymphocytes, and neutrophils in the bronchoalveolar lavage fluid were elevated markedly in response to bleomycin on d 7 (Table 1). LLDT-8 administration dose-dependently reduced the numbers of total cells, neutrophils, as well as lymphocytes, but weakly affected the macrophages number.

LLDT-8 reduced hydroxyproline and malondialdehyde production but enhanced SOD activity in bleomycin-treated mice To assess the total collagen content and fibrotic process, we determined the lung hydroxyproline level. Data are presented in Table 2. In the lung homogenates from the bleomycin control mice, the hydroxyproline level increased on d 7 and 14. Administration of LLDT-8 reduced the hydroxyproline production. On d 14, LLDT-8 at 2 mg/kg, 1 mg/kg, and 0.5 mg/kg decreased the hydroxyproline level by 30.4%, 23.9%, and 15.1%, respectively. Moreover, LLDT-8 at 2 mg/kg effectively reduced the hydroxyproline production to the basal level as that in the normal control.

The deprivation of antioxidant enzyme SOD indirectly reflects reactive oxygen species production in response to bleomycin. As shown in Table 2, LLDT-8 significantly prevented the decrease of SOD activity on d 7. On d 14, SOD activity tended to recover in the bleomycin control group; meanwhile, LLDT-8 still showed an enhancing effect on SOD activity.

Malondialdehyde is a marker of lipid peroxidation. In this study, malondialdehyde content in the lung tissue was remarkably elevated after bleomycin treatment. The increased percentages were 78.4% and 41.2% on d 7 and 14, respectively. LLDT-8 inhibited bleomycin-induced malondialdehyde production in a dose-dependent manner. In the 2 mg/kg LLDT-8-treated group, its production was reduced to the basal level as that in the normal control on d 7. On d 14, the beneficial effect of LLDT-8 was still observed when given at 2 mg/kg (Table 2).

LLDT-8 inhibited pro-inflammatory and pro-fibrotic cytokine production in lung homogenates To evaluate the roles of key cytokines in lung fibrosis, we detected TNF-α, IL-4, and TGF-β levels in lung homogenates. Data are presented in Figure 4. Administration of LLDT-8 significantly

Table 1. LLDT-8 decreased the inflammatory cell count in bronchoalveolar lavage fluid. On d 7 after intratracheal injection of bleomycin, mice were sacrificed and bronchoalveolar lavage was treated with saline solution. Data are expressed as mean±SEM, and are representative of 2 experiments (n=3). $^bP<0.05$ *vs* bleomycin control.

Group	Dose (mg/kg)	Total cells (1×10^5/mL)	Macrophages (1×10^4/mL)	Lymphocytes (1×10^4/mL)	Neutrophils (1×10^4/mL)
Control	–	1.13±0.1	8.1±0.8	2.8±0.3	0.3±0.0
Bleomycin	–	62.8±14.9	158.3±45.0	194.0±55.2	267.1±76.0
LLDT-8	2	24.2±6.0^b	140.1±34.9	65.5±16.4^b	37.1±9.3^b
	1	38.5±8.8	191.5±48.1	117.9±29.6	79.8±20.0^b
	0.5	46.7±14.2	208.6±70.4	107.3±36.2	136.7±46.1^b

Table 2. Effect of LLDT-8 on hydroxyproline, SOD, and malondialdehyde content in the lung tissues of bleomycin-treated mice. Animal was sacrificed on d 7 and 14 after bleomycin injection. Hydroxyproline production, SOD activity, and malondialdehyde production in lung homogenates were measured according to the manufacturer's instructions. Data are expressed as mean±SD, and are representative of 3 experiments (n=8). $^bP<0.05$, $^cP<0.01$ *vs* bleomycin control.

Group	Dose (mg/kg)	Hydroxyproline (mg/lung) D 7	Hydroxyproline (mg/lung) D 14	SOD (U/mg protein) D 7	SOD (U/mg protein) D 14	Malondialdehyde (nmol/lung) D 7	Malondialdehyde (nmol/lung) D 14
Control	–	23.3±1.88	29.3±4.52	173.3±4.8	171.3±3.0	138.3±17.6	154.8±14.1
Bleomycin	–	37.8±3.80	47.6±9.78	145.2±11.9	155.3±19.7	246.7±37.2	218.6±33.8
LLDT-8	2	31.2±3.85^c	31.2±6.52^c	160.8±6.8^c	184.3±16.1^c	132.0±28.8^c	180.2±27.7^b
	1	34.2±5.18	33.1±9.48^b	163.9±16.8^b	183.9±21.8^b	145.2±35.2^b	185.7±36.8
	0.5	33.7±6.59	40.4±12.2	166.7±13.8^c	181.5±18.6^b	160.1±29.4^c	192.1±41.1

inhibited TNF-α production on d 7 in a dose-dependent manner (Figure 4A). In addition, a remarkable increase of IL-4 levels was seen in the bleomycin control group on d 14 (Figure 4B), and LLDT-8 treatment effectively suppressed this increase.

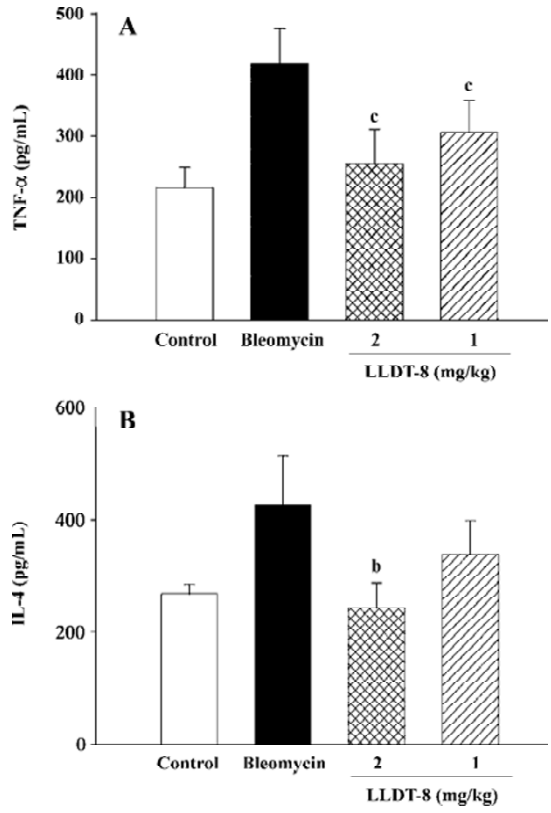

Figure 4. LLDT-8 inhibited cytokine production in lung homogenates of bleomycin-treated mice. Mice were sacrificed on d 7 and 14 after bleomycin exposure. Lungs were collected and the homogenates were prepared. Supernatants were analyzed for TNF-α level on d 7 (A) and IL-4 level on d 14 (B) by ELISA. Data are expressed as mean±SD (n=4). Three independent experiments were performed that gave similar results. $^bP<0.05$, $^cP<0.01$ *vs* bleomycin group.

In the Mv1Lu cell proliferation assay, TGF-β concentration in the lung tissue elevated from 580 pg/mL in the normal control group to 880 pg/mL on d 7, and reached 1012 pg/mL on d 14 after bleomycin injection (Figure 5). The increase of the TGF-β level was suppressed after LLDT-8 treatment. In addition, the anti-TGF-β1, 2, 3 antibody was taken to test the specificity of proliferative inhibition by TGF-β. As expected, the suppressed cell proliferation was almost completely restored by this antibody (data not shown).

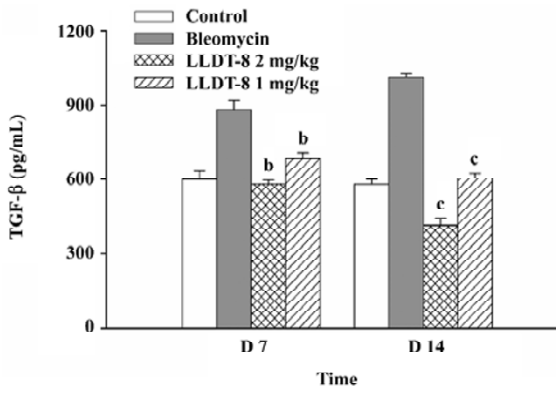

Figure 5. LLDT-8 inhibited TGF-β production in lung homogenates of bleomycin-treated mice. Mice were sacrificed on d 7 and 14 after bleomycin injection. Lung homogenates were tested in the Mv1Lu cell proliferation assay. TGF-β concentration was determined according to the rTGF-β inhibitory standard curve. Data are expressed as mean±SD (n=6). Three independent experiments were performed that gave similar results. $^bP<0.05$, $^cP<0.01$ *vs* bleomycin group.

Discussion

Idiopathic pulmonary fibrosis is a progressive lung dis-

ease with unknown pathogenesis. The role of the inflammation and anti-inflammatory strategy still remain controversial. It was suggested that in addition to inflammatory cells, alveolar epithelial cells, mesenchymal precursor cells, fibroblasts, and myofibroblasts played an important role in the diverse processes of fibrosis[2]. Thus, potential therapeutic strategies can be developed at any stage which result in idiopathic pulmonary fibrosis. These include agents that interfere with the action of inflammatory mediators, agents that prevent parenchymal cell damage, agents that prevent the proliferation of fibroblasts and collagen synthesis, agents that downregulate myofibroblast differentiation, and agents that intervene with 1 or more key events in the pathogenesis or signal transduction pathways of idiopathic pulmonary fibrosis[1]. In addition, it is unlikely that any single treatment can be sufficiently effective in the case of lung fibrosis.

Bleomycin-induced animal lung injury has been widely used as a model of human lung fibrosis because some biochemical and functional changes in the early stages in animals resemble that in humans. Other studies from long-term observations which reported the physiological and histological changes at late stages (d 120) in rats were very different from human disease[18], so that extrapolation of the data from the animal model to humans needs to be taken with caution. However some similarities exist between the bleomycin animal model and human lung fibrosis, and this model is informative for antifibrosis agent evaluation and potential mechanism research.

The inflammatory response to bleomycin is orchestrated partially by endogenous and migrating leukocytes, which is also well demonstrated in our present study. These activated leukocytes can synthesize and secrete various cytokines, chemokines, reactive oxygen species, and proteases that sustain the injury/repair processes. Neutrophils isolated from the bronchoalveolar lavage had a greater capacity to produce superoxide anion than those from the blood, and resulted in lung damage[19]. Moreover, these leukocytes together with lung epithelial and endothelial cells produced a feedback circle where stimuli from injury responses could activate alveolar and interstitial macrophages[20]. LLDT-8 ameliorated exudation in lung tissue and reduced the leukocytes number, which would result in a decrease in source of free radicals.

Substantial data prove the cellular redox state and the oxidant–antioxidant balance play a critical role in the pathogenesis of lung fibrosis in animal models and possibly in humans[21,22]. In addition to the inflammation mediator, the high level of oxidants may increase TGF-β release[23], activate protease, and enhance the fibrotic response; some antioxidants including N-acetylcysteine, and SOD can decrease collagen deposition and protect the lungs in a variety of animal models or even in clinical trials[24]. LLDT-8 served as a free-radical scavenger, enhanced SOD ability, and inhibited lipid peroxidation, which helped to ameliorate inflammatory reaction, meanwhile possibly contributing to decreased TGF-β production and alleviating fibrosis change.

Cytokines are involved in the fibrosis process. TNF-α induces adhesion molecule expression by vascular endothelial cells and intensifies the recruitment of inflammatory cells into the lungs. Moreover, TNF-α is relevant to the induction of fibrosis by augmenting synthesis of fibronectin, prostaglandin, and TGF-β. Administration of the anti-TNF-α antibody, soluble TNF-α is demonstrated to be beneficial in suppressing bleomycin-induced lung injury[25,26], and now a phase II clinical trial of soluble TNF-α receptor (Etanercept) is under way[1,27]. In our study, LLDT-8 markedly inhibited TNF-α production, providing 1 possible mechanism of its protective effect against lung injury.

The role of Th1 and Th2 cytokines in lung fibrosis remains controversial[28–32]. Recent studies disclosed that IL-4 might play a selective anti-inflammatory role during initial lung injury stages by limiting the early accumulation of T cells, but IL-4 promoted fibroblast proliferation and collagen deposition during the later stages of fibrosis[33–36]. The IL-4 level in lung tissues was detected on d 7 and 14; the IL-4 increase on d 7 was not evident, and the inhibitory effect of LLDT-8 was not significant (data not shown). However, LLDT-8 suppressed IL-4 production on d 14 after bleomycin injection, which possibly contributed to the blockade of lung injury.

Moreover, we detected IFN-γ and IL-10 levels in lung homogenates on d 7 and 14 after bleomycin injection. LLDT-8 did not affect these 2 cytokine production (data not shown). However, LLDT-8 decreased IFN-γ from concanavalin A or Sac-stimulated murine spleen cells in vitro[11]. The possible reason for this needs further investigation.

TGF-β is a pivotal mediator in lung fibrosis and has a broad spectrum of activities in pulmonary inflammation, tissue repair, and fibrosis. TGF-β can serve as a chemoattractant for fibroblasts and monocytes/macrophages and stimulate these cells to synthesize a number of pro-inflammatory and fibrogenic cytokines such as TNF-α, IL-1β, and TGF-β itself. At the same time, TGF-β is one of the most potent inducers of extracellular matrix production. TGF-β reduces the breakdown of collagen and other matrix proteins by inhibiting the generation of plasminogen activators, matrix metalloproteinase, and elastase, as well as by enhancing the expression of tissue inhibitors of metalloproteinases, plasminogen

activator inhibitor-1,2[1,37]. In our study, TGF-β production in lung homogenates elevated gradually after bleomycin treatment, while LLDT-8 potently suppressed TGF-β production and retained it at basal level as that in the normal control. Then, the reduced hydroxyproline production and lung injury by LLDT-8 was at least partially attributed to its inhibition of TGF-β production.

In conclusion, LLDT-8 demonstrated protective effects against bleomycin-induced murine pulmonary fibrosis. The beneficial effect of LLDT-8 might be closely associated with its activities of anti-inflammation, antioxidant, and cytokine inhibition.

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