Add-on Dextromethorphan improves the effects of pirfenidone in bleomycin-treated mice and patients with pulmonary fibrosis

Jie Huang[#a], Nana Liu[##a], Yueyue Jin[b], Shuangyu Han[a], Lian Li[b], Yunze Du[c], Luqing Wei[d], Dongsheng Li[e], Yan Zhang[f], Yubao Wang[a], Jau-Shyong Hong[g], Wen Ning[*b], Jing Feng[**a]

[a] Department of Respiratory and Critical Care Medicine, Tianjin Medical University General Hospital, Tianjin, 300052, China

[b] State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Tianjin Key Laboratory of Protein Sciences, Nankai University, Tianjin, 300071, China

[c] Department of Respiratory, Tianjin People's Hospital, Tianjin, 300122, China

[d] Department of Pulmonary and Critical Care Medicine, Tianjin Beichen Hospital, Tianjin, 300134, China

[e] Department of Respiratory and Critical Care Medicine, Tianjin Hospital of Integrated Traditional Chinese and Western Medicine Nankai Hospital, Tianjin, 300102, China

[f] Graduate School, Tianjin Medical University, Tianjin, 300070, China

[g] Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA

[#*]These authors contributed equally to this article

Corresponding authors

*Wen Ning,

Professor, State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences

Tianjin Key Laboratory of Protein Sciences, Nankai University, 94 Weijin Road, Tianjin, 300071, China

Email address: ningwen108@nankai.edu.cn

**Jing Feng, MD, PhD

Professor, National Key Clinical Specialty, Department of Respiratory and Critical Care Medicine

Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin, 300052, China

Email address: TMUGH_FJ@126.com

Supplementary Materials

Methods

Mice, cell lines, and reagents

C57BL/6J mice (males, 22–25 g, 6–8 weeks old) were provided by Vital River Laboratories (Beijing, China). The mice were housed in a pathogen-free facility at Nankai University (Tianjin, China) with a 12-h light/dark cycle, fed a standard diet, and kept in a temperature-controlled room (25 ℃). All the animal experiments were reviewed and approved by the Animal Care and Use Committee at Nankai University. Mouse lung fibroblast (MLg), human pulmonary epithelial (A549), and mouse lung epithelial (Mle-12) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MLg and A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Billings, MT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Mle-12 cells were cultured in DMEM/F12 (1:1) basic (1×) medium (Gibco) supplemented with 10% FBS (Gibco). All media contained 1% penicillin-streptomycin (Gibco). DM and PFD were purchased from Selleck Chemicals (Houston, TX, USA) and the Continent (Beijing, China), respectively. DM was dissolved in sodium chloride solution and prepared to the desired concentration either in *vivo* or in *vitro*; the desired concentration samples were step-like diluted with sodium chloride from highly concentrated solutions and administered three times a day in a BLM-induced pulmonary fibrosis mouse model. In *vivo*, PFD was prepared as a suspension via CMC-Na solution and given once a day. In *vitro*, PFD was dissolved in dimethyl sulfoxide solution at a working concentration of 1 mM Bleomycin

was purchased from Nippon Kayaku (Tokyo, Japan). All other chemicals used in this study were of analytical grade quality.

Bleomycin and drug administration

Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). DM and PFD were purchased from Selleck Chemicals (USA) and Continent (Beijing, China). Different dosages (1.5 U/kg, 2 U/kg, and 4 U/kg) of bleomycin were selected for pulmonary fibrosis models. In a bleomycin-induced pulmonary fibrosis mouse model, early alveolitis, pulmonary fibrogenesis, and late extracellular matrix deposition were distinguished in the timeline. The stages of alveolitis and pulmonary fibrogenesis overlapped each other, which were from day 0 to day 14. To achieve cost savings for mice, a bleomycin-induced 14-day pulmonary fibrogenesis mouse model was used for a more rapid evaluation of the antifibrotic effect of DM, and a 21-day pulmonary fibrosis mouse model was used to evaluate the efficacy of DM alone or combined with PFD next. Different dosages of bleomycin were selected for mouse models to successfully determine the model and mouse survival rate. Each dose of bleomycin corresponds to a different phase of fibrosis. Bleomycin at 1.5 U/kg was used to induce a 21-day advanced pulmonary fibrosis of extracellular matrix deposition in mice, 2 U/kg was used to induce a 14-day pulmonary fibrogenesis mouse model, and 4 U/kg was used to induce a 7-day alveolitis stage of pulmonary fibrosis.

In 14-day pulmonary fibrogenesis mouse model, mice were anesthetised with an abdominal injection of avertin and intratracheally injected with bleomycin (2 U/kg) or saline, Various

concentrations of dextromethorphan were administered subcutaneously three times daily within 14 days before sacrifice in mice. The control and BLM group received subcutaneous injection of the same volume of saline. In 21-day pulmonary fibrogenesis mouse model, after intratracheal injected with bleomycin (1.5 U/kg) or saline, 10 ng/kg DM were administered via subcutaneous injection three times daily and 100 mg/kg PFD was given by gavage once a day for 21 days. The control and BLM group received subcutaneous injection and gavag of the same volume of saline. In the late stage of pulmonary fibrogenesis mouse model, mice were anesthetized and intratracheally injected with bleomycin (1.5 U/kg) or saline. After two weeks, 10 ng/kg DM were administered via subcutaneous injection three times daily and 100 mg/kg PFD was given by gavage once a day for 7 days. The control and BLM group received subcutaneous injections and gavage of the same volume of saline. In 7-day alveolitis stage of pulmonary fibrosis mouse model, mice were anesthetised and intratracheally injected with bleomycin (4 U/kg) or saline, 10 ng/kg DM and 100 mg/kg PFD were administered via subcutaneously three times daily and gavag once a day within 7 days respectively. The control and BLM group received subcutaneous injection and gavag of the same volume of saline. In addition, DM only mice received intragastric NaCl injection and the PFD group received subcutaneous NaCl injection.

Hydroxyproline assay and histological examination

In the hydroxyproline assay, the right lungs were dried for 16 hours at 120 °C and subsequently digested with 6 N hydrochloric acid under the same condition. The lung

tissues were filtered through a 5.0-mm syringe filter and hydrolyzed using 2 N sodium hydroxide. Subsequently, 50 μL of lung tissue filtrates were oxidized with 1.4% chloramine T, and absorbances were measured spectrophotometrically. In histological examination, the left lung was inflated with 0.5 mL of 4% formalin in phosphate-buffered saline (PBS) and fixed overnight. Then, the lung tissues were embedded and cut for hematoxylin and eosin (H&E) and Masson trichrome staining to assess the fibrotic response. Digital image analysis was conducted using ImagePro Plus version 6.0 software (Media Cybernetics, Rockville, MD, USA). The histological analysis of haematoxylin and eosin (H&E)-stained lung sections was followed by quantification by a pathologist in a blind fashion. Specifically, the overall and fibrotic areas of the lung were delineated, and the pixels representing total versus fibrotic tissue were summed across each lung to obtain a percentage under blinded conditions.

Enzyme-linked immunosorbent assay and cell counting

The bronchoalveolar lavage fluid (BALF) was obtained by washing the lungs three times with 1 mL of sterile PBS through a tracheal cannula at room temperature. Cell-free supernatants and cell pellets were collected after centrifugation. Interleukin (IL)-1β and tumour necrosis factor (TNF)-α levels in the BALF supernatant were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA). Cell pellets were resuspended, and cell numbers were measured using a cell counter after mixing with equal volumes of supernatants and trypan blue stain (active cell dye). The resuspended cells were then smeared on a slide for differential cell counts.

Western blotting and immunofluorescence staining

In Western blotting, the following antibodies were used: FSTL1, collagen 1, α-SMA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-tubulin, β-actin (Cell Signaling Technology, Danvers, MA, USA), and E-cadherin and N-cadherin (Abcam, Cambridge, UK). For immunofluorescence staining, the lung tissue was pretreated with sodium citrate-hydrochloric acid buffer and subsequently blocked with 5% normal goat serum at room temperature. For immunofluorescence staining of α-SMA and collagen 1, slides were incubated with polyclonal anti-α-SMA (mouse, 1:200) and anti-Collagen 1 (Rabbit, 1:200) antibodies diluted in 5% normal goat serum (NGS). The tissue was washed at room temperature and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Cell proliferation and migration assays

For the cell proliferation assay, MLg cells were seeded in a 96-well plate at a density of 2000–3000 cells per well, with 5–6 replicates in each group. When the cell density reached 60%–70%, cells were starved for 24 hours. Cultures were pretreated with DM or PFD for 30 minutes before TGF-β1 was added. After 24 hours of TGF-β1 treatment, the CCK8 solution was added, and absorbance was measured at 450 nm. For the cell migration assay, MLg cells were seeded in 6-well plates. After the cells were starved for 24 hours, a vertical line was drawn from the top to the bottom of the well using a sterile 200-μL pipette tip. Drug treatment regimens were the same as those described earlier. Scratches were

photographed at different time points (8, 12, 24, and 36 hours) after TGF-β1 treatment using an inverted optical microscope (Olympus). Three images were taken from each group to record cell migration. The distance of scratches between the different groups was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

ROS detection

ROS levels were assessed using dihydroethidium (DHE) fluorescence. MLg cells were seeded in 24-well plates with 500 μL cell suspension per well. After 24 hours of starvation, the cells were pretreated with DM and PFD, and TGF-β1 was added 0.5 hours later. The ROS probe (DHE) was added at 1:1000 at different time points (12, 24, and 36 hours) after TGF-β1 treatment and incubated for 30 minutes. The images were acquired using an inverted fluorescence microscope (Olympus). Red-stained ROS-positive cells in the nuclear area were photographed under a 20× objective using a green excitation light. Three images were acquired from each group, and ROS-positive cells were counted using Photoshop software (Adobe, San Jose, CA, USA).

Clinical trial inclusion criteria

The inclusion criteria were as follows: (1) a diagnosis of IPF in accordance with the 2018 American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association guidelines[27]; (2) no administration of glucocorticoids or immunosuppressants were not administered within 1 month; (3) exclusion of influences from the occupational environment, connective tissue diseases, and

drugs; and (4) acute or chronic respiratory infection had been excluded. The chest high-resolution computed tomography (HRCT) findings of the patients were consistent with usual interstitial pneumonia (UIP) and probable UIP patterns, with two patients experiencing acute exacerbations of IPF (AE-IPF).

During follow-up, two patients in the PFD group died at 5 and 6 months after enrollment, and two patients in the PFD+DM group died at 4 and 5 months after enrollment. All four patients died due to viral infections rather than exacerbation of pulmonary fibrosis. Therefore, they were excluded from the study analysis.

Laboratory tests and Pulmonary function testing

Routine blood tests including C-reactive protein levels, liver and kidney function assessments were conducted before treatment. PFTs were performed using standard techniques with CHESTAC-33 (Chest MI Co., Tokyo, Japan) and Fudac-77 (Fukuda Denshi, Tokyo, Japan). The PFTs included several parameters. Forced vital capacity (FVC), which represents the maximum volume of air exhaled with maximal effort after full inspiration, was measured.[28] Forced expiratory volume in 1 second (FEV1), indicating the volume of air exhaled during the first second of the FVC manoeuvre,[28] was also assessed. Additionally, the diffusing capacity of the lung for carbon monoxide (DLCO), reflecting the amount of carbon monoxide gas diffusing from the alveoli to the capillaries per unit time and pressure difference, was measured as a percentage.[29] To standardize for variations owing to sex and age, the parameters above were expressed as percentages of predicted values. Thus, FVC%*pre* (FVC measured value divided by the predicted value), FEV1%*pre*

(FEV1 measured value divided by the predicted value), and DLCO%*pre* (DLCO measured value divided by the predicted value) were used. These values represent the measured parameters as a percentage of the predicted values based on sex and age. This approach enabled the comparison of lung function among patients while accounting for individual variations.

Computed tomography (CT) imaging score

HRCT was conducted using a Lightspeed-64 scanner (GE America, Chicago, IL, USA). CT images were acquired with a slice thickness of 0.5 mm or 1 mm, using settings of 140 kV and 200 mA. Each area of the normal lung and each specific lesion, including alveolar ground-glass opacities (GGOs), consolidation, reticular abnormalities, honeycombing, and emphysema, were recorded in three lung zones. The three zones were defined as follows: the upper zone (at or above the level of the aortic arch), the middle zone (between the level of the aortic arch and pulmonary veins), and the lower zone (at or below the level of the pulmonary veins). The extent of each abnormality, expressed as a percentage, was recorded in 5% increments. To obtain scores for alveolar ground glass and fibrosis, we divided the HRCT scans into three zones. Separate scores were obtained for the HRCT alveolar, interstitial, and honeycomb scores. For each patient, the average of the three regional scores was used for comparison.