Possible Involvement of Pirfenidone Metabolites in the Antifibrotic Action of a Therapy for Idiopathic Pulmonary Fibrosis

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Pirfenidone (PFD) is the first and only clinically used antifibrotic drug for the treatment of idiopathic pulmonary fibrosis (IPF). This study evaluated the antifibrotic effects of two metabolites of PFD, 5-hydroxypirfenidone (PFD-OH) and 5-carboxypirfenidone (PFD-COOH), on WI-38 cells in an in vitro lung fibroblast model. The inhibitory effects of PFD-OH and PFD-COOH on transforming growth factor-β1 (TGF-β1)-induced collagen synthesis in WI-38 cells were evaluated by measuring intracellular hydroxyproline, a major component of the protein collagen. PFD-OH and PFD-COOH at 300 and 1000 µM concentrations significantly decreased the TGF-β1-induced hydroxyproline content in WI-38 cells. These results indicate that PFD-OH and PFD-COOH have antifibrotic activities, which inhibit collagen synthesis in fibroblasts. This study suggests that the concentrations of PFD and its metabolites should be considered in clinical therapy for IPF.

Key words pirfenidone; 5-hydroxypirfenidone; 5-carboxypirfenidone; idiopathic pulmonary fibrosis; lung fibroblast

Fibrotic diseases occur in various tissue regions and can resemble scar tissue when they form in inappropriate locations such as lung, liver, heart, eye, and kidney. In particular, idiopathic pulmonary fibrosis (IPF) is devastating with an extremely low five-year survival rate (<50%). Currently, researchers are working to develop an antifibrotic drug that will improve the survival rate of patients with IPF.

Pirfenidone (PFD, Fig. 1), 5-methyl-1-phenyl-2-(1H)-pyridone, is the first and only clinically used antifibrotic drug for the treatment of IPF in Japan (Pirespa®), Europe (Esbriet®), and India (Pirfenex®). PFD has antifibrotic, anti-inflammatory, and antioxidative actions. In experimental animal models, PFD has demonstrated an antifibrotic effect in several tissues, such as lung, liver, and kidney. To date, clinical studies that have evaluated the PFD pharmacokinetics have been conducted in patients with IPF. After oral administration of PFD in humans, it is rapidly eliminated from plasma. Other researchers have shown that PFD is rapidly metabolized to 5-hydroxypirfenidone (PFD-OH) and 5-carboxypirfenidone (PFD-COOH) (Fig. 1), and the major metabolite PFD-COOH is eliminated in the urine (>87%). However, the antifibrotic effects of PFD-OH and PFD-COOH have not been reported. In the present study, we discussed the possible involvement of pirfenidone metabolites in the antifibrotic action of a therapy for IPF.

MATERIALS AND METHODS

Materials and Animals PFD was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PFD-OH and PFD-COOH were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). TGF-β1 was purchased from Peprotech Inc. (Rocky Hill, NJ, U.S.A.). All other reagents were commercially available and of analytical grade. Male SD rats, 8 weeks of age and 230–270 g body weight, were purchased from CLEA Japan, Inc. (Tokyo, Japan). The care and use of animals followed “The Guidelines for the Care and Use of Animals” approved by Ohu University in accordance with the principles of the NIH guidelines (Approval number: 2012–50).

Antifibrotic Experiments in Vitro WI-38 cells (Riken Gene Bank, Tsukuba, Japan), a human lung fibroblast cell line, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 40 µg/mL gentamicin in a humidified atmosphere of 5% CO2 at 37°C. Cells from passage numbers 12–13 were seeded (2.0 × 104 cells/well) on 24-well culture plates. At confluence, the medium was replaced with DMEM containing 0.4% FBS and 50 µg/mL of ascorbate. After a 24-h incubation, transforming growth factor (TGF)-β1 (10 ng/mL) and serial concentrations of PFD, PFD-OH, and PFD-COOH were added to the WI-38 cells, and subsequently the cells were incubated at 5% CO2 at 37°C for 24 h. After incubation, the medium was removed by aspiration and washed twice with ice-cold phosphate buffered saline (PBS). The cells were then extracted with 300 µL of 2 M NaCl, and the concentration of hydroxyproline in the cell extracts was measured by HPLC as described below. The DNA concentration in the cell extracts was determined using Fluorescent DNA Quantitation Kit (Bio-Rad, Hercules, CA, U.S.A.).

Pharmacokinetics Experiment in Vivo PFD dissolved in PBS was intravenously administered to rats at a dose of 30 mg/kg and the dosage volume was 1 mL/kg. At each design...
The concentrations of PFD, PFD-OH, and PFD-COOH detection (excitation wavelength of 250 nm and emission  

the eluate from the column was monitored by fluorescence  

tion was performed at a flow rate of 0.4 mL/min at 45°C, and  

was 85 m

GL Sciences, Inc., Torrance, CA, U.S.A.). The mobile phase  

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involving an InertSustain C18 (3.0

HPLC using a system (Jasco Corporation, Tokyo, Japan)  

were stable at 37°C in medium for 24 h (data not shown).  

These results indicate that PFD-OH and PFD-COOH have  

antifibrotic activities, which inhibit collagen synthesis in lung  

fibroblasts. In addition, after intravenous administration of  

PFD to rats, the PFD-COOH concentration in plasma was  

comparable to that of PFD (Fig. 3). The calculated terminal  

elimination half-life (T1/2) of PFD, PFD-OH, and PFD-COOH  

were 0.74±0.12, 0.79±0.26, and 0.84±0.26 h, respectively. The  

areas under the concentration–time curves (AUC) for PFD,  

PFD-OH, and PFD-COOH were 30.5, 3.9, and 24.2 µg*h/mL,  

respectively. These results indicate that the antifibrotic effect  

because of the presence of PFD-COOH in plasma cannot be  

neglected. The plasma concentrations of both PFD and PFD-  

COOH were lower than effective concentrations of those  

neglected. The plasma concentrations of both PFD and PFD-  

COOH were lower than effective concentrations of those  

injection of pentobarbital sodium at a dose of 40 mg/kg and  

blood was collected from the jugular vein. The concentration  

of PFD and its metabolites was measured in each sample by  

HPLC as described below. The pharmacokinetic analysis was  

performed using the non-compartment analytical method.  

Determination of Hydroxyproline by HPLC  

The concentrations of hydroxyproline in samples were measured by  

HPLC following fluorescent derivatization, using the method of Hutson et al.  

The derivative in samples was subjected  

HPLC following fluorescent derivatization, using the method  

of Hutson et al.  

HPLC as described below. The pharmacokinetic analysis was  

performed using the non-compartment analytical method.  

Determination of Hydroxyproline by HPLC  

The concentrations of hydroxyproline in samples were measured by  

HPLC following fluorescent derivatization, using the method  

Hutson et al.  

Determination of PFD and Its Metabolites by HPLC  

The concentrations of PFD, PFD-OH, and PFD-COOH in plasma were measured by HPLC, using the method by  

Wang et al.  

The prepared samples were subjected to HPLC using a system involving a MightySil RP-18GPII column (3.0×250 mm, Kanto Chemical Co., Tokyo, Japan). The mobile phase was 85 mM acetic buffer (pH 4.3)—acetonitrile (68:32). Separation  

was performed at a flow rate of 0.4 mL/min at 45°C, and  

the eluate from the column was monitored by fluorescence  

detection (excitation wavelength of 250 nm and emission  

wavelength of 310 nm).

Determination of PFD and Its Metabolites by HPLC  

The concentrations of PFD, PFD-OH, and PFD-COOH in plasma were measured by HPLC, using the method by  

Wang et al.  

The prepared samples were subjected to HPLC using a system involving a MightySil RP-18GPII column (3.0×250 mm, Kanto Chemical Co., Tokyo, Japan). The mobile phase was 0.2% acetic acid/methanol (74:26). The separation  

was performed at a flow rate of 0.4 mL/min at 45°C and the  

column was monitored by UV absorbance detection (absorbance wavelength of 310 nm).

Statistics  

Statistical analysis was performed using the Dunnnett’s t-test and SPSS software version 21 (IBM Inc., Armonk, NY, U.S.A.).

RESULTS AND DISCUSSION

The present study evaluated the antifibrotic effects of the  
PFD metabolites PFD-OH and PFD-COOH. The effects of  
different concentrations of PFD, PFD-OH, and PFD-COOH on hydroxyproline content, a major component of the protein  
collagen, in WI-38 cells, a human lung fibroblast cell line, are  

shown in Fig. 2. PFD at 100 µM (18.5 µg/mL), 300 µM (55.6 µg/  

mL), and 1000 µM (185 µg/mL), and PFD-OH at 300 µM  

(60.4 µg/mL) and 1000 µM (201 µg/mL), and PFD-COOH at  

300 µM (64.6 µg/mL) and 1000 µM (215 µg/mL) significantly  
decreased the TGF-β1-induced hydroxyproline content in  

WI-38 cells without cellular toxicity. PFD and its metabolites  

were stable at 37°C in medium for 24 h (data not shown).

**p<0.05 and ***p<0.01: significantly different from TGF-β1 alone.**
mig may differ in the mechanisms of PFD and its metabolites. Final stage in idiopathic pulmonary fibrosis (IPF), deposition of the excessive extracellular matrix (collagen) occurs via multiple pathways such as tissue injury, various mediators activation, and chemokine imbalance. Therefore, we evaluated first the inhibitory effects on the collagen synthesis of PFD and its metabolites. Further studies in lung fibroblasts are warranted to determine the contribution of PDGF and HSP47 expression in the antifibrotic effects of PFD-OH and PFD-COOH.

CONCLUSION

PFD-COOH, a main metabolite of PFD, reduced the hydroxyproline content in lung fibroblasts, which resulted in reduced collagen synthesis in lung fibrosis. This study suggests that not only the concentration of PFD but also that of its metabolite should be considered in clinical therapy of IPF.

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REFERENCES