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Superoxide scavenging activity of pirfenidone–iron complex

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ABSTRACT

Pirfenidone (PFD) is focused on a new anti-fibrotic drug, which can minimize lung fibrosis etc. We evaluated the superoxide ($O_2^{\cdot-}$) scavenging activities of PFD and the PFD–iron complex by electron spin resonance (ESR) spectroscopy, luminol-dependent chemiluminescence assay, and cytochrome *c* reduction assay. Firstly, we confirmed that the PFD–iron complex was formed by mixing iron chloride with threefold molar PFD, and the complex was stable in distillated water and ethanol. Secondary, the PFD–iron complex reduced the amount of $O_2^{\cdot-}$ produced by xanthine oxidase/hypoxanthine without inhibiting the enzyme activity. Thirdly, it also reduced the amount of $O_2^{\cdot-}$ released from phorbor ester-stimulated human neutrophils. PFD alone showed few such effects. These results suggest the possibility that the $O_2^{\cdot-}$ scavenging effect of the PFD–iron complex contributes to the anti-fibrotic action of PFD used for treating idiopathic pulmonary fibrosis.

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Pirfenidone (PFD) is a new anti-fibrotic drug. It has been reported that this compound could minimize lung fibrosis in drug–fibrotic models, including the bleomycin hamster model [1] and the cyclophosphamide mouse model [2]. PFD was also found to improve renal fibrosis in a rat model of unilateral urinary tract obstruction, reversed cardiac and renal fibrosis in streptozotocin-diabetic rats, and inhibited the development of hepatic fibrosis in rats [3–5]. Recent clinical trials have revealed that PFD also exhibits therapeutic effects in patients with idiopathic pulmonary fibrosis (IPF) [6,7]. These findings indicate the possible therapeutic effects of PFD against fibrosis.

The exact mechanism of action of PFD is not well understood; however, it is known that it regulates key fibrotic growth factors [8] and inhibits transforming growth factor$_{\beta1}$ (TGF$_{\beta1}$)-induced collagen synthesis [9]. Fibrosis is a reactive or reparative process characterized by the formation of excessive fibrous tissue. In the lungs, inflammation and immune processes are the major pathogenic mechanisms that injure tissue and stimulate fibrosis. There is considerable evidence that oxygen-free radicals play the major roles in inflammatory and immune-mediated tissue injury.

Nevertheless, these previous studies have not provided sufficient descriptions to elucidate the relationship between oxygen free radicals and PFD. Therefore, we hypothesized that PFD may exhibit antioxidant properties by scavenging reactive oxygen species (ROS), that is, not only hydroxyl radicals (·OH) but also superoxide anions ($O_2^{\cdot-}$). Because this property may be due to the PFD structure that seemingly forms a metal complex such as those formed by erythromycin and pyrimine with a metal, PFD–iron complex has $O_2^{\cdot-}$ scavenging activity as same as erythromycin–iron complex [10] and pyrimine–iron complex [11], which have scavenging activity for $O_2^{\cdot-}$. The major questions, we have addressed in the present study include the following: (1) Does PFD form an iron complex in a biological system? (2) If it does, does the PFD–iron complex scavenge $O_2^{\cdot-}$?

Materials and methods

Chemicals. PFD was purchased from Tocris Bioscience (Bristol, UK); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; purity, 99%), diethylene-triamine-pentaacetic acid (DTPA) and 1,10-phenanthroline from Dojindo Laboratories (Kumamoto, Japan); Cu, Zn-superoxide dismutase (SOD), hypoxanthine cytochrome *c*, and phorbol myristate acetate (PMA) from Sigma Chemical Co. (St. Louis, MO, USA); dimethyl sulfoxide, iron chloride hexahydrate ($FeCl_3$), and ethanol from Nakalai Tesque Inc., (Kyoto, Japan); Ascorbic acid, luminol (99%) from Wako; and xanthine oxidase (XO) from Roche (Basel, Switzerland).

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Preparation of cells. Human neutrophils were isolated by Ficoll–Hypaque density centrifugation followed by dextran sedimentation. Cells were suspended in Roswell Park Memorial Institute (RPMI) medium at 1×10^7 cells/ml [12].

*Formation of PFD–*iron *complex.* PFD powder or iron chloride was dissolved in 99% ethanol and then diluted with deionized water. To form the PFD–iron complex, PFD was mixed with iron in the final concentration of 3:1, respectively.

The spectra of PFD, iron chloride, and the PFD–iron complex were obtained using a Hitachi double wavelength spectrophotometer (Hitachi Ltd., Tokyo, Japan) at pH 6.8.

*Confirmation of chelating constant for PFD–*iron *complex.* The method is based on the 1,10-phenanthroline method. The reaction mixture contained 1 mM ascorbic acid and 1,10-phenanthroline and ethylene diamine triacetic acid (EDTA) or PFD in distilled water. The spectra of the formed 1,10-phenanthroline–iron complex were obtained using a Hitachi double wavelength spectrophotometer (Hitachi Ltd.) at 510 nm.

Superoxide production systems. Superoxide was produced by the XO/HPX system and PMA-stimulated neutrophils. The reaction mixture of the XO/HPX system contained 0.006 U/ml XO, 14.4 mM HPX, and 0.25 mM DTPA in 40 mM sodium phosphate buffer at pH 7.3. The scavenging activities of PFD, the PFD–iron complex, and SOD were estimated by adding them into the system at various concentrations. XO activity was assayed by monitoring uric acid production as indicated by an increase in absorbance at 290 nm on a spectrophotometer for 3 min at room temperature. The cells were suspended at $5–10 \times 10^5$ cells/ml with 5 ng/ml PMA, 20 mM DMPO, and 0.25 mM DTPA in 200 μl of RPMI, with or without PFD or the PFD–iron complex. Every buffer and solution of the reaction mixtures used was treated with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA, USA) before use to remove trace metals.

Electron spin resonance spectroscopy spin trapping. $O_2^{\cdot-}$ was assayed by ESR spin trapping with DMPO and $O_2^{\cdot-}$ scavenging activity was calculated from the relative intensity peak height of the DMPO–OOH ESR signal. The reacting mixtures were immediately transferred into quartz ESR flat cells (effective volume, 160 μl). ESR spectra were recorded at room temperature on a JES-TE 200 ESR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.03 mT; scanning field, 335.3 ± 5 mT; receiver gain, 300–500; response time, 0.3 s; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.43 GHz. The effect of PFD or the PFD–iron complex at various doses on $O_2^{\cdot-}$ release from PMA-stimulated neutrophils was observed by the signal intensity of DMPO–OOH adducts after the reaction proceeded. Computer simulation for experimental spectra was performed using the ESS-20 ESR simulation program (Labotec Co., Ltd., Tokyo, Japan) described previously. After recording the ESR spectra, the signal intensities of the DMPO–OOH adducts were normalized against that of a manganese oxide marker.

Chemiluminescence (CL) assay. The CL response induced by the $O_2^{\cdot-}$ released from neutrophils was measured using a luminometer (Advantec Co., Tokyo, Japan). The reaction was initiated by adding 5 ng/ml PMA to 1×10^5 neutrophils in RPMI containing 0.5 mg/ml luminol. The CL response was continuously recorded for 10 min at room temperature. We measured the 50% inhibitory concentration by adding various concentrations of PFD or SOD in the $O_2^{\cdot-}$ generation system for kinetic studies.

Cytochrome c reduction assay. The ferricytochrome *c* reduction rate of $O_2^{\cdot-}$ released from neutrophils was measured as reported previously at 540 and 550 nm using a Hitachi 557 double wavelength spectrometer equipped with a thermostat-controlled cell. The reaction mixture contained 5×10^5 neutrophils, 20 μM ferricytochrome *c*, and 5 ng/ml PMA in RPMI with or without various concentrations of PFD or the PFD–iron complex. Spontaneous $O_2^{\cdot-}$ release was measured for 10 min at 37 °C. PMA was added to the reaction mixture and ferricytochrome *c* reduction was observed for 10 min. Ferricytochrome *c* reduction was completely terminated by adding 500 U/ml Cu, Zn–SOD. The amount of reduced cytochrome *c* was calculated using a molar absorption coefficient of 19.1×10^3. The initial rate of $O_2^{\cdot-}$ release was expressed as nanomoles of cytochrome *c* reduced/min/10^7 cells. We measured the 50% inhibitory concentration by adding various concentrations of PFD or SOD in the $O_2^{\cdot-}$ generation system for kinetic studies.

Statistical analysis. We repeated each experiment at least three times to confirm whether similar data were obtained. The results are expressed as means ± SD of triplicate assays.

Results

Formation of PFD–iron complex

The spectra of PFD, the PFD–iron complex, and iron chloride at pH 6.8 are shown in Fig. 1. The UV/vis spectrum of the PFD–iron complex was distinct from that of PFD or iron chloride. To elucidate the binding abilities of PFD and iron, we used the competitive 1,10-phenanthroline method. As for the results, we confirmed that the chelating constant of the PFD–iron complex was 5.95. As this value was about half that of EDTA, this suggests that the PFD–iron complex might be formed in a biological system.

$O_2^{\cdot-}$ scavenging effects of PFD and PFD–iron complex in XO/HPX system

ESR spin trapping showed that DMPO–OOH (a_N = 1.49 mT, a_H^{β} = 1.15 mT, a_H^{γ} = 0.13 mT) adducts were produced by the XO/HPX system, and that DMPO–OH (a_N = 1.49 mT, a_H = 1.49 mT) adducts were formed by reaction with iron chloride. PFD did few change the control signal. However, the PFD–iron complex (250 μM) significantly decreased DMPO–OOH signals. The PFD–

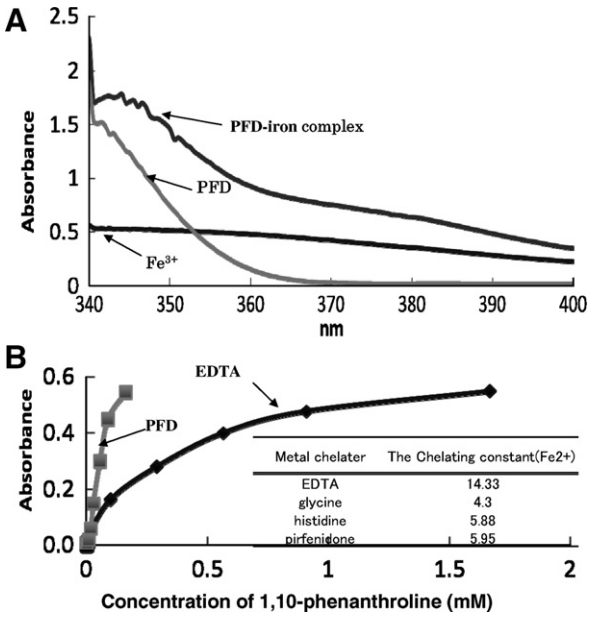

Fig. 1. Formation of PFD–iron complex. The PFD–iron complex was produced by mixing 600 μM PFD and 200 μM iron chloride in ethanol and deionized water at room temperature. The spectrum of the PFD–iron complex showed peaks at 345 nm and was distinct from the spectra of PFD and iron chloride at pH 6.8 (A). The formation of the PFD–iron complex was confirmed by the competitive 1,10-phenanthroline method (B). ■; EDTA, ■; PFD.

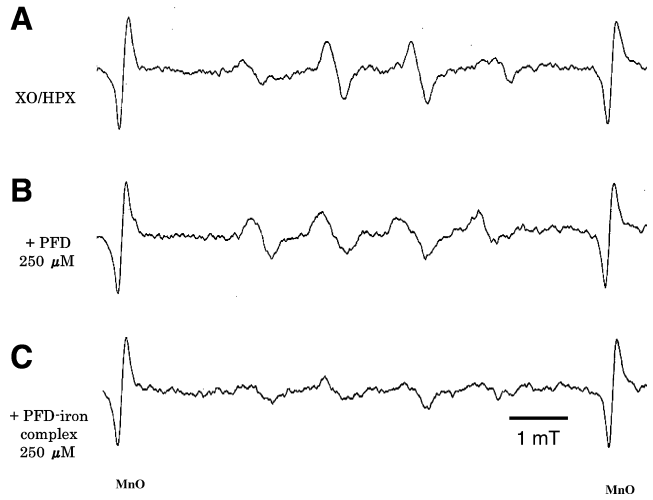

Fig. 2. ESR spin trapping for various radicals generated in XO/HPX reaction mixtures. The reaction mixture contained 0.006 U/ml XO and 14.4 mM HPX in 40 mM sodium phosphate buffer at pH 7.3. (A) DMPO–OOH adducts were produced by $O_2^{\cdot-}$ from the XO/HPX system and after addition of PFD (250 μM) (B) or the PFD–iron complex (C). ESR spectra were recorded at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.032 mT; scanning field, 335.2 ± 5 mT; receiver gain, (A–C) 500; response time, 0.3 s; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.431 GHz.

iron complex appeared to scavenge $O_2^{\cdot-}$ in a concentration-dependent manner but not PFD (Fig. 3). The concentration of the PFD–iron complex inducing a reduction of $[O_2^{\cdot-}]$ to 50% of the initial values (IC_{50}) was 250 μM. To estimate the relative scavenging activity of the PFD–iron complex, the IC_{50} values of known $O_2^{\cdot-}$ scavengers were determined. The inhibition of XO activity by the PFD–iron complex did not. The kinetics studies for PFD–iron complex were performed using XO/HPX system ($k_{\text{PFD–iron complex}} = 6.6 \times 10^4\ M^{-1}\ s^{-1}$ by ESR assay, $k_{\text{PFD–iron complex}} = 2.3 \times 10^4\ M^{-1}\ s^{-1}$ by cytochrome *c* reduction assay).

Effects of PFD and PFD–iron complex on $O_2^{\cdot-}$ release from neutrophils

The PFD–iron complex (150 μM) appeared to inhibit $O_2^{\cdot-}$ release from neutrophils stimulated with PMA (Fig. 4A). Concentration dependencies of the inhibition were also observed (Fig. 4B). The inhibition of $O_2^{\cdot-}$ release from PMN by the PFD–iron complex increased in a concentration-dependent manner and the IC_{50} was 150 μM. However, PFD (250 μM) inhibited only 28% of $O_2^{\cdot-}$ release from PMN. The effect of the PFD–iron complex was confirmed by CL and cytochrome *c* reduction assays. At concentrations of the PFD–iron complex greater than 50 μM, the CL response was inhibited in a concentration-dependent manner (data not shown). The rate of ferricytochrome *c* reduction by $O_2^{\cdot-}$ from PMA-stimulated PMN indicated that the PFD–iron complex (240 μM) inhibited 42% of $O_2^{\cdot-}$ release. The same concentration of PFD inhibited only 24% of $O_2^{\cdot-}$ release.

Discussion

In this study, we found the superoxide scavenging activity of the PFD–iron complex in enzymatic and cellular systems. These results suggest that the $O_2^{\cdot-}$ scavenging effect of the PFD–iron complex contributes to the anti-fibrotic action of PFD used for treating IPF.

The present study demonstrated that a stable PFD–iron complex was produced by the 3:1 reaction between PFD and iron chloride in ethanol and water, and its chelating constant enabled easy formation of the complex in a biological system (Fig. 1). The chemical structure of PFD is composed of 5-methyl-L-phenyl-2-(1*H*)-pyridone, and it appears to form metal complexes. We also confirmed that the resulting PFD–iron complex had radical scavenging properties in cell-free and human cell systems. The $O_2^{\cdot-}$ scavenging activity of the PFD–iron complex in the XO/HPX system was almost equal to that of ascorbic acid.

PFD reduces lung fibrosis induced by bleomycin or cyclophosphamide in experimental animals [1,2]; however, the mechanism of such cytoprotection has not yet been elucidated. Several studies have reported that PFD selectively regulates gene expression signaling from pro-fibrotic cytokines such as TGF-β1, PDGF, β-FGF, EGF, and TNF-α. In pre-clinical studies, PFD altered TGF-β transcription in a murine model of chronic cyclosporine nephrotoxicity [13], IL-10 expression in rat models of endotoxic shock [8], and the expression of ICAM-1 in cultured human fibroblasts [14]. In addition to these findings, interference with ROS [1,15–17] has been shown. ROS such as $O_2^{\cdot-}$ and ·OH causes cellular injury through lipid peroxidation which can decrease the function of alveolar-capillary beds leading to damage of the integrity of the cell membranes, acute increase in lung edema, and decrease in gas exchange [18–20]. Thus, ROS has been investigated in studies of fibrosis for many years. Regarding the relationship between PFD and ROS, several studies have reported that PFD is a potent scavenger of hydroxyl radicals and an inhibitor of membrane lipid peroxidation induced by ROS [17], and that PFD modulates oxidative stress indirectly by regulating enzymatic activities of pro-oxidant

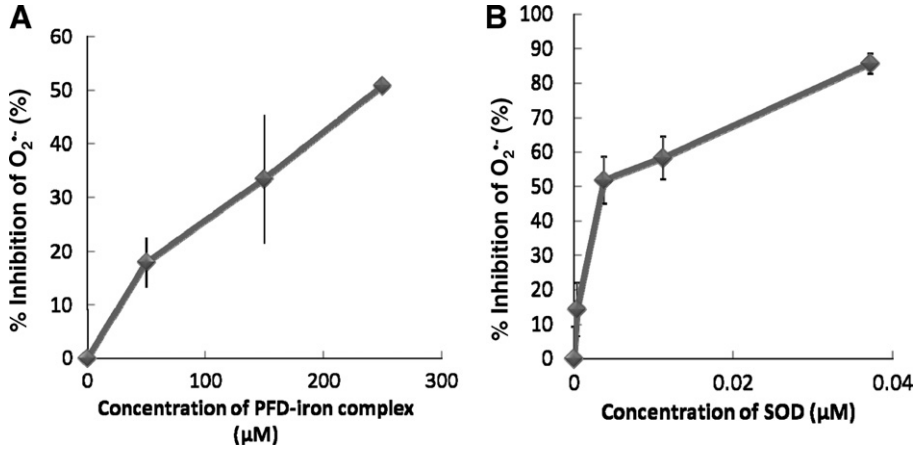

Fig. 3. Scavenging activity for $O_2^{\cdot-}$ by PFD–iron complex (A) and SOD (B). $O_2^{\cdot-}$ was generated by the XO/HPX reaction mixture. $O_2^{\cdot-}$ scavenging activity was calculated from the relative DMPO–OOH ESR spectrum. IC_{50} of $O_2^{\cdot-}$ were calculated by these graph for kinetic study of PFD–iron complex and SOD.

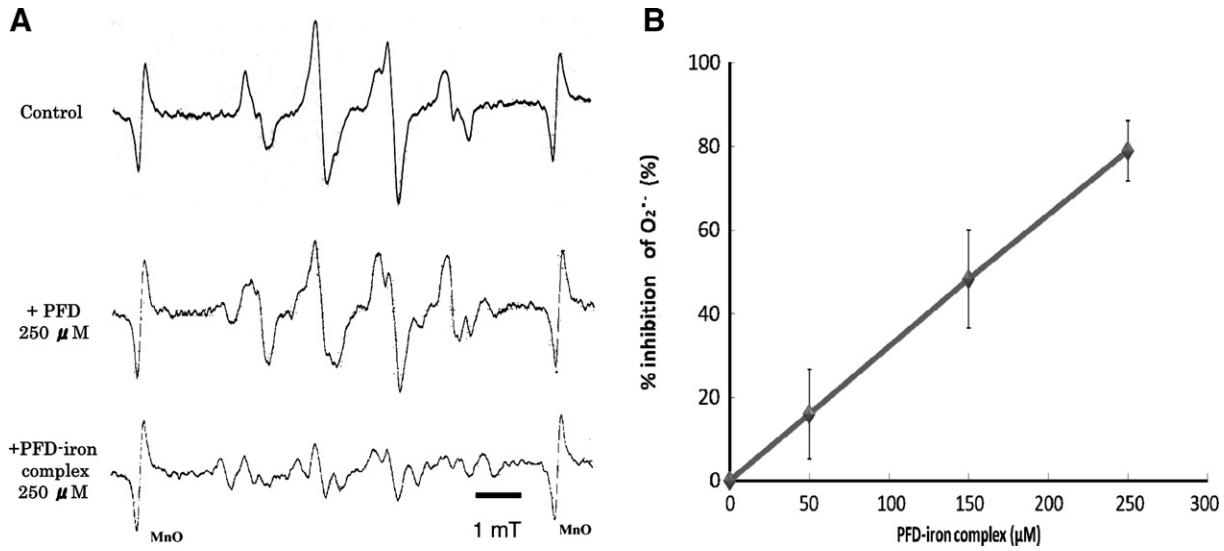

Fig. 4. Effects of PFD and PFD–iron complex on $O_2^{\cdot-}$ release from PMA-stimulated PMN observed by ESR spin trapping. (A) ESR spectra from $O_2^{\cdot-}$ release from PMA-stimulated PMN with or without PFD, PFD–iron complex. Cells were stimulated with 5 ng/ml PMA in RPMI containing 20 mM DMPO and 2 mM DTPA. ESR spectra were recorded under the same conditions as described for the XO/HPX system (Fig. 2). (B) Scavenging activity of $O_2^{\cdot-}$ by PFD–iron complex. Inhibition of $O_2^{\cdot-}$ release from PMN at 10 min after PMA addition by the PFD–iron complex was observed in a concentration-dependent manner, as shown in the inset.

and antioxidant enzymes [21]. An *in vivo* study also reported that the protective inhibition of free radical release by PFD serves as an important mechanism for reducing organ injury in acute respiratory distress syndrome [16]. However, it has also been reported that PFD is not an effective $O_2^{\cdot-}$ scavenger [17]. Therefore, we examined not only the $O_2^{\cdot-}$ scavenging activity of PFD but also that of the PFD–iron complex. The results obtained showed that the PFD–iron complex is an effective $O_2^{\cdot-}$ scavenger. These results were confirmed using three different assays.

The toxicity of $O_2^{\cdot-}$ and its role in deleterious processes in biology are well established. SOD is one of the most important antioxidant enzymes catalyzing superoxide neutralization by converting superoxide to hydrogen peroxide and oxygen. Metals are known to participate in reversible redox reactions, but free metals can be highly toxic. SODs are currently being investigated as useful pharmacological agents. The native enzyme, however, has several limitations, such as short shelf life, low lipid solubility, and poor penetration into cells. Therefore, attention is currently focused on SOD mimics with potent $O_2^{\cdot-}$ scavenging activity. Several copper and manganese complexes of important compounds have been shown to exhibit the ability to efficiently catalyze superoxide dismutation [22,23]. It has been suggested that metal complexes of antioxidants and other inflammatory drugs can be a better alternative to act as SOD mimics [10,11]. Our results indicate that only PFD does not scavenge $O_2^{\cdot-}$; however, it prevents $O_2^{\cdot-}$ production by chelating iron (Fig. 3), and its chelating constant indicates that PFD and iron easy form a complex in a biological system (Fig. 1.). Therefore, these results indicate that the beneficial effect of the PFD–iron complex is due to its behavior of acting as SOD mimics.

It is generally believed that ROS generation is the central mechanism for the bleomycin-induced lung damage/fibrosis model. In this model, $O_2^{\cdot-}$ ·OH can attack membrane and DNA via induction of lipid peroxidation [24]. This model has been widely used for studying the mechanisms of lung fibrosis and for screening potentially desirable anti-fibrotic drugs of therapeutic significance, including PFD. The present study was carried out to determine the radical scavenging effect of PFD using an *in vitro* system reflecting the bleomycin-induced lung injury model. Our result indicates that the PFD is effective in preventing bleomycin-induced lipid peroxidation via the reduction the trace metal by PFD to make complex with trace metal and scavenging of $O_2^{\cdot-}$ by its PFD–metal complex in inflammation site. Previously, there has been reported that PFD had a potent scavenger of ·OH [17]. According to our data, PFD has possibilities not only direct scavenging activities of ·OH radicals, and also the inhibition of the Fenton reaction by PFD by its chelating activity of trace metal. Based on all these findings, the antioxidant activity of PFD may partly explain its anti-fibrotic effects. Therefore, the present *in vitro* data reflect *in vivo* situations.

In conclusion, we demonstrated the $O_2^{\cdot-}$ scavenging activity of the PFD–iron complex using multiple assay systems *in vitro*. To confirm the anti-fibrotic action of this complex in chronic inflammatory lung disease, additional *in vivo* studies are necessary.

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