Pirfenidone Attenuates Bleomycin-Induced Changes in Pulmonary Functions in Hamsters (44187)

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Abstract. **The antifibrotic potential of pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) was examined in a single intratracheal bleomycin dose hamster model of pulmonary fibrosis. Bleomycin-induced fibrosis and the effectiveness of pirfenidone treatment were assessed by measuring pulmonary functions (Cqst, TLC, VC, IC, FRC, RV) and the level of hydroxyproline in whole lung homogenates. Thirty-five male golden Syrian hamsters were randomized into four experimental groups: saline instilled and fed a control diet of rat chow (SCD, $n = 8$); saline instilled and fed the control diet containing 0.5% (w/w) pirfenidone (SPD, $n = 8$); bleomycin instilled and fed the control diet (BCD, $n = 7$); and bleomycin instilled and fed the control diet containing 0.5% pirfenidone (BPD, $n = 10$). Twenty-one days following bleomycin instillation Cqst/TLC, TLC, VC, and IC were significantly reduced and total lung hydroxyproline levels were significantly increased in the BCD and BPD groups as compared with the SCD and SPD groups, respectively. Pirfenidone ingestion significantly attenuated these bleomycin-induced pertubations in pulmonary functions and lung hydroxyproline levels (BCD versus BPD). The data obtained in this study provide evidence of the benefical effects of pirfenidone in the hamster model of bleomycin-induced pulmonary fibrosis both at the functional and biochemical level.** [P.S.E.B.M. 1997, Vol 216]

Interstitial pulmonary fibrosis is one of many interstitial lung diseases that affect pulmonary function and the control of ventilation. These alterations in pulmonary function and breathing pattern are reflective of pulmonary inflammation and abnormal connective tissue remodeling and contribute to decrements in gas exchange and functional capacity. Most interstitial lung diseases are idiopathic and take months to years to develop. An exception to this scenario is the interstitial pulmonary fibrosis produced by the commonly used antineoplastic agent bleomycin (1). The pulmonary function and ventilatory changes associated with bleomycin treatment increase in a dose-dependent manner (2) and are similar to those present in patients with idiopathic pulmonary fibrosis (1). The pulmonary function changes include decreases in static lung compliance, vital capacity, and total lung capacity with variable changes in functional residual capacity and residual volume (3–5). The observed ventilatory changes consists of a rapid shallow breathing pattern that we have shown is partially mediated by vagal feedback from the lung (3) and in patients with pulmonary fibrosis is associated with a sensation of dyspnea.

Bleomycin-induced pulmonary fibrosis has been extensively studied in experimental animals because of its use as an antineoplastic agent in human cancer patients (1, 6). Bleomycin is thought to exert its cytotoxic effect on cells by cleaving DNA in the presence of molecular oxygen and a metal ion as well as causing lipid peroxidation of cell membranes (7–11). This cellular damage ultimately results in lung inflammation that culminates in pulmonary fibrosis (1). The fibrotic changes associated with bleomycin treatment are characterized by an increase in lung matrix, granu-

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lar tissue response, hyperplastic epithelium with cystic changes, and inflammatory cell infiltration (5). It is thought that the severity of pulmonary function changes is related to the severity and volume of the fibrotic lesion (3, 5, 12), though the relative contribution of altered lung matrix (i.e., excess collagen deposition) and cellularity (i.e., granulation and inflammation) to pulmonary function decrements has not been determined.

Numerous compounds have been tested as antifibrotic agents in rodent models of bleomycin-induced pulmonary fibrosis. However, the therapeutic usefulness of these compounds is severely limited by their systemic toxicity (13). Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is an investigational drug that we have shown attenuates markers of lung inflammation and fibrosis in a single-dose bleomycin hamster model of pulmonary fibrosis (12). In the present study, we examine the effects of pirfenidone treatment on bleomycin-induced pulmonary function and breathing pattern changes in hamsters in order to evaluate the possible beneficial effect of this experimental drug on the debilitating functional limitations associated with this model of pulmonary fibrosis.

Materials and Methods

Animals and Reagents. Male golden Syrian hamsters weighing between 90 and 110 g were purchased from Simonsen Inc. (Gilroy, CA). The animals were housed four per cage and had access to water and laboratory chow. Bleomycin sulfate (Blenoxane) was donated by Bristol Laboratories (Division of Bristol Meyers Co., Syracuse, NY). Pirfenidone was generously supplied by Dr. Margolin at Marnac Inc. (Dallas, TX). All other chemicals and reagents were of analytic grade and were obtained from standard commercial sources.

Treatment of Animals. Hamsters were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care. The hamsters were acclimatized in a room with constant temperature and filtered air flow for 1 week before the start of the experiment. A12:12-hr light:dark cycle was maintained.

The hamsters were given free access to either pulverized Rodent Laboratory Chow 5001 (Purina Mills Inc., St. Louis, MO) or the same pulverized rodent chow containing 0.5% pirfenidone (w/w). Pirfenidone was mixed with the laboratory chow in a planetary mixer and was stored at 4°C. The animals were fed these diets starting 3 days before the intratracheal instillation and continuing throughout the course of the study.

The hamsters were randomized into four experimental groups, as follows: saline instilled and fed the control diet (SCD, $n = 8$); saline instilled and fed the control diet containing pirfenidone (SPD, $n = 8$); bleomycin instilled and fed the control diet (BCD, $n = 9$); and bleomycin instilled and fed the control diet containing pirfenidone (BPD, $n = 10$). The hamsters were anesthetized with sodium pentobarbital (80–90 mg/kg, ip) and then bleomycin (5.5 U/4 ml of saline/kg) or pyrogen-free sterile isotonic saline solution (4 ml/kg) was instilled intratracheally. Bleomycin was freshly dissolved in isotonic sterile saline solution before intratracheal instillation.

Experimental Protocol. Twenty-one days after intratracheal instillation each hamster was anesthetized with a combination of urethane and α-chloralose (25% urethane, 2.5% α-chloralose IP [1.0 g urethane/kg; 0.1 g α-chloralose/kg]), and the trachea was cannulated. The hamster was then placed into a whole-body plethysmograph, and breathing pattern and pulmonary function measurements were performed. The hamster was attached *via* the tracheal cannula to a flow-by air stream in series with a pneumotachograph. Breathing patterns (tidal volume [V_T], frequency [f_R], inspiratory time [T_I], expiratory time [T_E], and minute ventilation [V_E] were recorded and quasistatic lung compliance [C_{qst}] and lung capacities (vital capacity [*VC*], inspiratory capacity [*IC*], and functional residual capacity [*FRC*]) measured. The lungs were then excised, frozen in liquid nitrogen, and stored at −80°C for later analysis of hydroxyproline.

Pulmonary Function Measurements. Pulmonary function measurements were performed using methods similar to those of Likens and Mauderly (14). In brief, a water-filled catheter was placed into the esophagus at the level of the chest. The hamster was placed in a whole-body plethysmograph and attached to a port linked to a flow-by air stream in series with a pneumotachograph (Hans Rudolph, Series 8300). Transpulmonary pressure was estimated by measuring the difference between the pressure in the tracheal cannula and mid thoracic esophagus using a differential pressure transducer (Validyne Model DP 15-26). Tracheal pressure was measured by a differential pressure transducer attached to the tracheal cannula (Validyne Model MP45-24-871). The lid of the box was secured in place, and the temperature within the box was allowed to equilibrate with box vented to atmosphere *via* a side port. While the hamster breathed spontaneously, the box was closed to the atmosphere and the changes in box pressure were measured using a differential pressure transducer (Validyne MP45-14). The hamster's tidal volume was obtained by integrating the respiratory flow signal from the pneumotachograph. All pressure and volume signals were recorded and saved for later processing using a MacLab 4e (AD Instruments) and Macintosh SE computer (Apple Computer, Inc.). The box pressure signal was corrected and converted to a volume while being calibrated against the integrated pneumotachograph signal. C_{qst} curves were obtained by deflating the lung from +30 cm H_2O to −30 cm H_2O tracheal pressure at a flow rate of 3–5 ml/sec while simultaneously measuring the changes in volume and transpulmonary pressures (the difference between tracheal and esophageal pressures). Quasistatic compliance was obtained by calculating the slope of the linear portion of the curve above *FRC*. *VC* was obtained by calculating the change in volume from +30 cm H_2O to lowest obtained transpulmonary pressure. *IC* was obtained by calculating the volume

change from +30 cm H_2O to *FRC*. *FRC* was calculated using Boyle's law from measurements obtained by occluding the hamster's airway at the end of expiration and measuring changes in tracheal pressure (Validyne MP45-24-871) and box pressure (representative of volume) while the hamster attempted to breath (15). Total lung capacity (*TLC*) was obtained by adding *FRC* and *IC*. Residual volume (*RV*) was obtained by subtracting *VC* from *TLC*. Expiratory reserve volume (*ERV*) was obtained by subtracting *RV* from *FRC*.

Tissue Processing. After measuring breathing pattern and pulmonary functions the hamsters were removed from the whole-body plethysmograph and killed by transecting the descending aorta and inferior vena cava. After the thoracic cavity was opened, the lungs were perfused *in situ* through the right side of the heart with 12 ml of ice-cold isotonic saline solution. All lung lobes were quickly excised free of nonparenchymal tissue, washed in ice-cold saline solution, and quickly frozen in liquid nitrogen before storage at −80°C. Subsequently, the frozen lungs were thawed and homogenized in 0.1 *M* KCL, 0.02 *M* Tris HCL buffer (pH 7.6) with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY). After the total homogenate volume was recorded (10–11 ml) it was thoroughly mixed and divided into several aliquots.

Determination of Hydroxyproline. Lung hydroxyproline, as a measure of collagen content, was assayed on the same day that the lungs were homogenized. One milliliter of whole homogenate was precipitated with 0.25 ml of ice-cold 50% (w/v) trichloroacetic acid, centrifuged, and the precipitate hydrolyzed in 2 ml of 6 *N* HCL for 18 hr at 110°C. The hydroxyproline content was then measured using the method of Woessner (16).

Calculations and Statistical Analysis of Data. In order to control for size differences between groups, C_{qst} was expressed as a ratio of the *%TLC* (C_{qst}/TLC) and lung volumes and capacities were expressed as ratios of body weight. In addition, hydroxyproline data was expressed on a per-lung basis in order to avoid artifactual lowering of values in bleomycin-treated hamsters caused by the presence of extrapulmonary proteins (16). The data were analyzed using a two-way analysis of variance (ANOVA) with bleomycin treatment and pirfenidone treatment being grouping factors (SuperANOVA; Abacus Software, Berkeley, CA). *Post hoc* analysis was done using repeated mean contrasts (SuperANOVA, Abacus Software). A value of $P \leq 0.05$ was considered statistically significant. Values are reported as mean ± SEM.

Results

Body Weight. The effect of bleomycin and pirfenidone treatment on the body weights of the four groups of hamsters are shown in Table I. There was no significant difference in body weight between any of the groups before bleomycin and pirfenidone treatment. Bleomycin treatment alone resulted in a weight loss in the BCD group with the BCD group having significantly lower body weights than the SCD group. There was no effect of pirfenidone alone on body weight as there was no significant difference between the SCD and SPD groups. Pirfenidone greatly attenuated the effect of bleomycin on body weight as the body weight for the BPD group was significantly greater than the BCD group, while there was no significant difference between the SPD and BPD groups.

Breathing Pattern and Cardiovascular Parameters. The effect of bleomycin and pirfenidone treatment on breathing pattern and cardiovascular parameters in the four groups of hamsters is shown in Table II. Bleomycin treatment alone resulted in significantly greater breathing frequency (*f*) and minute ventilation (V_E/BW) (SCD versus BCD). Pirfenidone treatment alone did not significantly affect either *f* or V_E/BW (SCD versus SPD). Pirfenidone treatment did not significantly reduce the bleomycin-induced tachypnea as there was a significantly greater *f* in the BPD versus SPD groups and there was no significant difference between the BCD and BPD groups. In contrast, pirfenidone slightly attenuated the bleomycin-induced increase in V_E/*BW* as there was no significant difference between the BPD and SPD groups but a trend for the BPD group to be less than the BCD group ($P = 0.09$). Neither bleomycin nor pirfenidone significantly effected V_T/BW, MAP, or HR.

Pulmonary Function. The effect of bleomycin and pirfenidone treatment on pulmonary function parameters in the four groups of hamsters is illustrated in Figure 1. Bleomycin significantly reduced chord compliance or C_{qst}/TLC in hamsters fed the control diet (SCD versus BCD) and the hamsters fed the pirfenidone diet (SPD versus BPD). Pirfenidone treatment alone did not significantly effect C_{qst}/TLC (SCD versus SPD). Most importantly, pirfenidone sig-

Table I. Body Weights Before and 21 Days after Instillation

Body weight	Saline + control diet (SCD)	Saline + pirfenidone diet (SPD)	Bleomycin + control diet (BCD)	Bleomycin + pirfenidone diet (BPD)
Before instillation (g)	144 ± 2	140 ± 2	143 ± 2	144 ± 2
21 days after instillation (g)	179 ± 4	169 ± 5	120 ± 8	157 ± 4
Absolute change (g)	35 ± 3	29 ± 4	−19 ± 6[a]	13 ± 3[b]

Note. Values represent mean ± SEM. Significance level set at $P \leq 0.05$.
[a] BCD significantly different from SCD.
[b] BPD significantly different from BCD.

Table II. Ventilatory and Cardiovascular Parameters

Parameters	Saline + control diet (SCD)	Saline + pirfenidone diet (SPD)	Bleomycin + control diet (BCD)	Bleomycin + pirfenidone diet (BPD)
V_T/BW (ml · kg^{-1})	3.97 ± 0.24	4.94 ± 0.35	4.72 ± 0.40	4.16 ± 0.42
f_R (br/min)	60.0 ± 2.8	65.4 ± 2.5	100.9 ± 11.9[a]	90.8 ± 12.3[b]
V_E (ml · [min · kg]$^{-1}$)	239 ± 19	328 ± 36	481 ± 87[a]	362 ± 44
HR (bpm)	404 ± 21	350 ± 44	333 ± 43	355 ± 40
MAP (mm Hg)	84.8 ± 6.0	81.5 ± 8.0	74.8 ± 9.7	84.0 ± 4.9

Note. Values represent mean ± SEM. Significance level set at $P \leq 0.05$. V_T, tidal volume; BW, body weight; f_R, respiratory frequency; V_E, minute ventilation; HR, heart rate; MAP, mean arterial pressure.
[a] BCD significantly different from SCD.
[b] BPD significantly different from SPD.

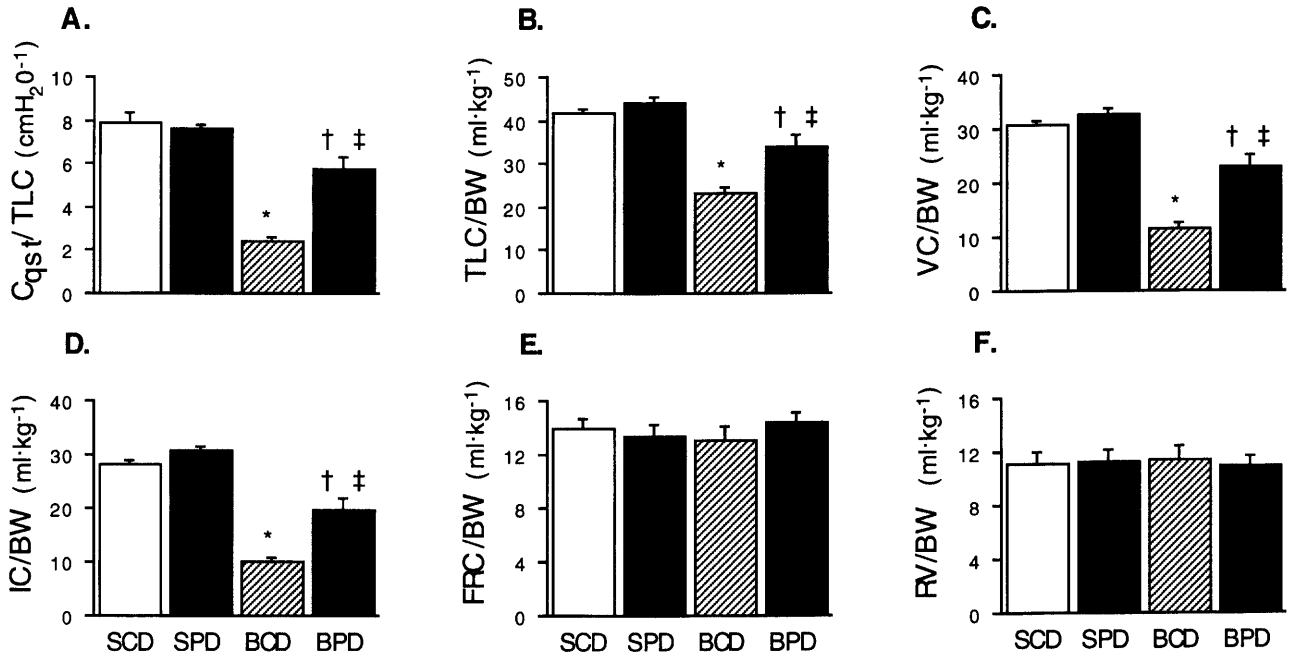

Figure 1. Effect of pirfenidone on bleomycin-induced pulmonary function decrements. There was a significant reduction in quasistatic compliance (C_{qst}), total lung capacity to body weight ratio (*TLC*/BW), vital capacity to body weight ratio (*VC*/BW) and inspiratory capacity to body weight ratio (*IC*/BW) between (i) the saline control diet (SCD) condition and bleomycin control diet (BCD) condition (*), (ii) the saline pirfenidone diet (SPD) condition and bleomycin pirfenidone diet (BPD) condition (†), and (iii) the bleomycin pirfenidone diet (BPD) condition and bleomycin control diet (BCD) condition (‡). This indicates that pirfenidone significantly attenuated the bleomycin-induced decrements in pulmonary function. Values are mean ± SEM ($P \leq 0.05$).

nificantly attenuated the bleomycin-induced decrease in C_{qst}/*TLC* (BCD versus BPD) (Fig. 1).

Paralleling the changes in C_{qst}/*TLC* bleomycin significantly reduced *TLC*/BW, *VC*/BW and *IC*/BW in hamsters fed the control diet (SCD versus BCD) and in hamsters fed the pirfenidone containing diet (SPD versus BPD). Pirfenidone treatment alone did not significantly effect *TLC*/BW, *VC*/BW and *IC*/BW (SCD versus SPD). As with C_{qst}/*TLC*, pirfenidone significantly attenuated the bleomycin-induced decrease in *TLC*/BW, *VC*/BW, and *IC*/BW (BCD versus BPD) (Fig. 1). Neither bleomycin nor pirfenidone significantly effected *FRC*/BW or *RV*/BW.

Lung Hydroxyproline. The changes in lung hydroxyproline induced by bleomycin and pirfenidone mirrored the changes in C_{qst}/*TLC* and lung capacities. Bleomycin significantly increased the amount of hydroxyproline in hamsters fed the control diet (SCD versus BCD). Pirfenidone treatment alone did not significantly affect the amount of hydroxyproline (SCD versus SPD). However, pirfenidone greatly attenuated the effect of bleomycin on the amount of hydroxyproline as the amount of hydroxyproline in the BPD group was significantly less than the BCD group, while there was still significant difference between the SPD and BPD groups (Fig. 2).

Discussion

In the present study, we observed that 21 days following a single intratracheal instillation of bleomycin C_{qst}/*TLC*, *TLC*, *VC*, and *IC* were significantly reduced and that pirfenidone ingestion significantly attenuated these bleomycin-induced decrements in pulmonary function. In contrast, pirfenidone treatment did not significantly attenuate the tachy-

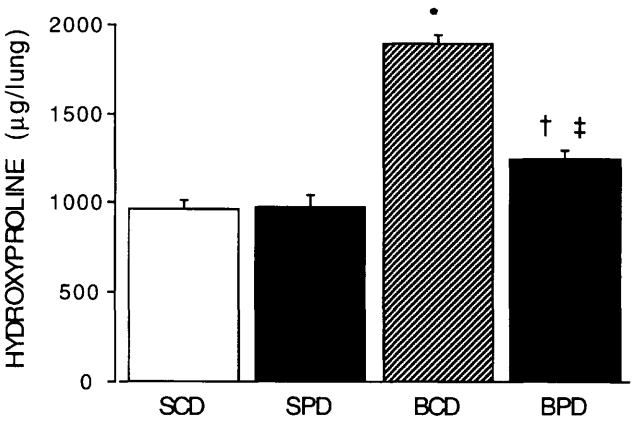

Figure 2. Effect of pirfenidone on bleomycin-induced increases in lung hydroxyproline content. There was a significant increase in lung hydroxyproline content between (i) the saline control diet (SCD) condition and bleomycin control diet (BCD) condition (*), (ii) the saline pirfenidone diet (SPD) condition and bleomycin pirfenidone diet (BPD) condition (†), and (iii) the bleomycin pirfenidone diet (BPD) condition and bleomycin control diet (BCD) condition (‡). This indicates that pirfenidone significantly attenuated the bleomycin-induced increases in lung hydroxyproline content. Values are mean ± SEM ($P \leq 0.05$).

pnea induced by bleomycin treatment, suggesting that pirfenidone, while attenuating the restrictive component of bleomycin-induced fibrosis, does not significantly alter other components of the bleomycin lesion that induces tachypnea and possibly alters pulmonary gas exchange. These results extend our previous observations that pirfenidone attenuates bleomycin-induced pulmonary fibrosis in hamsters (12) and illustrates the complex interaction of lung matrix and cellular abnormalities induced by bleomycin in producing functional decrements in pulmonary function and the control of ventilation.

Previously, Iyer *et al.* (12) found that pirfenidone treatment ameliorated the bleomycin-induced increase in lung hydroxyproline and produced a 75% reduction in the volume of fibrotic lesions in the lungs of bleomycin treated hamsters. In the present study, we observed a similar amelioration of bleomycin-induced increases in hydroxyproline. The mechanism by which pirfenidone attenuates bleomycin-induced fibrosis is not known; however, the data of Iyer *et al.* (12) does provide some insight. Bleomycin is known to bind to DNA chelating various divalent cations in turn generating reactive oxygen species (7–11). The free radicals produced in turn cause DNA-strand damage as well as lipid peroxidation. This DNA-strand damage and lipid peroxidation results in damage to lung connective tissue elements leading to lung inflammation that culminates in pulmonary fibrosis. Iyer *et al.* (12) suggest that the antifibrotic property of pirfenidone is related to its ability to ameliorate bleomycin-induced increases in superoxide dismutase and superoxide anion production. In turn, these reductions in superoxide dismutase and superoxide anion result in reduced reactive oxygen species and in turn less inflammation and fibrosis (12).

Fibrotic changes due to bleomycin treatment is well documented in both experimental animals (1, 6) and humans undergoing bleomycin therapy for cancer (1). Human subjects with idiopathic pulmonary fibrosis show decreases in lung compliance and *VC*, and small increases in RV that are highly variable (4). In patients with idiopathic pulmonary fibrosis, these alterations in pulmonary functions are associated with an increase in the work of breathing (6, 17). Similar changes in lung capacities and compliance following bleomycin treatment have been observed in rats (3), rabbits (18), and hamsters (19). We observed in our bleomycin-treated hamsters that C_{qst}/TLC, *TLC*, *VC*, and *IC* were significantly reduced and that these alterations in pulmonary functions were greatly attenuated by pirfenidone treatment. This would indicate that pirfenidone partially alleviated the respiratory stress in our bleomycin-treated hamsters by improving their respiratory reserves and thereby reducing their work of breathing.

Despite the significant improvement seen in pulmonary functions and hydroxyproline levels in the BPD group, breathing frequency remained elevated in this group when compared with the SPD and BCD groups (Table II). The mechanism leading to this persistent tachypnea cannot be determined from the data collected in this study. We would expect that the bleomycin-induced tachypnea would subside as the bleomycin-induced pulmonary functions improved with pirfenidone treatment. Iyer *et al.* (12) observed that following pirfenidone treatment lung inflammation persisted while bleomycin-induced elevations in hydroxyproline were ameliorated, suggesting that lung inflammation may provide a persistent stimulus for tachypnea in this model of lung fibrosis. This contention is supported by the observations that tachypnea has been observed in anesthetized rabbits intratracheally instilled with carrageenin (20) and in rats instilled with paraquat (21).

Both the pulmonary function and the hydroxyproline data obtained in this study provide further evidence for the antifibrotic effect of pirfenidone in the hamster model of bleomycin-induced pulmonary fibrosis. These data combined with the morphologic and biochemical data of Iyer *et al.* (12) establish pirfenidone as a potentially promising novel compound that may have therapeutic application in the treatment of life-threatening pulmonary fibrosis, for which there is currently no known drug for prevention or resolution. Despite the limitation that intratracheal administration of bleomycin does not duplicate the route of administration in human patients and as a result may not directly reflect the human clinical situation, further experiments are warranted for the evaluation of the antifibrotic effects of pirfenidone. Of greatest need are studies that evaluate the effect of pirfenidone on the antineoplastic potential of bleomycin in an appropriate animal model. In addition, studies are needed that evaluate the antifibrotic effects of pirfenidone at various stages of development of bleomycin-induced pulmonary fibrosis and that evaluate the most effective dose of pirfenidone administered by the oral route.

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