Pharmacological In Vivo Inhibition of S-Nitrosoglutathione Reductase Attenuates Bleomycin-Induced Inflammation and Fibrosis

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ABSTRACT

Interstitial lung disease (ILD) characterized by pulmonary fibrosis and inflammation poses a substantial biomedical challenge due to often negative disease outcomes combined with the need to develop better, more effective therapies. We assessed the in vivo effect of administration of a pharmacological inhibitor of S-nitrosoglutathione reductase, SPL-334 (4-[[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl](methyl)benzoic acid), in a mouse model of ILD induced by intratracheal instillation of bleomycin (BLM). Daily i.p. administration of SPL-334 alone at 0.3, 1.0, or 3.0 mg/kg had no effect on animal body weight, appearance, behavior, total and differential bronchoalveolar lavage (BAL) cell counts, or collagen accumulation in the lungs, showing no toxicity of our investigational compound. Similar administration of SPL-334 for 7 days before and for an additional 14 days after BLM instillation resulted in a preventive protective effect on the BLM challenge–induced decline in total body weight and changes in total and differential BAL cellularity. In the therapeutic treatment regimen, SPL-334 was administered at days 7–21 after BLM challenge. Such treatment attenuated the BLM challenge–induced decrease in total body weight, changes in total and differential BAL cellularity, and magnitudes of histologic changes and collagen accumulation in the lungs. These changes were accompanied by an attenuation of BLM-induced elevations in pulmonary levels of profibrotic cytokines interleukin-6, monocyte chemoattractant protein-1, and transforming growth factor-β (TGF-β). Experiments in cell cultures of primary normal human lung fibroblast have demonstrated attenuation of TGF-β–induced upregulation in collagen by SPL-334. It was concluded that SPL-334 is a potential therapeutic agent for ILD.

Introduction

Interstitial lung disease (ILD), a combination of pulmonary fibrosis and inflammation, is a serious complication of systemic and pulmonary diseases. ILD can also develop for unknown reasons as in idiopathic pulmonary fibrosis (IPF), a disease in which patients survive, on average, 2 to 3 years after being diagnosed (Raghu et al., 2011). In patients with systemic sclerosis, scleroderma lung disease is the leading cause of death (Steen and Medsger, 2007). ILD also contributes substantially to morbidity and mortality in other connective tissue diseases, such as rheumatoid arthritis (Marigliano et al., 2013) and idiopathic inflammatory myopathy (Hallowell et al., 2014); despite recent advances in developing new therapies (King et al., 2014; Richeldi et al., 2014), finding a cure and improving existing therapies for ILD is still challenging. Part of the reason for this challenge is that ILD is driven by numerous mechanisms including, but not limited to, disturbances in pulmonary epithelium, oxidative stress, exaggerated activation of clotting pathways, and immune inflammation (Todd et al., 2012). Due to the complexity of the disease and redundancy in underlying mechanisms, a rational drug design approach to new therapeutics has been challenging; the numerous pathways indicated previously have been explored via a trial and error approach. In this study, we considered the possibility that a prospective therapeutic developed by us, SPL-334 (4-[[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl](methyl)benzoic acid; SAJE Pharma, Baltimore, MD), is an effective agent for the treatment of ILD.

SPL-334 targets the nitric oxide signaling system and its modulation of inflammation, induction of antioxidant systems, vascular function, and pulmonary obstruction, by inhibiting the enzyme S-nitrosoglutathione (GSNO) reductase (GSNOR), also known as alcohol dehydrogenase 5, chi polypeptide and glutathione-dependent formaldehyde dehydrogenase. This enzyme is a member of the alcohol dehydrogenase family and is involved in the detoxification of nitric oxide. SPL-334 is a potent inhibitor of GSNOR and has been shown to attenuate the effects of bleomycin-induced lung injury in animal models. This study investigates the in vivo effects of SPL-334 on interstitial lung disease and inflammation, providing evidence for its potential use as a therapeutic agent for ILD.

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ABBREVIATIONS: ANOVA, analysis of variance; BAL, bronchoalveolar lavage; BLM, bleomycin; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosogluthione reductase; IFN-γ, interferon-γ; IL, interleukin; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; i.t., intratracheal; KC, keratinocyte chemotactant; MCP-1, monocyte chemotactant protein-1; MIP-1α, macrophage inflammatory protein-1α; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; SPL-334, 4-[[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl](methyl)benzoic acid; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.
family and is widely expressed in lung and other tissues (Foster et al., 2009). It has been shown to regulate the level of available endogenous S-nitrosothiols, the bioactive form of nitric oxide, through GSNO catabolism. GSNO is present in high levels in lung lining fluid and has been shown to exert bronchodilatory activity with a 100-fold higher potency than theophylline (Gaston et al., 1993, 1994). By inhibiting GSNOR, SPL-334 inhibits the catabolism of GSNO, which increases the cellular pool of GSNO and the level of nitroso groups on critical proteins, leading to smooth muscle relaxation, decreased inflammation via inhibition of the nuclear factor-κB (NF-κB) pathway, activation of antioxidant pathways via activation of Nrf-2, and increased mucin clearance (Nozik-Grayck et al., 2002, 2006; Que et al., 2005; Sanghani et al., 2009; Wu et al., 2010; Kelleher et al., 2011; Foster et al., 2012; Ferrini et al., 2013).

Inhibition of GSNOR activates Nrf-2, which is required for a spectrum of Nrf-2–dependent GSNOR inhibition–induced effects (Foster et al., 2012). Such inhibition may be protective against pulmonary fibrosis because Nrf-2 is by itself protective through several regulatory pathways (Cho et al., 2004, 2006; Sriram et al., 2009; Cho and Kleeberger, 2010; Kikuchi et al., 2010; Liu et al., 2013; Pekovic-Vaughan et al., 2014). This notion is consistent with the known decreased nuclear Nrf-2 expression in patients with IPF (Artaud-Macari et al., 2013). Similarly, GSNOR inhibition–driven suppression of the NF-κB pathway is predicted to be protective against pulmonary fibrosis (Zhang et al., 2000; Krug et al., 2010; Tully et al., 2013; Zhou et al., 2014).

Together, these considerations have suggested that SPL-334 may provide a multifactorial approach to the treatment of ILD as an inducer of antioxidant enzymes to limit oxidant damage (Chen and Kunsch, 2004), as an anti-inflammatory agent (Sanghani et al., 2009; Ferrini et al., 2013), and as a bronchodilator (Ferrini et al., 2013). To assess this possibility of a protective and curative effect of SPL-334 on ILD, we have tested preclinically the preventive and therapeutic effects of SPL-334 in vivo in the bleomycin (BLM) lung injury mouse model. Additionally, effects of SPL-334 on collagen production by cultured primary human pulmonary fibroblasts were evaluated.

Materials and Methods

Animals and Treatments. This study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland. Experiments were performed in 8–12 week old C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME). Animals were maintained in sterile microisolator cages with sterile rodent feed and water. Daily maintenance of mice was performed at the University of Maryland Animal Facility, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care, to model pulmonary inflammation and fibrosis, a single dose of 0.075 U of BLM (Sigma-Aldrich, St. Louis, MO) diluted in 50 μl of sterile phosphate-buffered saline (PBS) was delivered to mouse lungs intra-tracheally (i.t.) on day 0 as previously described (Luzina et al., 2006b, 2009, 2011a,b, 2012; Pochetuhen et al., 2007). Briefly, a minor anterior midline neck incision was made to make the trachea visible, a MicroSprayer (Penn-Century, Wyndmoor, Philadelphia) was inserted i.t. through the mouth, and the BLM solution was instilled. Control mice were instilled with 50 μl of sterile PBS with no additives. SPL-334 was administered i.p. in 50 μl PBS at doses of 0.3, 1.0, and 3.0 mg/kg daily. These doses were chosen based on the results of the previous studies in a mouse ovalbumin model (Ferrini et al., 2013) as well as our unpublished observations in a house dust mite model of asthma. In those studies, these doses were found to be effective yet safe. Preventive and therapeutic administration of SPL-334 was tested. In the preventive treatment experiments, mice received SPL-334 starting at day −7 through day +13 relative to day 0, on which day animals were challenged with BLM. The effects of preventive treatment were assessed on days 7 and 14. In the therapeutic treatment experiments, SPL-334 was administered on days 7–20, and the effects of the treatment were assessed on days 14 and 21. As a treatment control, pirfenidone (Tocris, Bristol, UK) diluted in 0.5% carboxymethyl cellulose in PBS was administered daily by oral gavage at 100 mg/kg of body weight. The timing of pirfenidone administration was similar to that of SPL-334 injections in the therapeutic treatment regimen.

Bronchoalveolar Lavage Analyses. The preparation and analyses of bronchoalveolar lavage (BAL) samples, including flow cytometry, were performed as previously described (Atamas et al., 1999, 2002, 2003; Luzina et al., 2002, 2003, 2006a,b, 2009, 2012; Pochetuhen et al., 2007; Mozaffarian et al., 2008). Briefly, the animals were euthanized, and lung lavage was performed immediately postmortem through an 18-gauge blunt-end needle secured in the trachea. Two instillations and withdrawals of 1 ml of PBS each time were performed in each animal. The 2 aliquots of BAL fluid were pooled, and the cells were separated by centrifugation. The BAL cells were stained with a Protocol Hema 3 staining set (Fisher, Kalamazoo, MI), and differential cell counts were performed. Flow cytometry of BAL cells stained for cell surface expression of CD3, CD4, CD8, and CD19 was performed by an investigator blinded to the histologic specimen grouping, using a 0–4 point scoring system, with a score of 0 denoting normal lung structure and 4 denoting maximal thickening of alveolar walls, accumulation of inflammatory cells, and deposition of collagen such that alveolar spaces are not seen. In each group, five mice were thus analyzed, with 10 randomly selected fields scored in each of the two lungs. Scores were presented as median (first quartile, third quartile) and groups were compared using Mann-Whitney test.

Assessment of Pulmonary Cytokine Levels. The levels of cytokines interleukin (IL)-1β, IL-4, IL-6, keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were measured in mouse lung homogenates utilizing multiplex assays (Luminex, Austin, TX). The levels of total transforming growth factor-β (TGF-β) were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

Assessment of Total Lung Collagen. Collagen content was measured in lung tissues based on detection of hydroxyproline, using the Total Collagen Assay kit from QuickZyme (Leiden, The Netherlands). Briefly, following hydrolysis of 50 mg lung tissue in 500 ml of 6M HCl for 20 hours at 95°C, the hydrolysate was diluted 10-fold with 4M HCl and measured against serial dilutions of collagen standard; the results were expressed as μg collagen per mg wet lung tissue.

Primary Human Pulmonary Fibroblast Cultures. Deidentified primary human fibroblasts from adult healthy donors and patients with IPF were derived, maintained, and tested as previously described (Luzina et al., 2006a, 2009, 2013). Cell viability/proliferation was assessed using CellTiter Aqueous (Promega,
SPL-334 Prevents BLM-Induced Changes. Daily i.p. administration of 0.3, 1.0, and 3.0 mg/kg of SPL-334, each dose for 7 and 14 days, caused no changes in animal body weight, appearance, behavior, or total and differential BAL cell counts in comparison with PBS-treated controls (Figs. 1A and 2A). To assess whether SPL-334 is protective in the preventive mode in the BLM model of pulmonary injury, mice were administered SPL-334 daily at 1.0 mg/kg i.p. for 7 days prior to instillation of BLM. Mice were then challenged with i.t. BLM at 0.075 U/mouse and started receiving i.p. SPL-334 at either 0.3, 1.0, or 3.0 mg/kg daily (Fig. 2A). As a positive control, pirfenidone was administered at 100 mg/kg by oral gavage daily.

Two-way ANOVA tests revealed significant effects of both time ($P < 0.05$) and treatment ($P < 0.01$), as well as a significant interaction between these effects ($P < 0.01$). One-way ANOVA tests for the effect of treatment on body weight were performed for each time point, revealing significant effects on days 10 and 14 ($P < 0.01$ in both cases) (Fig. 2B), and less significant effects on days 17 and 21 ($P < 0.05$ in both cases). Pairwise comparisons did not find differences between treatments with the tested doses of SPL-334 or pirfenidone; however, body weights of mice treated with these compounds were significantly higher than in the PBS-treated group at days 10, 14, 17, and 21 (probabilities varying from $P < 0.01$ to $P < 0.05$) (Fig. 2B).

Results

Daily i.p. administration of 0.3, 1.0, and 3.0 mg/kg of SPL-334, each dose for 7 and 14 days, caused no changes in animal body weight, appearance, behavior, or total and differential BAL cell counts in comparison with PBS-treated controls (Figs. 1A and 2A). To assess whether SPL-334 is protective in the preventive mode in the BLM model of pulmonary injury, mice were administered SPL-334 daily at 1.0 mg/kg i.p. for 7 days prior to instillation of BLM. Mice were then challenged with i.t. BLM at 0.075 U/mouse and received SPL-334 daily for an additional 14 days (Fig. 1A). Tests were performed on days 7 and 14 after BLM instillation, whereas body weight was measured more frequently (Fig. 1B).

Two-way ANOVA tests revealed significant effects of both time and treatment with SPL-334 on body weight ($P < 0.05$ and $P < 0.01$, respectively), as well as a significant interaction between these effects ($P < 0.01$). One-way ANOVA tests for the effect of treatment on body weight were performed for each time point, revealing significant effects on days 5, 7, 10, and 14 ($P < 0.01$ in each case) (Fig. 1B). Pairwise comparisons revealed similar ($P > 0.05$) body weight loss in response to BLM challenge in mice receiving PBS placebo and not receiving a treatment, whereas treatment with SPL-334 prevented body weight loss on days 5, 7, 10, and 14 ($P < 0.01$ in each case) (Fig. 1B). By day 7 after the BLM challenge, mice treated with SPL-334 but not PBS returned to their natural body weight, as was similarly observed in unmanipulated mice, and continued gaining weight (Fig. 1B) according to their age (8–12 weeks).

BAL cell counts showed that total pulmonary cellularity increased more than 2-fold in response to BLM challenge due to increases in all cell types, with a particularly notable contribution to the increase from lymphocytes (Fig. 1, C and D). There was no effect of preventive treatment with PBS, whereas similar treatment with SPL-334 resulted in a significantly lower accumulation of total BAL cells, particularly lymphocytes, especially on day 14 (Fig. 1D).

These findings suggested that preventive use of SPL-334 alleviates the severity of BLM injury. It was then considered that human patients with ILD, such as IPF, are usually diagnosed later in the disease process, when pulmonary changes have already occurred. To assess whether SPL-334 may have a therapeutic effect in the BLM model, the drug was administered to mice with already established pulmonary injury.

Therapeutic Effect of SPL-334 in the BLM Injury Model. Mice were challenged with i.t. BLM at 0.075 U/mouse on day 0, and were not treated until day 7, at which time they started receiving i.p. SPL-334 at either 0.3, 1.0, or 3.0 mg/kg daily (Fig. 2A). As a positive control, pirfenidone was administered at 100 mg/kg by oral gavage daily.

Two-way ANOVA tests revealed significant effects of both time ($P < 0.05$) and treatment ($P < 0.01$), as well as a significant interaction between these effects ($P < 0.01$). One-way ANOVA tests for the effect of treatment on body weight were performed for each time point, revealing significant effects on days 10 and 14 ($P < 0.01$ in both cases) (Fig. 2B), and less significant effects on days 17 and 21 ($P < 0.05$ in both cases). Pairwise comparisons did not find differences between treatments with the tested doses of SPL-334 or pirfenidone; however, body weights of mice treated with these compounds were significantly higher than in the PBS-treated group at days 10, 14, 17, and 21 (probabilities varying from $P < 0.01$ to $P < 0.05$) (Fig. 2B).

BAL cell counts showed that treatments with SPL-334 had an attenuating effect on total pulmonary cellularity, whereas treatment with PBS had no effect (Fig. 2, C and D). The attenuating effect on lymphocyte counts was particularly pronounced (Fig. 2, C and D). Of note, the highest tested dose (3.0 mg/kg per day) of SPL-334 caused an increase in pulmonary macrophages, contributing to an increase in total cellularity, while the attenuating effect on lymphocytes was still present. It remains unclear why this dose of SPL-334 had no such effect when administered to mice alone, whereas the effect was present in mice challenged with BLM.

Considering the previously suggested important roles of T lymphocytes (Luzina et al., 2008) and B lymphocytes (Komura et al., 2008; Yoshizaki et al., 2008) in the regulation of pulmonary inflammation and fibrosis (Luzina et al., 2008) in the BLM model, flow cytometry analyses assessed the lymphocyte population in the BAL samples, which were gated based on forward and side light scattering as previously described (Luzina et al., 2009). Cells stained for CD3 (T cells), CD4 and CD8 (helper and cytotoxic T cells, respectively), and CD19 (B cells) were evaluated. BAL samples were thus assessed in all treatment groups (preventive and therapeutic with all indicated SPL-334 doses), in 3–5 mice per group, at the time points indicated as tests in Figs. 1A and 2A. Consistent with previous reports (Thrall and Barton, 1984; Janick-Buckner et al., 1989), the majority of lymphocytes following BLM challenge were T cells, with CD4$^+$ T lymphocytes exceeding CD8$^+$ T lymphocytes by 1.5 to 2.5-fold. Statistical analyses revealed that despite the overall decrease in total lymphocytes in response to treatment with SPL-334 (Figs. 1, C and D, and 2, C and D), the relative contributions of T and B cells, or CD4$^+$ and CD8$^+$ cells within the CD3$^+$ populations, did not change in response to treatments ($P > 0.05$ in all cases). A conclusion was made that while decreasing the degree of the immune inflammation, SPL-334 does not change the nature of immune involvement in pulmonary pathology in this model.

In light of the central involvement of cytokines in the mechanisms of pulmonary inflammation and fibrosis (Luzina et al., 2015), levels of IL-1β, IL-4, IL-6, IFN-γ, and TNF-α were measured in mouse lung homogenates utilizing multiplex assays at days 14 and 21 in the therapeutic treatment regimen, whereas the levels of total TGF-β were measured by enzyme-linked immunosorbent assay. The levels of the majority of these cytokines were significantly elevated in the lung homogenates of BLM-challenged animals compared with PBS-challenged controls, and SPL-334 further significantly affected pulmonary levels of cytokines (Fig. 3).
The elevations of IL-6, MCP-1, and TGF-β, which are well known for their profibrotic activities (Luzina et al., 2015), were significantly attenuated by SPL-334, whereas the levels of protective cytokines IFN-γ and TNF-α (Redente et al., 2014; Luzina et al., 2015) were significantly increased or tended to be elevated, respectively, by SPL-334 (Fig. 3). The levels of IL-4 were too low to be reliably measured by multiplex assay and the levels of IL-1β, KC, and MIP-1α in the lungs of BLM-challenged mice were not significantly different between the therapeutic and placebo groups. Based on these data, it was concluded that SPL-334 effectively attenuates BLM-induced injury when used in either preventive or therapeutic modes.

**SPL-334 Attenuates BLM-Induced Histologic Changes and Accumulation of Collagen.** Histologic evaluation of the lungs revealed that BLM caused substantial accumulation of inflammatory infiltrates and collagen; however, therapeutic treatment with SPL-334 significantly attenuated this effect (Fig. 4). Considering the biomedical and socioeconomic importance of pulmonary fibrosis, more detailed assessment of pulmonary collagen levels based on hydroxyproline measurements in the lung tissue was performed (Fig. 5). In the preventive mode, there was no significant attenuation (P > 0.05) of BLM-induced moderate collagen accumulation by SPL-334 on day 7 (Fig. 5A).
However, SPL-334 attenuated BLM-induced collagen accumulation substantially and significantly ($P < 0.05$) on day 14 (Fig. 5A). In the therapeutic regimen (Fig. 5B), SPL-334 attenuated BLM-induced collagen accumulation at 1.0 and 3.0 mg/kg per day doses ($P < 0.05$), although there was only a tendency toward decrease, in which the threshold of statistical significance ($P > 0.05$) was not reached, at 0.3 mg/kg per day of SPL-334. Thus, SPL-334 alleviates not only inflammation but also fibrosis in the BLM injury model. Further tests evaluated the relevance of these observations to human health.

**SPL-334 Decreases TGF-β−Induced Collagen Production in Cultured Human Primary Fibroblasts.** To begin addressing the potential relevance of these findings to human health, experiments were performed with cultured primary human fibroblasts derived from adult healthy controls or patients with IPF. Western blotting assays assessed the basal levels of GSNOR in cultured fibroblast cell lysates. The antibody reacted with two adjacent bands in the vicinity of expected molecular weight of 40 kDa (Fig. 6A). The results showed that in this small set of primary fibroblasts, the cells from patients with IPF express higher levels of GSNOR compared with similar cells from healthy controls (Fig. 6A). Further experiments were performed, in which normal primary pulmonary fibroblasts from four separate donors (a representative example is shown in Fig. 6B) and primary pulmonary fibroblasts from two patients with IPF (an example is shown in Fig. 6C) were treated with 5 ng/ml of recombinant human TGF-β in the presence or absence of SPL-334 in the concentration of 1 μg/ml (corresponding to the in vivo dose of...
1 mg/kg). In all cases, stimulation with TGF-β led to a substantial increase in collagen levels in cell culture (Fig. 6, B and C). In three out of four of the tested primary normal cell cultures, as well as in both IPF-derived cultures, the presence of SPL-334 had an inhibitory effect on TGF-β--stimulated upregulation of collagen (examples are shown in Fig. 6, B and C), whereas SPL-334 alone had no toxic effect on cells based on microscopic appearance and cell viability assays. Parallel experiments utilizing cell proliferation/viability assays revealed that no changes to cell numbers occurred in these
cultures within the experimental time frame of 48 hours. These results in primary human cell culture form the basis for future mechanistic work in human cell culture as well as in the transition of SPL-334 to clinical trials in humans.

**Discussion**

The results of the experiments suggest that in vivo administration of SPL-334 has both a preventive (Fig. 1) and therapeutic (Fig. 2) effect in the BLM injury model of ILD, based on attenuation of the BLM challenge-induced decline in total body weight as well as changes in BAL cellularity. BLM challenge induced an increase in pulmonary lymphocytes (Figs. 1, C and D, and 2, C and D), an observation that is consistent with previous reports (Pochetuhen et al., 2007; Luzina et al., 2008, 2013) and models similar changes in human patients with ILD (Atamas et al., 1999; Luzina et al., 2003, 2008, 2009; Todd et al., 2013; Kropski et al., 2015) that are relevant to the pathophysiological mechanisms of the disease (Luzina et al., 2008). Administration of SPL-334 significantly attenuated such an increase, likely contributing mechanistically to the compound’s overall protective and therapeutic effects in this model. Potentially further contributing to the compound’s mechanism of action was the attenuating effect on BLM-induced increase in profibrotic and proinflammatory cytokines IL-6, MCP-1, and TGF-β; the stimulating effect on an antifibrotic cytokine IFN-γ; and the tendency, although not reaching statistical significance, to elevate a protective cytokine TNF-α (Fig. 3). The importance of the latter finding is in the central contribution of these cytokines to the mechanisms of ILD through their direct effect on fibroblasts and indirect effect through regulation of inflammation (Redente et al., 2014; Luzina et al., 2015).

SPL-334 has substantially attenuated BLM-induced histologic changes, including inflammatory infiltration and collagen accumulation, as assessed by H&E-based and trichrome-based staining of lung tissue (Fig. 4). Quantitative biochemical assessment of pulmonary collagen based on hydroxyproline measurements revealed that SPL-334 had both protective (Fig. 5A) and therapeutic (Fig. 5B) effects on BLM-induced increases in lung collagen. In light of the findings shown in Figs. 1–3, it is plausible that the attenuating effect of SPL-334 administration on collagen accumulation may be mediated through regulation of lymphocytic infiltration and the levels of proinflammatory/profibrotic cytokines.

It has been established that GSNOR contributes to asthma (Que et al., 2009), but it is currently unknown whether GSNOR is involved in the pathogenesis of ILD. Genomic profiling of lung tissues from patients with IPF in comparison with normal lung tissue samples [NCBI GEO accession GSE2052: IPF versus Control (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2052)] (Meltzer et al., 2011) showed no significant differences in the expression levels of GSNOR mRNA (data accessible at NCBI GEO database, GEO profiles 11087657 and 83315764; http://www.ncbi.nlm.nih.gov/geo/). However, these observations do not exclude the possibility that GSNOR may be increased in a particular cell type in patients with ILD, which is relevant to the disease process, with the increase masked by the lack of changes in GSNOR expression in other cells within the lung tissue samples. With relevance to this possibility, small sets of primary pulmonary fibroblasts from three patients with scleroderma lung disease and three patients with IPF were compared with similar cells from three healthy controls for global gene expression (Renzoni et al., 2004), with GSNOR mRNA among the targets tested (data accessible at NCBI GEO database, GEO profile 907518; http://www.ncbi.nlm.nih.gov/geo/). The comparison of the mRNA expression levels in the combined ILD group (scleroderma and IPF) versus healthy controls in that study revealed a significant 1.87-fold increase (P < 0.005) in fibroblasts from patients with ILD. Our findings in a similarly small set of primary fibroblast cultures (Fig. 6 and related text), combined with the findings in Renzoni et al. (2004), suggest that an increase in GSNOR expression in fibroblasts from patients with ILD occurs at the level of GSNOR mRNA and protein, and that GSNOR inhibition with SPL-334 attenuates the TGF-β-stimulated increase in collagen levels. In support of the therapeutic potential of SPL-334, its attenuating effect on the TGF-β-driven upregulation in collagen was observed in both

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![Graph](image-url)
normal lung fibroblasts and cells derived from the lungs of patients with IPF (Fig. 6, B and C). While somewhat differing phenotypically from pulmonary fibroblast in healthy individuals (Ramos et al., 2001), such cells in the lungs of patients with IPF remain similarly responsive to profibrotic stimulation, propelling the fibrotic process in the lungs of patients (Murray et al., 2008; Pechkovsky et al., 2012; Oruqaj et al., 2015). That SPL-334 effectively neutralizes the effect of TGF-β in IPF fibroblasts reinforces the notion that it can be therapeutically used in IPF and, perhaps, other ILDs.

The amplitude of the in vivo changes induced by SPL-334 was comparable to that induced by pirfenidone (Figs. 2 and 5), a compound that has been recently approved by the U.S. Food and Drug Administration (Silver Spring, MD) for treating IPF (King et al., 2014). One advantage of using pirfenidone is that it is orally available, and we are in the process of developing orally available analogs of SPL-334. The advantage of SPL-334 is that its mechanism of action is better understood because this compound was developed through rational design as an inhibitor of GSNOR. In addition to the ability of SPL-334 to reduce inflammation and relax smooth muscle, particular interest may be with its activation of the Nrf-2 antioxidant pathway, a major regulator of cytoprotective responses to oxidative stress (Chalupowicz and Sanchez Lopez-Boado, 2013). Reports in the literature have shown that Nrf-2 is important in IPF. In patients with IPF there is an increase in oxidative damage and an increase in exhaled nitric oxide levels (Saleh et al., 1997; Paredi et al., 1999; Pullamsetti et al., 2011). Fibroblasts from IPF patients exhibit decreased nuclear Nrf-2 expression, and IPF patients have shown diminished antioxidative capacity by having decreased expression of Nrf-2 target genes and decreased levels of glutathione (Beeh et al., 2002; Peltoniemi et al., 2004; Vuorinen et al., 2008; Ye et al., 2008; Artaud-Macari et al., 2013). In addition, preclinical models show that mice with a genetic deletion of Nrf-2 have decreased survival and increased inflammation following BLM-induced damage (Kikuchi et al., 2010). These data support the hypothesis that Nrf-2 may be important in IPF and that the activation of Nrf-2 by SPL-334, along with its anti-inflammatory and smooth muscle relaxation properties, may have an impact on reducing the effects of the disease.

Experimental validation of these mechanistic considerations goes beyond the translational scope of this paper. Future work will address exact molecular mechanisms mediating the protective effect of SPL-334 and, perhaps, will provide an explanation for the unexpected opposing effects on the BAL macrophage count seen with 3.0 mg/kg of SPL-334 compared with 1.0 mg/kg (Fig. 2). Deficiency of Nrf-2 does not, by itself, affect accumulation of macrophages in the lungs in response to BLM challenge (Cho et al., 2004), suggesting that other effects of GSNOR inhibition should be considered. For example, there is an unexplored possibility of a complex SPL-334 dose-dependent interplay between the significant decrease in the levels of MCP-1 and the significant increase in the levels of IFN-γ (Fig. 3), which are known to decrease (Saito et al., 2011) and increase (Kopach et al., 2014) accumulation of pulmonary macrophages, respectively. Such a mechanism would be further complicated by a difference between bone marrow–derived and resident pulmonary fibroblasts, which do and do not, respectively, depend on the MCP-1 receptor CCR2 (Opailek et al., 2007; Guiliams et al., 2013; Hashimoto et al., 2013). Moreover, fibroblasts (Murray et al., 2008) and fibrocytes (Moore et al., 2005) contribute to fibrosis directly in an MCP-1– and CCR2-dependent fashion. Further research is needed to clarify the contributions of these mechanisms to the effect of SPL-334.

In conclusion, SPL-334 is protective in a model of ILD and in primary human fibroblast culture through multiple pathways,
including the regulation of lymphocyte accumulation, levels of proinflamatory and proinflammatory cytokines, and the direct effect on collagen production. These findings form the basis for further work, which will address the development of orally available SPL-334 analogs and clinical trials in human patients with ILD. The underlying mechanisms for the aforementioned effects demonstrate that SPL-334 regulates multiple pathways and suggests that GSNOR inhibitors may be active in many other diseases, particularly those in which inflammation, oxidant damage, and the remodeling of fibrosis play a pathophysiological role.

**Authorship Contributions**

**Conducted experiments:** Luzina, Lockatell, Todd, Kopach.

**Performed data analysis:** Luzina, Pektilis, Atamas.

**Wrote or contributed to the writing of the manuscript:** Atamas, Luzina, Pentikis.

**References**


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