

ORIGINAL ARTICLE

Proteasomal inhibition after injury prevents fibrosis by modulating TGF- β_1 signalling

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ABSTRACT

Background The development of organ fibrosis after injury requires activation of transforming growth factor β_1 which regulates the transcription of profibrotic genes. The systemic administration of a proteasomal inhibitor has been reported to prevent the development of fibrosis in the liver, kidney and bone marrow. It is hypothesised that proteasomal inhibition would prevent lung and skin fibrosis after injury by inhibiting TGF- β_1 -mediated transcription.

Methods Bortezomib, a small molecule proteasome inhibitor in widespread clinical use, was administered to mice beginning 7 days after the intratracheal or intradermal administration of bleomycin and lung and skin fibrosis was measured after 21 or 40 days, respectively. To examine the mechanism of this protection, bortezomib was administered to primary normal lung fibroblasts and primary lung and skin fibroblasts obtained from patients with idiopathic pulmonary fibrosis and scleroderma, respectively.

Results Bortezomib promoted normal repair and prevented lung and skin fibrosis when administered beginning 7 days after the initiation of bleomycin. In primary human lung fibroblasts from normal individuals and patients with idiopathic pulmonary fibrosis and in skin fibroblasts from a patient with scleroderma, bortezomib inhibited TGF- β_1 -mediated target gene expression by inhibiting transcription induced by activated Smads. An increase in the abundance and activity of the nuclear hormone receptor PPAR γ , a repressor of Smad-mediated transcription, contributed to this response.

Conclusions Proteasomal inhibition prevents lung and skin fibrosis after injury in part by increasing the abundance and activity of PPAR γ . Proteasomal inhibition may offer a novel therapeutic alternative in patients with dysregulated tissue repair and fibrosis.

INTRODUCTION

After injury, the recovery of normal tissue function can be prevented or delayed by the development of fibrosis.¹ In animal models, activation of the cytokine transforming growth factor β_1 (TGF- β_1) is required and sufficient for the development of fibrosis in the lung and other organs.² In the lung, the intratracheal administration of bleomycin results in acute lung injury that peaks 3–5 days after the administration of bleomycin and is followed by TGF- β_1 -dependent lung fibrosis.² Active TGF- β_1 binds to specific membrane receptors inducing signalling cascades that

Key messages

What is the key question?

- Can the systemic administration of the proteasomal inhibitor bortezomib prevent the development of lung fibrosis after injury is established?

What is the bottom line?

- Bortezomib protects against the development of fibrosis in the lung and the skin by inhibiting transforming growth factor β_1 -mediated transcription.

Why read on?

- Bortezomib, a medication in widespread clinical use, may offer a therapeutic alternative for patients with lung fibrosis.

transcriptionally regulate myofibroblast differentiation, collagen expression and endothelial/epithelial to mesenchymal cell transition.³ The transcriptional programme activated by TGF- β_1 is therefore an attractive therapeutic target for the prevention of organ fibrosis after injury.²

In addition to its role in protein turnover, the ubiquitin/proteasome system plays a critical role in the modulation of critical cellular signalling pathways.⁴ Bortezomib is a proteasomal inhibitor approved for clinical use in the treatment of multiple myeloma and mantle cell lymphoma.^{5,6} In animal models, bortezomib and other proteasome inhibitors can prevent fibrosis in the liver, kidney, bone marrow and heart, suggesting that it acts to inhibit a common pathway involved in organ fibrosis.^{7–10} In this study we sought to determine whether the administration of bortezomib could prevent lung and skin fibrosis induced by bleomycin by inhibiting TGF- β_1 -mediated transcription.

METHODS

Animals and administration of bleomycin and bortezomib

The protocols for the use of animals were approved by the Northwestern University Animal Care and Use Committee. The protocol for bleomycin lung injury/fibrosis has been described elsewhere (see online supplement for details).¹¹ After conducting pilot experiments using different dosing schedules

of bortezomib (details in the online supplement), wild-type C57BL/6 mice were treated with intratracheal bleomycin (0.075 IU/mouse) followed 7 and 14 days later by bortezomib (120 µg/kg intraperitoneally) or saline and lung fibrosis was measured on day 21. For the skin fibrosis experiments, 6–8-week-old female BALB/c mice were treated with filter-sterilised bleomycin (20 µg/mouse, Mayne Pharma, Paramus, New Jersey, USA) or saline subcutaneously daily (27 gauge needle) into a shaved area of skin on the back of the animal. Bortezomib (400 µg/kg intraperitoneally) or vehicle was begun 7 days after the first dose of bleomycin and administered twice weekly until the animals were killed on day 40.

Cells and reagents

Normal human lung fibroblasts were obtained from Lonza (Basel, Switzerland). The cells were grown to 70% confluence for all conditions and were discarded after passage 5. Antibodies used include αSMA (R&D Systems, Minneapolis, Minnesota, USA), fluorescent anti-mouse antibody (Invitrogen, Carlsbad, California, USA), p-Smad3 (Cell Signaling, Boston, Massachusetts, USA), Smad1,2 3 (total Smad), CTGF and PPARγ (Santa Cruz, Santa Cruz, California, USA), collagen I (SouthernBiotech, Birmingham, Alabama, USA) and actin and tubulin (Sigma-Aldrich, St Louis, Missouri, USA). SBE-luciferase and PPRE-luciferase reporters have been previously described.^{12 13} Measurement of luciferase activity (Promega Dual-Luciferase

Reporter Assay System) was performed as previously described (see details in online supplement).¹⁴

Real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR was performed as previously described according to published guidelines and specific mRNA expression was normalised to that of the mitochondrial gene RPL19.¹⁵ Detailed protocols and primer sequences are given in the online supplement.

Immunoblotting and immunofluorescence

Immunoblotting and immunofluorescence were performed as previously described (see details in the online data supplement).¹⁶

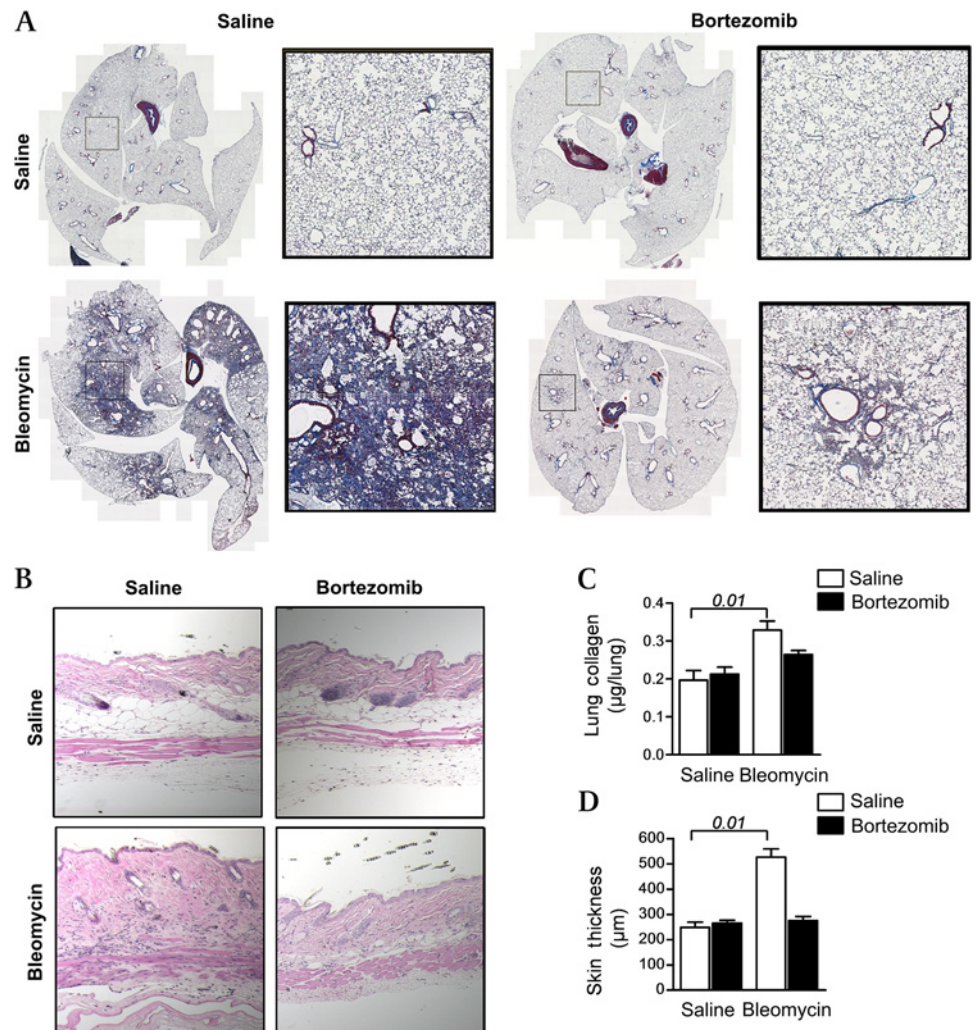
Measurement of active TGF-β₁

Active TGF-β₁ was measured from bronchoalveolar lavage (BAL) fluid in duplicate using the TGFβ₁ E_{max} ImmunoAssay System (ELISA) according to the manufacturer’s protocol (Promega, Madison, Wisconsin, USA). This assay only measures TGF-β₁ that has been cleaved and is biologically active.¹⁷

Histology and measurement of lung collagen

Lung and skin histology were performed as previously described.^{11 18} Lung collagen was measured using a modification of a previously described method.¹⁹ Details are provided in the online supplement.

Figure 1 Bortezomib abrogates pulmonary and skin fibrosis induced by bleomycin in mice. (A) Mice (C57BL/6, male) were treated with intratracheal bleomycin or saline (0.075 IU). Seven and 14 days later the mice were treated with bortezomib (120 µg/kg intraperitoneally) or saline. The mice were killed on day 21 and the lungs were examined by trichrome staining. (B) A separate group of mice (BALB/c) were treated with daily subcutaneous injections of bleomycin (20 µg) in the same region of the skin on their back and bortezomib (400 µg/kg intraperitoneally) twice weekly or saline was begun 7 days later. After 4 weeks, H&E-stained skin sections of the treated skin were obtained. The thickest regions of skin for each treatment are shown. (C) Total lung collagen was quantified by picosirius red collagen precipitation and (D) skin thickness was measured microscopically (n≥8 (lung) and n=5 (skin) for each treatment arm). p Values for comparisons between groups are indicated in italics above the bars.



Lentiviral PPAR γ shRNA

The pLKO.1 vector was used to express shRNA targeting PPAR γ 1 as described previously.¹⁹ The following sequence was used: 5'CCGGCTGGCCTCCTTGATGAATAAACTCGAGTTT-ATTCATCAAGGAGGCCAGTTTTT'3. The control shRNA was supplied by Sigma. Stable cell lines were generated by Virapower (Invitrogen) lentiviral infection using the 293FT packaging cell line and puromycin selection. Forty-eight hours after 293FT transfection, medium containing virus was supplemented with 8 μ g/ml polybrene (Sigma-Aldrich) for cell line infection and applied to normal human lung fibroblasts.

Clinical specimen collection

The collection of clinical data and specimens was approved by the Northwestern University Institutional Review Board. BAL fluid from 10 patients with a clinical and radiological diagnosis of lung fibrosis and eight control patients was used.¹⁴ The clinical details of the patients are given in the online supplement. Normal human lung fibroblasts were transiently transfected with the SBE-luciferase plasmid and incubated for 24 h with BAL fluid obtained from 10 patients with lung fibrosis or control patients without evidence of parenchymal lung disease in the presence or absence of bortezomib (200 nM).¹⁴ Samples of lung fibroblasts from fibrotic lungs and control lungs were cultured and stored as previously described.²⁰ Samples of skin fibroblasts were obtained by skin biopsy of an affected and unaffected area of the forearm of a patient with scleroderma as previously described.²¹

Statistical analysis

The data were analysed in Prism 4 (GraphPad Software Inc, La Jolla, California, USA). All data are shown as means with standard errors of the mean. Statistical significance was determined by ANOVA; when the ANOVA revealed a significant difference, individual differences were explored using t tests with the Bonferroni or Dunnett correction for multiple comparisons. p Values <0.05 were considered statistically significant.

RESULTS

Bortezomib inhibits bleomycin-induced fibrosis in lung and skin

Similar to previous reports, we observed that >70% of mice died when high- or low-dose bortezomib (400 or 120 μ g/kg intraperitoneally, respectively) was administered before or concomitant with bleomycin.²² We did not observe excess mortality in mice when bortezomib was given 7 days or more after bleomycin administration (a description of the dosing schedules employed is given in the online supplement). The lowest dose of bortezomib used (120 μ g/kg intraperitoneally) was sufficient to acutely inhibit the chymotrypsin-like activity of the proteasome in red blood cells and serum of control mice (see figure S1 in online supplement). Mice treated with bortezomib (120 μ g/kg intraperitoneally) 7 and 14 days after bleomycin administration were harvested for assessment of lung collagen assessed by histological examination of trichrome-stained lung sections, picrosirius red collagen precipitation (figures 1A and C) and immunoblotting using an antibody against type I collagen (see figure S2f in online supplement). Fibrosis was also attenuated in mice treated with the single dose of bortezomib on day 7 (see figure S3 in online supplement) and in a limited number of mice treated with bortezomib (120 μ g/kg intraperitoneally) 14 and 21 days after bleomycin administration and harvested for assessment of fibrosis at day 28 (see figure S4 in online supplement).

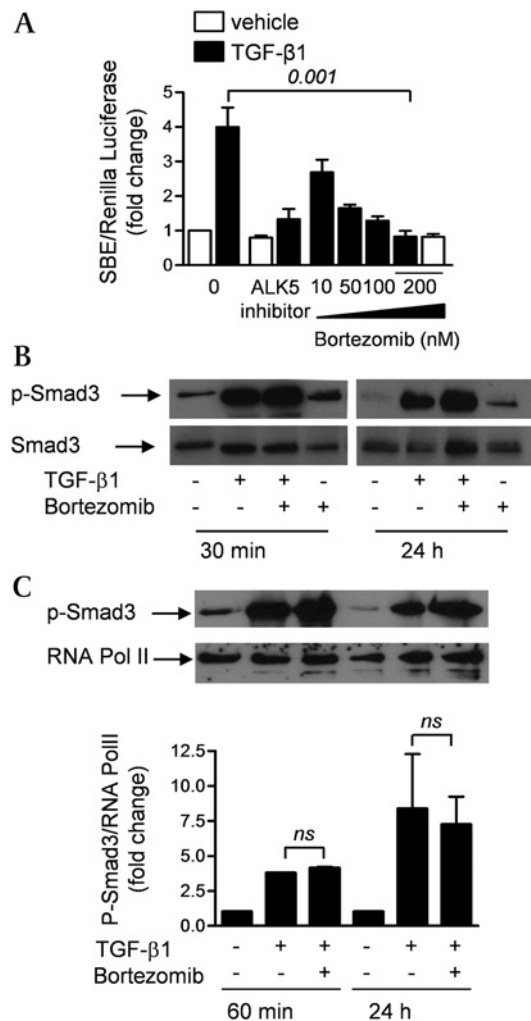


Figure 2 Bortezomib does not inhibit transforming growth factor β 1 (TGF- β 1)-induced Smad3 phosphorylation or nuclear translocation but inhibits Smad-mediated transcription. (A) Primary cultures of normal human lung fibroblasts were transfected with a plasmid containing SBE-luciferase and 24 h later treated with TGF- β 1 (5 ng/ml) with or without bortezomib. SBE-luciferase activity was measured 24 h later. Treatment with the ALK-5 inhibitor SB431542 (10 μ M) is shown as a control. *p*<0.0001 for difference in dose determined by one-way ANOVA; Bonferroni-corrected *p*<0.05 for comparisons between TGF- β 1 alone and all other conditions. (B, C) Primary cultures of normal human lung fibroblasts were grown to 70% confluence and incubated with TGF- β 1 (5 ng/ml) with or without bortezomib (0.2 μ M). The level of phosphorylated Smad3 was measured in (B) total cell lysates and (C) nuclear extracts using total Smad3 and RNA polymerase II (RNA Pol II) as loading controls, respectively. Phosphorylated-Smad3 expression was quantified using densitometry. *p* Values for comparisons are indicated in italics above the bars (N \geq 3 for all measures).

We also examined the effect of bortezomib in a murine model of scleroderma skin fibrosis.²³ Compared with saline, bortezomib (400 μ g/kg intraperitoneally twice weekly) substantially attenuated skin fibrosis in bleomycin-treated mice as assessed by histology and quantification of dermal thickness (figures 1B and 1D).

Bortezomib inhibits TGF- β 1-induced gene expression in fibroblasts by preventing Smad-mediated transcription

We transfected primary normal human lung fibroblasts with a Smad-responsive luciferase reporter construct.¹² Bortezomib resulted in a dose-dependent inhibition of Smad-mediated

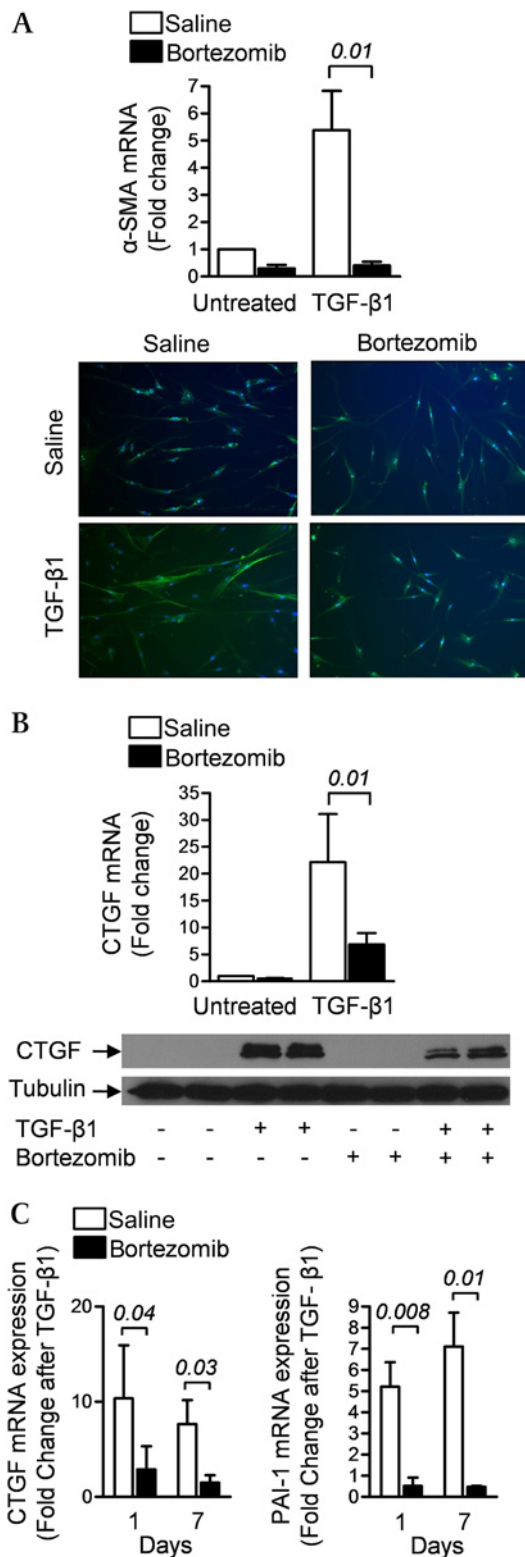


Figure 3 Bortezomib inhibits transforming growth factor β_1 (TGF- β_1)-induced gene expression in primary normal human lung fibroblasts. (A) Early passage primary cultures of normal human lung fibroblasts were grown to 70% confluence, serum-starved for 24 h and incubated with TGF- β_1 (5 ng/ml) with or without bortezomib (200 nM). After 24 h, α -smooth muscle actin (α -SMA) mRNA and protein were measured using RT-qPCR and immunohistochemistry, respectively. (B) Identically treated cells were harvested for measurement of connective tissue growth factor (CTGF) mRNA and protein expression using RT-qPCR and immunoblotting, respectively. (C) Normal human lung fibroblasts were

transcription in response to recombinant TGF- β_1 (figure 2A). At a dose of 200 nM, bortezomib was as effective as the ALK5 receptor inhibitor SB431542 (10 μ M) (figure 2A). No significant cell death was observed at the doses of bortezomib used (see figure S5 in online supplement). Following TGF- β_1 stimulation, bortezomib caused a non-significant increase in basal and phosphorylated Smad3 levels in whole cell lysates (figure 2B) and did not affect nuclear levels of phosphorylated Smad3 (figure 2C).

In normal human lung fibroblasts treated with recombinant TGF- β_1 , the increase in mRNA and protein levels of the TGF- β_1 target genes α -smooth muscle actin (α -SMA) and connective tissue growth factor (CTGF) were attenuated by treatment with bortezomib (200 nM) (figures 3A and 3B). The suppression of TGF- β_1 -mediated transcription of mRNA encoding CTGF and another TGF- β_1 target gene plasminogen activator-1 (PAI-1) persisted for at least 6 days after the removal of bortezomib from the culture medium (figure 3C). This was associated with a persistent reduction in the proteasomal activity of the cell lysates (see figure S3 in online supplement).

Bortezomib increases PPAR γ abundance and activity in lung fibroblasts

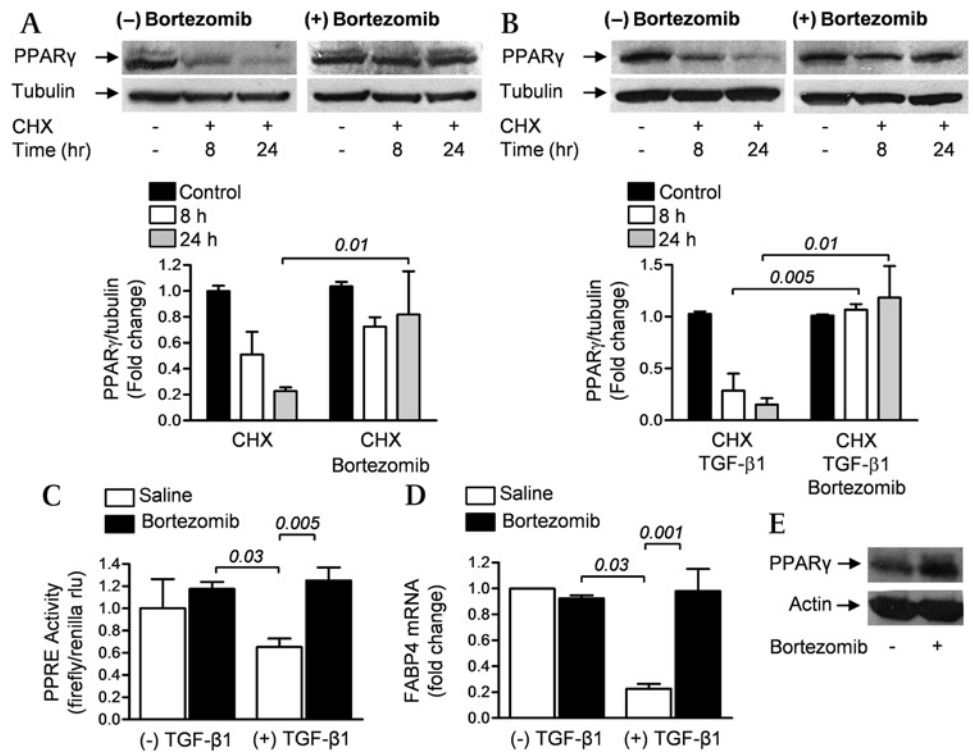
The apparent half-life of PPAR γ , a repressor of Smad-mediated transcription, in normal human lung fibroblasts was measured in the presence or absence of bortezomib.²⁴ Bortezomib increased the protein abundance and half-life of PPAR γ in the presence and absence of TGF- β_1 (figures 4A and 4B). Treatment with bortezomib prevented the TGF- β_1 -mediated repression of PPAR γ transcription as measured using a PPRE-luciferase reporter assay (figure 4C) and the transcription of the PPAR γ target gene fatty acid binding protein 4 (FABP4) (figure 4D) (positive (rosiglitazone) and negative (GW9662) controls in figure S6 in online supplement). Wild-type animals were also treated with bortezomib (120 μ g/kg intraperitoneally) and 24 h later lung homogenates were immunoblotted using an antibody against PPAR γ . Treatment with bortezomib resulted in an increase in total lung levels of PPAR γ (figure 4E).

We reasoned that, if the inhibition of TGF- β_1 -mediated transcription by bortezomib is in part due to an increase in PPAR γ , then PPAR γ agonists should potentiate and the loss of PPAR γ should attenuate the effects of bortezomib. We treated normal human lung fibroblasts with a dose of bortezomib that minimally inhibited TGF- β_1 -mediated transcription (10 nM, figure 5A), rosiglitazone (10 μ M) or the two together. Compared with either agent alone, the combination of low-dose bortezomib with rosiglitazone significantly attenuated the TGF- β_1 -mediated increase in CTGF mRNA (figure 5A). We used lentiviral shRNA to generate a line of normal human lung fibroblasts with a stable knockdown of PPAR γ (or a control lentivirus) and treated these cells with TGF- β_1 in the presence or absence of bortezomib. The inhibition of TGF- β_1 -mediated transcription of CTGF by bortezomib was significantly attenuated in the PPAR γ knock-down cells (figure 5B).

[Continued]

treated with bortezomib (200 nM) for 24 h, cultured in media without bortezomib for 5 days, treated with TGF- β_1 (5 ng/ml) in the absence of serum and 24 h later RNA was harvested for measurement of mRNA encoding the TGF- β_1 target genes CTGF and plasminogen activator-1 (PAI-1). The number of days after the single dose of bortezomib is indicated (N \geq 3 for all conditions). p Values are indicated in italics above the bars.

Figure 4 Bortezomib increases PPAR γ abundance and activity in normal human lung fibroblasts. (A) Primary normal human lung fibroblasts were cultured in the presence of cycloheximide (10 μ g/ml) with and without bortezomib (200 nM) and the protein abundance of PPAR γ was assessed by immunoblotting at the indicated times. (B) The same experiment to that described in (A) was performed in cells treated with transforming growth factor β_1 (TGF- β_1 , 5 ng/ml). (C) Primary normal human lung fibroblasts were infected with a plasmid containing PPAR γ response element (PPRE)-luciferase. Twenty-four hours after transfection the cells were treated with TGF- β_1 (5 ng/ml) with or without bortezomib and PPRE-luciferase activity was measured 24 h later. (D) Primary normal human lung fibroblasts were treated with TGF- β_1 (5 ng/ml) with or without bortezomib and the mRNA level of the PPAR γ target gene FABP4 was measured 24 h later by RT-qPCR. (E) Bortezomib (120 μ g/kg intraperitoneally) was administered to mice and 24 h later lung homogenates were immunoblotted using an antibody against PPAR γ . p Values are indicated in italics above the bars (N \geq 3 for all measures).



Bortezomib inhibits the autocrine release of TGF- β_1

Normal human lung fibroblasts were treated with TGF- β_1 in the presence or absence of bortezomib and TGF- β_1 mRNA was measured. The autocrine induction of TGF- β_1 was inhibited by bortezomib (figure 6A). To determine whether this mechanism might be important in vivo, BAL fluid levels of active TGF- β_1 were measured 14 days after the administration of bleomycin in mice that were treated on day 7 with either bortezomib (120 μ g/kg) or saline. The increase in active TGF- β_1 in BAL fluid observed 14 days after bleomycin was attenuated in mice that received a single dose of bortezomib at day 7 (figure 6B).

Bortezomib inhibits profibrotic gene expression in biological samples from patients with pulmonary fibrosis and scleroderma

We examined the effect of bortezomib on the profibrotic gene transcription induced by BAL fluid samples from patients with lung fibrosis. Consistent with our previous report, higher levels of active TGF- β_1 were seen in the BAL fluid from patients with lung fibrosis than controls (see figure S7 in online supplement).¹⁴ The administration of BAL fluid from 9 out of 10 patients with lung fibrosis increased SBE-luciferase activity in normal human lung fibroblasts and, for each of these nine patients, the increase was inhibited by bortezomib (figure 7A). We then compared the effect of bortezomib on TGF- β_1 -induced gene transcription in skin fibroblasts isolated from regions of fibrotic or normal skin from a patient with scleroderma.²¹ Baseline CTGF and PAI-1 mRNA levels were higher in fibroblasts isolated from regions of fibrotic skin than in those from normal skin. In both normal and fibrotic skin fibroblasts the TGF- β_1 -mediated transcription of these genes was significantly inhibited by bortezomib (figure 7B). Lastly, we examined the effect of bortezomib on TGF- β_1 -induced gene transcription in primary lung fibroblasts isolated from three patients with lung fibrosis. Both basal and

TGF- β_1 -induced transcription of CTGF and PAI-1 were inhibited by bortezomib in these cells (figure 7C).

DISCUSSION

This study shows that the systemic administration of the proteasomal inhibitor bortezomib to mice beginning 7 days after bleomycin administration prevented lung and skin fibrosis. In primary normal human lung fibroblasts, bortezomib inhibited the TGF- β_1 -mediated transcription of profibrotic genes downstream of the phosphorylation and nuclear translocation of Smads. Furthermore, bortezomib inhibited Smad-mediated transcription induced by BAL fluid from patients with lung fibrosis and prevented TGF- β_1 -induced gene transcription in skin and lung fibroblasts from patients with scleroderma and lung fibrosis, respectively. A bortezomib-induced increase in the abundance and activity of PPAR γ is an important mechanism contributing to this response.

In animal models, proteasomal inhibitors have been reported to ameliorate liver steatosis/fibrosis,⁷ myelofibrosis,⁸ cardiac fibrosis⁹ and renal fibrosis.¹⁰ In these reports, several distinct mechanisms were postulated to explain the antifibrotic effects of proteasomal inhibition including the induction of hepatic stellate cell apoptosis, activation of NF- κ B signalling and activation of matrix metalloproteinases. None of these mechanisms accounts for the broad antifibrotic effects of proteasomal inhibition in response to divergent profibrotic stimuli in multiple organs. Our finding that bortezomib inhibits TGF- β_1 -mediated transcription provides a common mechanism that is consistent with all of these findings.

Consistent with previous reports, we observed that bortezomib did not prevent the phosphorylation and nuclear translocation of Smad3 in response to TGF- β_1 in normal human lung fibroblasts.²² Despite this, the activity of a Smad reporter and the

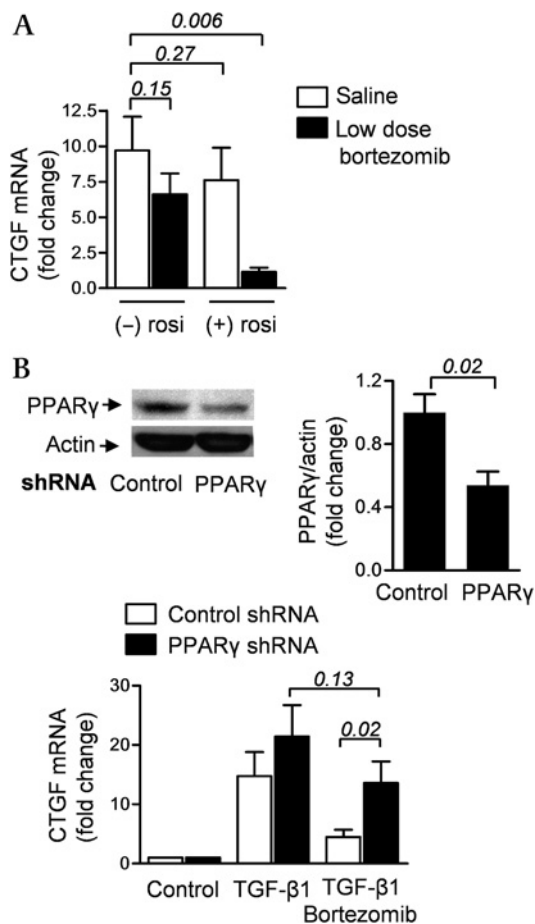


Figure 5 Bortezomib modulates fibrotic gene expression via PPAR γ . (A) Primary normal human lung fibroblasts were incubated with transforming growth factor β_1 (TGF- β_1 , 5 ng/ml) with or without low-dose bortezomib (10 nM) or rosiglitazone (10 μ M) and 24 h later connective tissue growth factor (CTGF) mRNA expression was measured using RT-qPCR. (B) Primary normal human lung fibroblasts were stably transfected with a shRNA against PPAR γ or a control lentivirus and PPAR γ levels were measured by immunoblotting (densitometry shown to the right of the blot). These cells were treated with TGF- β_1 (5 ng/ml) with or without bortezomib (200 nM) and CTGF mRNA was measured 24 h later (RT-qPCR). *p* Values are indicated in italics above the bars. In addition, *p*=0.026 for comparison between control transfected cells treated with TGF- β_1 or TGF- β_1 and bortezomib and *p*=0.12 for comparison between PPAR γ and control shRNA cells treated with TGF- β_1 alone.

expression of TGF- β_1 target genes were inhibited in cells treated with bortezomib. These findings suggest that bortezomib exerts its antifibrotic effects at the level of Smad-mediated transcription. The transcriptional response to activated Smads is controlled by both transcriptional co-activators (eg, p300) and co-repressors. The latter include PPAR γ , SnoN and Ski.^{5,25} PPAR γ is a nuclear hormone receptor and transcription factor essential for normal adipogenesis and glucose homeostasis that can be activated by endogenous lipids and eicosanoids or thiazolidinedione antidiabetic drugs such as rosiglitazone.²⁶ We have previously reported that PPAR γ inhibits TGF- β_1 signalling and target gene expression in fibroblasts by acting as a co-repressor of Smad-mediated transcription.²⁵ In this study we found that bortezomib increased the half-life and transcriptional activity of PPAR γ . In fibroblasts harbouring a stable knockdown of PPAR γ , the effect of bortezomib on TGF- β_1 -mediated transcription was

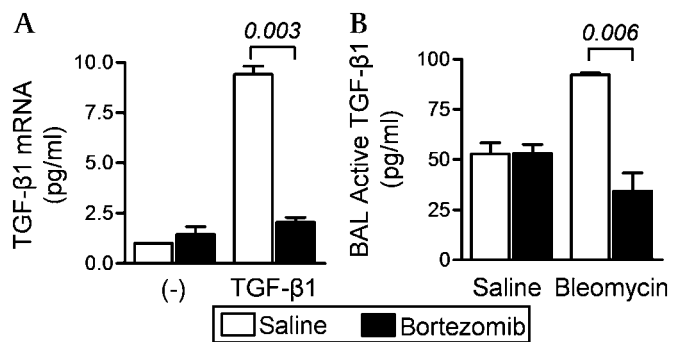


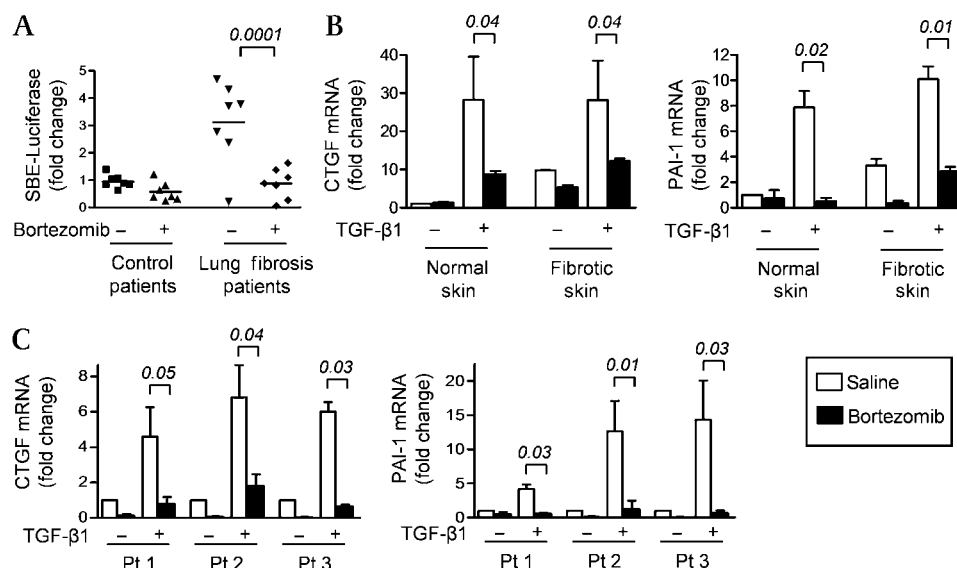
Figure 6 Bortezomib inhibits transforming growth factor β_1 (TGF- β_1) autocrine expression. (A) Primary normal human lung fibroblasts were incubated with TGF- β_1 (5 ng/ml) with or without bortezomib (200 nM) and 24 h later TGF- β_1 mRNA expression was measured using RT-qPCR. (B) Intratracheal bleomycin (0.075 IU) or saline was instilled into mice and intraperitoneal bortezomib (120 μ g/kg) or saline was administered 7 days later. Bronchoalveolar lavage was performed 14 days after bleomycin treatment for measurement of the level of active TGF- β_1 by ELISA. *p* Values are indicated in italics above the bars.

significantly diminished. Furthermore, bortezomib acted synergistically with the PPAR γ agonist rosiglitazone to inhibit TGF- β_1 -mediated transcription. Collectively these data suggest that, along with other mechanisms, an increase in the abundance and activity of PPAR γ contributes to the inhibition of TGF- β_1 -mediated transcription by bortezomib.

Similar to reports describing the pharmacodynamics of bortezomib in humans, we observed only modest reductions in serum and red blood cell proteasomal activity 3 h after administering the drug.²⁷ Nevertheless, we observed a profound antifibrotic effect when the drug was administered once weekly. This finding is similar to the results obtained in clinical trials using a weekly dosing schedule for the treatment of haematological malignancies.²⁸ It has been speculated that differential clearance of bortezomib from the serum and tissues explains the discrepancy between the kinetics of recovery of proteasomal activity in the blood and the frequency of dosing required to achieve an antineoplastic effect. Our observation that treatment of normal human lung fibroblasts with a single dose of bortezomib inhibited intracellular proteasomal activity and TGF- β_1 -mediated transcription for up to 7 days supports this hypothesis. Furthermore, TGF- β_1 has been reported to induce its own transcription and release from fibroblasts in an autocrine loop.²⁹ We found that bortezomib inhibited the autocrine production of TGF- β_1 in vitro and in vivo. We speculate that both the prolonged effect of bortezomib intracellularly and interruption of the autocrine production of TGF- β_1 explain its antifibrotic effects at the low doses and less frequent dosing schedules we employed.

The PPAR γ agonist rosiglitazone provides modest protection against bleomycin-induced lung fibrosis in mice when administered 3 days before bleomycin.³⁰ This contrasts with the nearly complete protection we observed in mice treated with bortezomib 7 or 14 days after bleomycin. We speculate this difference might result from accelerated proteasomal degradation of PPAR γ induced by treatment with rosiglitazone and other thiazolidinediones.³¹ Consistent with this mechanism, we observed a synergistic inhibition of TGF- β_1 -mediated transcription in mice treated with a combination of rosiglitazone and bortezomib; the concentration of bortezomib required to completely suppress TGF- β_1 -mediated transcription was 20-fold lower when it was administered in combination with rosiglitazone.

Figure 7 Bortezomib inhibits profibrotic gene expression in biological samples obtained from patients with lung fibrosis or scleroderma. (A) Primary cultures of normal human lung fibroblasts transfected 24 h earlier with a plasmid containing SBE-luciferase were incubated with bronchoalveolar lavage fluid obtained from 10 patients with lung fibrosis or eight patients without evidence of lung fibrosis with or without bortezomib (200 nM). SBE-luciferase activity was measured 24 h later. (B) Primary cultures of skin fibroblasts obtained from a region of normal or fibrotic skin from a patient with scleroderma and (C) primary lung fibroblasts cultured from three patients (Pts 1, 2 and 3) with lung fibrosis were treated with transforming growth factor β_1 (TGF- β_1 , 5 ng/ml) with or without bortezomib or bortezomib alone and the levels of connective tissue growth factor (CTGF) and plasminogen activator-1 (PAI-1) mRNA were measured using RT-qPCR. p Values are indicated in italics above the bars.



Fineschi *et al* reported that bortezomib failed to protect against lung fibrosis in bleomycin-exposed mice; however, these investigators administered bortezomib before or concomitant with the administration of bleomycin.²² Consistent with their findings, we observed excess mortality when bortezomib and bleomycin were administered concomitantly. As the administration of bleomycin results in acute lung injury that peaks 3–5 days later and slowly resolves thereafter, these findings may provide insight into the sporadic reports of patients who have developed pulmonary toxicity, including fatal interstitial pneumonias, while receiving twice weekly bortezomib for haematological malignancies.^{32–34} While speculative, it may be that the fatal pulmonary events associated with bortezomib resulted from its administration to patients with untreated pneumonia. In support of this hypothesis, therapeutic guidelines developed in response to the reports of bortezomib-related pulmonary complications recommend avoiding bortezomib in patients with evidence of pneumonia. The implementation of these guidelines has been associated with a reduction in bortezomib-related pulmonary toxicity.^{27, 35}

Our finding that bortezomib is effective when started 7 days after bleomycin administration is in sharp contrast to other pharmacological therapies that prevent bleomycin-induced lung fibrosis. For example, treatment of mice with imatinib (an inhibitor of Smad-independent signalling by TGF- β_1) prior to the administration of bleomycin prevented the subsequent development of lung fibrosis³⁶ but was ineffective when administered after bleomycin.³⁷ A recent clinical trial of imatinib in patients with idiopathic pulmonary fibrosis failed to show improvement in mortality or in lung physiological parameters.³⁸ Based on these and other data, some investigators have advocated that only drugs that are effective in preventing fibrosis when administered after bleomycin should be considered for clinical development.³⁹

CONCLUSIONS

Bortezomib, a drug with a well-defined and acceptable toxicity profile and clear mechanism of action,^{5, 6} prevents bleomycin-induced lung fibrosis in mice. It differs from other therapeutic

strategies shown to inhibit bleomycin-induced lung fibrosis as it was effective when given after bleomycin-induced acute lung injury had peaked and TGF- β_1 was active in the lung. The administration of bortezomib was similarly effective in preventing bleomycin-induced skin fibrosis. Bortezomib effectively inhibited TGF- β_1 signalling in primary normal human lung fibroblasts and in fibroblasts from the lungs and skin of patients with lung fibrosis or scleroderma, respectively, and inhibited the profibrotic activity induced by BAL fluid from patients with lung fibrosis. Our results suggest that the bortezomib-mediated increase in the abundance and activity of the TGF- β_1 transcriptional repressor PPAR γ contributes to its antifibrotic activity. Given the dearth of proven antifibrotic therapies, clinical trials assessing the efficacy of bortezomib alone or in combination with PPAR γ agonists in patients with tissue fibrosis may be justified.

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Competing interests None.

Ethics approval This study was conducted with the approval of Northwestern University Institutional Review Board.

Contributors GMM, GRSB and MJ designed the experiments, reviewed the data and wrote the manuscript. MW, APL, AZ, SR, DU, SEC, LHTG and AKG conducted the experiments and assisted with the data analysis. MS and AP provided cell lines from patients with lung fibrosis. JV provided skin fibroblasts, designed the skin fibrosis experiments and reviewed the data. DWK, NSC and JIS assisted with the design and interpretation of the experiments.

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Proteasomal inhibition after injury prevents fibrosis by modulating TGF- β 1 signaling

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Materials and Methods

Animal protocol and the administration of bleomycin and bortezomib. The protocols for the use of animals were approved by the Northwestern University Animal Care and Use Committee. The results of experiments to determine the dosing schedules for the administration of bleomycin are described below. We treated wild-type C57BL/6 mice with intratracheal bleomycin (0.075 IU/mouse) followed 7 and 14 days later by bortezomib (120 µg/kg, ip) or saline and measured lung fibrosis on Day 21. We also performed experiments in which bortezomib or saline was administered on days 14 and 21 (120 µg/kg, ip) following intratracheal bleomycin administration and the lungs were harvested on day 28. For measurement of proteasomal activity, blood was collected 3 hours after bortezomib administration.

For the skin fibrosis experiments, we treated 6-8-week-old female BALB/c mice with filter-sterilized bleomycin (20 µg/mouse, Mayne Pharma, Paramus, NJ) or saline subcutaneously daily (27 gauge needle) into a shaved area of skin on the back of the animal. Bortezomib (400 µg/kg, ip) or vehicle was begun 7 days after the first dose of bleomycin and administered twice weekly until sacrifice on day 40.

Proteasomal Activity. Anesthetized mice underwent thoracotomy and blood was obtained via right atrial puncture using a 30 gauge needle. The blood was centrifuged (5,000 X g) for serum separation and the chymotrypsin-like activity activity of the proteasome was measured by examining the degradation of fluorescently labeled Suc-LLVY-AMC in the presence or absence of the proteasome inhibitor MG-132 as previously described.¹ All measurements were performed in duplicate and averaged for each animal.

Cell Culture. Normal human lung fibroblasts (NHLF) were obtained from Lonza (Basel, Switzerland) and grown in FGM-2 media supplemented with SingleQuots (Lonza) with in a humidified incubator (5% CO₂) at a temperature of 37°C. The cells were grown to 70% confluence and discarded after their fourth passage.

SBE-luciferase and PPRE-luciferase transfections. Transfections to assess Smad and PPAR γ mediated expression were performed by using the Mirus *TransIT* Transfection reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's protocol. The SBE-luciferase is a pGL2 vector containing smad binding response elements upstream of firefly luciferase.² The PPRE-luciferase is a vector which contains 3 copies of the PPRE from the acyl-coenzyme A (acyl-CoA) oxidase gene linked to a luciferase genes.³ TK-Renilla luciferase was co-transfected to control for transfection efficiency. Luciferase activity was measured using luminometry according to standard protocols for the Promega Dual-Luciferase Reporter Assay System.

Real Time quantitative PCR. mRNA expression was determined by real-time quantitative-PCR (RT-qPCR) using SYBR green chemistry The following primer sequences were used: α -SMA – GGCGGTGCTGTCTCTCTAT and CCAGATCCAGACGCATGATG; RPL19 (control) – AGTATGCTCAGGCTTCAGAAGA and CATTGGTCTCATTGGGGTCTAAC; CTGF – GGCTTACCGACTGGAAGAC and AGGAGGCGTTGTCATTGG; PAI-1 - AAGACTCCCTTCCCCGACTC and GGTCTGTCCATGATGATCTCCTC; FABP4- TCAAGAGCACCATAACCTTAG and GTGGAAGTGACGCCTTTC. Normal human lung fibroblasts were serum starved overnight and then incubated with TGF- β 1 with/without

bortezomib for 24 hours. Total RNA from cells was isolated after 24 hours of incubation using Triazol (Invitrogen, Carlsbad, CA). cDNA was synthesized from 2 μ g of total RNA using M-MLV Reverse Transcriptase (Ambion, Austin, TX) with random decamer primers. Real-time quantitative PCR was carried out using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). cDNAs were amplified using the Biorad iCycler iQ system. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial gene RPL19.⁴

Immunoblotting. Immunoblotting was performed as previously described.⁵ Recombinant active human TGF- β 1 (5ng/ml) was purchased from Calbiochem (Gibbstown, NJ). Whole cell lysates and nuclear lysates were analyzed by immunoblotting with phospho-Smad3 antibody (Cell Signaling, Danvers, MA) (1:1000) and to control for loading total Smad (Santa Cruz, Santa Cruz, CA) (1:200) and RNA Pol II antibody (Santa Cruz) (1:200) were used for cell and nuclear extracts, respectively. For the CTGF and PPAR γ immunoblots, primary normal human lung fibroblasts were serum starved for 24 hours before treatment and whole cell lysates were analyzed by immunoblotting with either a CTGF (Santa Cruz) (1:200) antibody or a PPAR γ antibody (Santa Cruz) (1:200). Actin and tubulin antibodies (Sigma-Aldrich, St. Louis, MO, 1:1500) were used to control for loading.

Cell Death Assay. Cell death was assessed by the release of lactate dehydrogenase (LDH) into the surrounding medium using a cytotoxicity detection kit (Roche Applied Sciences). Percentage of cell death was calculated by the amount of LDH released in the medium, divided by the total LDH released after treatment of cells with 1% Triton X-100 as previously described.⁶

Immunofluorescence. NHLFs were serum-starved for 48 hrs and incubated with TGF- β 1 (5 ng/mL) with or without bortezomib for 48 hrs. The cells were fixed and permeabilized with

50%:50% methanol: acetone solution at -4°C and washed three times with PBS. The cells were blocked for 1 hr (PBS with 2% bovine serum albumin and 0.1% Triton) and then incubated overnight with αSMA antibody (5 ng/mL, 1:200) (R&D Systems, Minneapolis, MN). Following three washes, cells were incubated for 45 mins with fluorescent anti-mouse antibody (Invitrogen) (1:100). After washes in phosphate buffered saline, the coverslips were fixed in Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

Measurement of active TGF- β 1. Active TGF- β 1 was measured from BALF in duplicate using the TGF β ₁ E_{max}[®] ImmunoAssay System (ELISA) according to manufacturer protocol (Promega, Madison, WI). This assay only measures TGF- β 1 that has been cleaved and is biologically active.

Histology. A 20 gauge angiocath was sutured into the trachea, the lungs and heart were removed en bloc and the lungs inflated to 15 cm H₂O with 4% paraformaldehyde. The heart and lungs were fixed in paraffin and 5 μM sections were stained with hematoxylin and eosin. Lesional skin tissue was embedded in paraffin, and consecutive 4- μm serial sections were stained with hematoxylin and eosin (H&E). Dermal thickness, defined as the distance between the epidermal-dermal junction and the dermal-adipose layer junction, and the adipose layer, defined as the distance between the dermal-adipose junction and the muscle, was determined in H&E-stained sections at x100 microscopic magnification.

Measurement of lung collagen. Lung collagen was measured using a modification of a previously described method for the precipitation of lung collagen using picosirius red as previously described.⁷ The lungs were harvested and suspended in 0.5 N acetic acid and

homogenized first with a tissue homogenizer (30 seconds on ice) and then using 12 strokes in a Dounce homogenizer (on ice). The resulting homogenate was spun ($>10,000 \times g$) for 10 minutes and the supernatant was used for subsequent analysis. Collagen standards were prepared in 0.5N acetic acid using rat tail collagen (Sigma-Aldrich). Picrosirius red dye was prepared by mixing 0.2 g of Sirius Red F3B (Sigma-Aldrich) with 200 ml of saturated picric acid in water (solid picric acid maintained at the bottom of the flask to insure saturation). One ml of the Picrosirius red dye was added to 50 μ L of the collagen standard or the lung homogenates and they were mixed continuously at room temperature on an orbital shaker for 30 minutes. The precipitated collagen was then pelleted and washed once with 0.5 N acetic acid ($>10,000 \times g$, 10 minutes). The resulting pellet was resuspended in 500 μ L of 0.5 M NaOH and Sirius red staining was quantified spectrophotometrically (540 nm) using a colorometric plate reader (BioRad).

Lentiviral PPAR γ knockdown. The pLKO.1 vector was used to express shRNA targeting PPAR γ as previously described.⁷ Constructs were ordered from Sigma-Aldrich and we screened five validated hairpin sequences and the following sequence had the most efficient knockdown, 5'CCGGCTGGCCTCCTTGATGAATAAACTCGAGTTTATTCATCAAGGAGGCCAGTTTT T'3 . The non-silencing (control) shRNA was ordered from Sigma. Stable cell lines were generated by Virapower (Invitrogen) lentiviral infection using the 293FT packaging cell line and puromycin selection. 48 hours post 293FT transfection, medium containing virus was supplemented with 8 μ g/ml polybrene (Sigma-Aldrich) for cell line infection and applied to normal human lung fibroblasts.

Clinical specimen collection. The collection of clinical data and specimens was approved by the Northwestern University Institutional Review Board. Bronchoalveolar lavage fluid samples from ten patients with a clinical and radiological diagnosis of pulmonary fibrosis and eight control patients were used.⁸ The pulmonary fibrosis patients were recruited from the pulmonary clinic prior to undergoing diagnostic bronchoscopy or VATS biopsy. The diagnosis was based on clinical and HRCT findings or histologic findings on subsequent VATS biopsy.⁹ The control subjects were patients who underwent bronchoscopy who did not have evidence of parenchymal lung disease (i.e. mediastinal lymphadenopathy, unexplained hemoptysis etc.). A fiberoptic bronchoscope was wedged and sterile saline was instilled in 60cc aliquots and then aspirated. The fluid was centrifuged at 1500 rpm within 30 minutes of collection for 10 minutes, aliquoted and frozen at -80° C. A fiberoptic bronchoscope was wedged into position of a distal bronchus of an affected lobe and sterile saline was instilled in 60 ml aliquots and then aspirated and collected. This was repeated up to 3 times. The fluid was centrifuged at 1500 rpm within 30 minutes of collection for 10 minutes, aliquotted and frozen at -80° C. Samples of lung fibroblasts from fibrotic lungs and control lungs were cultured and stored as previously described.¹⁰ Samples of skin fibroblasts were obtained by skin biopsy of an affected and unaffected area of the forearm of a patient with scleroderma as previously described.¹¹

Statistical Analysis. The data were analyzed in Prism 4, GraphPad Software, Inc., (La Jolla, CA). All data are displayed as means with standard errors of the mean. Statistical significance was determined by ANOVA; when the ANOVA revealed a significant difference, individual differences were explored using t-tests with the Bonferroni or Dunnett correction for multiple comparisons. A $p < 0.05$ was considered statistically significant.

RESULTS

We measured proteasomal activity in the blood following treatment with bortezomib. Mice were treated with bortezomib (3 µg/kg, ip) and 3 hours later blood was collected via right atrial puncture. Compared with saline treated mice, mice treated with bortezomib exhibited reduced proteasomal activity in both RBCs and the serum (Figure S1A). In order to determine the duration of the effect of bortezomib on intracellular proteasomal activity, we treated primary normal human lung fibroblasts with bortezomib (200 nM, single dose) for 24 hours after which total cell lysates were collected and proteasomal activity was measured. In parallel experiments, after 24 hours of bortezomib exposure, the cells were changed to bortezomib-free media and maintained in culture for 6 more days after which total cell lysates were collected and proteasomal activity was measured (Figure S1B).

In selecting the dose and dosing schedule for bortezomib, our initial plan was to mirror the dosing schedule used for the treatment of patients with multiple myeloma. Our initial dose of 400 µg/kg is similar, adjusted for body surface area, to the recommended human dose.¹² We began our initial studies by administering bortezomib concomitantly or beginning 1 week prior to bleomycin administration. Both these dosing schedules were associated with an unexpected >70% mortality in the mice which received bortezomib prior to or concomitant with bleomycin. All of this mortality occurred in the first 5 days after treatment with bleomycin. There was no unexpected mortality in mice that received bortezomib or bleomycin alone. Similar mortality was observed when mice were given bortezomib 120 µg/kg, ip before or concomitant with the administration of bleomycin. Based on our *in vitro* findings that bortezomib inhibited TGF-β1-

mediated transcription, we reasoned that it might be more effective if administered at a time when lung injury was improving and TGF- β 1 was active. Therefore, we conducted studies in which bortezomib was administered at a dose of 400 μ g/kg, ip twice weekly, beginning 7 days after the administration of bleomycin. At this dose, fibrosis was completely inhibited at 21 days. We then reduced the dose to 400 μ g/kg, ip once weekly and again observed nearly complete protection against fibrosis at 21 days. We then further reduced the dose to 120 μ g/kg on Day 7 and Day 14 after the administration of bleomycin. This dose was associated with nearly complete protection against bleomycin induced fibrosis as measured by examination of Trichrome stained lung sections, picrosirius red collagen precipitation from whole lung homogenates and immunoblotting of whole lung homogenates using an antibody against type I collagen (Figure 1A and C and Figure S2). The administration of a single dose of bleomycin administered on Day 7 or Day 14 was associated with only partial protection against bleomycin-induced fibrosis. Only data for the lower dose are shown as the data for the higher dose are qualitatively similar but lack the statistical power required to determine equivalence.

To determine whether bortezomib was effective after lung fibrosis had begun, mice were given bortezomib (120 μ g/kg, ip) 14 and 21 days after bleomycin was administered and harvested for assessment of fibrosis 28 days after bleomycin administration. Lung fibrosis at 28 days was substantially attenuated in the bortezomib treated mice (Figure S2).

To determine whether bortezomib induces cell death at the concentrations used for our in vitro assays, normal human lung fibroblasts (NHLFs) were treated with bortezomib (200 nM) in the presence or absence of recombinant human TGF- β 1 (5 ng/ml) and cell death was measured (LDH release) 24 hours later. No significant cell death was observed in cells treated with

bortezomib with or without TGF- β 1. Treatment with H₂O₂ (500 μ M) was used as a positive control (Figure S4).

To determine whether increased PPAR γ in bortezomib treated cells was associated with increase PPAR γ transcriptional activity, we transiently transfected NHLF with a plasmid encoding a PPAR γ response element linked to a luciferase reporter (PPRE-luciferase)³. The PPAR γ agonist rosiglitazone alone or with the PPAR γ antagonist GW9662 were used as controls. Rosiglitazone induced a significant increase in PPRE-luciferase activity which was inhibited by GW9662 (Figure S6).

Using an ELISA which only detects active TGF- β 1, we then measured active TGF- β 1 levels in BAL fluid from patients with lung fibrosis and control subjects. Patients with lung fibrosis had significantly higher BAL levels of active TGF- β 1 levels compared to control subjects (Figure S7).

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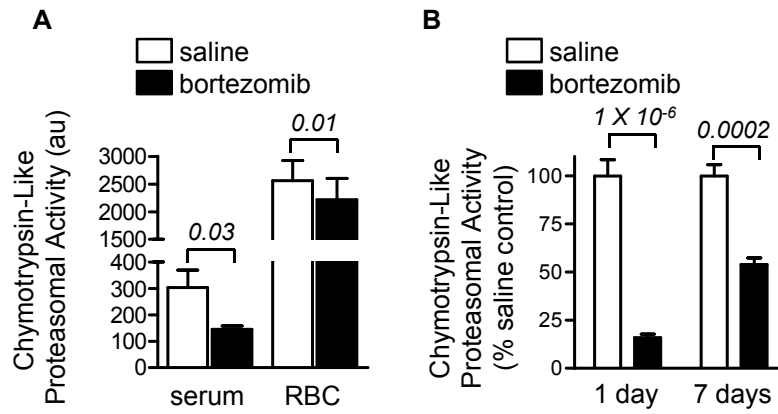


Figure S1. Bortezomib inhibits blood and cellular proteasomal activity. (A) Mice were treated with bortezomib (120 $\mu\text{g}/\text{kg}$) and 24 hours later blood was collected for measurement of the chymotrypsin-like proteasomal activity of the serum and the red blood cells. Each bar represents between 4-8 animals. (B) Primary normal human lung fibroblasts were treated with media supplemented with saline or bortezomib (200 nM). After 24 hours the media was changed and the cells were cultured for 6 days in media free of bortezomib after which the chymotrypsin like proteasomal activity of the cell lysates were measured. N = 3 for all measures. P values are indicated in italics above the bars.

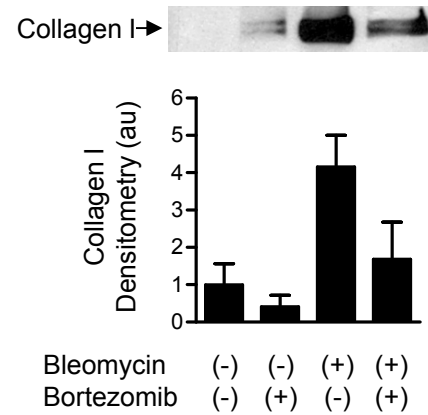


Figure S2. Mice were treated with intratracheal bleomycin or saline and 7 and 14 days later were treated with intraperitoneal bortezomib (120 $\mu\text{g}/\text{kg}$) or saline. After 21 days, the lungs were perfused with PBS via the right ventricle and homogenized in 0.5 N HCl. Equal volumes of lung homogenates were immunoblotted using an antibody against Type I collagen. A representative immunoblot (top) and densitometry from 4 replicates (bottom) are shown. * indicates $P < 0.05$ for Dunnett corrected comparison with the double-vehicle control.

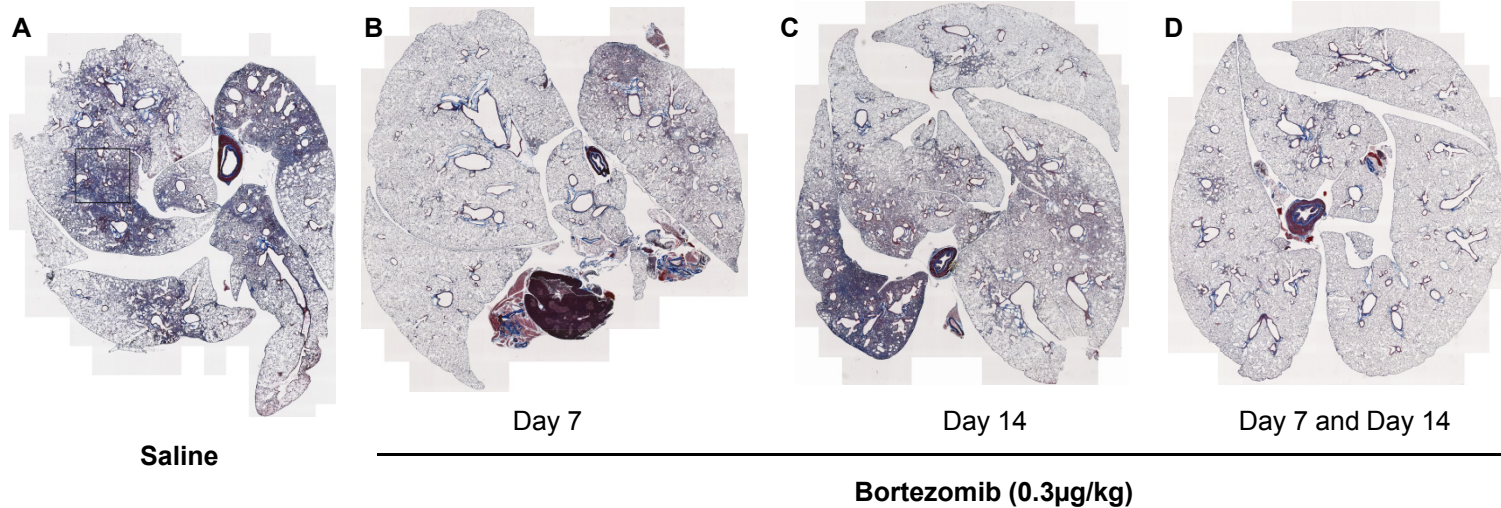


Figure S3. Bortezomib prevents bleomycin induced lung fibrosis. Representative lung sections from mice, all of which were treated with intratracheal bleomycin (0.07 IU/ mouse) followed by (A) intraperitoneal saline on Day 7 and 14, (reproduced from Figure 1) or (B) bortezomib (120 µg/kg) on Day 7, (C) Day 14 or (D) both Day 7 and 14 (D, reproduced from Figure 1). Trichrome stained lung sections 21 days after the instillation of bleomycin are shown, N > 4 for all treatment groups.

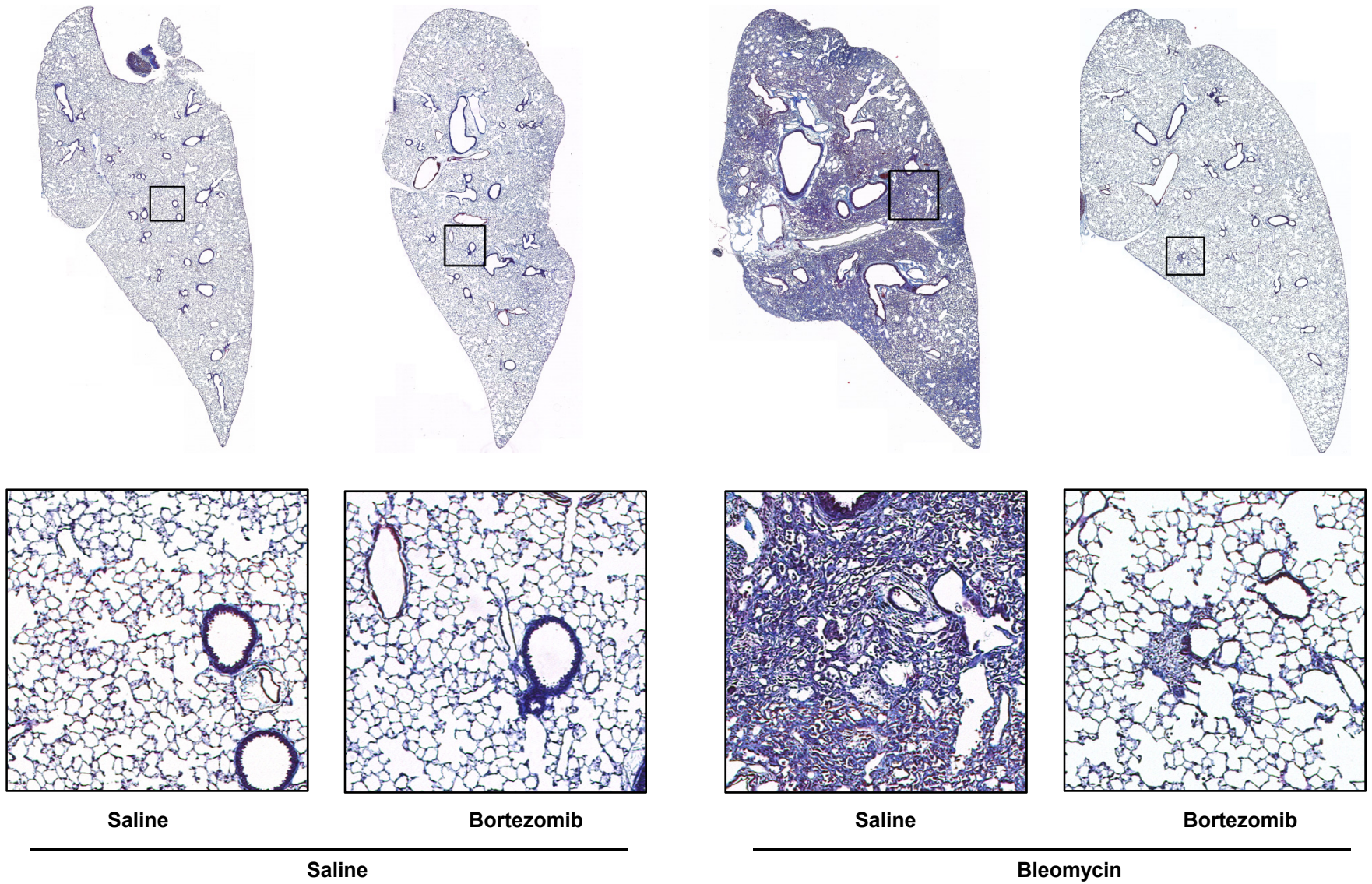


Figure S4. Bortezomib prevents lung fibrosis in mice when administered 14 and 21 days after bleomycin. Mice were treated with intratracheal saline or bleomycin (0.07 IU/ mouse) (Day 0). On Day 14 and Day 28, the mice were treated with saline or bortezomib (120 µg/kg). Representative Trichrome stained lung sections from 1 animal in the bleomycin followed by saline group and 3 animals in the bleomycin followed by bortezomib group 28 days after the instillation of bleomycin are shown,.

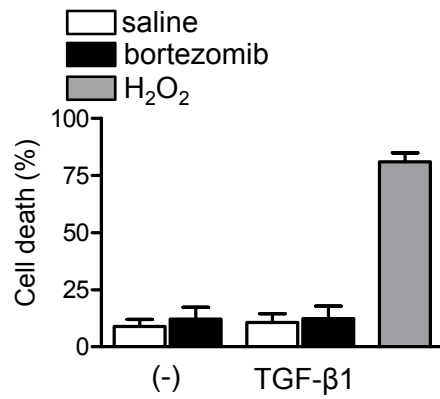


Figure S5. Treatment with bortezomib does not cause cell death. Primary normal human lung fibroblasts were treated with saline, bortezomib (200 nM) or H₂O₂ (400 μM, positive control) in the presence or absence of TGF-β1 (5 ng/ml) and 24 hours cell death was assessed (LDH release).

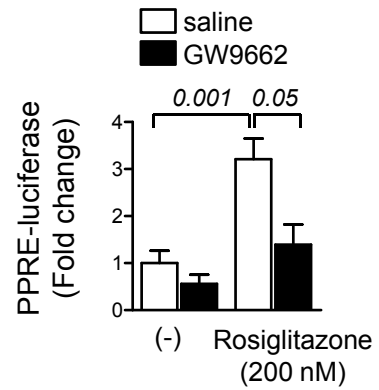


Figure S6. Controls for the PPRE-luciferase transfection. Primary normal human lung fibroblasts were transfected with a PPAR γ responsive luciferase reporter (PPRE-luciferase) and a Renilla luciferase reporter and treated with vehicle or the PPAR γ antagonist GW9662 (10 μ M) in the presence or absence of the PPAR γ agonist rosiglitazone (10 μ M) and luminescence (firefly/Renilla) was measured 24 hours later. $N \geq 4$ for all conditions. P values are indicated in italics above the bars.

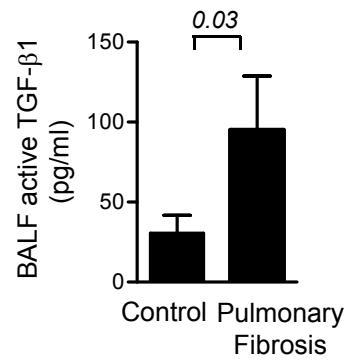


Figure S7. The levels of active TGF-β1 are increased in BAL fluid from patients with pulmonary fibrosis. The levels of active TGF- β1 in the BAL fluid from control patients and patients with pulmonary fibrosis (Figure 7A) were measured by ELISA. The P value is indicated in italics above the bars.