

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

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Data Supplement

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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 picrosirius 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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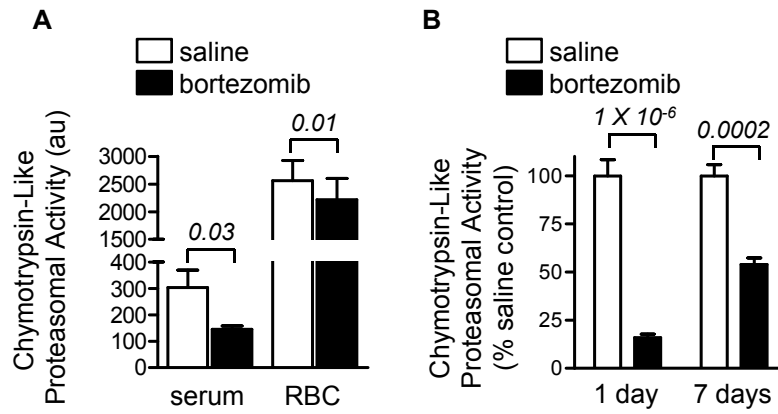


Figure S1. Bortezomib inhibits blood and cellular proteasomal activity. (A) Mice were treated with bortezomib (120 $\mu\text{g}/\text{kg}$) and 24 hours later blood was collected for measurement of the chymotrypsin-like proteasomal activity of the serum and the red blood cells. Each bar represents between 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.

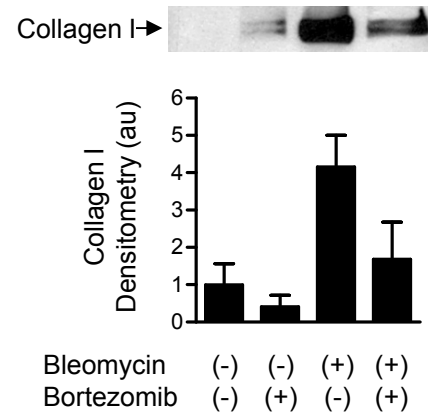


Figure S2. Mice were treated with intratracheal bleomycin or saline and 7 and 14 days later were treated with intraperitoneal bortezomib (120 $\mu\text{g}/\text{kg}$) or saline. After 21 days, the lungs were perfused with PBS via the right ventricle and homogenized in 0.5 N HCl. Equal volumes of lung 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.

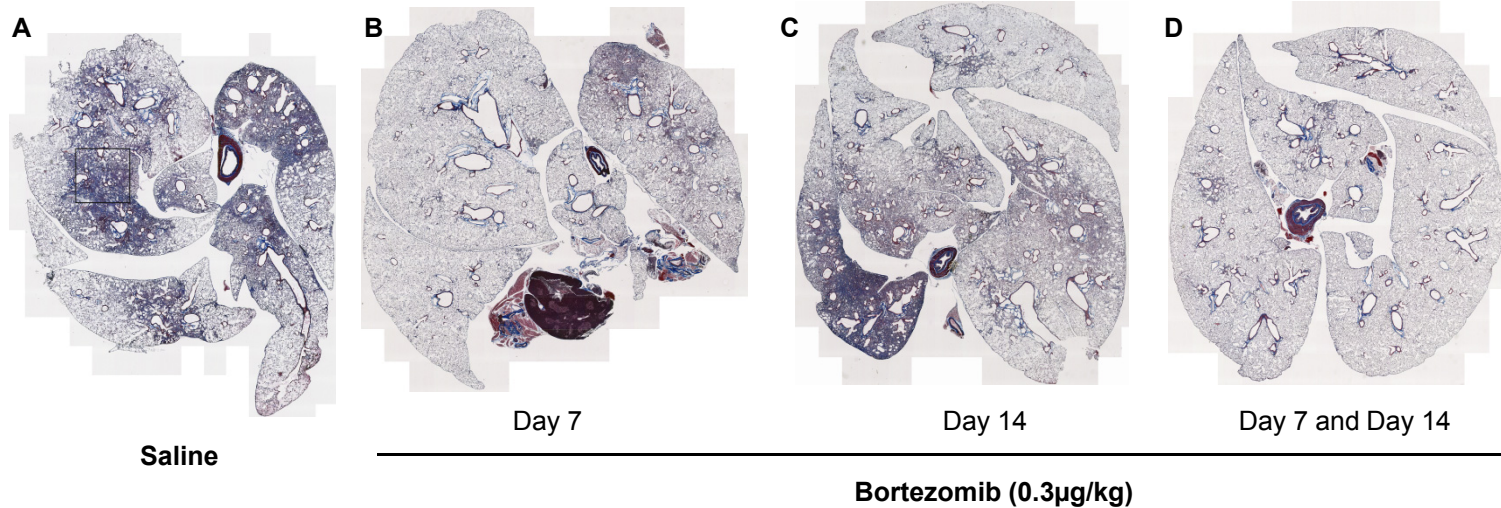


Figure S3. Bortezomib prevents bleomycin induced lung fibrosis. Representative lung sections from mice, all of which were treated with intratracheal bleomycin (0.07 IU/ mouse) followed by (A) intraperitoneal saline on Day 7 and 14, (reproduced from Figure 1) or (B) bortezomib (120 µg/kg) on Day 7, (C) 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.

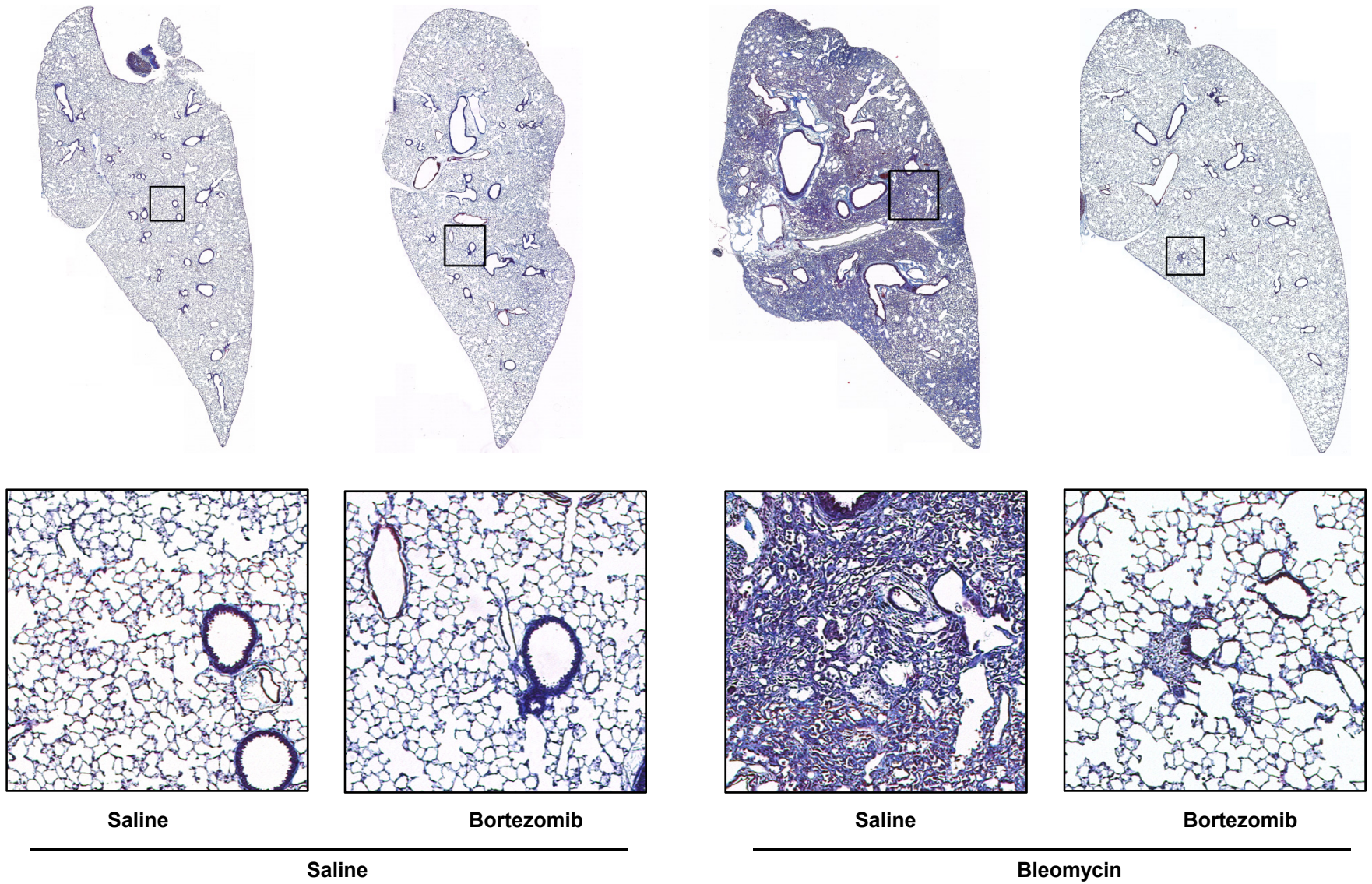


Figure S4. Bortezomib prevents lung fibrosis in mice when administered 14 and 21 days after bleomycin. Mice were treated with intratracheal saline or bleomycin (0.07 IU/ mouse) (Day 0). On Day 14 and Day 28, the mice were treated with saline or bortezomib (120 µg/kg). Representative Trichrome stained 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,.

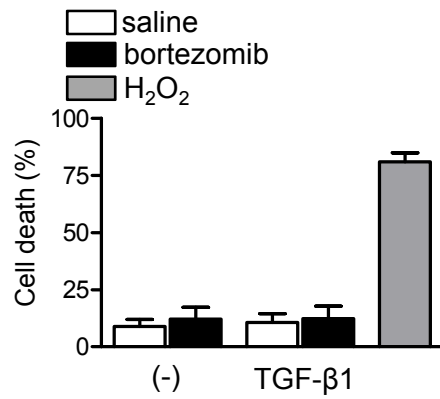


Figure S5. Treatment with bortezomib does not cause cell death. Primary normal human lung fibroblasts were treated with saline, bortezomib (200 nM) or H₂O₂ (400 μM, positive control) in the presence or absence of TGF-β1 (5 ng/ml) and 24 hours cell death was assessed (LDH release).

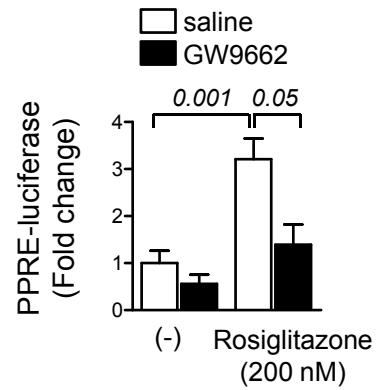


Figure S6. Controls for the PPRE-luciferase transfection. Primary normal human lung fibroblasts were transfected with a PPAR γ responsive luciferase reporter (PPRE-luciferase) and a Renilla luciferase reporter and treated with vehicle or the PPAR γ antagonist GW9662 (10 μ M) in the presence or absence 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.

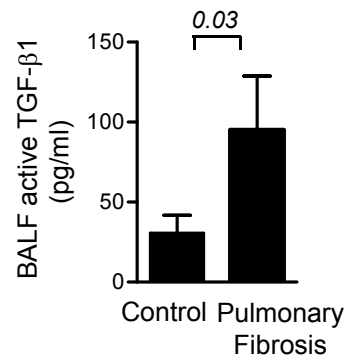


Figure S7. The levels of active TGF-β1 are increased in BAL fluid from patients with pulmonary fibrosis. The levels of active TGF- β1 in the BAL fluid from control patients and patients with pulmonary fibrosis (Figure 7A) were measured by ELISA. The P value is indicated in italics above the bars.