Published in final edited form as:

Int J Radiat Oncol Biol Phys. 2017 October 01; 99(2): 353–361. doi:10.1016/j.ijrobp.2017.02.216.

Inhibition of Bcl-2/xl With ABT-263 Selectively Kills Senescent Type II Pneumocytes and Reverses Persistent Pulmonary Fibrosis Induced by Ionizing Radiation in Mice

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Abstract

Purpose: Ionizing radiation (IR) induced pulmonary fibrosis (PF) is an irreversible and severe late effect of thoracic radiation therapy. The goal of this study was to determine whether clearance of senescent cells with ABT-263, a senolytic drug that can selectively kill senescent cells, can reverse PF.

Methods and Materials: C57BL/6J mice were exposed to a single dose of 17 Gy on the right side of the thorax. Sixteen weeks after IR, they were treated with 2 cycles of vehicle or ABT-263 (50 mg/kg per day for 5 days per cycle) by gavage. The effects of ABT-263 on IR-induced increases in senescent cells; elevation in the expression of selective inflammatory cytokines, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinases; and the severity of the tissue injury and fibrosis in the irradiated lungs were evaluated 3 weeks after the last treatment, in comparison with the changes observed in the irradiated lungs before treatment or after vehicle treatment.

Results: At 16 weeks after exposure of C57BL/6 mice to a single dose of 17 Gy, thoracic irradiation resulted in persistent PF associated with a significant increase in senescent cells.

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Conflict of interest: Y.W. and D.Z. filed a patent application for the use of ABT-263 as an antiaging agent. A potential royalty stream to Y.W. and D.Z. may occur consistent with University of Arkansas for Medical

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Treatment of the irradiated mice with ABT-263 after persistent PF had developed reduced senescent cells and reversed the disease.

Conclusions: To our knowledge, this is the first study to demonstrate that PF can be reversed by a –senolytic drug such as ABT-263 after it becomes a progressive disease. Therefore, ABT-263 has the potential to be developed as a new treatment for PF.

Introduction

Thoracic radiation therapy is an essential treatment modality for lung, breast, and esophageal cancers, as well as various mediastinal tumors. Despite significant improvements in radiation therapy delivery methods, many thoracic cancer patients remain at risk of the development of ionizing radiation (IR) induced pulmonary fibrosis (PF) after radiation therapy (1–3). It is important to note that PF is a progressive disease that not only affects long-term quality of life but also can result in fatal respiratory insufficiency. The pathogenesis of PF remains unclear, and effective interventions that can be used to treat PF once it becomes a fully developed disease are still lacking in the clinic, leading to a poor prognosis for PF patients (3–7). Therefore, new strategies that can reverse the course of the disease are urgently needed.

Accumulating evidence suggests that induction of cellular senescence may play an important role in the pathogenesis of various fibrotic lung diseases, including PF (8) and those induced by bleomycin (9), telomere dysfunction (10), and idiopathic PF (11, 12). Senescence of alveolar epithelial cells in fibrotic foci has been described in a number of settings, including in the lungs of idiopathic PF patients (11, 12). Similarly, mice treated with bleomycin (9) or thoracic irradiation (8) have demonstrated senescence in type II alveolar epithelial cells (AECIIs), the putative alveolar stem cells. In contrast, senescent cells (SCs) were barely detectable in normal lungs (8, 9, 11, 12). SCs can potentially promote PF via multiple mechanisms, including induction of alveolar epithelial stem cell exhaustion, induction of chronic oxidative stress and inflammation, and disruption of normal tissue structure and function (13–16). Therefore, we hypothesize that induction of senescence by IR not only mediates the initiation of PF but also plays a critical role in the progression of the disease.

Recent studies aimed at preventing and/or mitigating PF after DNA damaging insults induced by IR or bleomycin have focused on inhibiting pathways associated with induction of cellular senescence by targeted inhibition of nicotinamide adenine dinucleotide phosphate oxidases (8, 9) or treatment with a recombinant truncated plasminogen activator inhibitor 1 protein (rPAI-1₂₃) (17) or rupertadine (18). These interventions have to be applied before and/or shortly after the insults and cannot be used as a treatment to reverse PF. Therefore, in this study we investigated whether clearance of SCs with a pharmacologic agent, when PF has already developed and become a persistent disease long after exposure to IR, can still reverse the disease using a mouse model.

Methods and Materials

Mice

Male C57BL/6J mice were used as described in Appendix E1 (available online at www.redjournal.org). The Institutional Animal Care and Use Committee at Institute of Radiation Medicine, Peking Union Medical College and Chinese Academy of Medical Sciences approved all experimental procedures used in this study.

Irradiation (IR) and ABT-263 administration

Mice were exposed to a single dose of 17 Gy of radiation on the right side of the thorax, and 16 weeks after IR, they were treated with vehicle (ethanol/polyethylene glycol 400/Phosal 50 PG (Fisher, MA, USA) at 10:30:60) or ABT-263 (Active Biochem, Maplewood, NJ) (50 mg/kg per day for 5 days per cycle for 2 cycles, with a 2-week interval between cycles) by gavage, as we reported recently (19) and as described in Appendix E1 (available online at www.redjournal.org).

Histopathologic evaluation

Histopathologic examination was performed as described previously (8) and in Appendix E1 (available online at www.redjournal.org). A professional pathologist who was blinded to the animal groups semiquantitatively assessed the histologic changes of the lung tissues based on alveolar septum width, fibrous exudates, and inflammatory cell infiltration. Each section was scored from 0 (no pathologic changes) to 4 (marked pathology approaching maximal) (20). To evaluate collagen deposition, the cross-sectional area of lung tissues with positive Masson trichrome staining was measured by Image-Pro Plus software (version 5.1; Media Cybernetics, Rockville, MD). The data are expressed as a percentage of the collagen deposited area relative to the total lung tissue area examined under 4 to 5 microscopic fields for each tissue section as described previously (18).

Quantitative polymerase chain reaction assay

Lung tissue was homogenized in Trizol (ThermoFisher Scientific, Pittsburgh, PA) to isolate RNA according to the manufacturer's instructions. The expression of p16^{Ink4a} (*Cdkn2a*) messenger RNA (mRNA) and *Bcl2* mRNA was determined by quantitative polymerase chain reaction as previously reported (19). The expression of selective genes related to the induction of fibrosis—including interleukin 1 α (*Il1a*), interleukin 1 β (*Il1b*), tumor necrosis factor α (*Tnf*), tumor growth factor β (*Tgfb1*), matrix metalloproteinase 13 (*Mmp13*), tissue inhibitor of matrix metalloproteinase 1 (*Timp1*), type I α 2 collagen (*Col1a2*), and type III α 1 collagen (*Col3a1*)—was analyzed by the PAMM-120Z Mouse Fibrosis PCR Array (Qiagen, Germantown, MD). The data for each gene expressed in an experimental group are presented as relative expression (fold) in comparison with the Ct value for the control mice from each experiment.

Measurement of lung hydroxyproline content

Frozen lung tissues were homogenized in water. Collagen content in the homogenates was measured with a hydroxyproline colorimetric assay kit (BioVision, Milpitas, CA) according

to the manufacturer's instructions. The data are presented as micrograms of hydroxyproline per milligram of protein of the lung homogenates.

Senescence-associated β -galactosidase staining

Frozen lung sections (thickness of 6 μ m) were stained by use of a β -galactosidase staining kit (Beyotime Biotechnology, Beijing, China) as described in Appendix E1 (available online at www.redjournal.org).

In vitro studies

Primary pneumocytes were isolated from C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) as described previously (8) and in Appendix E1 (available online at www.redjournal.org). Enriched pneumocytes were exposed to 17 Gy of IR or were sham irradiated 24 hours after plating. Four days after IR, irradiated and control pneumocytes were incubated with vehicle or ABT-263 for 3 days to assess their viability and to quantify the percentage of senescent AECIIs (eg, senescence-associated β -galactosidase [SA- β -gal]-positive and prosurfactant protein C(Pro-SpC)-positive cells) as we reported previously (8) and in Appendix E1 (available online at www.redjournal.org).

Lung index

The lung index was determined by dividing the lung weight (in milligrams) by the body weight (in grams).

Statistical analysis

The data were analyzed by analysis of variance with the GraphPad Prism program (version 5.0; GraphPad Software, San Diego, CA). In the event that analysis of variance justified post hoc comparisons between group means, these were conducted by use of the Tukey multiple comparisons test. In addition, the Student t test was used for the data analysis between 2 groups. $P < .05$ was considered significant.

Results

Exposure of mice to thoracic irradiation induces persistent PF

As shown in our recent study (8), exposure of mice to a high dose (17 Gy) of thoracic irradiation induced persistent PF (Fig. 1), as evidenced by significant increases in the alveolar septum width, fibrous exudates, inflammatory cell infiltration (Figs. 1A and 1B; Fig. E1; available online at www.redjournal.org), the lung areas with collagen deposition (Figs. 1C and 1D; Fig. E2; available online at www.redjournal.org), and the hydroxyproline content in the irradiated lungs (Fig. 1E). The induction of persistent PF by IR was associated with upregulation of collagens, such as *Col1a2* and *Col3a1* (Figs. 1F and 1G). In addition, dysregulation of collagen degradation may have contributed to the persistent fibrosis after IR, because the expression of *Mmp13* (a major collagen-degradation enzyme) mRNA in the irradiated lungs was initially upregulated from 8 to 16 weeks after IR but then subsided at 23 weeks after IR (Fig. 1H). In contrast, the increased expression of *Timp1* (an inhibitor of matrix metalloproteinases) mRNA persisted until the end of the experiment (Fig. 1I).

Exposure of mice to thoracic irradiation induces persistent increase in SCs in lungs

In agreement with our previous findings (8), exposure of mice to 17 Gy of thoracic irradiation induced a persistent increase in SA- β -gal⁺ SCs in the lungs (Figs. 2A and 2B; Fig. E3; available online at www.redjournal.org). The increase in SCs was also confirmed by the upregulation of *Cdkn2a* mRNA expression (Fig. 2C). In addition, increased expression of *Bcl2* mRNA was detected in the irradiated lungs 16 and 23 weeks after IR (Fig. 2D), which is consistent with the observation that SCs, including those in fibrotic mouse lungs, are resistant to apoptosis in part via upregulation of *Bcl2* and/or Bcl-xl (9, 19). Because SCs also express the senescence-associated secretory phenotype (SASP) by producing increased levels of various inflammatory and profibrotic cytokines that are known to participate in the induction of pulmonary inflammation and fibrosis after IR, we measured the expression of SASP. The levels of *Tnf*, *Il1a*, and *Il1b* mRNA in the irradiated lungs were increased 8 weeks after IR and returned to control levels at 16 weeks after IR. The levels of *Il1a* and *Il1b* mRNA, but not those of *Tnf* mRNA, were also elevated in the irradiated lungs 23 weeks after IR. In contrast, the levels of *Tgfb1* mRNA in the irradiated lungs were significantly elevated only at 16 weeks after IR. Various types of cells including senescent AECIIs could contribute to the upregulation of some of these inflammatory cytokines (8, 17, 21).

ABT-263 can selectively kill IR-induced senescent AECIIs in vitro and clear SCs in mice after exposure to thoracic irradiation

Our recent studies showed that exposure of mice to a high dose of thoracic irradiation induced cellular senescence primarily in AECIIs (8, 17). Inhibition of IR-induced AECII senescence by administration of an nicotinamide adenine dinucleotide phosphate oxidase inhibitor immediately before or rPAI-1₂₃ prior to thoracic irradiation protected mice from PF (8, 17), suggesting that senescent AECIIs play an important role in mediating the induction of PF. However, whether senescent AECIIs also play a role in promoting the progression of PF is not known and therefore was investigated. First, we examined whether IR-induced senescent AECIIs are more sensitive to ABT-263 than nonsenescent AECIIs. Our group recently identified that ABT-263 is a senolytic drug, which can selectively kill various types of SCs via inhibition of the members of the *Bcl2* family. As shown in Figure 3A, the viability of nonirradiated mouse AECIIs was slightly reduced after they were cultured with 3- and 5- μ mol/L ABT-263. In contrast, incubation of irradiated AECIIs with 3- and 5- μ mol/L ABT-263 resulted in 50% and 75% reduction of viable cells, respectively, indicating that irradiated AECIIs are more sensitive to ABT-263 cytotoxicity than nonirradiated AECIIs. To determine whether the enhanced toxicity of ABT-263 in irradiated AECIIs is attributable to the induction of senescence after irradiation, we performed SA- β -gal and Pro-SpC staining on these cells after incubation with vehicle or 3- μ mol/L ABT-263 (Fig. 3B). As shown in Figures 3B and 3C, >40% of AECIIs stained positive for SA- β -gal 4 days after IR, whereas about 17% of nonirradiated AECIIs were positive for SA- β -gal staining, demonstrating that exposure to IR induced AECII senescence. After incubation with 3- μ mol/L ABT-263, the percentage of SA- β -gal⁺ cells in irradiated AECIIs was reduced to a level similar to that of nonirradiated cells. These findings suggest that IR-induced senescent AECIIs are more sensitive to ABT-263 than nonsenescent AECIIs.

To determine whether ABT-263 could clear IR-induced SCs in fibrotic lungs, we treated the irradiated mice with vehicle or ABT-263, 16 weeks after IR, when the mice exhibited significant increases in SCs in the irradiated lungs and persistent PF had already developed (Fig. 3D). The treatment with ABT-263 substantially reduced the numbers of SA- β -gal⁺ SCs in the irradiated lungs compared with the lungs from various control mice (Figs. 3E and 3F; Fig. E4; available online at www.redjournal.org). In addition, ABT-263 treatment significantly reduced the expression of *Cdkn2a*, *Bcl2*, *Il1a*, and *Il1b* mRNA (Fig. 3G–J). These findings suggest that ABT-263 can clear SCs in the fibrotic lungs induced by IR and that SCs contribute to the increased expression of *Il1a* and *Il1b* mRNA in the irradiated lungs at 23 weeks after IR.

ABT-263 treatment reversed PF

Finally, we examined whether clearance of SCs by ABT-263 treatment can delay the progression of PF or even reverse PF in mice. The mice were sham irradiated or were irradiated and then treated with vehicle or ABT-263 as shown in Figure 3D and euthanized at 23 weeks after IR, along with a group of mice that were euthanized 16 weeks after IR without any treatment. As shown in Figure 4A–G, as well as Figures E5 and E6 (available online at www.redjournal.org), 16 weeks after IR, severe PF developed in the irradiated mice, which continued progressing in vehicle-treated mice until the end of the observation. To our surprise, ABT-263 treatment reversed PF (Fig. 4A–G; Figs. E5 and E6; available online at www.redjournal.org). The reversal was attributable not only to the downregulation of *Col1a2* and *Col3a1* but also to the partial restoration of collagen degradation as evidenced by a reduction in *Timp1* expression with a simultaneous increase in expression of *Mmp13* after ABT-263 treatment compared with that observed in irradiated, vehicle-treated mice (Figs. 4J and 4K).

Discussion

PF represents one of the most deleterious late effects of thoracic radiation therapy. Many radioprotectors and mitigators, such as amifostine (22), enalapril (an angiotensin-converting enzyme inhibitor) (23), superoxide dismutase and catalase and superoxide dismutase—catalase mimetics (24–26), triptolide (27), MSX-122 (a C-X-C chemokine receptor type 4 antagonist) (28), and LY2109761 (a *Tgfb1* receptor 1 inhibitor) (29), can prevent or mitigate PF in rodents. However, these agents must be given to animals either before or shortly after IR to be effective. At present, there are no therapeutic agents that can be used to treat PF once PF becomes persistent. Therefore, the finding that treatment of irradiated mice with ABT-263 many weeks after exposure to IR can reverse PF is highly significant.

The mechanism by which ABT-263 reverses PF may be attributable primarily to its senolytic activity. This is because PF is associated with increases in SCs, particularly senescent AECIIs, in the irradiated lungs, and inhibition of senescence induction prior to thoracic irradiation prevents PF (8, 17). AECIIs are considered alveolar epithelial stem cells (30), and loss of AECIIs is sufficient to cause PF (31). When AECIIs become senescent after IR, they lose their ability to repair the damaged alveolar epithelium induced by IR. In addition, SCs produce increased levels of reactive oxygen species and secrete a plethora of inflammatory

mediators (eg, cytokines and chemokines) and extracellular proteases and tissue inhibitors—termed SASP—which may contribute to the pathogenesis of PF by causing chronic oxidative stress and inflammation and causing dysregulation of the extracellular matrix composition, structure, and function (13–16). Furthermore, SCs can induce senescence in neighboring cells through a paracrine mechanism, which can result in the self-propagation of PF (8). Therefore, senescent AECIIs may be the primary perpetrators of PF. We and other authors have shown that SCs, including senescent AECIIs, express a higher level of *Bcl2* and other Bcl family members and depend on these antiapoptotic proteins for survival (8, 9, 19, 32, 33). Therefore, SCs are more sensitive to *Bcl2* inhibition with an inhibitor such as ABT-263 than their nonsenescent counterparts as shown in this study. By removing SCs in the irradiated lungs, ABT-263 can potentially stop the perpetuation of PF. However, the optimal timing for the initiation of ABT-263 treatment and whether ABT-263 can also be used to prevent or mitigate PF have yet to be determined.

Using a senolytic drug such as ABT-263 to treat PF has several other potential advantages over known radiation protectants and mitigators. First, senolytic drugs target SCs, which may be the primary perpetrators that are fundamentally responsible for initiating and driving PF. Therefore, senolytic drugs should be more effective than radiation protectants and mitigators to prevent and mitigate PF, because most of the known radiation protectants and mitigators only target individual harmful molecules (eg, reactive oxygen species, a cytokine, or a chemokine) that may be produced by SCs to mediate PF. Second, SC accumulation may also be responsible for many other IR-induced late effects (34–37), including IR-induced skin fibrosis (38, 39) and cardiovascular diseases (40, 41), that can occur after thoracic radiation therapy as well. These late effects can also benefit from senolytic drug treatment similar to PF. In addition, unlike inhibition of cellular senescence, which may increase the risk of cancer and make tumor cells more resistant to IR, clearance of already formed SCs has the potential to lower the risk of secondary cancer induced by IR and improve the therapeutic efficacy of radiation therapy by permanently removing cells with oncogenic mutations and inhibiting SASP that can promote malignant transformation in neighboring cells and stimulate residual tumor cell regrowth and metastasis (13–16, 42, 43). Therefore, senolytic drug treatment may confer multiple benefits against PF. Although the finding that ABT-263 can reverse PF in a mouse model is very exciting, translating our finding into the clinic requires overcoming several obstacles. For example, ABT-263 was developed as an anticancer agent that has some toxic side effects (44). We have to develop a safer Bcl-2/xl inhibitor for the treatment of PF in the future.

Supplementary Material

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Acknowledgments—

This work was supported in part by grants from National Natural Science Foundation of China (81129020, 81372928, and 81573094) to A.M. and grants from NCI/NIH (R01CA122023) to D.Z. and NIGMS/NIH (P20GM109005) to D.Z. and M.H.-J. E.J.C. and D.E.C. were supported by the Intramural Research Program of NCI at NIH. Microarray experiments were performed by KangChen Bio-tech, Shanghai, China.

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Summary

Ionizing radiation-induced pulmonary fibrosis (PF) is an irreversible and severe late effect of thoracic radiation therapy. Our study shows that ABT-263, a specific Bcl-2/xl inhibitor and a newly identified senolytic drug that can selectively kill senescent cells, could reverse PF even when the PF had already become persistent in mice after thoracic irradiation. This finding suggests that ABT-263 has the potential to be used as an effective treatment for PF.

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Fig. 1.

Induction of pulmonary fibrosis (PF) in mice. (A) Representative images of hematoxylin-eosin staining of lung tissue sections. (B) Pathologic scores. (C) Representative images of Masson trichrome staining of lung tissue sections. Collagen appears blue; nuclei, purple; and cytoplasm of epithelial cells, pink. (D) Areas with collagen deposition after Masson trichrome staining of lung tissue sections. (E) Collagen content in lungs. (F-I) Expression of type I $\alpha 2$ collagen (*Col1a2*), type III $\alpha 1$ collagen (*Col3a1*), matrix metalloproteinase 13 (*Mmp13*), and tissue inhibitor of matrix metalloproteinase 1 (*Timp1*) messenger RNA (mRNA) in lung tissues. All data in the bar graphs are presented as mean $\pm$ standard error of the mean (with 6 mice per group for B and 3 mice per group for D-I). The scale bar equals 50 μ m. A letter *a* indicates $P < .05$ versus control (CTL); *b*, $P < .05$ versus 8 weeks after ionizing radiation (IR); and *c*, $P < .05$ versus 16 weeks after IR.

Fig. 2.

Exposure of mice to a high dose of thoracic irradiation induces a persistent increase in senescent cells in the lungs. (A) Representative images of senescence-associated β -galactosidase (SA- β -gal) staining of lung tissue sections. (B) Numbers of SA- β -gal⁺ cells per field. (C-H) Expression of *Cdkn2a*, *Bcl2*, tumor necrosis factor α (*Tnf*), interleukin 1 α (*Il1a*), interleukin 1 β (*Il1b*), and tumor growth factor β (*Tgfb1*) messenger RNA (mRNA) in lung tissues presented as relative expression (fold change) in comparison with lungs from control mice. All data in the bar graphs are presented as mean $\pm$ standard error of the mean with 3 mice per group. The scale bar equals 50 μ m. A letter *a* indicates $P < .05$ versus control (CTL); *b*, $P < .05$ versus 8 weeks after ionizing radiation (IR); and *c*, $P < .05$ versus 16 weeks after IR.

Fig. 3.

ABT-263 can selectively kill ionizing radiation (IR)-induced senescent type II alveolar epithelial cells (AECIIs) in vitro and clear senescent AECIIs in mice after exposure to thoracic irradiation. **Abbreviations:** EC₅₀ Z concentration for 50% of maximal effect; DAPI Z 4', 6-diamidino-2-phenylindole; ABT Z ABT-263; CTL Z control; PRE Z pretreatment; VEH Z vehicle. (A) Effect of ABT-263 on the survival of nonirradiated and irradiated AECIIs in vitro in comparison with vehicle-treated cells. (B) Representative images of senescence-associated β-galactosidase (SA-β-gal) and prosurfactant protein C (Pro-SpC) immunofluorescent co-staining of nonirradiated and irradiated AECIIs after incubation with vehicle or 3-μmol/L ABT-263. The blue color-stained cells in the bright field images represent SA-β-gal⁺ senescent cells. The red color-stained cells in the immunofluorescent images represent Pro-SpC⁺ AECIIs with DAPI nuclear staining in blue. (C) Percentages of SA-β-gal⁺ cells in nonirradiated and irradiated AECIIs after incubation with vehicle or 3-μmol/L ABT-263. (D) Diagram illustrating experimental design. (E) Representative images of SA-β-gal staining of lung tissue sections. (F) Numbers of SA-β-gal⁺ cells per field. (G-J) Expression of *Cdkn2a*, *Bcl2*, interleukin 1α (*Il1a*), and interleukin 1β (*Il1b*) messenger RNA (mRNA) in lung tissues presented as relative expression (fold change) in comparison with lungs from control mice. All data in the bar graphs are presented as mean ± standard error of the mean, with 5 samples for C and 3 mice per group for F through J. The scale bar equals 50 μm. The pound sign indicates *P* < .05 versus control; ampersand, *P* < .05 versus IR plus vehicle; *a*, *P* < .05 versus control; *b*, *P* < .05 versus pretreatment; and *c*, *P* < .05 versus vehicle.

Fig. 4.

ABT-263 treatment reversed pulmonary fibrosis in mice. C57BL/6J mice were irradiated and treated as described in Figure 3D. (A) Representative photographs of freshly collected and inflated mouse whole lungs. *Abbreviations:* ABT = ABT-263; CTL = control; PRE = pretreatment; VEH = vehicle. (B) Lung index. (C) Representative images of hematoxylin-eosin staining of lung tissue sections. (D) Pathologic scores. (E) Representative images of Masson trichrome staining of lung tissue sections. Collagen appears blue; nuclei, purple; and cytoplasm of epithelial cells, pink. (F) Areas with collagen deposition after Masson trichrome staining of lung tissue sections presented as a percentage of the total lung tissue areas examined. (G) Collagen content in the lungs expressed as micrograms of hydroxyproline per milligram of lung protein homogenates. (H-K) Expression of type I $\alpha 2$ collagen (*Col1a2*), type III $\alpha 1$ collagen (*Col3a1*), matrix metalloproteinase 13 (*Mmp13*), and tissue inhibitor of matrix metalloproteinase 1 (*Timp1*) messenger RNA (mRNA) in lung tissues presented as relative expression (fold change) in comparison with lungs from control mice. All data in the bar graphs are presented as mean $\pm$ standard error of the mean (with 6 mice per group for B and D and 3 mice per group for F-K). The scale bar equals 2 mm for A and 50 μ m for C and E. A letter *a* indicates $P < .05$ versus CTL; *b*, $P < .05$ versus PT; and *c*, $P < .05$ versus VEH.