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Research Article

Anti-Fibrotic Effect of REV-5901 in Bleomycin-Induced Pulmonary Fibrosis

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Abstract

Pulmonary fibrosis (PF) is a progressive lung disease and has a bad prognosis with median survival ranging from 2.5 to 3.5 years. Leukotrienes which are eicosanoid lipid mediators synthesized from arachidonic acid by 5-lipoxygenase (5-LOX) enzyme have been associated with PF. REV-5901 is potent 5-LOX inhibitor; however, its role in PF has not been studied. We demonstrate that REV-5901 has an inhibitory effect on bleomycin (BLM)-induced PF in both in vitro and in vivo models. REV-5901 reduced expression of BLM induced key fibrotic markers in both cells and animal samples. Histopathological analysis of lung tissue demonstrated an alleviating effect on fibrosis with inhibitor treatment. We also observed downregulation of 5-LOX, pAkt and VEGF expressions upon treatment with inhibitor indicating involvement of both lipid and angiogenic pathways in the regulatory effect of REV-5901. Overall, our data suggests REV-5901 demonstrates an anti-fibrotic effect on BLM-induced PF, which warrants further investigation.

Keywords: Pulmonary fibrosis, Bleomycin, Rev-5901, 5-lipoxygenase, VEGF

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Idiopathic pulmonary fibrosis (IPF) is a chronic fibrotic disease of the lung. A variety of insults to the lung such as toxic,[1] autoimmune,[2] drug-induced e.g. bleomycin (BLM),[3] infectious,[4] or traumatic injuries[5] results in IPF. The constant duress from any of these factors triggers deregulated pro-inflammatory and pro-fibrotic healing response involving cytokine production and activation of fibroblasts leading to fibrotic tissue development in the interstitial lung spaces. This constant build of the scar tissue results in progressive decline in pulmonary function and ultimately respiratory failure.

Several lipids and their metabolites have been shown to play a critical role in the IPF.[6, 7] Aberration in the metabolism of fatty acids, phospholipids, prostaglandins (PDGs) especially prostaglandin 2 (PDG2) and prostaglandin E2 (PGE2), leukotrienes (LT), and sphingolipids is associated with IPF.[8] Elevated levels of palmitic acid were detected in the lungs of patients with IPF compared with control subjects.[9] Depletion of hematopoietic PGD synthase (H-PGDS) which synthesizes PDG2 in hematopoietic lineage cells were reported to accelerate BLM-induced PF and increase in vascular permeability.[10] A recent study found secretion of LTs from senescent lung fibroblasts promotes lung fibrosis. The LT-rich conditioned media of senescent lung fibroblasts (IMR-90) induced pro fibrotic signaling in naive fibroblasts.[11] LTs have been associated with progression of fibrosis in various fibrosis models.[12-14]

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LTs are eicosanoid lipid mediators synthesized from arachidonic acid. This biosynthesis is catalyzed by 5-lipoxygenase (5-LOX) mediated by 5-lipoxygenase activating protein (ALOX5AP/FLAP) which is believed to selectively translocate arachidonic acid to 5-LOX.[15] 5-LOX is a nonheme iron dioxygenase which depending on cell type is either located in nucleus or cytosol. Arachidonic acid is oxygenated to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) by 5-LOX which is further dehydrated to an unstable epoxy leukotriene LTA4. It is then hydrolyzed to pro-inflammatory LTB4. In another metabolic transformation LTA4 is conjugated with glutathione to form LTC4 by LTC4 synthase. LTC4 is subjected to extracellular metabolism to form LTD4 and LTE4.[16-18] These three LTs are also known as cysteinyl leukotrienes.

Role of LTs in IPF is understudied. However, there is evidence that suggests that they may be playing a part in IPF making 5-LOX a therapeutic target for IPF treatment.[11] There are several 5-LOX inhibitors which can be divided in four categories based on their inhibitory mechanism – a) redox (Zileuton, Atreleuton etc.), b) non-redox (PF-4191834, Setileuton etc.), c) iron chelators (Zileuton, Atreleuton, Fenleuton (A-76745) etc.), and d) allosteric.[19] Zileuton is the only FDA approved 5-LOX inhibitor drug for asthma. REV-5901 is a quinolin based non-redox 5-LOX inhibitor.[20] It has been shown to have inhibitory effects on the release of LTs and histamine from human lung tissue in-vitro indicating it may be useful in lung disorders such as asthma.[21] Another study demonstrated inhibitory effect of REV-5901 on the proliferation of RAW 264.7 macrophage cells through regulation of LTs.[22] Even though there is clear evidence of regulation of LTs by REV-5901 via 5-LOX, its use as a potential IPF therapeutic intervention has not been studied.

In this study, we investigated the role of REV-5901 in BLM-induced pulmonary fibrosis. We demonstrate that REV-5901 significantly inhibited BLM-induced fibrosis in both in vitro and in vivo models. We observed significant amelioration of key fibrogenesis markers upon treatment with REV-5901 in CRL-1490 cells. Histopathological analysis of mice lung tissue demonstrated a clear and significant reduction in bleomycin-induced fibrosis with REV-5901 co-treatment. In addition, REV-5901 significantly reduced 5-LOX levels and VEGF levels in animal samples. Also, a significant reduction in the pAkt expression upon treatment with the inhibitor was observed in in vitro samples. We had previously reported that VEGF plays a key role in regulating BLM - induced PF via PI3K/Akt pathway. Results of this study indicate involvement of multiple cellular pathways including alteration of lipid metabolism and modulation of angiogenesis as potential mechanisms involved in protective effect of REV-5901.

Methods

Chemicals and Reagents

REV-5901 was purchased from Santa Cruz Biotechnologies (Dallas, TX) and BLM was obtained from Sigma-Aldrich (St Louis, MO). Antibodies for Col1A1, α-SMA, Akt, pAkt and GAPDH were obtained from Cell Signaling Technology (Danvers, MA). Antibody for Fibronectin-1 was purchased from Abnova (Taipei City, Taiwan) and 5-Lipoxigenase (5-LOX) antibody was obtained from Santa Cruz Biotechnologies (Dallas, TX). Human VEGF, Fibronectin-1 and pro Col1A1 ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Cell Culture

Human lung CRL-1490 fibroblasts (ATCC; Manassas, VA) were maintained in Eagle's Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin (Hyclone), 100 mg/ml streptomycin (Hyclone) and Amphotericin B (Invitrogen). Cells were cultured at 37°C in 5% CO2 incubator, and were passaged at preconfluent densities using a solution containing 0.25% trypsin (Sigma).

Animal Model

For the animal studies, 6–8 weeks old C57BL/6 mice were used (Jackson Laboratories, Bar Harbor, ME). Mice were housed in a barrier facility with specific pathogen-free conditions, and all experiments were performed using protocols approved by the Old Dominion University (ODU) animal facility. Mice were divided in groups of five animals each based on number of treatments. First animal study was performed to access the time and dose dependent fibrotic response of BLM treatment on mice. Various groups were either treated with saline (control) or 0.3, 1 and 3 U of BLM dissolved in saline for 2, 4 and 6 weeks respectively. To assess the effect of REV-5901, mice were treated with assigned doses of REV-5901 +/- BLM for either 14 or 28 days. In both of these studies prior intranasal instillation of BLM or saline (control) was carried out for animals under isoflurane anesthesia. Starting from Day 8, animals in groups designated for REV-5901 treatment received intraperitoneal injections of respective doses of Rev-5901 every day (14 day study) or every other day (28 day study). On the last day of all the studies, all animals were euthanized. Lungs were perfused with 5 ml of cold saline through the left ventricle and surgically removed. The left lungs were used to evaluate the fibrotic score by histological examination and for immunohistochemistry staining and the right lungs were homogenized to analyze for protein expression levels and other biochemical assays.

Histopathology

Mice were euthanized and the left lung was fixed with 10% formalin overnight and embedded in paraffin. Paraffin sections (4 µm thick) were stained with hematoxylin-eosin (H&E) and Masson Trichrome stain. All sections were studied using light microscopy (EVOS XL Core) using 10x objective. The severity of fibrosis was semi-quantitatively assessed using Ashcroft method.[23] The grade of lung fibrosis was assigned on a scale of 0-8 by examining six randomly chosen fields per sample.

Western Blot Analysis

Western blot analysis was performed as described previously.[24] Briefly, Mice lung homogenates or cell lysates were resolved on a 6% and 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDSPAGE) and transferred onto a nitrocellulose membrane. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL), and equal amount of protein was loaded per sample. The membrane was blocked with TBS-T (0.1% Tween-20 in TBS) containing 5% dry milk, and incubated with primary antibody overnight at 4°C. After three washes with TBS-T, the membrane was incubated with HRP-conjugated secondary antibody for 1 h and then washed with TBS-T. Immunoreactive proteins were detected by chemiluminescence (Supersignal1 West Pico, Pierce, Rockford, IL) and quantified by imaging densitometry, using myImageAnalysis Software (Thermo). Mean densitometry data from independent experiments was normalized to results obtained from untreated control cells.

Enzyme-Linked Immunosorbent Assays (ELISA)

The expression levels of Collagen-1, Fibronectin-1 and VEGF proteins were quantified using ELISA essay. Supernatant from treated cells or tissue lysates from saline, BLM and REV-5901 treated mice were collected and analyzed for Collagen-1, Fibronectin-1 and VEGF protein levels using respective Quantikine ELISA kits (R&D Systems, Minneapolis, MN) per manufacturer's protocol. Briefly, samples or reference standards (100 µl) were added to each well of a microplate pre-coated with monoclonal antibody specific to the target proteins being analyzed and incubated overnight at 4°C. After washing out unbounded proteins, an HRP-conjugated polyclonal secondary antibody was added to the wells and incubated for 2 h at room temperature. After washing and adding 100 µl of substrate solution, optical density was determined at 450 nm (Synergy HI Hybrid Reader, BioTek).

Statistical Analysis

The data represent mean±S.E.M from three or more independent experiments. Statistical analysis was performed using Student's t-test to evaluate the measurements at a significance level of $p<0.05$.

Results

BLM Induces Fibrosis in vitro and in vivo

CRL-1490 cells were treated with different doses of BLM (1 - 25 mU) for 24h and cell lysates were analyzed by Western blotting (WB) for key fibrotic markers such as Fibronectin-1, Col1A1 and α-SMA (Fig. 1A). BLM clearly induced fibrosis as observed by increased expression of the aforementioned proteins upon BLM treatment. This effect may not necessarily follow a dose response trend as a clear dose dependent change in expression was not observed for all the proteins. Time and dose dependent fibrotic effects of BLM were accessed in in vivo model. Mice were treated with saline (control) or 0.3, 1 and 3 U BLM for 2, 4 and 6 weeks. At the end of the trial animals were sacrificed and lung tissue was analyzed histopathologically by H&E staining to deter-

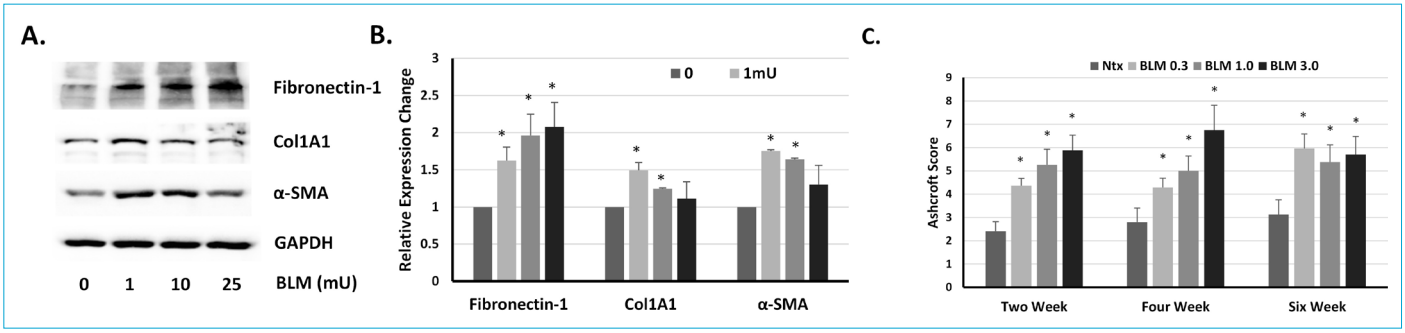

Figure 1. BLM induces fibrosis in both in vitro and in vivo models. **(a)** CRL-1490 cells were incubated with indicated doses of BLM for 24 hrs and cell lysates were analyzed for key fibrogenesis markers by WB. The blot is representative of at least three independent experiments. **(b)** Densitometric analysis of immunoblots to determine the relative expression levels of proteins in response to BLM treatment **(c)** Mice were treated with 0.3, 1 and 3 U/kg doses of BLM or saline control for 2, 4 and 6 weeks. Lung tissues were analyzed by H&E staining and images were semi quantified by assigning Ashcroft scores to determine induction of fibrosis in response to BLM treatment. At least five different images per group were analyzed for quantification. Data are mean ± S.E.M (n≤3). *, $p < 0.05$ versus non-treated control.

mine the extent of fibrosis. These images were semi quantified and assigned a fibrotic score between 0-8 by Ashcroft method (Fig. 1B). We observed a time and dose dependent increase in fibrosis in mice with BLM treatment.

REV-5901 Alleviates BLM-Induced Fibrosis in CRL-1490 Cells

CRL-1490 cells were pretreated with REV-5901(1, 10 and 25 μM) for an hour followed by treatment with BLM (10 mU) for 24h and 72 h respectively (Fig. 2A and 2B). WB analysis of important fibrosis markers clearly demonstrated dose-dependent down regulation in response to REV-5901 treatment. Only a single dose of 10uM REV-5901 was used for 72h treatment which reproduced anti fibrotic effects of 24h treatment with the inhibitor. To further validate these results the supernatant of CRL-1490 cells treated for 72 h was analyzed for soluble fibrotic markers Fibronectin-1 and Col1A1 by ELISA essay (Fig. 2C). A significant reduction in the levels of these important fibrotic markers was observed with REV-5901 treatment supporting the anti-fibrotic efficacy of the inhibitor observed by WB analysis.

REV-5901 Demonstrated a Significant Anti-Fibrotic Effect in Mouse Model

Inhibitory effect of REV-5901 on BLM-induced pulmonary fibrosis was verified in an animal model. C57BL/6 mice

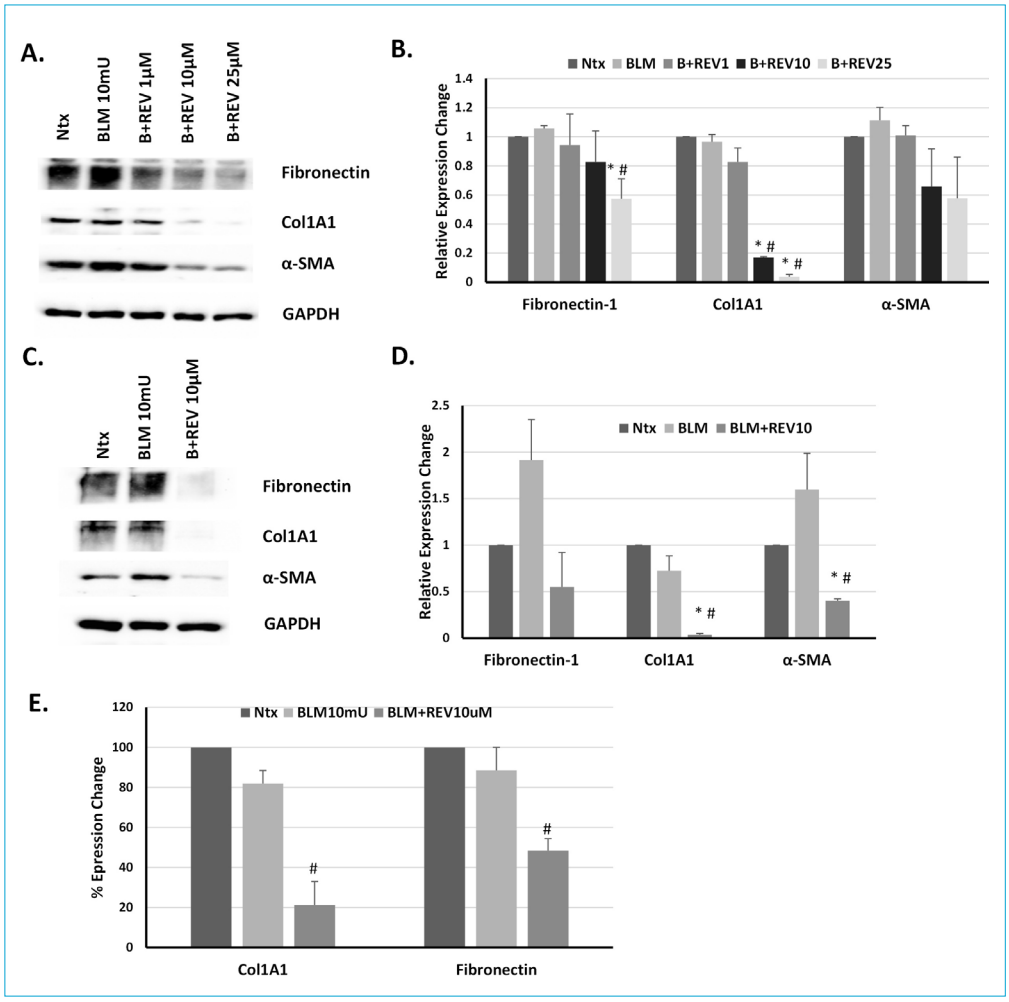

Figure 2. REV-5901 alleviates BLM-induced fibrogenesis in CRL-1490 cells. CRL-1490 cells were pretreated with indicated doses of REV-5901 for 1 hr followed by 24 hr treatment with BLM. **(a)** Samples were collected and analyzed for fibrogenesis marker proteins. **(b)** Densitometric analysis of immunoblots to determine the relative expression levels of proteins in response to BLM treatment **(c)** Cell lysates were collected after 72 hrs of treatment and analyzed by WB. **(d)** Densitometric analysis of immunoblots to determine the relative expression levels of proteins **(e)** Supernatant of the cells treated with indicated doses of BLM and REV-5901 was analyzed to determine the levels of soluble fibrogenesis markers by ELISA assay. Data are mean±S.E.M (n≤3). *, p<0.05 versus non-treated control. #, p<0.05 versus BLM treatment.

were treated with BLM 1U/Kg followed by treatments with two different doses of REV-5901(1mg/Kg and 10mg/Kg). Animals were sacrificed on day 28 and histopathological analysis of tissue was performed. Trichrome staining (Fig. 3A) of lung tissue clearly indicated induction of fibrosis on treatment with BLM (Fig. 3A-ii) and a significant reduction in fibrotic tissue upon treatment with the REV-5901(Fig. 3A-iv and 3A-vi). The extent of lung fibrosis was determined by quantitative histology according to Ashcroft's method which indicated a much potent and significant anti-fibrotic response for 1mg/kg dose of REV-5901 compared to 10mg/kg dose (Fig. 3B). A short 14 day animal trial was carried out to evaluate time dependent antifibrotic potency of REV-5901. Only 1mg/Kg dose of inhibitor was used for this study as this dose showed better results in 28 day study. Histopathology and Ashcroft quantification (Fig. 4A and 4B) of lung tissue demonstrated a significant reduction in fibrosis in BLM treated mice upon treatment with inhibitor further establishing REV-5901 1mg/Kg treatment as a potential standard dose. All the treatments were well tolerated by mice as no significant loss in body weight was observed during the course of treatment (Fig. 3C and 4C).

PI3K/Akt> VEGF Pathway May be Involved in REV-5901 Induced Anti-Fibrotic Effects

REV-5901 is a known 5-LOX inhibitor. In fact, WB analysis of tissue lysates from animal trials indicated a reduction in 5-LOX expression in animals treated with REV-5901, however, this effect was not as consistent in the BLM+REV-5901 treated animals (Fig. 5a and 5b). We also observed a reduced expression of pAkt in CRL-1490 cells treated with the inhibitor (Fig. 5c). ELISA essay evaluation of tissue lysates showed a significant reduction in Col1A1 levels (Fig. 5D) on treatment with REV-5901 as observed in in vitro samples (Fig. 2C) further verifying our in vitro findings. We also observed a significant reduction in the levels of VEGF in the tissue lysates with the treatment of REV-5901 (Fig. 5E). VEGF is a key angiogenic marker and have been shown to be associated with pulmonary fibrosis via PI3K/Akt pathway.[24] It is plausible that an altered lipid metabolism in conjunction with reduction in angiogenesis via PI3K/Akt>VEGF pathway may be involved in the anti-fibrotic response of REV-5901.

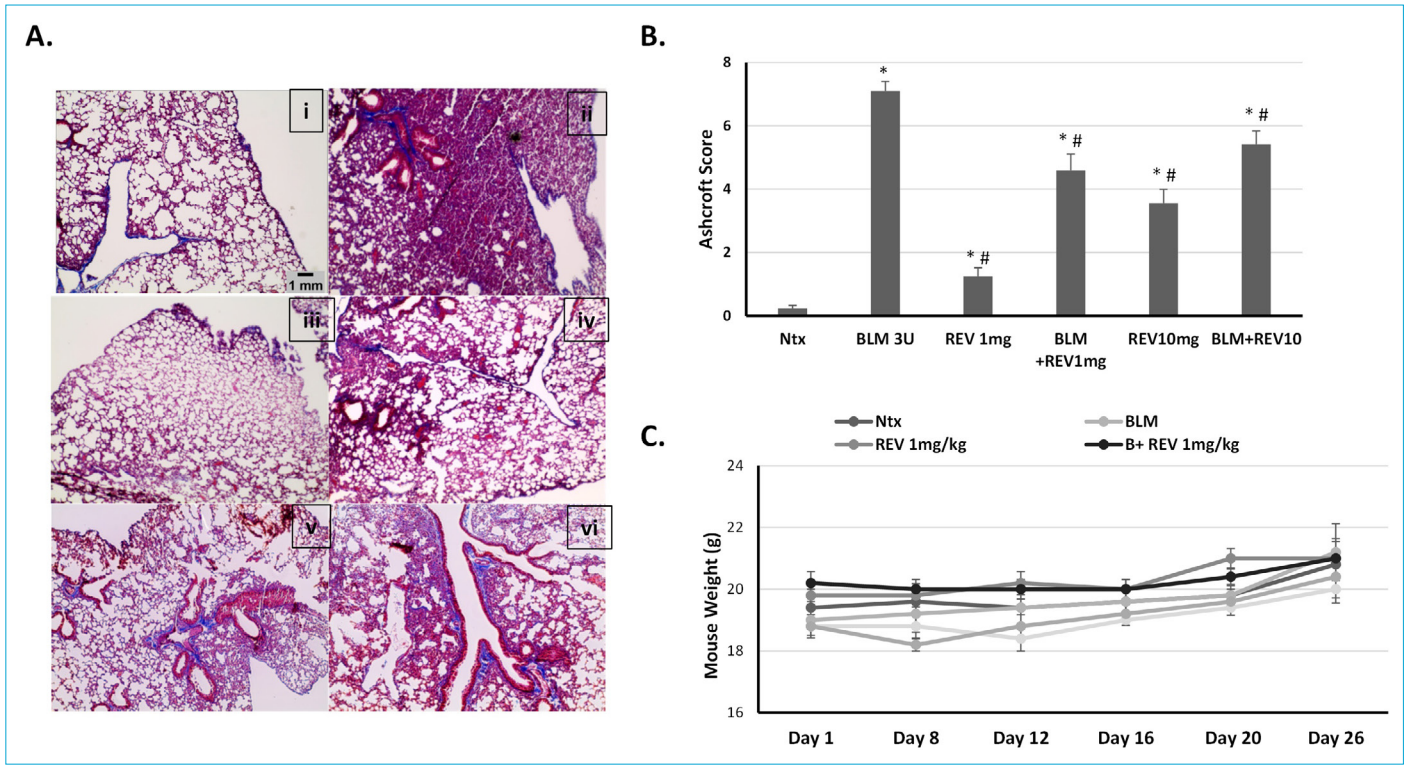

Figure 3. Rev-5901 alleviates BLM-induced lung fibrosis in C57BL/6 mice in 28 day trial. **(a)** Representative trichrome stained lung sections of mice treated with i) saline ii) bleomycin 1U/kg iii) Rev-5901 1 mg/kg iv) bleomycin 1U/kg + Rev-5901 1 mg/kg v) Rev-5901 10 mg/kg vi) bleomycin 1U/kg + Rev-5901 10 mg/kg **(b)** Semi quantitative analysis of fibrosis by Ashcroft method for mice treated for 28 days and **(c)** Mouse weight variation in different treatment groups indicating no serious weight loss in response to treatments . Data are mean ± S.E.M (n≤3). *, $p < 0.05$ versus non-treated control. #, $p < 0.05$ versus BLM treatment.

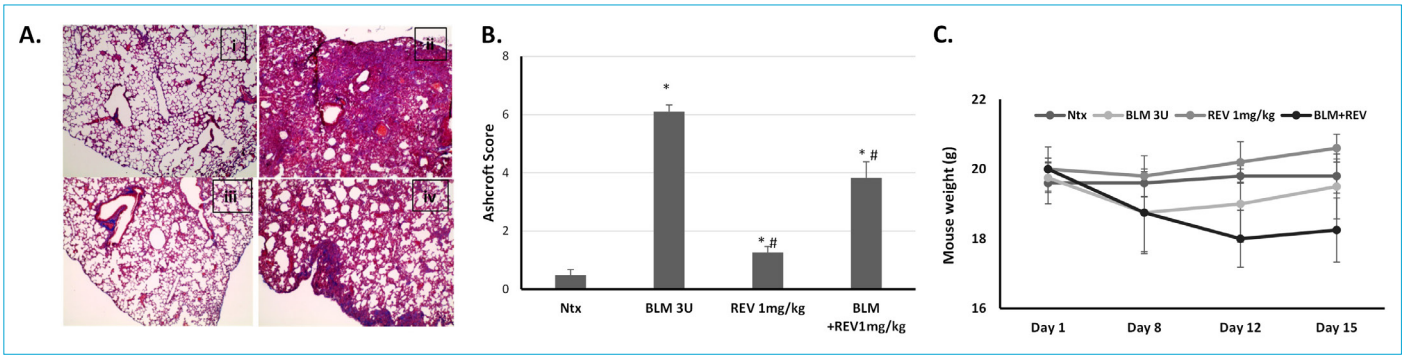

Figure 4. Rev-5901 alleviates bleomycin-induced lung fibrosis in C57BL/6 mice in 14 day trial. **(a)** Representative trichrome stained lung sections of mice treated with i) saline ii) bleomycin 1U/kg iii) Rev-5901 1 mg/kg iv) bleomycin 1U/kg + Rev-5901 1 mg/kg **(b)** Semi quantitative analysis of fibrosis by Ashcroft method for mice treated for 14 days and **(c)** Mouse weight variation in different treatment groups indicating no serious weight loss in response to treatments for 14 days. Data are mean±S.E.M (n≤3). *, p<0.05 versus non-treated control. #, p < 0.05 versus BLM treatment.

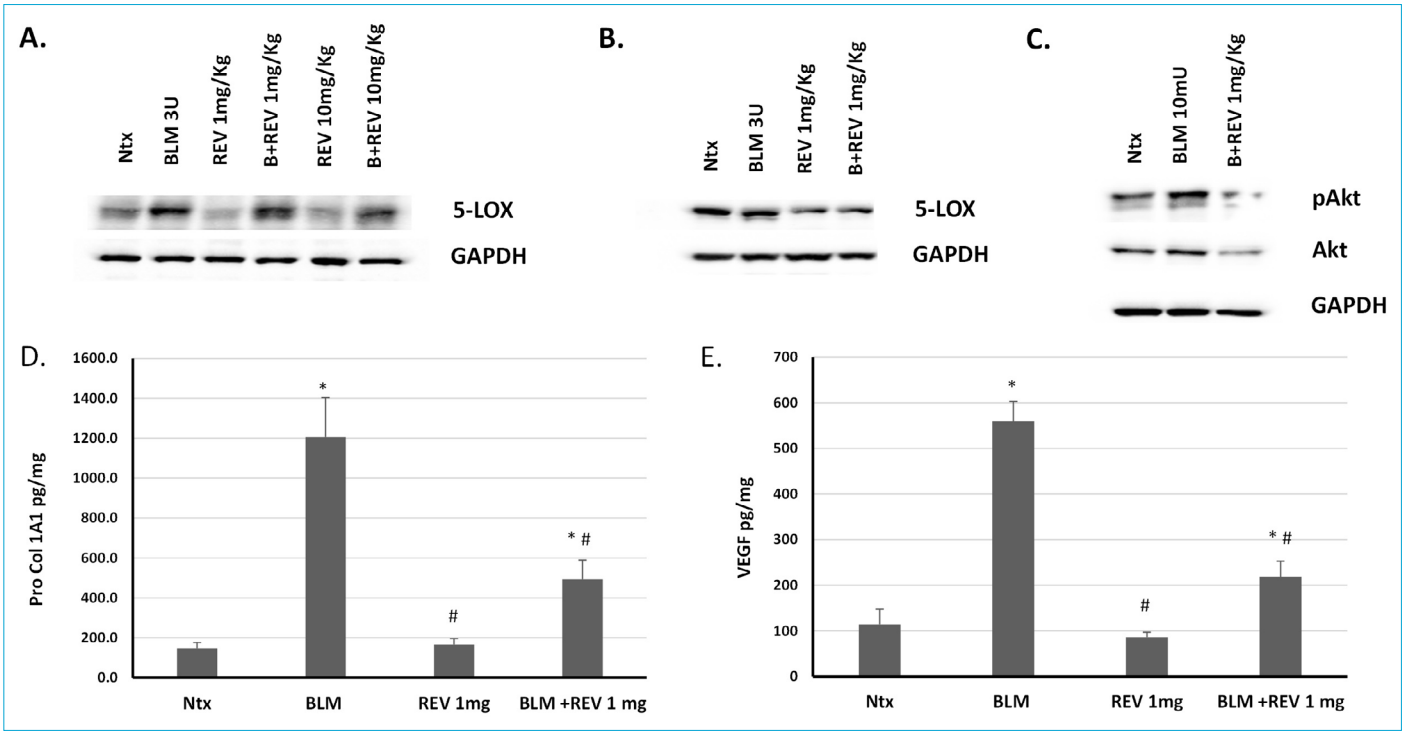

Figure 5. Mechanistic evaluation of anti-fibrotic effects of REV-5901 on BLM-induced fibrosis. REV-5901 targeted 5-LOX in animal trials and inhibited its expression. This inhibition was very clear in REV-5901 treated mice, however it was not as obvious in BLM+REV-5901 treated animals **(a)** 28 day trial **(b)** 14 day trial **(c)** PI3K/Akt pathway may be involved in curative response upon treatment with REV-5901 as a reduction in pAkt level was observed in the CRL-1490 cells treated with the inhibitor **(d)** Inhibitory effect was mediated by significant reduction in Col1A1 levels in the tissue lysates (14 Day trial) as observed by ELISA essay **(e)** VEGF an important angiogenic marker is also involved in REV-5901 induced anti-fibrotic response as evaluation of tissue lysates (14 Day trial) by ELISA assay showed a significant reduction in VEGF levels on treatment with the inhibitor. Data are mean ± S.E.M (n≤3). *, p < 0.05 versus non-treated control. #, p < 0.05 versus BLM treatment.

Discussion

Several studies indicated a potential role played by aberrant lipid metabolism in progression of IPF.[8, 25] Various lipid metabolites such as fatty acids, phospholipids, prostaglandins (PDGs), leukotrienes (LT), and sphingolipids have been shown to be prominently involved in IPF.[26] LTs are eicosanoid lipid mediators synthesized from arachidonic acid by 5-LOX and have been shown to play some role in IPF development.[12] However, role of 5-LOX as potential therapeutic target in IPF has not been explored much. In this study REV-5901 which is a known 5-LOX inhibitor was investigated to study its potential inhibitory effect on IPF. Our data indicates a time and dose dependent amelioration of

fibrogenetic effect of BLM in CRL-1490 cells upon treatment with REV-5901. A significant reduction of key fibrogenic markers such as Fibronectin 1 and Col1A-1 was observed by both WB and ELISA assays (Fig. 2). These results were replicated in animal trials as well. Histopathological analysis of lung tissue indicated a significant reduction of fibrotic legions in the REV-5901 treated animals both in 14 and 28 day trials (Fig. 3a and 4a). A dose of 1mg/Kg of REV-5901 was found to be more effective than a higher dose in 28 day animal trial indicating saturation of dose response at a lower concentration (Fig. 3b). Same dose was found to be very effective against fibrosis in a shorter 14 day study (Fig. 4b). REV-5901 is well tolerated by the animals as no significant weight loss was observed in 14 day as well as 28 day animal trial (Fig. 3c and 4c).

REV-5901 definitely targeted 5-LOX as we observed a clear reduction in 5-LOX expression for the animals treated with the inhibitor alone, however, it may not be the only reason for the remission of fibrosis in the animals as we did not observe a clear reduction in 5-LOX expression in BLM+REV-5901 treated animals (Fig. 5a and 5b). Consistent with our in vitro data, analysis of lung tissue lysates by ELISA assay demonstrated a significant reduction in a key fibrotic marker Col1A1-1 on inhibitor treatment (Fig. 5d). Interestingly, we also observed a significant reduction in the expression of VEGF in tissue lysates (Fig. 5e). VEGF is a significant player in angiogenesis and several studies have indicated that an increase in VEGF expression played an instrumental role in IPF progression.[27] In one of our previous studies we showed that BLM induced angiogenesis played a key role in fibrosis via the regulation of VEGF through PI3K/Akt pathway.[24] We also observed a reduction in pAkt expression in CRL-1490 cells upon treatment with REV-5901(Fig. 5c) indicating a role of PI3K/Akt>VEGF pathway in the REV-5901 mediated regulation of fibrosis.

Overall, REV-5901 alleviated BLM induced fibrosis via down regulation of key fibrotic markers both in in vitro and in vivo models. This effect may be, in part, due to the regulation or alteration of lipid metabolism by the inhibition of 5-LOX on REV-5901 treatment. However, it may not be the only regulatory mechanism and inhibitory effects of REV-5901 may also be supplemented by regulation of VEGF PI3K/Akt pathway as we observed a significant reduction in the VEGF and pAkt expressions on treatment with the inhibitor. To our knowledge, this is the first report showing role of a 5-LOX inhibitor in bleomycin-induced pulmonary fibrosis and may pave way for future investigations on the roles of lipids in pulmonary fibrosis. Further understanding of the underlying mechanism for anti-fibrotic effects of REV-5901 is warranted. An in depth inhibitory mechanistic analysis will provide more insight in to the role of lipoxygenases in pulmonary fibrosis.

Disclosures

Ethics Committee Approval: This study was approved by Old Dominion University (ODU) Institutional Animal Care and Use Committee. (Date: August 16, 2021 IACUC protocol #21-008).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – N.A.; Design – N.A., K.F., J.S.Y., A.K.V.I.; Supervision – N.A.; Materials – K.F., J.S.Y., A.K.V.I.; Data collection &/or processing – V.K.; Analysis and/or interpretation – K.F., J.S.Y., A.K.V.I.; Literature search – K.F., J.S.Y., A.K.V.I.; Writing – V.K.

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