HDAC Inhibition Promotes Fibroblast Apoptosis and Ameliorates Pulmonary Fibrosis in Mice

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MATERIALS AND METHODS

Cell Culture and Treatments

This study was approved by the UAB Institutional Review Board. Primary IPF fibroblasts were from the University of Alabama at Birmingham Tissue Procurement Facility, or as generous gifts from Dr. Carol Feghali-Bostwick (Univ. of Pittsburgh). Non-IPF control cells were from the University of Alabama at Birmingham Tissue Procurement Facility. The cells were cultured in 10% fetal bovine serum (FBS) Dulbecco's modified Eagle medium (Invitrogen), with 1% penicillin/streptomycin. The cells were seeded at a density of $3x10^4$ cells/well in 6-well plates or as indicated, when cells were near 80% confluent, the culture medium were changed into 1% FBS medium, and SAHA of 100nM or 200nM or as indicated were added in the culture for 60h. SAHA was purchased from Sigma Aldrich (St Louis, MO), dissolved in dimethyl sulfoxide (DMSO) and stored at -80C. For control cells, DMSO only (0 SAHA) was added.

Apoptosis assays: Annxin-V FITC

The cells were prepared as above and when 80% confluence was reached, cells were changed to 1% FBS medium, and SAHA or DMSO was added as indicated. Cells were collected at the end of 60h of SAHA treatment. Cells were evaluated for apoptosis by flow cytometry using an Annexin-V FITC kit (MBLI, Woburn, WA) as previously described (11).

Cell Proliferation Assay

A cell proliferation assay kit (MBLI, Woburn, MA) was used to assess the proliferation of IPF lung fibroblasts without or with SAHA treatment according to the manufacturer protocol.

Caspase-3 Assay

Caspase-3 activity assay was measured by using an established kit at the end of 60h treatment of SAHA as above, according to the manufacturer's protocol (MBLI, Woburn, WA).

DNA/RNA/Protein/Nuclear Extract and Quantitative real-time RT-PCR

DNA, RNA and protein were extracted by Allprep kit (Qiagen, Valencia, CA). 1µg RNA was transcribed to cDNA using a synthesis kit (Clontech, Mountain View, CA). Nuclear proteins were extracted by using the EpiQuick Nuclear extraction kit (Epigentek).

All real-time RT-PCR were performed in triplicates and normalized to 18S with ΔΔCt method as previously done (12).

Antibodies

Antibodies against Bid (#2002), Bcl-2 (#2870), β-actin (#3700) were from Cell signaling, anti-Bok (sc-11424), anti-Col1A1 (sc-28657) from Santa Cruz Biotech, p16 (cat#554079) from BD Biosciences. β-tubulin or β-actin was used as loading control for whole cell lysate. Total histone H3 or H4 was used as loading control for nuclear extracts.

Immunofluorescence staining

Fibroblasts were cultured on coverslips, grown to 50% confluence, SAHA then added at 100nM for 60 h. Control cells had a similar amount of vehicle (DMSO) without SAHA. The cells were then fixed, permeabilized, washed, blocked, then incubated with anti-H3K9Ac or anti-H3K9Me3 antibody at 1:800, followed by FITC-conjugated secondary antibody as previously done (11). The slides were examined with a Zeiss Axiovert 200M fluorescence/phase microscope and analyzed with Axiovision LE software (Carl Zeiss International, Germany).

DNA methylation changes in apoptosis related genes

Briefly, one part of the DNA was digested with BstuI and HapII restriction enzymes (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. At the promoter region where the CpG islands are located, at least 3 cutting sites from the restriction enzymes were found between the PCR primers. Another equal part of DNA was used as mock digestion

for control according to the OneStep qMethyl kit. Specific primers were designed for selected methylated regions of DNA to have at least 3 restriction enzyme-cutting sites to measure the difference of cycle threshold value depending on methylation status. After digestion, the DNA from both samples was amplified using real-time PCR and percentage of methylation changes were calculated following the protocol.

Animal Model, lung function analysis, immunohistochemistry and Immunoblotting

Eight mice were initially allocated to each group. Bleomycin sulfate (Almirall, Reinbek, Germany) was dissolved in sterile normal saline and administered at a single dose of 3U/kg body weight intratracheally, by instillation with a Stepper Repetitive Pipette (Tridak, DYMAX Corporation, Torrington, CT). SAHA dissolved in HOP-β-CD solution (15) was fed at 20mg/kg every other day by mouth with a pipette tip starting day 10 till day 27 post bleomycin injury to a group of mice subject to bleomycin injury, and another group of mice subject to saline at the same dosage and schedule for control; vehicle (HOP-β-CD) only was given on the same schedule as SAHA to another group of bleomycin injured mice.

Right before sacrifice, mice were sedated with ketamine/xylazine and pulmonary function was evaluated on a flexivent apparatus (SCIREQ, Montreal, Canada). Briefly, an 18-G blunt needle was inserted into the trachea and fixed with a ligature of 3-0 silk. The flexiVent equipped with a module 1 was used to perform measurement maneuvers including perturbations (predefined pressure of volume waveforms) such as forced oscillations, using room air in the closed-chest animal. The tidal volume was set at 6 ml/kg, with a respiratory rate of 150/min. Measurements made included compliance (C), airway resistance (Rn or Raw; Newtonian resistance, which is primarily the resistance of the central or upper airways). Calibration of the flexiVent was done

using the tracheal cannula to be used, before each experiment. Lung volumes were measured by volume displacement after completion of the flexiVent measurements.

Mice were then euthanized and the lungs were either inflation fixed for histology, or lysate for immunoblots. The lungs were inflation fixed via the trachea with 10% formalin at 20 cm H_2O pressure, and the right ventricle was perfused with formalin at the same pressure until the effluent was clear to flush out the blood in the pulmonary vessels. After formalin fixation for 24h, samples were changed to 70% ethanol to avoid over-fixation. The lung tissues were then sent for paraffin-embedment. Antigen retrieval was performed on paraffin-embedded sections by heating in pH6.0 citrate buffer for 20min. The collagen III primary antibody (anti-Col3A1, cat#49-394, from ProSci Incorporated, Poway, CA) was used at 1:600; H3K9Ac was used at 1:1600 dilutions; Bcl-xL at 1:800. For Col3A1, staining was developed with biotinylated anti-rabbit secondary antibodies, after washes, alkaline phosphatase-conjugated streptavidin was added. Color development was performed using vector red AP substrate (Vector Labs, Burlingame, CA). Slides were counterstained with hematoxylin QS. For H3K9Ac or Bcl-xL, staining was developed with biotinylated anti-rabbit secondary antibody, after washes, peroxidase-conjugated streptavidin was added; 100ul DAB was added to develop brown coloring. Nuclei were counterstained with hematoxylin QS. Images were obtained with a Nikon TE2000U microscope equipped with a QiCam Fast Cooled high-resolution CCD camera with MetaMorph software (v.6.2r4, Universal Imaging, West Chester, PA).

Figures

Figure S1. Characterization of non-IPF and IPF primary fibroblasts, apoptosis analysis and apoptotic gene expressions in non-IPF/IPF fibroblasts

A. Whole cell lysate from primary non-IPF or IPF fibroblasts in full culture medium were collected, and subject to immunoblots with antibodies against α-SMA, collagen 1A1(Col1A1), Bcl-xL and Bak. β-tubulin as loading control. The figures presented in the text were mainly from IPF#2. Cell lines n=3 of non-IPF control or IPF.

B. SAHA induced apoptosis in non-IPF and IPF fibroblasts (n=3 for normal or IPF cell lines) with 2μM SAHA for 60 h. Bars indicate the mean±SD. *p<0.001: apoptotic cells *vs.* non-apoptotic cells in the same group. †$\boldsymbol{p}$<0.001: apoptotic cells in SAHA-treated *vs.* untreated (-) group

C. The expression of Bak, Bcl-xL and loading control of β-tubulin of non-IPF fibroblasts (#2 of A) treated without (0) or with SAHA (at 100 nM or 200 nM) for 60h.

D. The expression of Bak, Bcl-xL, α-SMA, DNMT1 and DNMT3a in other two IPF fibroblasts (IPF#1 and #3) treated without (SAHA 0) or with SAHA at 100 nM or 200 nM for 60h (see text for detailed protocols).

Figure S2. Effect of SAHA on apoptosis related genes, Collagen 1A1, and p16 expression, in addition to proliferation of IPF fibroblasts. IPF cells were treated without or with SAHA at 100 nM or 200 nM for 60 h.

A. Western blots for Bcl-2, Bok and Bid. Protein lysates of IPF control (SAHA 0), or SAHA at 100nM or 200nM were subjected to immunoblots, β-tubulin was used as a loading control. Right Panel: Real-time RT-PCR of Bcl-2, using $2^{-\Delta\Delta Ct}$ method, all normalized to 18S. p>0.05, SAHA100nM or 200nM *vs.* untreated control (SAHA 0)

B. Protein lysates of IPF control (SAHA 0), or SAHA at 100nM or 200nM were subjected to western blots for Col1A1 and β-tubulin. Right Panel: densitometric quantification of western

blots like B, average of at least 3 independent experiments. * $p<0.05$, SAHA100 nM or 200 nM treated compared to untreated control (SAHA 0).

C. Western blots for p16 and β-tubulin of IPF primary fibroblasts without (IPF Cont) or treated with 200 nM SAHA for 24h or 48h. Right Panel: densitometric quantification of western blots like C, average of at least 3 independent experiments. * $p<0.05$ SAHA 200 nM treated for 48h compared to untreated control (IPF Cont).

D. Cell proliferation assays of IPF fibroblasts without (SAHA 0) or treated with SAHA for 100 nM or 200 nM for 48h. *$p<0.05$ SAHA treated groups compared to untreated control (SAHA 0)

Figure S3. Photomicrographs of lung sections stained using H&E (A) or Trichrome (B) of mice exposed to Normal Saline (Saline), Bleomycin (Bleo), or Bleomycin treated with SAHA (Bleo/SAHA) (See text for details). **A**: Same scale as Figure 7C in text, photomicrographs taken at 200x. **B**: Photomicrographs taken at 200x.

C. Low magnification of lung histology by H&E of Saline, Bleomycin (Bleo), and Bleomycin treated with SAHA (Bleo/SAHA), taken at 100x. Scale bar: 150μm.

D. Low magnification images (100x) of lung tissues stained with Collagen III (red). See text for details.

E. Immunohistochemistry of H3K9Ac of lung sections from mice exposed to Normal Saline (Saline), Bleomycin (Bleo) or Bleomycin with SAHA (Bleo/SAHA) treatment. H3K9Ac was stained with peroxidase-conjugated streptavidin (brown), while nuclei stained with hemoxylin (blue). Scale bar (bottom right corner): 100μm. a, b, and c on the left panel are enlargements of the marked squares from the right panel.

Figure S4. A. Representative immunoblots of Bak, Bcl-xL from mice whole lung lysate 28-days after normal saline (Saline), bleomycin (Bleo), or bleomycin with SAHA (Bleo/SAHA) treatment. β-actin used as a loading control. **B.** Densitometry of Western blots of Bak or Bcl-xL. n≥3. Bars indicate the mean±SD. **p* <0.05, Bleo/SAHA *vs.* Bleo. **C.** Representative immunohistochemistry of Bcl-xL (1:800) in normal saline (Saline), bleomycin (Bleo), or bleomycin with SAHA (Bleo/SAHA) mice lung. Bcl-xL was stained with peroxidase-conjugated streptavidin (brown), while nuclei were stained with hemoxylin (blue). Scale bar : 80μM.