

Pharmacological inhibition of PAI-1 alleviates cardiopulmonary pathologies induced by exposure to air pollutants PM_{2.5}

Asish K Ghosh,*[‡] Saul Soberanes,[#] Elizabeth Lux,* Meng Shang,* Raul Piseaux-Aillon,[#] Mesut Eren,* G.R. Scott Budinger,[#] Toshio Miyata,[§] Douglas E Vaughan*[‡]

*Feinberg Cardiovascular and Renal Research Institute, [#]Pulmonary and Critical Care Division, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA. [§]United Centers for Advanced Research and Translational Medicine, Tohoku University, Miyagi, Japan

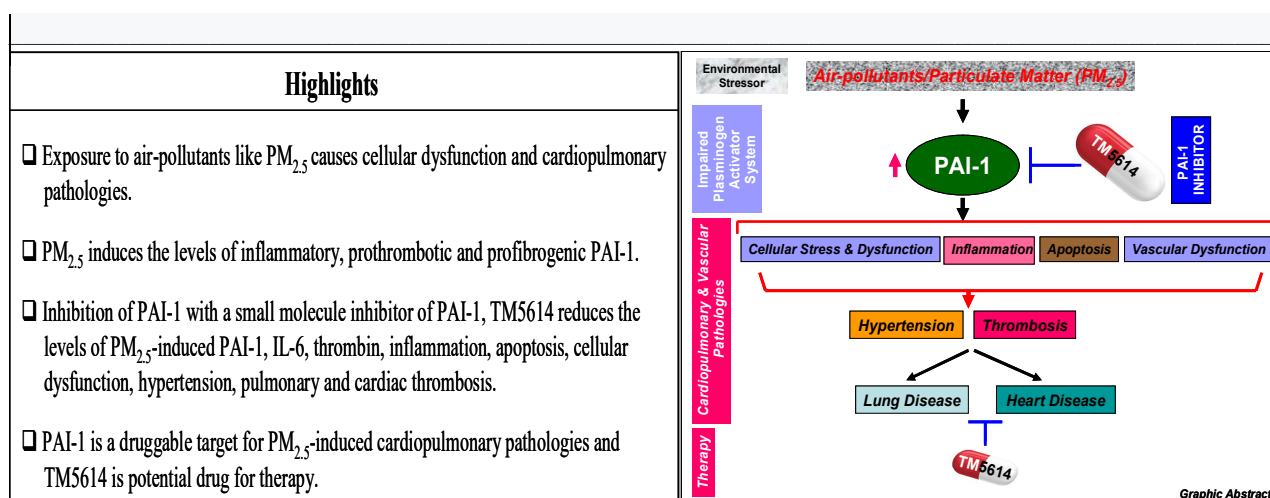
Correspondence to: a-ghosh2@northwestern.edu or d-vaughan@northwestern.edu

OBJECTIVE: Exposure to air pollutants leads to the development of pulmonary and cardiovascular diseases, and thus air pollution is one of the major global threats to human health. Air pollutant particulate matter 2.5 (PM_{2.5})-induced cellular dysfunction impairs tissue homeostasis and causes vascular and cardiopulmonary damage. To test a hypothesis that elevated plasminogen activator inhibitor-1 (PAI-1) levels play a pivotal role in air pollutant-induced cardiopulmonary pathologies, we examined the efficacy of a drug-like novel inhibitor of PAI-1, TM5614, in treating PM_{2.5}-induced vascular and cardiopulmonary pathologies.

APPROACH AND RESULTS: Results from biochemical, histological, and immunohistochemical studies revealed that PM_{2.5} increases the circulating levels of PAI-1 and thrombin and that TM5614 treatment completely abrogates these effects in plasma. PM_{2.5} significantly augments levels of pro-inflammatory cytokine IL-6 in bronchoalveolar lavage fluid, and this also can be reversed by TM5614, indicating its efficacy in amelioration of PM_{2.5}-induced increases in inflammatory and pro-thrombotic factors. TM5614 reduces PM_{2.5}-induced increased levels of inflammatory markers Mac3 and pSTAT3, adhesion molecule VCAM1, and apoptotic marker cleaved caspase 3. Longer exposure to PM_{2.5} induces pulmonary and cardiac thrombosis, but TM5614 significantly ameliorates PM_{2.5}-induced vascular thrombosis. TM5614 also reduces PM_{2.5}-induced increased blood pressure and heart weight. *In vitro* cell culture studies revealed that PM_{2.5} induces the levels of PAI-1, type I collagen, fibronectin, and SREBP-1/2, a transcription factor that mediates profibrogenic signaling, in cardiac fibroblasts. TM5614 abrogated that stimulation, indicating that it may block PM_{2.5}-induced PAI-1 and profibrogenic signaling through suppression of SREBP-1. Furthermore, TM5614 blocked PM_{2.5}-mediated suppression of Nrf2, a major antioxidant regulator in cardiac fibroblasts.

CONCLUSIONS: Pharmacological inhibition of PAI-1 with TM5614 is a promising therapeutic approach to control air pollutant PM_{2.5}-induced cardiopulmonary and vascular pathologies.

Key words: Air-pollutants, Particulate Matter PM_{2.5}, PAI-1, TM5614, Cardiopulmonary pathologies, Thrombosis



INTRODUCTION:

Air pollution-associated toxicity is a major threat to human health. Exposure to air pollutants leads to development of pulmonary and cardiovascular disease-related morbidity and mortality. Environmental pollutants like ozone, nitrogen oxide, and particulate matter (PM) induce oxidative stress, which leads to cardiopulmonary vascular diseases (CPVDs) including thrombosis, hypertension, arteriosclerosis, arrhythmias and, myocardial infarction—affecting millions of people worldwide.^{1,2} PM is the most dangerous environmental pollutant, as these solid particles contain a wide variety of toxic substances including sulfates, nitrates, hydrocarbons, heavy metals, and bacteria. Based on its aerodynamic diameter, PM has been classified as coarse (PM₁₀), fine (PM_{2.5}), and ultrafine (PM_{0.1}).^{3,4} The detrimental effects of PM on the cardiopulmonary/vascular system stem from chronic or acute exposure-induced oxidative stress and systemic inflammation, cellular dysfunction, and impaired tissue homeostasis.¹⁻⁸ While the acute effects of PM may be direct, involving rapid crossover from the lung epithelium into the circulation, the chronic effects of PM involve generation of pulmonary oxidative stress, systemic inflammation, and cellular dysfunction.⁹ With time, both direct and indirect effects of PM facilitate the onset and progression of CPVD pathologies. However, the exact molecular mechanism by which environmental pollutants like PM_{2.5} contribute to cardiopulmonary vascular diseases is not fully understood.

Plasminogen activator inhibitor-1 (PAI-1), a secretory protein that inhibits t-PA/uPA serine proteases, regulates the tissue fibrinolytic system, and its deregulation contributes to different diseases, including CPVDs like thrombosis and accelerated cellular senescence and aging.¹⁰⁻¹⁴ Here, we tested a hypothesis that elevation of PAI-1 plays a pivotal role in PM-induced CPVDs by inhibiting t-PA, increasing oxidative stress, and promoting cellular dysfunction and cardiopulmonary vascular injury; conversely, pharmacological inhibition of PAI-1 ameliorates PM-induced CPVDs. The strong rationale behind formulating this hypothesis comes from the previous reports that (i) the levels of PAI-1 are significantly elevated in oxidative stress-induced cardiac cells, (ii) elevated PAI-1 is a key

determinant of premature cellular senescence and dysfunction in different pulmonary and cardiac cells, and (iii) pharmacological inhibition of PAI-1 effectively blocks oxidative stress-induced cellular dysfunction through suppression of key senescence mediators.¹²⁻¹⁴ In addition, the levels of PAI-1 are significantly elevated in bronchoalveolar lavage fluid (BALF) in mice, and in lung and heart tissues from rats, exposed to PM_{2.5} and are associated with inflammation and apoptosis.^{15,16} These findings strongly predict a therapeutically relevant function of PAI-1 inhibition in preventing PM_{2.5}-induced CPVDs. In this study, we tested the efficacy of a novel drug-like small molecule inhibitor of PAI-1, TM5614, in amelioration of air pollutant PM_{2.5}-induced CPVD pathologies. The results of the present investigation reveal that pharmacological inhibition of PAI-1 in mice is associated with suppression of PM_{2.5}-induced inflammatory and prothrombotic factors and thus of CPVD pathologies including vascular thrombosis.

MATERIALS AND METHODS

All data and supporting materials have been provided with the published article and in the data supplement.

Animal studies: Wild-type, PAI-1 heterozygous, and PAI-1 knockout C57Bl/6 mice (9-12 weeks old) were used for this study. All mouse protocols were approved by the Northwestern University Animal Care and Use Committee. Wild-type male mice were divided into 4 groups. Group 1: mice were fed with regular chow and received 50 µl of phosphate-buffered saline (PBS as control) (25 µl X2) by intratracheal instillation. Group 2: mice were fed with regular chow and received particulate matter 2.5 (PM_{2.5} in PBS) (the National Institutes of Standards and Technology, Gaithersburg, MD, USA; NIST SRM 1649) (50 µg-200µg/mouse; 50 µg or 100 µg or 200 µg in 50 µl PBS; 25 µl X2) by intratracheal instillation. Doses of PM_{2.5} were selected based on well accepted published literature.¹⁵⁻²¹ Group 3: mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS (25 µl X2 control) and TM5614 chow. Group 4: mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (50-200 µg/mouse in 50 µl PBS; 25 µl X2) and TM5614

chow. After 24 h or 72 h (short-term exposure to PM_{2.5}: 50 and 200 µg/mouse/week or PBS in the presence or absence of TM5614) or 4 weeks (long-term exposure to PM_{2.5}: 50-200 µg/mouse/week or PBS in the presence or absence of TM5614), mice were sacrificed and plasma and BALF (short-term exposure only) were collected and processed for biochemical analysis. For PAI-1 genetic deficiency study, batches of wild-type, PAI-1 heterozygous, and PAI-1 knockout male and female mice were fed with regular chow and treated either with PM_{2.5} (50 µg/mouse/week for 4 weeks) or PBS as controls. Hearts and lungs from short-term exposed mice (72 h to study the early pathological events) and long-term-exposed mice (4 weeks to study late pathological events) were collected and used for histological and immunohistochemical analysis.

Measurement of PAI-1 and thrombin-antithrombin (TAT) complex levels in plasma:

Plasma was collected from the 4 groups of wild-type mice, and 50 µl of TAT standard and each sample were used in duplicate for TAT assays using a mouse ELISA kit for TAT (cat no ab137994, Abcam). Similarly, PAI-1 standard and each plasma sample were used in duplicate for PAI-1 assays using a mouse PAI-1 ELISA kit (cat no ab197752, Abcam).

Measurement of IL-6 in BALF: BALF was collected from each group of mice after 24 h of treatment as indicated above, and 50 µl of IL-6 standard and each sample were used in duplicate for IL-6 protein measurement using a Quantikine IL-6 mouse ELISA kit (cat no M6000B, R&D).

Blood pressure and echocardiography: To monitor the effect of PM_{2.5} exposure on blood pressure, both systolic and diastolic blood pressure were measured in conscious mice using a non-invasive tail-cuff system (CODA Non-Invasive Blood Pressure, Kent Scientific Corp.). Trans-thoracic two-dimensional M-mode echocardiography was performed using a Vevo 3100 device (VisualSonics, Toronto, Canada) equipped with a MX550D transducer. Echocardiographic studies were performed after 4 weeks of PM_{2.5} exposure following manufacturer's instruction. M-mode tracings were used to measure left ventricular (LV) wall thickness, LV dimensions, LV volume, and LV mass. Percent fractional shortening (% FS) and

ejection fraction (% EF) were also determined. The mean value of at least 3-5 cardiac cycles was used to determine the measurements for each animal in all four groups. Post-mortem heart weight and body weight of each mouse in all four groups were recorded and compared.

Histology: Heart and lung tissues were collected and fixed in 10% formalin overnight at room temperature, followed by fixation in 70% ethanol at 4°C overnight, and then processed for embedding in paraffin. Paraffin-embedded heart and lung tissues were subjected to microtome sectioning and processed for histological analysis. For morphology studies, the tissues were processed for hematoxylin and eosin (H&E) staining. The H&E staining of specimens was performed on a fully automated platform (Leica Autostainer XL) using Harris hematoxylin (Fisher-brand cat no.245-651) and eosin (Leica cat no. 3801600). The levels of collagen deposition and thrombotic blood vessels in lung and heart tissues were determined by H&E and Masson trichrome staining. Photographs were taken with an Olympus DP71 camera. The levels of matrix deposition were estimated by Fiji-ImageJ software.

Immunohistochemistry: All the immunohistochemistry staining was completed using chromogenic enzyme substrate reactions with DAB (DAKO, cat no.K3468). Formalin-fixed paraffin-embedded tissue sections (4 µm) were first deparaffinized on an automated platform (Leica Autostainer XL). After deparaffinization, slides were subjected to an antigen retrieval step using a sodium citrate solution at pH 6 in a pressure cooker (Biocare). Primary antibody was then added manually and incubated at 4 °C overnight in a humid chamber. Secondary antibody incubation and chromogenic reactions were then performed using an automated system (Biocare Intellipath). Once the staining was complete, specimens were counterstained with hematoxylin. Slides were then coverslipped using a xylene-based mounting medium (Leica Micromount). The antibodies used in this study are as follows: Mac3: BD Biosciences cat no.553322; concentration 1:5000. Antigen retrieval: pH 6 sodium citrate, 11°C for 10 min. pSTAT3: Cell Signaling, cat.no.9145; concentration 1:250. Antigen retrieval: pH 6 sodium citrate, 11 °C for 10 min. Cleaved Caspase 3: Cell Signaling cat

.no.9661; Concentration 1:500; Antigen Retrieval: Ph6 Sodium Citrate, 11°C for 10min.VCAM1: Abcam ab134047, concentration 1:500. Antigen retrieval: pH 6 sodium citrate, 11 °C for 20 min. Photographs were taken using a light microscope. The sum of several fields per heart and lung tissue from each mouse were used for staining intensity measurement using ImageJ software, and the levels of statistical significance were determined.

Cell cultures and treatment with PM_{2.5} and TM5614: Primary cultures of human cardiac fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. To test the efficacy of PAI-1 inhibitor TM5614 in suppression of PM_{2.5}-induced cardiac fibroblast dysfunction, confluent cultures of human cardiac fibroblasts were subcultured in 12-well clusters. After 24 h, media were replaced with 0.1% FBS-containing DMEM for 3 h. Cells were pretreated with TM5614 (10 μM) or DMSO (vehicle control) for 2 h followed by treatment with PM_{2.5} (50 μg/ml) in triplicate for another 2 h. For the other set of experiments, cells were pretreated with TM5614 (10 μM) or DMSO in triplicate for 24 h. After 24 h, media were replaced with 0.1% FBS-containing DMEM and treated with TM5614 (10 μM) or DMSO and PM_{2.5} (50 μg/ml) in triplicate for another 24 h.

Preparation of cell lysates and western blot analysis: At the end of treatment with PM_{2.5} in the absence and presence of PAI-1 inhibitor TM5614, supernatants from control and treated wells were collected. The cell lysates were prepared using RIPA lysis buffer containing protease inhibitor cocktail (cOmplete) and phosphatase inhibitor cocktail (PhosSTOP) (Sigma). Cell lysates were pooled from 3 wells for each control and treatment group and equal amounts of protein were subjected to gel electrophoresis, transferred to PVDF membranes, and subjected to western blotting using antibodies against PAI-1 (Molecular Innovations, Inc.), type I collagen (Southern Biotech), fibronectin, SREBP1, SREBP2, Nrf2 (Abcam), and α-tubulin (GenScript), with HRP-tagged corresponding secondary antibodies. The membranes were developed with enhanced chemiluminescence reagents (Luminata Forte, Millipore), and images of protein bands were

captured using a BIO-RAD ChemiDoc XRS system (BIO-RAD, CA).

Statistical analysis: Data are presented as mean ± SEM. The significance of differences between controls and experimental groups was determined by statistical analysis using Student's t-test, where a value of P<0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

RESULTS:

PAI-1 inhibitor TM5614 reduces PM_{2.5} exposure-induced plasma levels of PAI-1 and thrombin in mice: Four groups of mice described in the Methods section were used in this study: group 1, controls; group 2, PM_{2.5} only; group 3, TM5614 only; and group 4, TM5614 and PM_{2.5}. After 24 h or 72 h, mice were sacrificed, and plasma was used for PAI-1 and TAT assays. The results revealed that exposure to PM_{2.5} (50 μg/mouse/once) for 24 h increases the levels of PAI-1 significantly, and TAT complex insignificantly in plasma. Importantly, pretreatment of mice with PAI-1 inhibitor TM5614 completely abrogates the PM_{2.5}-induced increased levels of PAI-1 and TAT in plasma (Figure 1A, B). Similarly, exposure to PM_{2.5} (200 μg/mouse/once) for 72 h significantly stimulates the levels of TAT in plasma, and TM5614 abrogates that stimulation

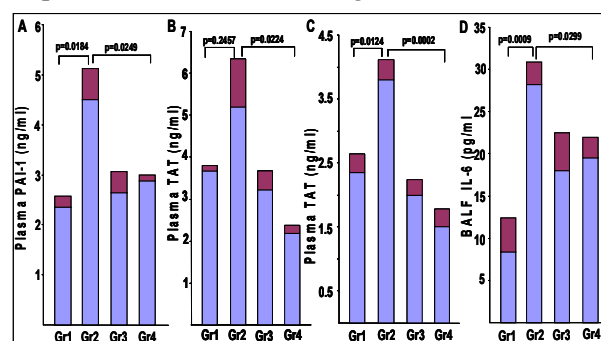


Figure 1. PAI-1 inhibitor TM5614 inhibits the levels of PM_{2.5}-induced PAI-1 and TAT in plasma and IL-6 in BALF. Plasma collected from 4 groups of mice (n=4-8) was used in duplicate for PAI-1 and TAT assays. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (50 μg/mouse) or PBS by intratracheal instillation; Day 8: Plasma collected and processed for PAI-1 (A) or TAT (B) assay using an ELISA kit. (C) TAT levels in plasma from mice after 72 h treatment with PM_{2.5} (200 μg/mouse) and TM5614 (10 mg/kg) (n=4-7). (D) IL-6 levels in BALF from mice after 24 h treatment with PM_{2.5} (50 μg/mouse) and TM5614 (10 mg/kg) were determined in duplicate using an ELISA kit (n=7-8). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

(Figure 1C). These results indicate that PAI-1 inhibitor TM5614 is highly efficient in reduction of PM_{2.5}-induced PAI-1 and thrombin, which contribute to vascular pathologies like thrombosis.

PAI-1 inhibitor TM5614 significantly inhibits PM_{2.5}-induced IL-6 in BALF: BALF collected from the four groups of mice was used for determination of IL-6 levels as described in the Methods. Administration of PM_{2.5} (50 µg/mouse/once) for 24 h significantly increases the levels of pro-inflammatory cytokine IL-6 in BALF and most importantly, PM_{2.5} fails to stimulate the levels of IL-6 in mice fed with TM5614, indicating the efficacy of PAI-1 inhibitor TM5614 in amelioration of PM_{2.5}-induced lung inflammation (Figure 1D). The levels of IL-6 in BALF from TM5614-treated (Group 3) and PBS control (Group 1) mice are not significantly increased (p=0.1301).

PAI-1 inhibitor TM5614 alleviates PM_{2.5}-induced lung inflammation, apoptosis, and vascular pathologies: Wild-type mice were

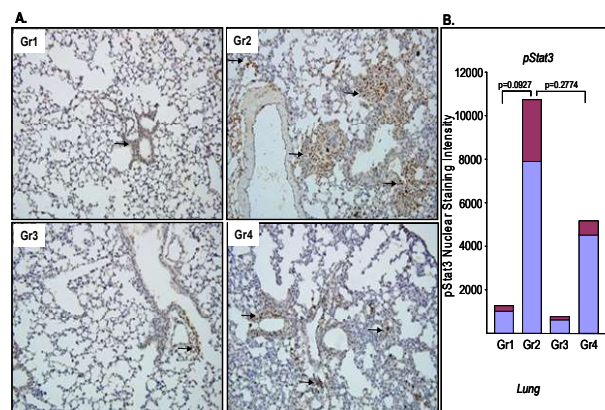


Figure 2. Effect of PAI-1 inhibitor TM5614 on PM_{2.5}-induced inflammatory factor pSTAT3 in lungs. Lungs collected from 4 groups of mice (n=4-6) were processed for immunohistochemistry using an anti-pSTAT3 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation; Day 10: Lungs were collected and processed for immunohistochemistry. Representative images are shown in (A). Images are reduced to 15% of original 20X images. The levels of nuclear pSTAT3 in several fields of each lung section were determined by ImageJ followed by statistical analysis. Quantitative data are shown in (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

exposed to a single dose of PM_{2.5} (200 µg/mouse). After 72 h, lung tissues were collected and

processed for morphological analysis by H&E staining and immunohistochemical analysis using antibodies against inflammatory markers Mac3 and pSTAT3, apoptotic marker cleaved caspase 3, and adhesion molecule VCAM1. Morphologically, PM_{2.5}-exposed lungs (4 out of 7) are relatively denser compared to PBS controls (1 out of 4) and importantly, TM5614 partially reverses the PM_{2.5}-induced lung morphological changes (Supplemental Figure s1, representative images are presented). Immunohistochemistry results revealed the presence of low levels of inflammation in PBS treated lungs. However, administration of PM_{2.5} increases the staining intensity of pSTAT3 and Mac3 compared to controls and mice treated with

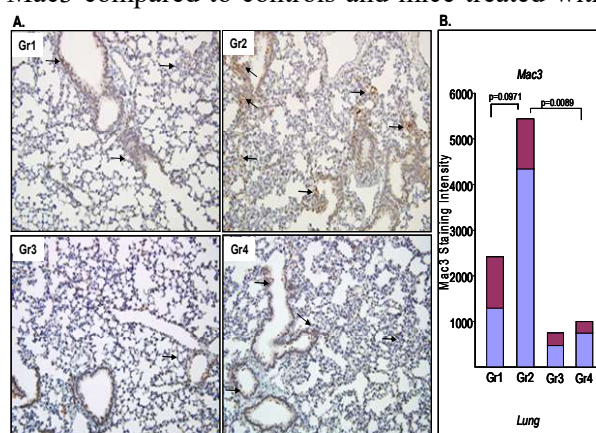


Figure 3. PAI-1 inhibitor TM5614 reduces PM_{2.5}-induced inflammation in lungs. Lungs collected from 4 groups of mice (n=4-6) were processed for immunohistochemistry using an anti-Mac3 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation. After 72 h, lungs were collected and processed for immunohistochemistry. Representative images are shown in (A). Images are reduced to 15% of original 20X images. The levels of Mac3 intensity in several fields of each lung section were determined by ImageJ software. Quantitative data are presented in (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

TM5614 alone. Treatment with PAI-1 inhibitor TM5614 modestly reduces PM_{2.5}-induced pSTAT3 without reaching statistical significance (Figure 2A, representative images are presented and quantitative data are shown in Figure 2B) and significantly reduces the Mac3 (Figure 3A, representative images are presented and quantitative data are shown in Figure 3B) levels in lung tissues. These results are consistent with our observation that PM_{2.5} induces the level of pro-inflammatory cytokine IL-6 in BALF and TM5614

completely abrogates the PM_{2.5}-induced increased levels of IL-6 in lungs.

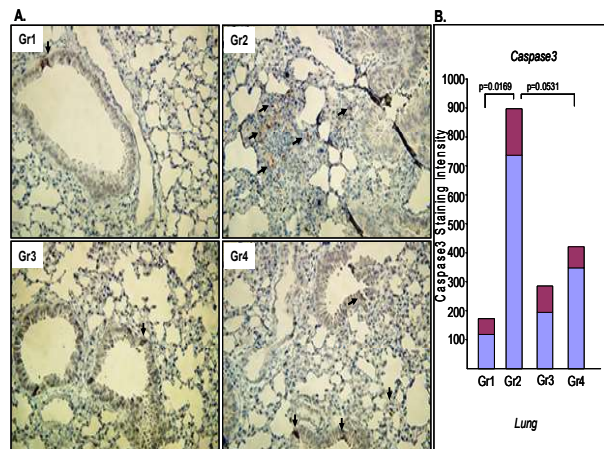


Figure 4. Effect of TM5614 on PM_{2.5}-induced cellular apoptosis in lungs. Lungs collected from 4 groups of mice (n=4-6) were processed for immunohistochemistry using an anti-cleaved caspase 3 antibody. Mice were fed with TM5614 (10 mg/kg) for 6 days and on day 7, PM_{2.5} (200 µg/mouse) instillation or PBS was used as control. After 72 h, lungs were processed for immunohistochemistry. Representative images are reduced to 15% of original 40X images (A). The levels of cleaved caspase 3 in several fields of each lung were determined by ImageJ software. Quantitative data are shown in (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

Exposure to PM_{2.5} for 72 h significantly increases the levels of cleaved caspase 3 positivity in lungs (Figure 4A, representative image from each group; quantitative data are shown in Figure 4B). Importantly, TM5614 treatment reduces the levels of cleaved caspase 3 positivity in lungs. Next, we determined the levels of adhesion molecule VCAM1, known to be involved in inflammation and vascular dysfunction, in PM_{2.5}-exposed murine lungs. Exposure to PM_{2.5} induces the levels

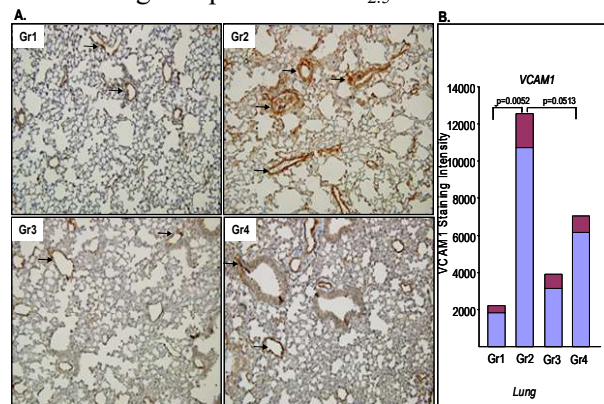


Figure 5. TM5614 reduces PM_{2.5}-induced adhesion molecule VCAM1 in lungs. Lungs collected from 4 groups of mice (n=4-6) were processed for immunohistochemistry using an anti-VCAM1 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation; Day 10: Lungs were processed for immunohistochemistry. Representative images are reduced to 15% of original 20X images (A). The levels of VCAM1 in several fields of each lung were determined by ImageJ. Quantitative data are shown (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

of VCAM1, consistent with the previous observation that PM_{2.5}-induced stress increases VCAM1 in endothelial cells.^{22,23} Most importantly, TM5614 prevents PM_{2.5}-induced induction of VCAM1 (Figure 5A, representative images are presented from each group; quantitative data are shown in Figure 5B). Collectively, these results indicate that pharmacological inhibition of PAI-1 is an ideal approach to control air pollutant-induced inflammation and cellular apoptosis in murine lungs and that small molecule TM5614 effectively prevents air pollutant-induced lung pathological events.

Effect of PAI-1 inhibitor TM5614 on PM_{2.5}-induced cardiac inflammation and apoptosis:

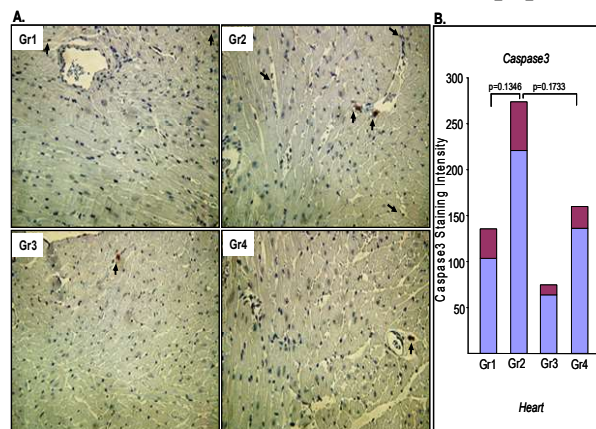


Figure 6. Effect of PAI-1 inhibitor TM5614 on PM_{2.5}-induced cellular apoptosis in hearts. Hearts collected from 4 groups of mice (n=4-6) were processed for immunohistochemistry using an anti-cleaved caspase 3 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation. After 72 h, hearts were collected and processed for immunohistochemistry. Representative images are reduced to 15% of original 40X images (A). The levels of cleaved caspase 3 in several fields of each heart section were determined by ImageJ. Quantitative data are shown in (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

Hearts collected from the four groups of mice were processed for H&E staining and immunohistochemical analysis using antibodies against pSTAT3, Mac3, cleaved caspase 3, and VCAM1. There were no morphological changes in PM_{2.5}-exposed hearts (Supplemental Figure s2). Immunohistochemistry results revealed that exposure to PM_{2.5} (200 µg/mouse/once) for 72 h increases the levels of cleaved caspase 3 in myocardial tissues and that TM5614 treatment reduces the levels of cleaved caspase 3 without attaining statistical significance (Figure 6A, representative image from each group; quantitative data are shown in Figure 6B). The levels of PM_{2.5}-induced apoptotic cells in hearts are lower compared to lungs. While administration of PM_{2.5} significantly increases cardiac inflammation as evidenced by increased Mac3 staining, TM5614 modestly reduces inflammation (Figure 7A, representative image from each group; quantitative data are shown in Figure 7B). There is no difference in the levels of pSTAT3 in cardiac tissues derived from control and treated mice (Supplemental Figure s3A, representative image of each group, and quantitative data are shown in

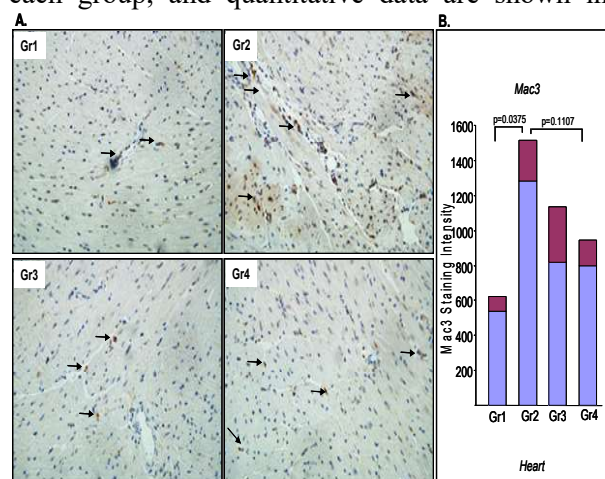


Figure 7. PAI-1 inhibitor TM5614 reduces PM_{2.5}-induced inflammation in murine hearts. Hearts were collected from 4 groups of mice (n=4-6) and processed for immunohistochemistry using an anti-Mac3 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation. After 72 h, hearts were processed for immunohistochemistry. Representative images are shown in (A). Images are reduced to 15% of original 40X images. The levels of Mac3 intensity in several fields of each heart were used for quantification using ImageJ. Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

Figure s3B). While exposure to PM_{2.5} modestly stimulates the levels of VCAM1 in myocardial

tissues, in the TM5614-treated mice, PM_{2.5} fails to stimulate adhesion molecule VCAM1 (Figure 8A, representative image from each group; quantitative data are shown in Figure 8B). These results signify the efficacy of PAI-1 inhibitor TM5614 in suppression of air pollutant-induced cardiovascular pathologies.

Effect of long-term exposure to PM_{2.5} on blood pressure, cardiac hypertrophy, and cardiac matrix remodeling: Mice were divided into 4 groups. In Group1, mice were fed with regular

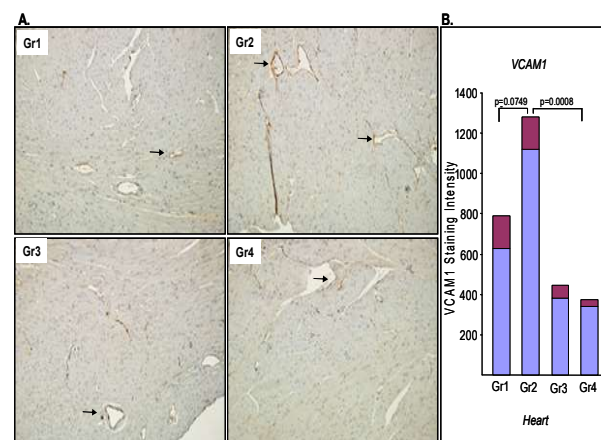


Figure 8. TM5614 reduces PM_{2.5}-induced adhesion molecule VCAM1 in hearts. Hearts collected from 4 groups of mice (n=4-6) as indicated were processed for immunohistochemistry using an anti-VCAM1 antibody. Day 1-6: TM5614 (10 mg/kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation; Day 10: Heart sections were processed for immunohistochemistry. In (A), representative images are shown. Images are reduced to 15% of original 20X images. The levels of VCAM1 in several fields of each heart were determined by ImageJ software. Quantitative data are shown in (B). Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: TM5614+PM_{2.5}.

chow and received PBS. Group 2, mice were fed with regular chow and received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). Group 3, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS for 4 weeks. Group 4, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). At the end of the 4 weeks, blood pressure was determined, and each mouse was subjected to echocardiography. The results revealed that 4 weeks of PM_{2.5} exposure increases systolic pressure non-significantly and diastolic blood pressure significantly. Importantly, treatment of mice with PAI-1 inhibitor TM5614

reduces the PM_{2.5}-induced blood pressure increase (Supplemental Figure s4A,B). Analysis of echocardiography data revealed that there is no significant differences in EF, FS, left ventricular anterior or posterior wall thickness-systolic or diastolic (LVAWs, LVAWd, LVPWs and LVPWd) within control and treated groups (data not shown). Post-mortem heart weight analysis reveals that PM_{2.5} (100 µg/mouse once per week for 4 weeks) induces cardiac hypertrophy based on increased heart weight/body weight ratio and that TM5614 decreases the PM_{2.5}-induced cardiac hypertrophy (Supplemental Figure s4C). Collectively, these results suggest that TM5614 is effective in amelioration of PM_{2.5}-induced cardiac pathologies including hypertension and cardiac hypertrophy. However, PM_{2.5} fails to stimulate fibrogenesis in heart tissue after 4 weeks of treatment (100 µg once per week for 4 weeks) (data not shown).

PAI-1 inhibitor TM5614 reduces long-term PM_{2.5} exposure-induced pulmonary and cardiac thrombosis in mice: Mice were divided into 4 groups and treated for 4 weeks as described above. At the end of treatment, mice were sacrificed, and heart and lungs were collected. Lung and heart tissues were processed for H&E staining and Masson's trichrome staining. Exposure to PM_{2.5} for 4 weeks significantly induces pulmonary thrombogenesis in mice (incidence of thrombus formation: 4 out of 7) compared to PBS controls (incidence of thrombus formation: 0 out of 4) and mice treated with TM5614 alone (incidence of thrombus formation: 0 out of 5). Most importantly, PM_{2.5} failed to induce pulmonary thrombosis in mice pretreated with PAI-1 inhibitor TM5614 (incidence of thrombus formation: 0/4) (Figure 9, representative image of each group). This result is highly significant, as it clearly indicates the beneficial effect of PAI-1 inhibitor TM5614 in improving vascular thrombosis, a major pulmonary pathology, in mice exposed to air pollutant PM_{2.5}. However, PM_{2.5} fails to stimulate the pulmonary fibrogenesis significantly after 4 weeks of treatment (Supplemental Figure s5).

Analysis of hearts from each group revealed that longer exposure to PM_{2.5} modestly induces cardiac thrombosis in mice, to a lesser extent than in lungs. However, PM_{2.5} failed to induce cardiac

thrombosis in mice pretreated with PAI-1 inhibitor TM5614 (Supplemental Figure s6). These results are consistent with our observation that PM_{2.5} induces expression of PAI-1 and thrombin, the major contributors to thrombogenesis, and that TM5614 completely abrogates the PM_{2.5}-induced increased levels of PAI-1 and thrombin in plasma (Figure 1). Additionally, these results further indicate that PAI-1 is an ideal druggable target for the treatment of air pollutant-induced cardiopulmonary pathologies, including vascular thrombosis.

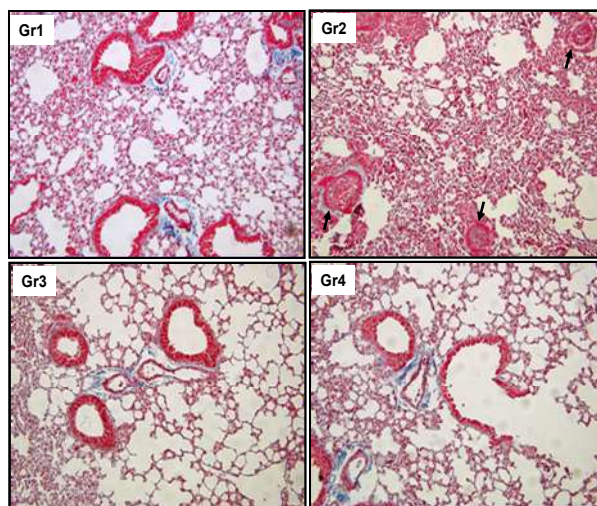


Figure 9. PM_{2.5} induces pulmonary thrombosis and PAI-1 inhibitor TM5614 prevents lung thrombogenesis. Mice were divided into 4 groups (n=4-7). In Group 1, mice were fed with regular chow and received PBS. In Group 2, mice were fed with regular chow and received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). In Group 3, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS. In Group 4, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). Representative image from each group are shown. Images reduced to 15% of original 20X images. An arrow indicates the presence of a thrombus.

PAI-1 heterozygous mice are partly protected from PM_{2.5}-induced pulmonary thrombosis: As a complementary study to pharmacological inhibition of PAI-1 and its beneficial effect on air pollutant-induced cardiopulmonary and vascular pathologies, next we investigated the effects of haplo- and complete genetic deficiency of PAI-1 on air pollutant PM_{2.5}-induced cardiopulmonary pathologies. Wild-type, PAI-1 heterozygous, and PAI-1 knockout mice were treated with PM_{2.5} (50 µg/mouse/week for 4 weeks) or PBS as a control.

All mice were fed with regular chow. Four weeks of PM_{2.5} exposure induces pulmonary thrombosis in both wild-type and PAI-1 knockout mice, indicating complete deficiency of PAI-1 does not protect against developing PM_{2.5}-induced pulmonary pathologies. Interestingly, PAI-1 heterozygous mice are partially protected from PM_{2.5}-induced pulmonary thrombotic pathogenesis, indicating that, like pharmacological inhibition of PAI-1, PAI-1 haplodeficiency, but not complete absence of PAI-1, is beneficial for air pollutant-induced pulmonary pathologies (Supplemental Figure s7). However, we did not observe any difference in pulmonary matrix remodeling in PM_{2.5}-treated wild-type, PAI-1 heterozygous, and knockout groups compared to controls and TM5614-treated groups, as evidenced by Masson's trichrome staining (*data not shown*).

TM5614 reduces PM_{2.5}-induced cellular stresses and abnormalities: As PM_{2.5} induces the levels of reactive oxygen species, TGF- β , and matrix remodeling, and fibroblasts are the major contributing cell type in matrix protein production, we have developed an *in vitro* model system to study the effects of PM_{2.5} on cellular processes in cardiac fibroblasts and the efficacy of TM5614 in ameliorating PM_{2.5}-induced cellular abnormalities. Our results showed that PM_{2.5} modestly induces

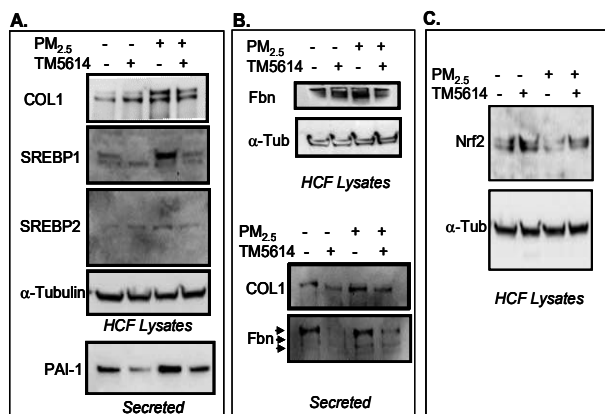


Figure 10. TM5614 blocks PM_{2.5}-induced cellular stress and profibrogenic responses. Human cardiac fibroblasts were cultured in 12-well clusters. After 24 h, media were replaced with 0.1% FBS containing DMEM for 3 h. Cells were pretreated with TM5614 (10 μ M) or DMSO (vehicle control) for 2 h followed by treatment with PM_{2.5} (50 μ g/ml) in triplicate for another 2 h. Supernatants and cell lysates were used for western blotting using antibodies as indicated (A). In (B) and (C), cells were pretreated with TM5614 (10 μ M) or DMSO in triplicate. After 24 h, media were replaced with 0.1% FBS containing DMEM and treated with TM5614 (10 μ M) or DMSO and PM_{2.5} (50 μ g/ml) in triplicate for another 24 h. Supernatants and cell lysates were processed for western blotting using antibodies as indicated (B,C).

the levels of PAI-1, type I collagen, and fibronectin and that TM5614 reduces the PM_{2.5}-induced stimulation of these fibrogenic factors (Figure 10A-C). The PM_{2.5}-induced stimulation of PAI-1 and its normalization by TM5614 *in vitro* is consistent with the *in vivo* observation on plasma levels of PAI-1 in PM_{2.5}- and TM5614-treated mice (Figure 1). As SREBP1, a basic-helix-loop-helix leucine zipper family transcription factor, mediates profibrogenic signaling and fibrogenesis,²⁴ and air pollutants induce the expression of SREBP1,²⁵ we measured the levels of SREBP1 and SREBP2 in control, PM_{2.5}- and TM5614-treated cardiac fibroblasts. Interestingly, PM_{2.5} induces the expression of SREBP1/2 in human cardiac fibroblasts and most importantly, TM5614 completely abrogates that stimulation, indicating that TM5614 effectively blocks PM_{2.5}-induced profibrogenic signaling through suppression of SREBP1 and SREBP2 (Figure 10A). As PM_{2.5} induces reactive oxygen species²⁶ and Nrf2, a basic leucine zipper (bZip) transcription factor, is a master regulator of antioxidant genes and signaling,²⁷ we measured the effect of PM_{2.5} on Nrf2 expression in human cardiac fibroblasts and the efficacy of TM5614 in normalizing the Nrf2

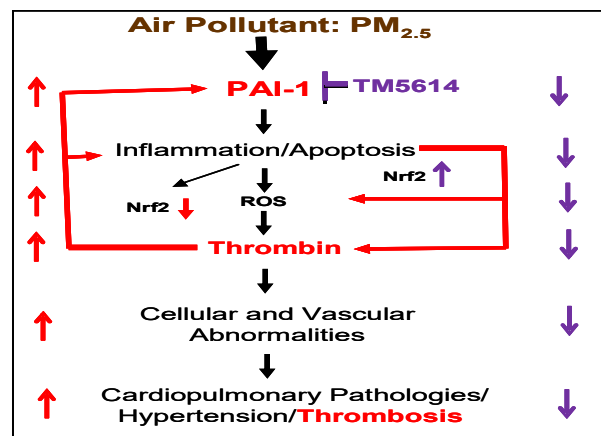


Figure 11. Possible pathological pathways driven by PM_{2.5}-induced PAI-1 and the beneficial effect of PAI-1 inhibitor TM5614. Air pollution stressors increase the levels of proinflammatory and prothrombotic mediators/regulators that cause cellular and vascular dysfunction and contribute to cardiopulmonary vascular pathologies. Neutralization of PM_{2.5}-induced PAI-1 with TM5614 reduces PM_{2.5}-induced cardiopulmonary vascular pathologies. expression. Our results revealed that PM_{2.5} reduces the levels of Nrf2 in cardiac fibroblasts and importantly, that TM5614 reverses the suppression of Nrf2 by PM_{2.5} (Figure 10C). Collectively, these

in vitro results indicate the efficacy of PAI-1 inhibitor TM5614 in improvement of cardiac cellular stresses and abnormalities induced by exposure to air pollutant PM_{2.5}.

DISCUSSION:

Environmental pollutants like PM are a global threat to organismal growth and health. Short-term or long-term exposure to environmental pollutants like PM causes devastating CPVDs. CPVDs including pulmonary inflammation, vascular dysfunction, hypertension, arteriosclerosis, arrhythmias, and myocardial infarction cause an estimated 60-80% of environmental pollutant-associated deaths worldwide.¹⁻⁸ However, the underlying molecular basis of PM-induced CPVDs is not well understood, and an effective therapeutic approach to block PM-induced CPVD-related pathologies is not available. As exposure to environmental pollutants deregulates the plasminogen activator system,^{15,16} and PAI-1 plays pivotal roles in initiation and progression of CPVDs,¹⁰⁻¹⁴ we initiated the present study in order to delineate the role of PAI-1 in air pollutant PM_{2.5}-induced CPVD-related pathologies, and assess the potential of PAI-1 as a druggable target for CPVD therapy. Using the small molecule inhibitor TM5614, which targets PAI-1, and mouse strains with PAI-1 haplo- or complete deficiency, the present study demonstrated that PAI-1 plays a key role in air pollutant PM_{2.5}-induced CPVD pathologies and that pharmacological inhibition of PAI-1 ameliorates PM_{2.5}-induced CPVD pathologies.

In this study, we demonstrated that short-term exposure to air pollutant PM_{2.5} increases the levels of pro-inflammatory and pro-thrombotic PAI-1 and thrombin in plasma and inflammatory cytokine IL-6 in lungs, consistent with previous observations.¹⁵ The levels of TAT complex serve as an indicator of coagulation activation. Thrombin is a serine protease that converts soluble fibrinogen to an insoluble fibrous protein, fibrin, that polymerizes and becomes a key component of blood clots. Antithrombin, an endogenous inhibitor of thrombin, forms the TAT complex with thrombin. As thrombin is highly unstable and has a short half-life in plasma, the TAT complex is measured as an indicator of thrombin levels and

activation of coagulation that contributes to thrombosis.²⁸

Most importantly, here we have presented data showing that the stimulation of PAI-1, thrombin, and IL-6 in air pollutant-exposed mice is abrogated by treatment with PAI-1 inhibitor TM5614. The elevated levels of PAI-1 and IL-6 clearly indicate increased inflammation by exposure to air pollutant PM_{2.5}, and early inflammation plays a pivotal role in the development of downstream inflammatory signals and organ pathologies, especially in the lungs and cardiovascular system, including vascular occlusion and disruption of lung and heart tissue homeostasis (Figure 11). Suppression of IL-6 by PAI-1 inhibitor TM5614 can be explained in the light of previous observations made by Kubala and colleagues,²⁹ who demonstrated that while elevated PAI-1 stimulates the levels of IL-6 and activates downstream pSTAT3, depletion of PAI-1 is associated with down-regulation of IL-6 and pSTAT3 activation in response to inflammatory signals.²⁹

Next, we investigated the effect of short-term exposure to PM_{2.5} on lung and heart inflammation and apoptosis and showed that the lung morphology is abnormal in several PM_{2.5}-exposed mice (200 µg/mouse) in terms of its tissue density, consistent with previous observations that PM_{2.5} causes damage in lung morphological structure.³⁰ These results indicate that acute exposure to air pollutant is able to damage lung tissue architecture. However, in the presence of PAI-1 inhibitor TM5614, PM_{2.5} fails to induce pulmonary tissue damage. Furthermore, TM5614 reduces air pollutant PM_{2.5}-induced inflammation, apoptosis, and vascular dysfunction as evidenced by the decreased levels of inflammation- and apoptosis-associated factors in lungs derived from mice exposed to PM_{2.5} in the presence of TM5614. Similarly, TM5614 is also effective in suppression of PM_{2.5}-induced modestly elevated inflammatory and apoptotic events in murine hearts. These results signify that pharmacological neutralization of PM_{2.5}-induced PAI-1 is an effective approach for suppression of early events of air pollutant-induced cardiovascular and pulmonary pathological events. Previous studies demonstrated that PM_{2.5} induces the levels of PAI-1^{15,16} and that

elevated PAI-1 plays a key role in a number of subclinical and clinical pathologies, including inflammation, atherosclerosis, insulin resistance, obesity, and multimorbidities.^{10,11,14,31} The elevated PAI-1 may also contribute to cellular apoptosis in a context-dependent manner. For example, the elevated PAI-1 in alveolar type II cells derived from idiopathic pulmonary fibrosis patients is associated with elevated levels of cleaved caspase 3 and thus increased apoptosis.³² Therefore, pharmacological suppression or neutralization of PAI-1 is an ideal approach to control pathological/sustained inflammation and disease development like air pollutant-induced increased inflammation and apoptosis-driven cardiopulmonary diseases.

The most notable observation we made in the present study is the induction of significant levels of pulmonary and, to a lesser degree, cardiac vascular thrombosis in mice exposed to air pollutant PM_{2.5}, and that air pollutant exposure fails to develop pulmonary and cardiac thrombosis in the mice treated with PAI-1 inhibitor TM5614. This result is consistent with our observation that TM5614 significantly reduces PM_{2.5}-induced increased levels of inflammation and pro-thrombotic mediators IL-6, PAI-1, and thrombin. Furthermore, normalization of PM_{2.5}-induced increased blood pressure and heart weight by TM5614 treatment are also significant in the context of the pivotal role of PAI-1 in PM_{2.5}-induced vascular pathologies. In this context, it is important to note that previous studies implicated PM and higher levels of PAI-1 in high blood pressure.^{33,34} Like wild-type mice, several PAI-1 knockout mice also developed pulmonary thrombosis upon longer exposure to air pollutant PM_{2.5}. Interestingly, PAI-1 haplodeficient mice exposed to PM_{2.5} are partly protected from developing pulmonary thrombosis, indicating that, like pharmacological inhibition of PAI-1, partial genetic deficiency of PAI-1 or low levels of PAI-1 are beneficial for organisms exposed to air pollutants. Therefore, it is apparent that normalization of the levels of PAI-1, but not complete depletion, is favorable to prevent air pollutant-induced pulmonary pathologies. Further studies are necessary to understand the underlying molecular basis. Although previous reports suggested significant cardiac matrix remodeling in

mice and rats in response to exposure to air pollution,³⁵⁻³⁷ under our experimental setup (100 µg/once/week/4 weeks), we did not observe any significant change in collagen deposition in PM_{2.5}-exposed murine lungs and hearts. However, our *in vitro* study showed up-regulation of profibrogenic signal transducers and matrix proteins in cardiac fibroblasts exposed to PM_{2.5}, and importantly, TM5614 is effective in the suppression of these PM_{2.5}-induced profibrogenic responses. Further studies are required to investigate the effect of PM_{2.5} exposure with an increased number of doses/week for 3-6 months on pulmonary and cardiac pathological matrix remodeling and whether TM5614 can effectively block PM_{2.5}-induced pulmonary and cardiac fibrogenesis on that timescale. Together, these results signify the pivotal role of PAI-1 in air pollutant-induced cardiopulmonary vascular pathologies and establish the potential therapeutic efficacy of PAI-1 inhibitor TM5614, an FDA-approved drug for clinical trials of other PAI-1 related pathologies, in amelioration of air pollutant exposure-mediated cardiopulmonary pathologies.

In search of cellular and molecular events induced by exposure to air pollutant PM_{2.5}, we studied the effects of PM_{2.5} on cultured human cardiac fibroblasts in the presence and absence of TM5614. The *in vitro* results suggest that air pollutant PM_{2.5} modestly induces profibrogenic markers and regulators, namely profibrogenic PAI-1, matrix protein collagen, and transcriptional regulators SREBP1 and SREBP2; and most importantly, that PAI-1 inhibitor efficiently blocks air pollutant-induced increases in PAI-1, profibrogenic regulators, and target matrix proteins. The rescue of PM_{2.5}-induced suppression of Nrf2 levels by TM5614 is interesting, as it clearly indicates that suppression of Nrf2, a major transcriptional regulator of antioxidant genes, and augmentation of oxidative stress by PM_{2.5} can be prevented by pharmacological inhibition of PAI-1. This result is consistent with our previous observations showing PAI-1 inhibition is associated with the rescue of homocysteine-mediated suppression of the antioxidant regulator Nrf2¹³ and of doxorubicin-mediated suppression of antioxidant catalase,¹² as well as with prevention of accelerated cellular aging.

In conclusion, PAI-1 is a potentially druggable target for the treatment of air pollutant-induced cardiopulmonary vascular pathologies including inflammation, thrombosis, hypertension, and cardiac hypertrophy; and TM5614, a drug-like small molecule inhibitor of PAI-1, effectively ameliorates these cardiopulmonary vascular pathologies by suppression of air pollutant PM_{2.5}-induced regulators of inflammation, prothrombotic factors, profibrogenic regulators, and by rescue of PM_{2.5}-induced suppression of a key antioxidant regulator (Figure 11). Further *in vitro* studies are needed to test the efficacy of TM5614 in amelioration of other air pollutant-induced signaling pathways involved in cardiopulmonary vascular pathologies, including inflammatory signaling, canonical or non-canonical TGF- β signaling, and pro-apoptotic signaling pathways in endothelial cells and macrophages. Future expansion of the present study will be helpful to design clinical trials of TM5614 for the treatment of air pollutant-induced CPVDs.

Acknowledgments: We thank Northwestern University's Mouse Histology and Phenotyping Laboratory for technical support.

Source of Funding: This work is supported by the American Heart Association-Innovative Project Award (18IPA34170365) and by a grant from the National Institutes of Health (5R01HL051387).

Disclosures: None

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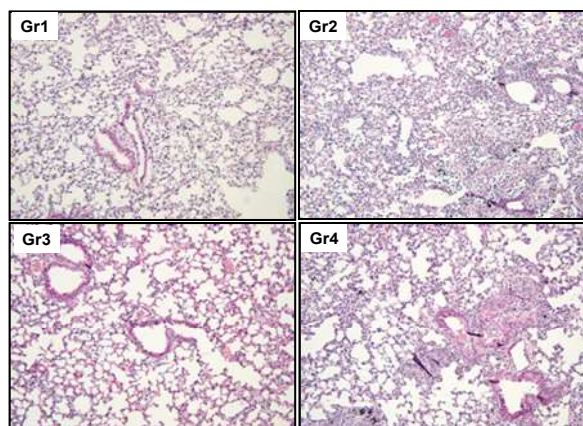
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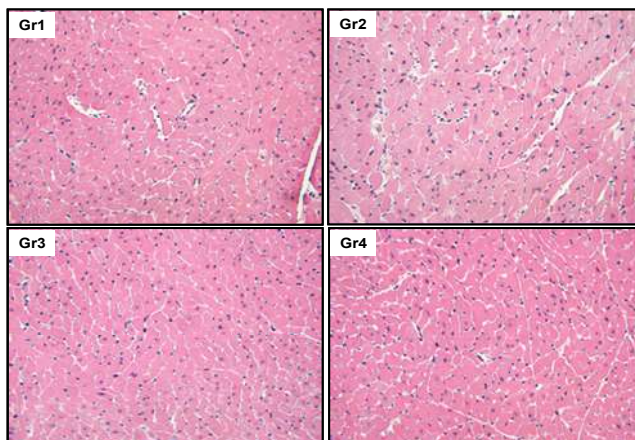
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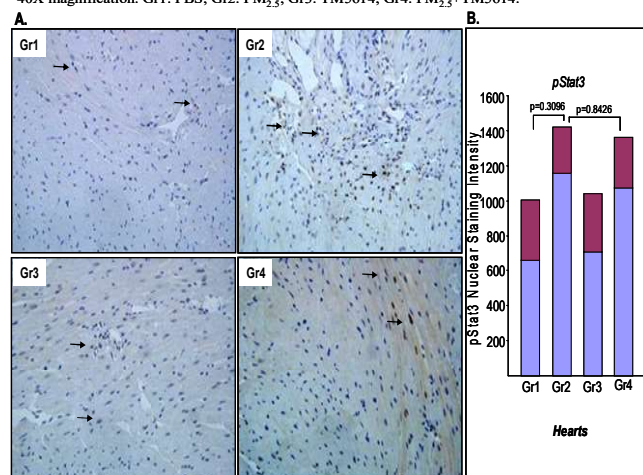
Ghosh et al. Supplemental Data



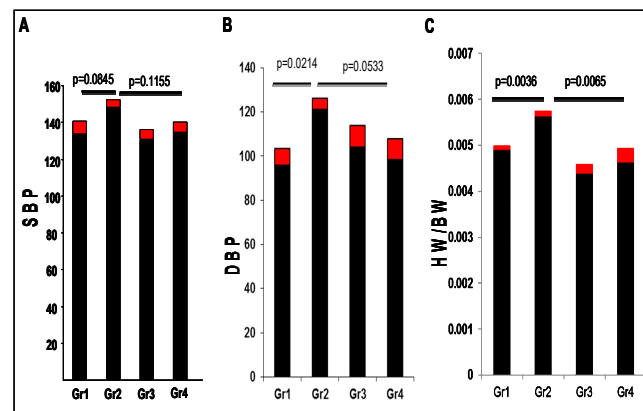
Supplemental Figure s1. Effects of PM_{2.5} and PAI-1 inhibitor TM5614 on lung morphology. Lungs collected from 4-groups were processed for H&E staining (n=4-7). Day 1-6: TM5614 (10 mg/Kg) or normal diet; Day 7: PM_{2.5} (200 µg/50 µl/mouse) or PBS instillation; Day 10: Lungs collected were processed for morphology study. Images reduced to 15% of original 20X images. Gr1 (PBS); Gr2: (PM_{2.5}); Gr3: TM5614; Gr4: PM_{2.5}+TM5614. Morphology denser: Gr1: 1/4; Gr2: 4/7; Gr3: 1/4; Gr4: 2/6



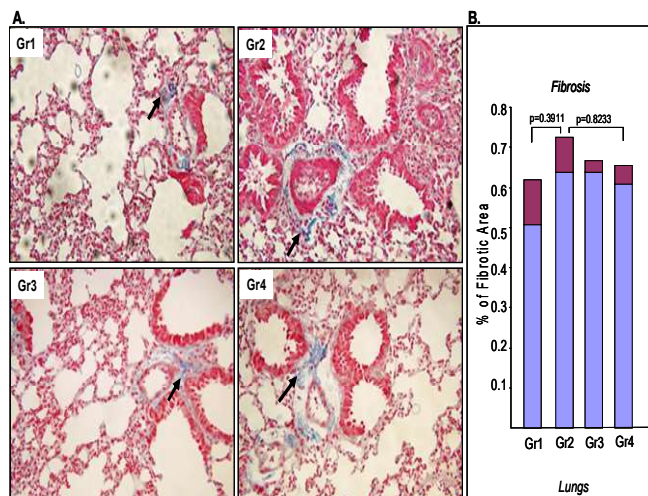
Supplemental Figure s2. Effect of PM_{2.5} and PAI-1 inhibitor TM5614 on cardiac morphology. Hearts collected from 4-groups (n=4-7) as indicated were processed for haematoxylin and eosin staining. [Day 1-6: TM5614 (10 mg/Kg); Day 7: PM_{2.5} (200 µg/mouse) or PBS instillation. Day 10: Hearts collected and processed for histological analysis. Images reduced to 15% of original 40X magnification. Gr1: PBS; Gr2: PM_{2.5}; Gr3: TM5614; Gr4: PM_{2.5}+TM5614.



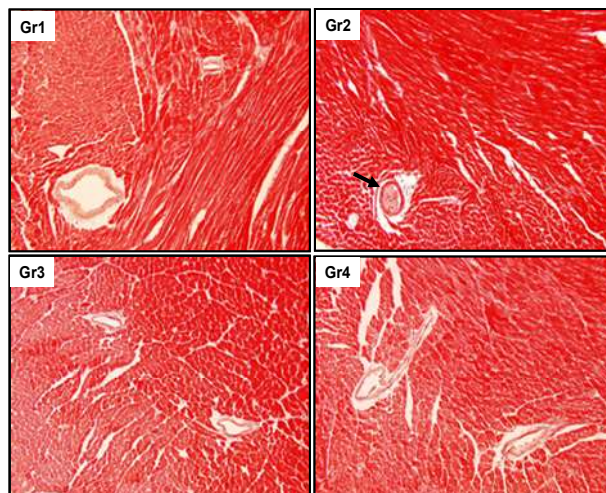
Supplemental Figure s3. Effect of PM_{2.5} and PAI-1 inhibitor TM5614 on inflammatory factor pStat3 in Hearts. Hearts were collected from 4-groups (n=3-6) and formalin fixed. Heart tissues were processed for immunohistochemistry using inflammatory marker pSTAT3 antibody. [Day 1-6: TM5614 (10 mg/Kg); Day 7: PM_{2.5} (200 µg/50 ml/mouse/once) or PBS intubation. Day 10: Hearts were collected and processed for immunohistochemistry. The representative image from each group was presented in (A). The levels of nuclear pSTAT3 in several fields of heart sections were determined by ImageJ software followed by statistical analysis (B). Representative images reduced to 15% of original 40X magnification. Gr1(PBS); Gr2 (PM_{2.5}); Gr3 (TM5614); Gr4 (TM5614+PM_{2.5}).



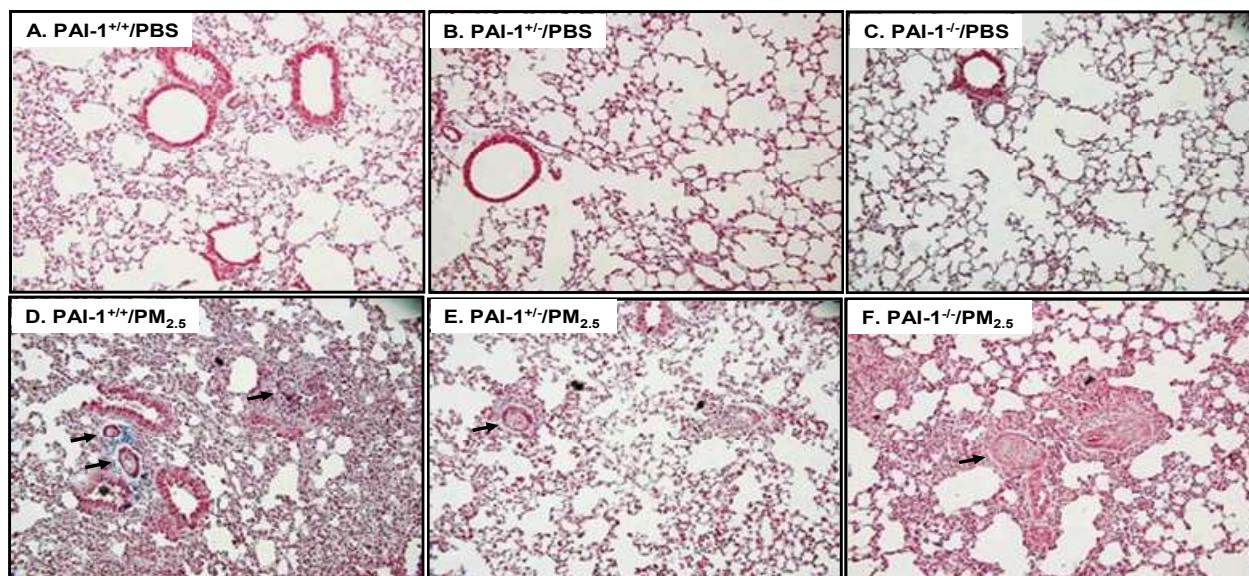
Supplemental Figure s4 . Effect of long-term exposure to PM_{2.5} on blood pressure and cardiac hypertrophy: Mice were divided into 4 groups. In Group 1, mice were fed with regular chow and received PBS. In Group 2, mice were fed with regular chow and received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). In Group 3, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS. In Group 4, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). Systolic (A) (n=3-7) and diastolic (B) (n=3-7) blood pressure in four groups. Heart weight/body weight in four groups are shown in (C), n=4-7.



Supplemental Figure s5. Effects of PM_{2.5} and PAI-1 inhibitor TM5614 on pulmonary matrix remodeling. Lungs collected from 4-groups (n=4-7) were processed for Masson's trichrome staining to determine the levels of pulmonary fibrosis. Mice were divided into 4 groups. In Group1, mice were fed with regular chow and received PBS as control. In Group 2, mice were fed with regular chow and received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). In Group 3, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS. In Group 4, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). Lungs collected were processed for Masson's trichrome staining. Representative images are reduced to 15% of original 40X images (A). Quantitative data of fibrosis using Fiji is just ImageJ software are shown in B.



Supplemental Figure s6. Effects of TM5614 on PM_{2.5}-induced cardiac thrombosis. Mice were divided into 4 groups. In Group1, mice were fed with regular chow and received PBS. In Group 2, mice were fed with regular chow and received PM_{2.5} (100 µg/mouse/once per week for 4 weeks). In Group 3, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PBS. In Group 4, mice were fed with chow containing TM5614 (10 mg/kg) for 6 days and then received PM_{2.5} (100 µg/mouse/once per weeks for 4 weeks). Analysis of hearts from each group reveal that exposure to PM_{2.5} modestly induces cardiac microthrombi with lesser extent compared to lungs in mice (microthrombi in Gr1: 0/4 mice; Gr2: 4/7 mice; Gr3: 0/5 mice; Gr4:0/4 mice). PM_{2.5} fails to induce cardiac thrombosis in mice treated with PAI-1 inhibitor TM5614 (Gr4). Images reduced to 15% of original 20X images are shown.



Supplemental Figure s7. Effects of PAI-1 haplo and complete deficiency on air pollutant PM_{2.5}-induced pulmonary pathologies. The wildtype, PAI-1 heterozygous and PAI-1 knockout mice were treated with PM_{2.5} (50µg/mouse/week for 4 weeks) or PBS as controls. All mice were fed with regular chow. Each group of mice viz. wildtype (A,D), PAI-1 heterozygous (B,E) and PAI-1 global knockout mice (C,F) were divided into 2 groups. While Group1 mice received 50 µl of PBS (A, B, C), Group 2 mice received PM_{2.5} (50 µg/mouse/once per week for 4 weeks) (D, E, F). There are total 6 groups. Representative images showing microthrombi are presented in A. Images reduced to 15% of original 20X magnification. Arrows indicate PM_{2.5}-induced microthrombi in lungs. Microthrombi in A:0/5; B:0/6; C: 0/4; D: 4/5; E:1/4; F: 3/6 mice.