Therapeutic potential of an orally effective small molecule inhibitor of plasminogen activator inhibitor for asthma


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Running title: PAI-1 and asthma

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Abstract

Asthma is one of the most common respiratory diseases. Although progress has been made in our understanding of airway pathology and many drugs are available to relieve asthma symptoms there is no cure for chronic asthma. Plasminogen activator inhibitor 1 (PAI-1), a primary inhibitor of tissue type and urokinase type plasminogen activators (tPA and uPA, respectively), has pleiotropic functions besides suppression of fibrinolysis. In this study, we show that administration of TM5275, an orally effective small molecule PAI-1 inhibitor, 25 days after ovalbumin (OVA) sensitization-challenge, significantly ameliorated airway hyper-responsiveness in an OVA-induced chronic asthma model. Furthermore, we show that TM5275 administration significantly attenuated OVA-induced infiltration of inflammatory cells (neutrophils, eosinophils, and monocytes), the increase in the levels of OVA-specific IgE and Th2 cytokines (IL-4 and IL-5), the production of mucin in the airways, and airway subepithelial fibrosis. Together, the results suggest that the PAI-1 inhibitor, TM5275, may have therapeutic potential for asthma through suppressing eosinophilic allergic response and ameliorating airway remodeling.

Key words: Asthma; PAI-1; PAI-1 inhibitor; animal model; therapeutic
**Introduction**

Asthma is one of the most common respiratory diseases affecting approximately 5% of population in the US. The pathological features of chronic asthmatic airways include infiltration of eosinophils, hyper-responsiveness, mucus hypersecretion, and subepithelial fibrosis. Despite intensive studies, the precise mechanisms underlying the pathogenesis of chronic asthma remain unclear. Most importantly, there are no effective treatments to cure chronic asthma, although anti-inflammatory steroids plus bronchodilators provide temporal relief of the symptoms during asthma exacerbation. Understanding the pathogenesis and developing effective treatments for chronic asthma have been and will continue to be a major challenge for clinicians and basic science researchers.

Plasminogen activator inhibitor 1 (PAI-1) is a primary inhibitor of tissue type and urokinase type plasminogen activators (tPA and uPA, respectively). Besides suppressing fibrinolysis through inhibition of tPA and uPA and thereby plasminogen activation, PAI-1 has pleiotropic functions, including inhibition of extracellular protein degradation, modulation of cell attachment and migration, and regulation of cell senescence and apoptosis processes (25). Importantly, it has been reported that PAI-1 protein is elevated in sputum of allergic asthmatic patients (1-3, 21, 22) and in experimental asthma models (23, 31). Individuals carrying polymorphic allele of the PAI-1 gene (4G instead of 5G), which leads to increased plasma PAI-1 level, have higher risk to develop asthma and show more severe asthmatic symptoms than 5G/5G carriers (28, 30, 32). Most importantly, it has been reported that intratracheal administration of small interfering RNA targeting PAI-1 (29) or a PAI-1 inhibitor tiplaxtinin (24) attenuated allergen-induced
pathological changes in experimental asthma models. Together, the data suggest that PAI-1 plays a critical role in the pathogenesis of asthma (3, 4, 28, 37), and therefore may serve as an ideal therapeutic target for the treatment of asthma. Nonetheless, despite strong evidence supporting the role of PAI-1 in asthma pathogenesis, no therapeutic drugs that target PAI-1 for the treatment of chronic asthma have yet been developed.

TM5275 is an orally effective small molecule PAI-1 inhibitor. It has potent anti-thrombotic activity in both rodents and nonhuman primates (cynomolgus monkey) (18). Importantly, TM5275 does not interfere with other serpin/serine protease systems such as $\alpha_1$-antitrypsin/trysin and $\alpha_2$-antiplasmin/plasmin, causes no obvious toxicity to the liver, kidney, hematopoietic system, central nervous system, or cardiovascular system of rats and monkeys when given in doses up to 2000 mg/kg/day for two weeks (18). It has no significant effect on activated partial thromboplastin time, prothrombin time, or bleeding time, the most common side effects of anticoagulation agents (18). These data suggest that TM5275 is relatively specific for PAI-1 with low toxicity. Therefore, we explored, in this study, the therapeutic potential of TM5275 for asthma in an OVA-induced chronic asthma model. Our results show that oral administration of TM5275, 25 days after OVA sensitization and challenge, significantly ameliorated OVA-induced airway hyper-responsiveness, eosinophil and neutrophil infiltration, mucin production, and subepithelial fibrosis. The results suggest that TM5275 may have therapeutic potential for asthma.
Materials and methods

Mouse model of chronic allergic asthma: A well-established chronic asthma model induced by ovalbumin (OVA) was used in this project to test the therapeutic value of TM5275 for chronic asthma (10, 11). Specifically, 6-8 weeks old female BALB/cJ mice were divided into three groups, 16 mice per group. Group 1 (non-asthmatic) mice were sensitized and challenged with saline. Group 2 (asthmatic) mice were sensitized with 50 µg of alum-precipitated chicken egg OVA by intraperitoneal injection twice (day 0 and day 14) and then challenged with OVA by aerosol inhalation, 5 mg/ml, 30 min/day, daily from day 21 to day 25, and 10 min/day every other day from day 26 to day 35. This group of mice were also treated with vehicle (20% DMSO in saline) by oral gavage daily from day 26 to day 35. Group 3 (asthmatic plus TM5275 treatment) mice were sensitized and challenged with OVA as the group 2 mice and treated with 40 mg/kg TM5275 (dissolved in 20% DMSO) by oral gavage daily from day 26 to day 35. The dose of 40 mg/kg TM5275 was chosen based on our previous study, in which we showed that oral administration of 40 mg/kg TM5275 for 10 consecutive days almost completely blocked TGF-β1-induced lung fibrosis with no obvious toxicity (no body weight loss) (13). A schematic diagram with the detailed information about sensitization, challenge, and treatment is presented in Fig 1. The whole experiment has been repeated once. Twenty-four hours after final challenge and TM5275 treatment, 8 mice from each group were used for lung function test using SCIREQ FlexiVent system, whereas another 8 mice from each group were sacrificed for histology and biochemistry analyses. All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and conducted at the UAB animal facilities under specific pathogen-free conditions.
Measurement of airway hyperresponsiveness. Airway hyper-responsiveness (AHR) was measured using FlexiVent system upon challenging with increasing concentrations of methacholine as we described previously (11). Briefly, 48 hours after the last OVA challenge and TM5275 treatment, mice were anesthetized with ketamine (450 mg/kg) and a tracheotomy tube (18G) was inserted and connected to the inspiratory and expiratory ports of a ventilator (Flexivent; SCIREQ, Montreal, PQ, Canada). Mice were mechanically ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml with a positive end-expiratory pressure of 2 to 4 cm H$_2$O. Methacholine (0, 5, 10, 15, and 20 mg/ml) were administered via aerosolization. From 20 seconds up to 3 minutes after each methacholine aerosol challenge, resistance, elastance, tissue damping, and total lung capacity were recorded continuously. Average value of each parameter was taken to express changes in murine airway function.

Zymography analysis of uPA and tPA activities: The activities of tPA and uPA in lung tissue were determined by zymographic analysis as we have described before (13, 39). Briefly, equal amounts of proteins were loaded onto 12% polyacrylamide gel containing 2 mg/ml casein in the presence of 5 μg/ml plasminogen. After electrophoresis, the enzyme reaction was initiated by incubating the gel in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 hours and the lytic areas were developed by staining the gel with a solution containing 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie blue G250. The gels were destained in the same solution without dye and scanned using Bio-Rad Fluor-s MultImaging system. To avoid the possible interference of matrix metalloproteinases (MMPs), EDTA (2 mM) was included in the Glycine-NaOH buffer during the incubation period. The tPA and uPA bands were identified based on the mobility of
the molecular weight markers. The photo-negative images of the gels are presented in the figures and assessed semi-quantitatively using the Image J analyzing software (NIH website).

**Bronchoalveolar lavage (BAL), BAL cell counting, and lung tissue processing:** Bronchoalveolar lavage (BAL) was performed with 0.8 ml of phosphate buffered saline (PBS). BAL fluid (BALF) was spun down at 400 g for 10 min; the supernatants were collected and the cells were re-suspended uniformly in saline. The BAL cells were centrifuged onto a microscope slide using a CytoSpin and stained with Diff-Quik™ stain set (B4132-1A, Siemens Healthcare Diagnostics Inc. Newark, DE, USA). Total cell numbers were counted and calculated based on the volume of the BAL fluid; differential cell counts were performed by counting 300-600 cells on each slide using oil immersion (100X) lens of Zeiss microscope and the percentages of monocytes, neutrophils, lymphocytes, and eosinophils calculated. After lavage, pulmonary artery vascular beds were perfused and then left lung fixed with 10% PBS buffered formalin for histology and immunochemistry analysis as we have described previously (27). The rest of the lung was frozen immediately in liquid nitrogen for biochemistry analyses.

**Measurement of urea concentrations in the plasma and BALF:** Concentrations of urea in BALF and in the plasma were measured using a commercially available kit (Teco Diagnostics, Anaheim, CA) following the protocol provided by the manufacturer. The ratio of urea concentrations in the plasma and in BAL fluid (dilution factor) is calculated for each mouse.

**Measurement of OVA specific IgE in plasma and BALF:** OVA specific IgE in mouse plasma and BALF were measured by anti-OVA mouse-IgE ELISA kit from BioVendor (Gunma, Japan)
following the instruction provided by the manufacturer. The results were normalized with urea dilution factors and the results are expressed as ng/ml of ELF.

**Measurement of cytokine/chemokines in BAL fluid:** The protein levels of cytokines and chemokines in mouse BAL fluid were analyzed using a Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) according to the manufacturer's instructions (26). The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). The results were normalized with urea dilution factors and expressed as ng/ml of ELF.

**Measurement of goblet cell hyperplasia:** Lung tissue fixed in 10% paraformaldehyde were stained with periodic acid-Schiff (PAS) to reveal mucin production in goblet cells as described previously (24). Goblet cell hyperplasia was quantified by determining the percentage of PAS-positive cells/length of bronchial basal membrane in 10 sites/mouse by quantitative morphometry techniques using Image J software as we have described previously (13).

**Measurement of mucus hypersecretion:** The amounts of mucin 5 subtype AC (MUC5AC) in BAL fluid were measured using a mouse Mucin 5 AC ELISA kit (Biomtik, Ontario, Canada) following the instruction provided by the manufacture. The results were normalized with urea dilution factors and expressed as ng/ml of ELF.

**Collagen staining:** Collagen deposition was revealed by Sirius red staining. High quality images were taken at 40 x magnifications. The collagen deposition around large airways (including the bronchioles and tertiary bronchi, identified by their ciliated pseudostratified
columnar epithelium and relative thick smooth muscle layer in the walls with no cartilage) and small airways (including the terminal bronchioles, identified by their low cuboidal epithelium and intact walls, and the respiratory bronchioles, identified by their low cuboidal epithelium and occasional mural alveoli) (19) was quantified by quantitative morphometry techniques using Image J software as we have described previously (13).

**Statistical analysis**

Airway hyperresponsiveness data (tissue damping, resistance, elastance, and total lung capacity) were analyzed by two-way analysis of variance (ANOVA) trend test to compare the slopes of the methacholine dose-response curves from three treatment groups (saline, OVA, and OVA + TM5275). Post-hoc analyses were conducted with Tukey test. The data presented in other figures were evaluated by one-way ANOVA and statistical significance was determined post-hoc by Fisher LSD test wherein p<0.05 was considered significant.
Results

**TM5275 attenuates OVA-induced airway hyperresponsiveness**

Asthmatic airways are highly sensitive to allergens or irritants (airway hyper-responsiveness, AHR). To elucidate whether TM5275 has therapeutic potential for asthma, we first assessed the effect of TM5275 on OVA-induced airway hyper-responsiveness induced by methacholine, using a FlexiVent system. Two-way ANOVA trend tests were conducted to compare the slopes of the methacholine dose-response curves from 3 treatment groups (saline, OVA, and OVA + TM5275). The results show that there were statistically significant differences in the slopes among 3 groups (Table 1) and between any two groups (Table 2) for the tissue damping, resistance, elastance, or total lung capacities. Post-hoc analyses further show that OVA sensitization-challenge significantly increased the resistance, tissue damping, and elastance, and reduced total lung capacity; treatment with TM5275, on the other hand, significantly attenuated OVA-mediated changes in these parameters under high methacholine concentration condition (Fig 2). No obvious toxicity, evaluated by body weight loss (data not shown), was observed with TM5275 treatment. The results suggest PAI-1 inhibitor mitigates allergy-induced airway hyper-responsiveness. Our results further confirm that TM5275 is relatively safe to use.

**OVA suppresses whereas TM5275 restores the activities of uPA and tPA in mouse lung**

To confirm the inhibitory effect of TM5275 on PAI-1 in mouse lung, the activities of uPA and tPA in mouse lung homogenates were assessed by zymographic analysis as we have described
previously (13). The results show that OVA sensitization-challenge led to decreases in the activities of both tPA and uPA in mouse lung tissue; administration of TM5275, on the other hand, partially protectd against OVA-induced inactivation of uPA and tPA in mouse lung (Fig 3). The results suggest that oral administration of TM5275 is effective.

**TM5275 suppressed OVA-induced infiltration of inflammatory cells in mouse lung**

Inflammatory response in asthmatic airways is characterized by infiltration and activation of eosinophils, which in turn contributes importantly to the pathophysiology of asthma. OVA-induced chronic asthma model is featured with eosinophilic inflammatory cell infiltration. To determine whether TM5275 can suppress OVA-induced inflammatory response, we counted the total cell numbers as well as the numbers of neutrophils, lymphocytes, monocytes, and eosinophils in BAL fluid after Diff-Quik staining. The results show that OVA sensitization-challenge significantly increased total cell numbers as well as the numbers of neutrophils, monocytes, and eosinophils in BAL fluid (Fig 4). Of the total cells found in BAL fluid of OVA challenged mice, 62% are eosinophils, confirming an eosinophilic inflammatory response in our asthma model. Treatment with TM5275, 25 days after OVA sensitization-challenge, significantly mitigated OVA-induced infiltration of inflammatory cells (Fig 4). Differential cell count results further show that TM5275 treatment significantly reduced OVA-induced infiltration of neutrophils (reduced by 80%), eosinophil (reduced by 57%), and monocytes (reduced by 43%), although it had no significant effect on OVA-induced lymphocyte infiltration. The results suggest that PAI-1 is involved in inflammatory cell infiltration and that TM5275 has potent anti-inflammatory function.
**TM5275 suppressed OVA-induced IgE production**

IgE plays a critical role in allergy and is produced mainly by eosinophils. To further determine whether PAI-1 inhibitor TM5275 suppresses eosinophilic allergic response, the amounts of OVA-specific IgE in the plasma and BAL fluid was assessed by ELISA. The results show that OVA sensitization-challenge significantly increased OVA-IgE in the plasma and in BAL fluid. TM5275 treatment, on the other hand, partially blocked the OVA-stimulated increase in OVA-IgE in the BAL fluid, although it had no significant effect on the level of plasma OVA-IgE (Fig 5). The results further confirm that PAI-1 inhibitor TM5275 suppressed OVA-induced eosinophilic inflammatory response in mice.

**Effects of TM5275 on OVA-induced cytokine production**

Increased Th2 cytokine production is associated with asthma and contributes importantly to asthmatic airway pathology. To further elucidate the mechanism whereby TM5275 mitigates OVA-induced airway hyperresponsiveness, the amounts of Th1 and Th2 cytokines in BAL fluid were measured by Bio-Plex mouse cytokine ELISA kit. The results show that OVA sensitization-challenge significantly increased the amounts of IL-4 and IL-5 in BAL fluid, although it had no significant effect on other cytokines including IL-2, IL-10, IL-12, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-a), and granulocyte macrophage colony stimulating factor (GM-CSF) (Fig 6). TM5275 treatment, on the other hand, reduced OVA-stimulated IL-4 and IL-5 production (Fig 6).
**TM5275 reduced OVA-stimulated mucin production**

Mucin overproduction, one of the pathological features of asthmatic airways, was assessed by Periodic Acid Schiff (PAS) staining and by measuring mucin 5 subtype AC (MUC5AC), a major form of mucin secreted by airway epithelial cells and associated with asthma, in BAL fluid by ELISA. PAS staining results show that OVA sensitization-challenge significantly increased the amount of mucin in airway epithelial cells whereas treatment with TM5275 partially blocked OVA effect (Fig 7A&B). MUC5AC ELISA results further show that OVA sensitization-challenge significantly increased the amount of MUC5AC in BAL fluid; TM5275 administration again reduced the amount of secreted MUC5AC stimulated by OVA (Fig 7C). Together, the results suggest that TM5275 may have therapeutic potential for asthma through reducing allergy-induced airway mucin production.

**TM5275 reduced subepithelial collagen deposition in airways**

Collagens are the major type of extracellular matrix. Subepithelial collagen deposition (airway remodeling) leads to airway narrowing and airflow obstruction. As PAI-1 plays a critical role in the regulation of collagen degradation, we further examined whether TM5275 treatment reduces collagen deposition in large and small airways by Sirius Red staining. The results show that mice sensitized and challenged with OVA had significantly increased amount of collagen deposition around large airways, although OVA sensitization-challenge had no significant effect on collagen deposition in small airways. Oral administration of TM5275, 25 days after OVA sensitization and challenge, significantly reduced the amount of collagen in the large airways.
The results suggest that TM5275 may have therapeutic potential for chronic asthma by reducing allergy-induced subepithelial collagen deposition.
Discussion

PAI-1 expression is increased in the sputum of asthmatic patients and in experimental asthma models, and has been shown to play a critical role in the pathogenesis of asthma (1-3, 21-24, 28-32). Although PAI-1 siRNA and a small molecule PAI-1 inhibitor, tiplaxtinin, have been tested in animal models, no therapeutics that target PAI-1 for the treatment of asthma has been yet developed (24, 29). TM5275, 5-chloro-2-[(2-[4-(diphenylmethyl) piperazin-1-yl]-2-oxoethoxyl}(28)acetyl]amino] benzoate, is an orally effective small molecule PAI-1 inhibitor. It has potent antithrombotic activity in rat and monkey thrombosis models (18). Most importantly, it does not interfere with other serpin/serine protease system nor prolong bleeding time or cause obvious toxicity in rats or monkeys when administrated with up to 2000 mg/kg for 2 weeks (18), suggesting that TM5275 is relatively specific for PAI-1 and safe to use (low toxicity). In this study, we show that oral administration of 40 mg/kg of TM5275, a dose that is much lower than its No Observed Adverse Effect Level (NOAEL, 2000 mg/kg), 25 days after OVA sensitization and challenge, ameliorated OVA-induced airway hyperresponsiveness and suppressed OVA-induced eosinophilic inflammatory response, mucin production, and airway remodeling. The results suggest that TM5275 may have therapeutic potential for chronic asthma. It should be pointed out that, although tiplaxtinin, another small molecule PAI-1 inhibitor, has been shown previously to ameliorate airway pathological changes when given simultaneously with OVA in a similar asthma model (24), TM5275 may be a better candidate drug as its toxicity is lower (NOAEL of TM5275 in monkey is >2,000 mg/kg, whereas NOAEL of tiplaxtinin in dogs is 100 mg/kg) and the bioavailability is higher (96% in monkeys for TM5275 vs. 36% in dogs for tiplaxtinin), compared to tiplaxtinin (9, 18).
One of the pathological features of asthmatic airways is inflammation. Eosinophils play important role in allergic response and in asthma pathology. In this study, we show that challenge with OVA dramatically increased the number of eosinophils and the amount of OVA-IgE in BAL fluid, confirming the nature of allergic response in our OVA-induced asthma model. Treatment with TM5275 significantly reduced the number of eosinophils (by 57%) and the amount of OVA-IgE in BAL fluid of OVA challenged mice, indicating that PAI-1 is involved in eosinophilic allergic response in asthma. Besides eosinophils, TM5275 administration also significantly reduced the numbers of neutrophils and monocytes in BAL fluid of OVA challenged mice. It should be pointed out that, although the percentages of cell types in BAL fluid are similar between OVA and OVA plus TM5275 groups (4%, 29.3%, 62.4%, and 4.3% in OVA challenged mice vs. 6.4%, 35.4%, 56.4%, and 1.8% in OVA plus TM5275 treated mice for lymphocytes, monocytes, eosinophils, and neutrophils, respectively), TM5275 had more dramatic effect on BALF cell counts of neutrophils (reduced by 80%), eosinophils (reduced by 57%), and monocytes (reduced by 43%) than on lymphocytes (no significant decrease). The mechanism and biological significance underlying the selective effect of TM5275 on BAL cells is unclear and warrants further investigation. Associated with suppression of inflammatory cell infiltration, TM5275 reduced the production of OVA-IgE as well as IL-4 and IL-5, two Th2 cytokines that promote eosinophil response and IgE production. The results suggest that TM5275 has potent anti-inflammatory effect. Similar effects on inflammatory cell infiltration have been reported by other groups with another PAI-1 inhibitor, tiplaxtinin (24), and PAI-1 siRNA (29). Together, the results suggest that PAI-1 inhibitors may have therapeutic potential for asthma through suppressing inflammatory response, specifically eosinophilic response.
The mechanism whereby TM5275 reduces the numbers of neutrophils, eosinophils, and monocytes in BAL fluid is unknown at moment. PAI-1 has pleiotropic functions besides inhibiting the activities of uPA and tPA. It binds to extracellular matrix protein vitronectin and thereby blocks the binding of vitronectin to integrins and uPAR on cell surface, leading to cell detachment/migration (6, 36, 38). PAI-1 can also affect cell migration and function through binding to uPA-uPAR and then low-density lipoprotein receptor-related protein (LRP) on cell surface (7, 16). Interestingly, it has been reported that macrophage migration depends on the interaction between PAI-1 and LRP, and that TM5275 inhibits macrophage migration through interfering such interaction (16). Therefore, one of the potential mechanisms whereby TM5275 reduces the numbers of neutrophils, eosinophils, and monocytes in BAL fluid is to block PAI-1-mediated cell migration (infiltration) through interfering with the interaction between PAI-1 and vitronectin and/or LRP. Emerging evidence also shows that PAI-1 modulates survival/apoptosis process in different types of cells (12, 13, 34, 41). Previous study from this lab showed that TM5275 induces p53, a master apoptosis inducer, and apoptosis in primary human (13) and mouse (12) lung fibroblasts. A previous study from this group also showed that treatment with PAI-1 protein inhibited spontaneous and TNF-α-induced apoptosis of neutrophils, whereas knockout of the PAI-1 gene (PAI-1−/− mice) promoted neutrophil apoptosis induced by LPS (41). Therefore, another potential mechanism whereby TM5275 reduced the numbers of eosinophil, neutrophils, and/or monocytes in BAL fluid of OVA challenged mice is to induce apoptosis of these inflammatory cells. More studies are needed to decipher the mechanisms whereby TM5275 reduces inflammatory cell numbers in BAL fluid.
Hypertrophy and hyperplasia of goblet cells, which leads to overproduction of mucin, is another important pathological feature of asthmatic airways. Using periodic acid-Schiff (PAS) staining technique, Lee et al. showed that treatment with PAI-1 inhibitor tiplaxtinin reduced OVA-induced mucin overproduction, although the mechanism by which tiplaxtinin reduces mucin overproduction was not explored (24). In this study, we show that OVA challenge dramatically increased, whereas TM5275 administration significantly reduced OVA-stimulated, mucin production in airways by PAS staining and by measuring the amount of MUC5AC in BAL fluid. We further show that TM5275 administration attenuated OVA-mediated increase in IL-4 and IL-5, two Th2 cytokines that have been shown to stimulate mucin production in goblet cells (17, 20, 35). Together, our results suggest that TM5275 administration ameliorated OVA-induced airway mucin production/accumulation probably through inhibition of inflammatory response and Th2 cytokine production.

Airway remodeling or subepithelial fibrosis is another important pathological feature of chronic asthmatic airways. Collagens are the major types of extracellular matrix proteins and the major component of fibrotic tissue. Previous study from this lab and others has shown that PAI-1 inhibited collagen degradation in cultured fibroblasts (39) and plays an important role in the development of lung fibrosis (5, 8, 13, 33, 40). In this study, we show that OVA sensitization-challenge significantly increased collagen deposition in large airways, whereas treatment with TM5275 partially blocked OVA effect. These results are consistent with our previous findings, although in a different disease model (13). In that study, we showed that treatment of mice with TM5275 almost completely blocked collagen and hydroxyproline accumulation in the lung in a mouse lung fibrosis model induced by AdTGF-β1^{223/225}, an adenovirus expressing constitutively
active TGF-β1, when given 4 days after AdTGF-β123/225 instillation (13). It is unclear at moment whether TM5275 reduces collagen deposition/accumulation by stimulating collagen degradation and/or by suppressing collagen synthesis. As previous studies from this lab and others have shown that PAI-1 suppresses collagen degradation (14, 15, 39), it is speculated that TM5275 reduces collagen accumulation in large airways in part by increasing collagen degradation.

In summary, our data suggest that orally effective small molecule PAI-1 inhibitor TM5275 attenuates OVA-induced pathophysiological changes in mouse airways and may have therapeutic potential for chronic asthma.
Acknowledgements

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Table 1. Two-way ANOVA trend test of the slopes of the methacholine dose-response curves among 3 treatment groups

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Table 2. Two-way ANOVA trend test of the slopes of the methacholine dose-response Curves between two treatment groups

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Fig 1. Schematic diagram of experimental design.

Fig 2. Effects of TM5275 on OVA-induced airway hyperresponsiveness. Airway hyperresponsiveness was assessed using a FlexiVent system with increasing concentrations of methacholine as described in the Materials and Methods section. The average tissue damping, resistance, elastance, and total lung capacity in each concentration of mechacholine were calculated from the record data of 12-14 mice. The statistical results were from Tukey post-hoc analyses. α, Significantly different from same concentration of methacholine-stimulated saline control mice; β, significantly different from same concentration of methacholine-stimulated OVA alone challenged mice (p<0.05, n=12-14).

Fig 3. Effect of TM5275 on the activities of uPA and tPA in mouse lung. The activities of tPA and uPA in lung tissue were determined by zymographic analysis as described in the Material and Method section. The photo-negative images of the gels are presented in the figures and the band intensities were semi-quantified using the Image J analyzing software. α, Significantly different from saline control mice; β, significantly different from OVA alone group (p<0.05, n= 4-5)

Fig 4. Effect of TM5275 on OVA-induced inflammatory responses in mouse lung. Total cell numbers as well as differential cell counts in BAL fluid were determined after the cells were centrifuged onto a microscope slide and stained with Diff-Quik™ as described in the Materials
and Methods section. Top panels: representative BAL cell staining pictures; bottom panel, quantification data of BAL cells. ND, not detected; α, significantly different from saline control mice; β, significantly different from OVA alone group (p<0.05, n=5-8).

**Fig 5. Effect of TM5275 on OVA-induced IgE in the plasma and BAL fluid.** OVA specific IgE in the plasma and BAL fluid was measured using anti-OVA mouse-IgE ELISA kit and the results normalized by urea dilution factors as described in the Materials and Methods section. α, Significantly different from saline control mice; β, significantly different from OVA alone group (p<0.05, n=7).

**Fig 6. Effect of TM5275 on OVA-stimulated production of cytokine/chemokines in BAL fluid.** The protein levels of cytokines/chemokines in mouse BAL fluid were analyzed using a Bio-Plex multiplex suspension cytokine array and the data analyzed using Bio-Plex Manager software as described in the Materials and Methods section. α, Significantly different from saline control mice; β, significantly different from OVA alone treated mice (p<0.05, n=11-13).

**Fig 7. Effect of TM5275 on OVA-induced mucin production.** Mucin production in airway epithelial cells were revealed by periodic acid-Schiff (PAS) staining and by measuring MUC5AC in BAL fluid using an ELISA kit as described in the Materials and Methods section. Left panels: representative pictures of PAS staining; the middle panel, semiquantified results of PAS staining; the right panel, ELISA results of MUC5AC in BAL fluid. α, Significantly different from saline control mice; β, significantly different from OVA alone group (p<0.05, n=4-7).
Fig 8. Effect of TM5275 on OVA-induced collagen deposition in small and large airways. Collagen deposition in the large and small airways in mouse lung was revealed by Sirius red staining and quantified by morphometric techniques using Image J software as described in the Materials and Methods section. The results are expressed as area of collagen/mm² basement membrane. α, Significantly different from saline control mice; β, significantly different from OVA alone group (p<0.05, n=11-13).

Fig 9. Potential mechanisms whereby TM5275 ameliorates OVA-induced airway pathophysiological changes.
OVA (50 μg) or saline i. p. on Day 0.

OVA (50 μg) or saline i. p. on Day 14.

OVA aerosol exp. 5 mg/ml, 30 min/day (day 21 to day 25).

OVA aerosol exp. 5 mg/ml, 10 min every other day; TM5275 40 mg/kg or solvent, gavage daily (day 26 – day 35).

Day 36: Sacrifice (histologie & biochemistry analyses).

Day 37: Lung function test.

Fig 1
Fig 2
Fig 3

Activities (arbitrary units)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>OVA</th>
<th>OVA+TM5275</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA</td>
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<tr>
<td>uPA</td>
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</tbody>
</table>
Fig 4

Saline | OVA | OVA+TM5275

<table>
<thead>
<tr>
<th>Cell numbers in BALF ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Lymph.</td>
</tr>
<tr>
<td>Monocy.</td>
</tr>
<tr>
<td>Eosino.</td>
</tr>
<tr>
<td>Neutro.</td>
</tr>
</tbody>
</table>

- Saline: ND
- OVA: $\alpha$
- OVA+TM5275: $\beta$
Fig 5

Plasma Anti-OVA IgE (U/ml)

- Saline
- OVA
- OVA + TM5275

Anti-OVA IgE (U/ml)

Plasma

ELF

Fig 5
Fig 6

Cytokines in BALF (ng/ml)

- IL-4
- IL-5
- IL-10
- IFN-γ
- TNF-α
- IL-2
- IL-12
- GM-CSF

Saline
OVA
OVA + TM5275
Fig 7

Saline

OVA

OVA + TM5275
Fig 8

Area of collagen (mm² basement membrane)

- Large airways
- Small airways

Saline
OVA
OVA + TM5275
Inflammatory cells infiltration

Cytokines/chemokines (IL-4, IL-5…)

Epithelial hypertrophy mucin overproduction

Airway obstruction/ hyperresponsiveness

Subepithelial fibrosis

Increases ECM degradation

Fig 9