Tryptase Inhibition Blocks Airway Inflammation in a Mouse Asthma Model


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Tryptase Inhibition Blocks Airway Inflammation in a Mouse Asthma Model


Release of human lung mast cell tryptase may be important in the pathophysiology of asthma. We examined the effect of the reversible, nonelectrophilic tryptase inhibitor MOL 6131 on airway inflammation and hyper-reactivity in a murine model of asthma. MOL 6131 is a potent selective nonpeptide inhibitor of human lung mast cell tryptase based upon a β-strand template (Kᵢ = 45 nM) that does not inhibit trypsin (Kᵢ = 1,061 nM), thrombin (Kᵢ = 23, 640 nM), or other serine proteases. BALB/c mice after i.p. OVA sensitization (day 0) were challenged intratracheally with OVA on days 8, 15, 18, and 21. MOL 6131, administered days 18–21, blocked the airway inflammatory response to OVA assessed 24 h after the last OVA challenge on day 22; intranasal delivery (10 mg/kg) had a greater anti-inflammatory effect than oral delivery (10 or 25 mg/kg) of MOL 6131. MOL 6131 reduced total cells and eosinophils in bronchoalveolar lavage fluid, airway tissue eosinophilia, goblet cell hyperplasia, mucus secretion, and peribronchial edema and also inhibited the release of IL-4 and IL-13 in bronchoalveolar lavage fluid. However, tryptase inhibition did not alter airway hyper-reactivity to methacholine in vivo. These results support tryptase as a therapeutic target in asthma and indicate that selective tryptase inhibitors can reduce allergic airway inflammation. The Journal of Immunology, 2002, 168: 1992–2000.

Asthma is a chronic inflammatory disease of the airways, characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyper-responsiveness to both inhaled allergens and non-specific stimuli. Mast cells are important in the pathogenesis of asthma, with release of inflammatory mediators such as histamine, leukotrienes, cytokines, and serine proteases after exposure to allergens. The serine protease tryptase, localized in mast cell granules, constitutes up to 25% of the total mast cell protein (1, 2) and may play a key role in the pathogenesis of asthma and other allergic disorders (3, 4). Unlike most trypsin-like serine proteases, tryptases are activated intracellularly. As a consequence of IgE-mediated mast cell degranulation, tryptase is exocytosed from secretory granules together with histamine and other preformed mediators (5). After release extracellularly, tryptases are resistant to circulating inhibitors of other tryptase-like proteases and may remain active for a longer period after release than other proteases (5). Tryptase levels are elevated in the bronchoalveolar lavage (BAL) fluid from atopic asthmatics compared with nonasthmatic controls at baseline (6, 7) and are significantly increased shortly after Ag challenge in patients with allergic asthma (8).

Tryptase has potent biologic activities that may contribute to the inflammatory response and airway hyper-reactivity seen in asthma. Tryptase inactivates the airway-relaxing activity of vasoactive intestinal peptide (9), which is released from airway motor nerves; bronchorelaxation in guinea pigs is blocked by enhanced degradation of vasoactive intestinal peptide (10). Tryptase hydrolyzes calcitonin gene-related peptide (11), which has endogenous vasodilatory activity. Tryptase promotes human mast cell degranulation (12); induces eosinophil and neutrophil migration (13); amplifies the bronchoconstrictor effects of histamine on lung tissue (9); stimulates the growth of airway fibroblasts, smooth muscle cells, and epithelial cells (14); and activates stromelysin (15). Tryptase activates proteinase-activated receptor-2 (16–18), a G protein-coupled receptor that is important in the control of inflammation and proliferation (19). In vivo inhalation of tryptase into the airways results in bronchoconstriction and development of airway hyper-reactivity in allergic sheep (20). Recently, tryptase inhibitors have been shown to reduce Ag-induced airway hyper-reactivity in allergic guinea pigs and sheep (21–23).

Our study goal was to determine the therapeutic potential of the selective small molecule inhibitor of tryptase, MOL 6131, on allergic airway inflammation and airway hyper-reactivity in a mouse model of asthma. We found that tryptase inhibition inhibits eosinophil infiltration into the lungs and airway mucus release, but does not alter airway hyper-reactivity to methacholine.
Materials and Methods

Tryptase inhibitor MOL 6131

A β strand template library (24–26) was used to develop the tryptase inhibitor MOL 6131 (MW 756.90 with C₈H₁₄N₃O₂). A molecular docking model of MOL 6131 into two adjacent active sites (i.e., A and D or B and C monomers) of human β-tryptase (pdb1a01.ent) (27, 28) was generated by Insight II software (MSI, San Diego, CA) based on the x-ray structure of similar compounds bound to the homologous serine protease, thrombin (29).

In vitro serine protease inhibition assays

All serine protease inhibition assays were performed at room temperature in 96-well microplates using a Bio-Rad model 3550 (Bio-Rad, Cambridge, MA). SpectroMax (model 250, Molecular Devices, Sunnyvale, CA) or Fluoroscan Ascent fluorescence (Labsystems, Helsinki, Finland) plate reader. Either 1-mM solutions of test compounds in water or 10-nM solutions of test compounds in DMEM served as the stock solution for each inhibition assay. For tryptase assays, the release of pNA from the chromogenic substrate S-2366, p-N-p-tosyl-Gly-Pro-Arg-pNA (Sigma, St. Louis, MO) or the fluorogenic substrate N-p-tosyl-Gly-Pro-Arg-AMC (Molgen, MA), SpectroMax (model 250, Molecular Devices, Sunnyvale, CA) or Graphpad PRISM (Graphpad Software, San Diego, CA). The determined initial velocities were then non-linear least squares fitted against the concentrations of a tested compound using either GraFit (Erithacus Software, London, U.K.) or GraphPad PRISM (Graphpad Software, San Diego, CA).

The general format of the assays is as follows. One hundred microliters of an inhibitor solution and 50 µl enzyme solution were placed in a microplate well and incubated at room temperature for 30 min, and then 100 µl substrate solution was added to initiate the reaction. In the trypsin assay, 0.2 nM human lung trypsin (Elastin Products, Owensville, MO) and 200 µM S-2366 were used in Tris buffer, pH 8.0. In thrombin assays, 0.025 nM human thrombin (Sigma) and 40 µM N-p-tosyl-Gly-Pro-Arg-pNA or 20 nM N-p-tosyl-Gly-Pro-Arg-AMC were used in Tris buffer, pH 8.0. In trypsin assays, 0.5 nM bovine trypsin (Sigma) and 40 µM N-p-tosyl-Gly-Pro-Arg-pNA or 20 nM N-p-tosyl-Gly-Pro-Arg-AMC were used in Tris buffer, pH 8.0. In coagulation factor VII assays, 100 µl inhibitor in 50 nM Tris buffer, pH 8.0, and 50 µl 10 nM factor VIIa in Dade Innovin (recombinant human tissue factor thromboplastin, Dade International, Miami, FL) were added in a microplate well. In factor Xa assays, 0.25 nM factor Xa (Hematologic Technologies, Essex Junction, VT) and 40 µM S-2765 were used in Tris buffer, pH 8.0. In factor Xa assays, 0.2 nM factor Xa (Hematologic Technologies) and 200 µM S-2366 were used in Tris buffer, pH 8.0. In factor Xa assays, 1 nM human plasma factor Xa (Calbiochem, San Diego, CA) and 75 µM Z-FR-AMC were used in Tris buffer, pH 8.0. In urokinase assays, 160 nM human urokinase (ICN Pharmaceuticals, Costa Mesa, CA) and 40 µM S-2444 were used in Tris buffer, pH 8.0.

To determine whether MOL 6131 is a competitive inhibitor of human trypsin, MOL 6131 was serially diluted from a 10-nM stock solution to 600, 300, 150, and 75 nM final concentrations. At each MOL 6131 concentration, trypsin substrate, S-2366, was titrated at 800, 600, 400, 300, 200, 150, 100, and 50 µM. Initial rates at each concentration of S-2366 and MOL 6131 were determined as described above and transformed to 1/V, the inverse of initial rates vs the inverse of S-2366 concentrations were plotted (Leweineber-Burk plot).

Allergen sensitization/challenge protocol

OVA (500 µg/ml; Pierce, Rockford, IL) in PBS was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma) in distilled water and incubated for 60 min at room temperature after adjustment to pH 6.5 using 10 N NaOH. After centrifugation at 750 × g for 5 min, the OVA/alum pellet was resuspended to the original volume in distilled water. Mice received an i.p. injection of 100 µg OVA (0.2 ml of 500 µg/ml in normal saline) complexed with alum on day 0. Intratracheal (i.t.) challenges were performed as previously described by Andrea et al. (30). Mice were anesthetized by i.p. injection of a 0.2-mM mixture of ketamine and xylazine (0.44 and 6.3 mg/ml, respectively) in normal saline and were placed on a board in the supine position. Two hundred fifty micrograms (100 µl of a 2.5 mg/ml of OVA (on day 8) and 125 µg (50 µl of 2.5 mg/ml of OVA (on days 21) were placed on the back of each animal. The deposition pattern of the OVA by this i.t. delivery was examined using toluidine blue dye. OVA (2.5 mg/ml) was mixed in toluidine blue, and 100 µl was administered by i.t. delivery. The majority of the toluidine blue dye staining was seen in the lumen and interstitium of the tracheal wall, with the remainder in the lumen and interstitium of the small airways. Toluidine blue dye staining was not detected in the esophagus or stomach. Control mice received i.p. saline with alum on day 0 and i.t. saline on days 8, 15, 18, and 21.

Drug treatment

MOL 6131 was dissolved in PBS (pH 7.4) and given by i.n. (10 mg/25 ml/kg) under ketamine/xylazine anesthesia or oral (10 mg/10 ml/kg or 25 mg/10 ml/kg) administration. Mice received MOL 6131 on days 18 through 21, once daily. On days 18 and 21 they received MOL 6131 30 min before OVA challenge. Control groups received PBS only.

Pulmonary function testing

In vivo airway responsiveness to methacholine was measured 24 h after the last OVA challenge in conscious, freely moving, spontaneously breathing mice using whole body plethysmography (model PLY 3211; Buuxo Electronics, Sharon, CT) as previously described by Hamelmann et al. (31). Mice were challenged with aerosolized saline or increasing doses of methacholine (5 and 30 mg/ml) generated by an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA) for 2 min. The degree of bronchoconstriction was expressed as enhanced pause (Penh), a calculated dimensionless value, which correlates with the measurement of airway resistance, impedance, and intrapulmonary pressure in the same mouse. Penh readings were taken and averaged for 4 min after each nebulization challenge. Penh was calculated as follows: Penh = [(Tf/Ti) – 1] × (PEF/PFI), where Ti is expiration time, Tf is relaxation time, PEF is peak expiratory flow, and PFI is peak inspiratory flow × 0.67 coefficient. The time for the box pressure to change from a maximum to a user-defined percentage of the maximum represents the relaxation time. Tf measurement begins at the maximum box pressure and ends at 40%.

Bronchoalveolar lavage

After measurement of airway hyper-reactivity, the mice underwent exanguination by cardiac puncture, and then BAL was collected (0.4 ml saline, three times) from the right lung after tying off the left lung at the mainstem bronchus. Total BAL fluid cells were counted from a 0.05-m1 aliquot, and the remaining fluid was centrifuged at 200 × g for 10 min at 4°C. Cell pellets were resuspended in saline containing 10% BSA with smears made on glass slides.

Eosinophils were stained for 5 min with 0.05% aqueous eosin and 5% aqueous distilled water, rinsed with distilled water, and counterstained with 0.07% methylene blue (32).

Lung histopathology

After BAL, the trachea and upper and lower lobes of the left lung were removed and fixed for 24 h in 10% neutral buffered formalin solution. The tissues were embedded in paraffin and cut into 5-µm sections. The tissues sections were stained with Diamine’s solution to identify eosinophils, with H&E to identify neutrophils/other inflammatory cells and edema, and with Alcian Blue, pH 2.5, and Nuclear Fast Red counterstaining to identify airway goblet cells and mucus. The degree of airway inflammatory cell infiltration (0–4+), the number of eosinophils and neutrophils per unit airway area (2200 µm²; goblet cell number (percentage of airway cells), mucus occlusion of airway diameter (0–4+), and airway edema (0–4+) were determined by morphometry; morphometric analyses were performed by three blinded to the experimental design blinded to the experimental design (33). VCAM-1 was localized in the lung tissue by immunocytochemistry using rat anti-mouse VCAM-1 mAb (BD Pharmingen, San Diego, CA) as previously described (34).
Cytokine assays

BAL fluid levels of IL-4 (≥2 pg/ml), IL-13 (≥1.5 pg/ml), and eotaxin (≥3 pg/ml) were determined by ELISA (R&D Systems, Minneapolis, MN).

Statistical analyses

The data are reported as the mean ± SEM. Differences were analyzed for significance (p < 0.05) by either Student’s two-tailed t test or ANOVA using the protected least significant difference method as indicated.

Results

MOL 6131 is a potent selective human tryptase inhibitor

The β strand template structure of MOL 6131 and a molecular model of the interaction of MOL 6131 with two adjacent active sites (i.e., S1 pocket of monomers A and D) of human β-tryptase are shown in Figs. 1 and 2 respectively. The selectivity profile of MOL 6131 against serine proteases is shown in Table I. MOL 6131 is a potent inhibitor of human mast cell tryptase, with a Ki of 45 nM. MOL 6131 is a highly selective, reversible competitive inhibitor of tryptase compared with trypsin (Ki = 1,061 nM) and thrombin (Ki = 23, 640 nM). In addition, MOL 6131 does not inhibit other serine proteases (Table I; Ki >40,000 vs factor VIIa, urokinase, plasma kallikrein, factor Xa, and factor Xla). Line-weaver-Burk plot analysis indicates that MOL 6131 is a competitive inhibitor of tryptase (Fig. 3).

Effect of MOL 6131 on eosinophil recruitment into BAL fluid

On day 22, 24 h after the final i.t. OVA or saline treatment in mice from each experimental group, BAL was performed, and lung tissue was obtained to assess inflammatory cell infiltration and mucus release. The effect of the selective tryptase inhibitor MOL 6131 on allergic airway inflammation was determined.

i.t. OVA challenge on days 8, 15, 18, and 21 in mice sensitized previously by i.p. OVA on day 0 caused a marked influx of leucocytes into the BAL fluid 24 h after the last i.t. challenge with Ag (Fig. 4). The OVA-sensitized/challenged mice had a 10.3-fold increase in total cells recovered from BAL fluid compared with the saline group (Fig. 4A; p = 0.0004, OVA vs saline); 59.4% of the BAL fluid cells were eosinophils in the OVA-treated mice compared with 3.7% of total cells in saline-treated controls (Fig. 4B; p < 0.0001, OVA vs saline). The mean number of eosinophils in the BAL fluid in the controls was 0.023 ± 0.016 × 10^5 cells (Fig. 4C). The OVA-treated mice had a 191.2-fold increase in total eosinophils recovered in the BAL fluid to 4.4 ± 0.7 × 10^5 cells (Fig.

![FIGURE 1. Chemical structure of MOL 6131.](image)

![FIGURE 2. Docking model of MOL 6131 into two adjacent active sites of human tryptase. The interaction of MOL 6131 with the two ASP-189 residues at the bottom of the S1 pockets from tryptase monomers A and D is shown in this stick model. The structure of the reversible tryptase inhibitor, 4-amidophenylpyruvic acid (APPA), is also shown.](image)

<table>
<thead>
<tr>
<th>Enzyme</th>
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<td>Tryptase</td>
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<td>Urokinase</td>
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<td>Plasma kallikrein</td>
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*Each enzyme was of human origin except for trypsin which was of bovine origin.
4C; \( p = 0.0003 \), OVA vs saline). Treatment with MOL 6131 at an i.n. dose of 10 mg/kg decreased the influx of eosinophils into the BAL fluid by 64.1\% (Fig. 4C; \( p = 0.0072 \), 10 mg/kg 6131/OVA i.n. vs OVA). BAL fluid eosinophilia in OVA-treated mice was not affected by oral delivery of MOL 6131 at a dose of 10 mg/kg (Fig. 4C). A 49.1\% reduction in BAL fluid eosinophils was seen in OVA-sensitized/challenged mice after oral administration of MOL 6131 at a dose of 25 mg/kg.

The saline-treated controls had airways of normal appearance by light microscopy; inflammatory cells were absent in the lung interstitium (Fig. 5A), and airway mucus release was not present (Fig. 5B). In contrast, OVA-sensitized/challenged mice had a dense, mixed cellular infiltrate in the lung interstitium composed of eosinophils, neutrophils, and mononuclear cells (Fig. 5C). By morphometry, a 14.2-fold increase in total inflammatory cells infiltrating the lung interstitium was observed in OVA-sensitized/challenged mice compared with saline controls (Fig. 6A; \( p < 0.0001 \), OVA vs saline). In OVA-treated mice, airway eosinophil and neutrophil infiltration increased markedly from 0.0 ± 0.0 eosinophils/2200 \( \mu m^2 \) lung tissue in control animals to 27.0 eosinophils/2200 \( \mu m^2 \) lung tissue (Fig. 6B; \( p < 0.0001 \), OVA vs saline) and from 0.3 ± 0.2 neutrophils/2200 \( \mu m^2 \) lung tissue in controls to 18.4 ± 1.3 neutrophils/2200 \( \mu m^2 \) lung tissue in OVA-treated mice (Fig. 6C; \( p < 0.0001 \), OVA vs saline) as determined by morphometry.

The tryptase inhibitor MOL 6131 administered i.n. (10 mg/kg) significantly reduced infiltration into the lung interstitium of total inflammatory cells and eosinophils and neutrophils (Fig. 5, E vs C, and Fig. 6, A (\( p = 0.0001 \), 10 mg/kg i.n. 6131/OVA vs OVA for total cells), B (\( p = 0.0241 \), 10 mg/kg i.n. 6131/OVA vs OVA for eosinophils), and C (\( p = 0.0015 \), 10 mg/kg i.n. 6131/OVA vs OVA for neutrophils)). MOL 6131 (administered at a dose of 25 mg/kg orally) significantly reduced the total inflammatory cell (Fig. 6A) and neutrophil (Fig. 6C), but not the eosinophil (Fig. 6B), infiltration of lung tissue in OVA-sensitized/challenged mice.

**Effect of MOL 6131 on airway mucus and edema**

Airway goblet cell hyperplasia and mucus hypersecretion were observed in the OVA-treated mice (Fig. 5D), and both were reduced by tryptase inhibitor MOL 6131 (Fig. 5, F vs D). By morphometric analysis, 45.0 ± 1.8\% of the airway cells were goblet cells in the OVA-treated mice compared with 0.6 ± 0.2\% in saline controls (Fig. 7A; \( p < 0.0001 \), OVA vs saline). A 23.0\% reduction in airway goblet cell hyperplasia was observed in OVA-sensitized/challenged mice treated with i.n. MOL 6131 at a dose of 10 mg/kg (Fig. 7A; \( p = 0.0082 \), 10 mg/kg 6131/OVA i.n. vs OVA). MOL 6131, administered either i.n. (10 mg/kg) or orally (25 mg/kg), decreased the airway mucus occlusion scores in OVA-treated mice by 50.4\% (Fig. 7B; \( p < 0.0001 \), 10 mg/kg 6131/OVA i.n. vs OVA) and 48.2\% (Fig. 7B; \( p = 0.0011 \), 25 mg/kg 6131/OVA orally vs OVA), respectively. Similarly, the airway edema observed in OVA-treated mice was significantly reduced by either i.n. or oral administration of MOL 6131 (Fig. 7C; \( p < 0.0001 \), 10 mg/kg 6131/OVA i.n. vs OVA; \( p = 0.0001 \), 25 mg/kg 6131/OVA orally vs OVA).

**Effect of MOL 6131 on pulmonary cytokine release and VCAM-1 expression**

Significant levels of IL-4 (147.4 ± 91.6 pg/ml), IL-13 (58.3 ± 26.2 pg/ml), and eotaxin (21.2 ± 5.4 pg/ml) were found in the
FIGURE 5. Effect of tryptase inhibitor MOL 6131 on airway inflammation in OVA-treated mice. Lung tissue (upper and lower lobes of left lung) was obtained from saline-treated mice (A and B) and OVA-sensitized/challenged mice in the absence (C and D) or the presence of 10 mg/kg MOL 6131 i.n. (E and F), stained with H&E (A, C, and E) or Alcian Blue with Nuclear Fast Red counterstaining (B, D, and F), and examined by light microscopy. Bars =
BAL fluid of the OVA-sensitized/challenged mice. These cytokines were not detected in the saline-treated controls. The increased IL-4 levels in the BAL fluid of the OVA-treated mice were decreased 48.3 and 60.4%, respectively, by i.n. (10 mg/kg) and oral (25 mg/kg) administration of MOL 6131. MOL 6131 also reduced the increased levels of IL-13 in the BAL fluid of OVA-treated mice (49.8 and 67.0% reductions by i.n. (10 mg/kg) and oral (25 mg/kg) administration of MOL 6131, respectively). BAL fluid levels of eotaxin in the OVA-treated mice were unaffected by either i.n. (10 mg/kg) or oral (25 mg/kg) treatment with MOL 6131.

By immunocytochemistry, VCAM-1 expression was greatly increased in pulmonary blood vessel endothelial cells of OVA-treated mice compared with saline controls (Fig. 8, A vs C). This increased expression of VCAM-1 in the pulmonary vasculature of OVA-sensitized/challenged mice treated with MOL 6131 have a marked reduction in the cellular infiltration around blood vessels (BV) and airways (AW). F, Scant mucus release is seen in the airways (AW) of the mice receiving tryptase inhibitor MOL 6131.

100 μm. A, The control mice have airways (AW) and blood vessels (BV) of normal appearance without inflammatory cell infiltration. B, Little airway (AW) mucus is seen in the saline-treated mouse lungs. C, OVA-treated mice have a dense, mixed inflammatory cell infiltrate composed predominantly of eosinophils in the lung interstitium (arrows) surrounding the airways (AW) and blood vessels (BV). D, Airway (AW) goblet cell hyperplasia with increased mucus release (arrows) is seen in the OVA-treated mice. E, Compared with OVA treatment alone, OVA-sensitized/challenged mice treated with MOL 6131 have a marked reduction in the cellular infiltration around blood vessels (BV) and airways (AW). F, Scant mucus release is seen in the airways (AW) of the mice receiving tryptase inhibitor MOL 6131.
the OVA-treated mice was markedly reduced by administration of MOL 6131 (Fig. 8).

Effect of tryptase inhibition on allergen-induced airway hyper-reactivity to methacholine

Airway hyper-reactivity to aerosolized methacholine was determined by noninvasive in vivo plethysmography 24 h following the last i.t. challenge with OVA. Airway hyper-reactivity was observed in the OVA-treated mice after challenge with methacholine at 5 and 20 mg/ml, with significant increases in $P_{enh}$ (percentage of air) compared with the saline-treated control group (Fig. 9).

MOL 6131 after i.n. (10 mg/kg) or oral (25 mg/kg) delivery did not alter airway hyper-reactivity in OVA-treated mice compared with saline controls (Fig. 9).

Discussion

In a murine model of asthma, the tryptase inhibitor MOL 6131 significantly reduced the following features of allergic airway inflammation: eosinophil infiltration in BAL fluid and lung tissue, goblet cell hyperplasia and mucus occlusion of airways, peribronchial edema, and IL-4 and IL-13 release in BAL fluid. Delivery of MOL 6131 by the i.n. route was superior to oral dosing in decreasing airway eosinophil infiltration and goblet cell hyperplasia. Examination of x-ray crystal structures of proteolytic enzymes and their endogenous inhibitors (i.e., serpins, Kunitz inhibitors) has demonstrated that an extended strand motif is uniformly adopted by the inhibitor/pseudo-substrate in the enzyme-active site (35, 36). A strand template library enables development of potent specific inhibitors of proteolytic enzymes, such as thrombin, factor VIIa, urokinase-type plasminogen activator, hepatitis C virus protease, and caspase 3 (24, 37). Using a template library of strand mimetics, we found that the low m.w. MOL 6131 is a potent (45 nM) and selective noncovalent, reversible competitive inhibitor of human mast cell tryptase in an in vitro chromogenic assay. The high degree of selectivity achieved is probably due to the unique tetrameric structure for active tryptase and the ability of an aniline ring in the R2 substituent of MOL 6131 to interact with the S1 pocket (i.e., ASP-189 residue) of an adjacent tryptase monomer (i.e., monomers A and D (as shown in Fig. 2) or monomers B and C (not shown)) (28). By comparison, previous inhibitors that have been evaluated in preclinical models of asthma have significant drawbacks. APC 366 ($N$-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide hydrochloride) (21) exhibits low selectivity and a time-dependent, irreversible inhibition. Bis(5-amidino-2-benzimidazolyl)methane (38) requires a relatively high concentration of zinc.
to achieve potency and specificity, and AMG-126737 (1,5-bis-[4-[[3-carbamimidoyl]-benzenesulfonylamine](methyl)phenoxyl]pentane) (22) has limited selectivity with respect to plasmin, a key component in the fibrinolytic cascade. Lactoferrin is a 78-kDa protein and, although potent and selective against trypstatin (39), has no oral availability.

Tryptase has potent chemoattractant activity for leukocytes. Human mast cell tryptase induces infiltration of neutrophils and eosinophils into the skin of guinea pigs and the peritoneum of mice after intradermal and i.p. administration, respectively (40). Tryptase, isolated from human lung tissue, has been previously demonstrated to stimulate IL-8 production and ICAM-1 expression in the human epithelial cell line H292 (14). IL-8, a potent neutrophil chemoattractant and activator (41), also promotes eosinophil chemotaxis through endothelium and epithelium (42). An IL-8 homologue in mice has not been identified; macrophage inflammatory protein-2 and CXC chemokine ligand 1, also known as KC, which interact with the mouse IL-8R homologue, promote neutrophil, but not eosinophil, chemotaxis in pulmonary inflammation (43). Although neutrophils increased in the lungs of OVA-treated mice compared with controls, eosinophils were the predominant inflammatory cell infiltrating both lung interstitium and BAL fluid in our murine asthma model.

The movement of eosinophils into the lungs of the OVA-sensitized/challenged mice in this asthma model was inhibited by the selective tryptase inhibitor MOL 6131. In OVA-treated mice, the increased BAL fluid levels of IL-4 and IL-13, but not eotaxin, were decreased by tryptase inhibitor MOL 6131. These data suggest that tryptase may act via proteinase-activated receptors to affect the transcription of a subset of Th2 cytokines. IL-4 may play an important role in mediating airway eosinophilia in the murine asthma model used. The expression of VCAM-1 (CD106) is increased in airway tissue by IL-4 (44). VCAM-1 binds VLA-4 (α4β1; CD49d/CD29), the integrin heterodimer expressed on eosinophils and lymphocytes, but not on neutrophils, and is probably important for the selective movement into allergic airways of eosinophils and lymphocytes (45). The increased VCAM-1 expression in the blood vessel endothelial cells in the lungs of the OVA-treated mice was inhibited by MOL 6131 to indicate an additional mechanism for trypstatase inhibition to block airway eosinophil infiltration.

IL-4 also induces airway mucus accumulation (46, 47). In this murine asthma model, soluble IL-4R, which inhibits the biologic actions of IL-4, blocks airway mucus hypersecretion in OVA-sensitized/challenged mice. BAL fluid release of IL-13, a potent mediator of allergic inflammation, was also inhibited by MOL 6131. IL-13 promotes goblet cell hyperplasia and mucus glycoprotein accumulation in the airways after exogenous administration in mice (46, 48) or increased endogenous production in IL-13 transgenic (49).

Thus, inhibition of IL-4 and IL-13 production by the trypstatase inhibitor MOL 6131 may be an important anti-inflammatory mechanism for decreasing airway goblet cell hyperplasia and mucus release.

In vitro and in vivo studies have suggested a role for trypstatase in mediation of airway hyper-reactivity in asthma. Tryptase, a mitogen for cultured dog tracheal smooth muscle cells (50), potentiates the histamine-induced contractile response in isolated canine (51) and human (52, 53) bronchial tissue. In sheep, inhaled trypstatase induces bronchoconstriction and airway hyper-responsiveness. Tryptase inhibitors block airway hyper-reactivity in some animal models of asthma. For example, the selective trypstatase inhibitor AMG-126737 inhibits allergen-induced airway hyper-responsive-ness in guinea pigs and sheep (22). The allergen-induced increase in specific lung resistance in sheep is also reduced by the trypstatase inhibitors, APC 366 (21), bis-(5-amidino-2-benzimidazolyl) methane (21), and lactoferrin (39). However, in our murine asthma model, trypstatase inhibition by MOL 6131 did not reduce airway hyper-reactivity to inhaled methacholine in the OVA-treated mice. This was unexpected, since C57BL/6 mice that are deficient in the tryptic protease, mouse mast cell protease 7 (mMCP-7), have decreased airway responsiveness to methacholine (54, 55), although they have increased levels of airway eosinophils, total IgE, and Ag-specific IgE compared with BALB/c mice treated similarly. The situation is complicated by the fact that human α- and β-trypstatase are not the equivalents of mMCP-6 and mMCP-7 (56, 57), and although mMCP-6 and mMCP-7 exhibit homologies with human β-trypstatase (57), the true orthodoxes may await discovery (58). These and additional studies (59) confirm that the genetic background has a striking and selective effect on the phenotype of murine pulmonary disease and that the development of AHR cannot be predicted by levels of airway inflammation or IgE. Species and allergen sensitization/challenge protocol differences may account for the different results between our murine model and the guinea pig and sheep models of asthma. Our results are consistent with prior studies demonstrating discordance between inhibition of airway inflammation and hyper-reactivity. In OVA-treated mice, leukotriene synthesis inhibitors block airway tissue and BAL fluid eosinophilia, but not airway hyper-reactivity to methacholine (32). Similarly, soluble IL-4R blocks pulmonary eosinophilia without affecting airway hyper-reactivity in OVA-treated mice (34). Whereas i.n. administration of CD49 mAb blocks both airway inflammation and hyper-reactivity, systemic delivery of CD49d mAb by i.p. administration blocks only the influx of eosinophils into the lungs and does not affect airway hyper-reactivity to methacholine in OVA-sensitized/challenged mice (45).

In summary, our data indicate an important role of trypstatase in the pathogenesis of allergic airway inflammation and demonstrate the potential benefit of trypstatase inhibitors in asthma therapy.

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