The Mast Cell-restricted Tryptase mMCP-6 Has a Critical Immunoprotective Role in Bacterial Infections*

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Although it has been shown that mast cell-deficient mice have diminished innate immune responses against bacteria, the most important immunoprotective factors secreted from activated mast cells have not been identified. Mouse mast cell protease 6 is a tetramer-forming tryptase. This serine protease is abundant in the secretory granules and is exocytosed upon bacterial challenge. Here we have described the generation of a mast cell protease-6-null mouse. Our discovery that mice lacking this neutral protease cannot efficiently clear Klebsiella pneumoniae from their peritoneal cavities reveals an essential role for this serine protease, and presumably its human ortholog, in innate immunity.

Approximately 50% of the weight of a mature tissue mast cell (MC) consists of protease-serglycin proteoglycan complexes stored in the secretory granules. In humans, β tryptases are the most abundant MC-restricted neutral proteases (1–3). The corresponding tryptases in mice are mouse MC protease (mMCP)-6 (4, 5) and mMCP-7 (6), with mMCP-6 being the most similar in amino acid sequence and substrate specificity to human tryptase (hTryptase) β1 (7–9). MCs are the only cells that express mMCP-6, and this serine protease is particularly abundant in those MCs that reside in the peritoneal cavity, skin, and lung (4, 5, 10).

Numerous biochemical studies have been carried out to understand the biosynthesis and substrate preference of mMCP-6. This tryptase is initially translated as a zymogen with a 245-mer mature domain. When the signal and propeptides are proteolytically removed, the mature protease spontaneously forms tetramers with the active site of each monomer facing the central core of the tetramer unit, as first described for its human ortholog (11). A positively charged face forms on the surface of each monomer, thereby allowing mature mMCP-6 to interact with negatively charged serglycin proteoglycans in the Golgi complex. The resulting tryptase-serglycin macromolecular complexes are then targeted and packaged in the cell secretory granules. When exocytosed, these complexes are retained in connective tissues for hours because of their large sizes (12). Protease inhibitors are abundant in blood. Nevertheless, no circulating protease inhibitor has been identified that rapidly inactivates mMCP-6 or hTryptase β1. Substrate specificity studies carried out using varied peptide combinatorial libraries revealed that recombinant mMCP-6 and hTryptase β1 prefer to cleave peptides having a Pro at residues P2 to P5 and a Lys or Arg at residue P1. However, due to the unique structural constraints of the tetramer unit, the abilities of mMCP-6- and hTryptase β1 to cleave large-sized proteins are very limited. Thus, the importance of these evolutionarily conserved enzymes in MC-dependent reactions remains to be determined.

MC development in vivo is highly dependent on the cytokine kit ligand/stem cell factor on the surface of mesenchymal cells and its tyrosine kinase receptor c-Kit/CD117 on the surface of MC-committed progenitors. Signaling through c-Kit results in the translocation of microphthalmia transcription factor (MITF) into the nucleus. WBB6F1/KitW/KitW-v (W/Wv) mice are MC-deficient secondary to a point mutation in the intracellular domain of c-kit, which makes their MCs and progenitors less responsive to kit ligand. WBB6F1/KitW/KitW-v (W/Wv) mice, on the other hand, have reduced numbers of MCs, because they express a mutated isofrom of MITF. Thus, transcription of the mMCP-6 gene and certain other MC-restricted genes are greatly diminished in MITF-deficient tg/tg mice (13).

In 1996, Echtenacher et al. (14) noted that W/Wv mice quickly die from septic peritonitis after their caecum is ligated and punctured. Malaviya et al. (15) reported at the same time that W/Wv mice cannot efficiently combat a Klebsiella pneumoniae infection of their peritoneal cavities or lungs. The same phenomenon was observed in tg/tg mice (16), confirming the importance of MCs in innate immunity. The latter data suggest that a MC-restricted gene whose transcription is highly dependent on MITF plays an essential immunoprotective role in bacterial infections. Although it has been concluded that MC-derived tumor necrosis factor-α (TNF-α) is needed to con-
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trol bacterial infections (14, 15), the number of TNF-α-expressing lipopolysaccharide-responsive macrophages in the lungs and peritoneal cavities of bacteria-infected mice greatly exceeds the number of MCs. There is also no evidence that MITF regulates the transcription of the TNF-α gene in any cell. Thus, additional factors more restricted to MCs must be required for an optimal antibacterial response in vivo to explain why MC-deficient mice are so susceptible to bacterial challenge. In support of this conclusion, Maurer et al. (17) note that MC activation significantly improves host defenses in TNF-α−/− mice, and Jiippo et al. (16) have found that the injection of TNF-α protein into the peritoneal cavities of tg/tg mice fail to completely restore their MC-dependent antibacterial responses.

We have here described the generation of an mMCP-6−/− mouse strain. Using these mice, we have shown that mMCP-6 is required to efficiently combat K. pneumoniae infections.

EXPERIMENTAL PROCEDURES

Targeting of the mMCP-6 Locus in Mouse Embryonic Stem Cells and Generation of the mMCP-6−/− Mouse Strain—The mMCP-6 gene (GenBank™ gene identification number 17229) resides in the tryptase locus on mouse chromosome 17A3.3 (18). Using the bacterial artificial chromosome clone RP24–263I14 (Children’s Hospital Oakland Research Institute, CA) and oligonucleotides based on the genomic sequence of the chromosomal locus at GenBank™ accession number NT_039649, a PCR approach was carried out to obtain the mMCP-6-specific nucleotide sequences for the targeting vector shown in Fig. 1A. Employing the translation initiation site of the mMCP-6 transcript as a reference point (defined here as residue +1), a 2970-bp 5′ homology arm (nucleotides −2966 to +3) was cloned into the BglII-HindIII sites of the pKO Scrambler NTK-1907 plasmid (Stratagene) downstream of the phosphoglucomutase promoter-driven neomycin resistance gene (PGK-Neo). A 3196-bp 3′ homology arm (nucleotides +897 to +4092) was then cloned into the Sall-NotI sites upstream of PGK-Neo. The herpes simplex virus thymidine kinase cDNA driven by a mutant polyomavirus enhancer (MC1-TK) is present in this plasmid for negative selection.

A mouse expressing Cre recombinase (Cre) under the control of an MC-specific promoter has not yet been created to selectively diminish the expression of ubiquitously expressed genes using a Cre/loxP approach. Because mMCP-6 is highly restricted to MCs, a knock-in approach was carried out by placing Cre with a SV40 poly(A) signal at the 3′ end of the 5′ homology arm using HindIII and XhoI restriction sites. The linearized targeting vector was electroporated into 129svJ embryos and ES cell clones that underwent homologous recombination were identified by blot analysis of BglII-digested ES cell DNA hybridized with a radiolabeled probe generated with Klenow polymerase as use template a 1-kb 3′ external fragment (nucleotides +4285 to +5283). Positive clones were confirmed by PCR with a primer located upstream of the 5′ homology arm (5′-GATCCCCGACTTGAGATAGGC-3′) and another located in the Cre coding region (5′-GAGACAGAGCATTTCCAGTATGC-3′).

Selected ES cell clones were microinjected into C57BL/6-Tyr−/− (The Jackson Laboratory) blastocysts, which were then implanted into pseudopregnant CD-1 foster mothers. Resulting male chimeric mice were crossed with C57BL/6-Tyr−/− females, and non-albino pups from this cross were screened for germ line transmission of the mutant mMCP-6 allele. Genotyping was carried out on tail biopsies by multiplex PCR using oligonucleotides within the 5′ homology arm (P1, 5′-CCTGGAGCAGATACCAAGC-3′), within the deleted portion of the mMCP-6 gene (P2, 5′-AGGGCTCAAGACAATCTACGAG-3′), and with the Cre coding region (P3, 5′-CTGACCATTTGCTGATACG-3′). mMCP-6 heterozygotes were backcrossed to C57BL/6 mice to eliminate any mutated background gene in our mMCP-6−/− mice. Using a marker-assisted selection protocol/speed-congenic approach, a male mMCP-6−/− mouse expressing 100% of C57BL/6 polymorphisms at 50 chromosomal loci was identified in the third backcrossed generation. This mouse was selected as the founder of a line that was backcrossed three more times before generating the mMCP-6−/−, mMCP-6+/−, and mMCP-6+/+ littermates used in this study. All mice were maintained in a BL2 animal facility of The University of Texas M. D. Anderson Cancer Center according to their animal care guidelines.

Isolation and Purification of In Vivo Differentiated MCs—MCs were isolated from the peritoneal cavity. After euthanasia with CO2, 10 ml of modified Tyrode’s buffer was injected into the peritoneal cavity. Eight to nine ml of the injected solution was recovered in each instance, and the resulting exudates were centrifuged at 450 × g for 5 min. The pelleted cells were resuspended in 1 ml of modified Tyrode’s buffer and layered over a 2-ml solution of 22.5% metrizamide (Nycomed/Accurate Chemical) in modified Tyrode’s buffer. After centrifugation at 400 × g for 15 min, peritoneal MCs were recovered at the bottom of the gradient. Purification was confirmed by toluidine blue staining or by flow cytometry with anti-c-kit/CD117 (Pharmingen) and anti-FceRα (eBioscience) antibodies.

Immunoblot Analysis—Peritoneal MCs were lysed by freeze thawing in phosphate-buffered saline supplemented with a protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce). Proteins were resolved by SDS-PAGE with 4–15% linear gradient Ready Gels (Bio-Rad). The resulting blots were probed with affinity-purified rabbit anti-peptide antibodies specific for mMCP-4, mMCP-5, and mMCP-6 (12, 19–21). Peroxidase-conjugated, goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories) and SuperSignal West Pico ECL substrate (Pierce) were used for protein detection. Membranes were stripped at 65 °C for 15 min in 50 mM Tris-HCl, 0.2 M 2-mercaptoethanol, and 2% SDS; they were then reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Abcam) to serve as a protein loading control.

Electron Microscopy—Isolated peritoneal MCs from four mMCP-6+/− mice and four mMCP-6−/− mice (5 × 104 cells/sample) were separately fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.2) for 2 h followed by 1% osmium tetroxide for 1 h. Samples were dehydrated, embedded in Araldite™ (Huntsman Advanced Materials), sectioned at
100 nm with an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a JEOL 200CX electron microscope. Cell profiles from each section were photographed using an unbiased random sampling technique. Twenty cell profiles from seven samples from each group were examined. The fraction of cell profiles identified morphologically as MCs (e.g. by the presence of their characteristic electron-dense granules, non-segmented nucleus, and surface microvilli) was assessed by unbiased stereology (22). The volume fraction (which represents the fraction of the total volume of the cell occupied by granules) and the surface density (which is directly proportional to the membrane surface of the granules per unit of volume) were calculated with the point-counting method using a cycloid grid. The area of the cell profiles was measured using a point grid.

Cell Count and Differential—Peritoneal lavages from four mMCP-6+/+ mice and four mMCP-6−/− mice were obtained but not subjected to MC purification. The nucleated cells in each sample were counted with a Neubauer chamber, and the cell differential was determined on cytospins of 400 μl of each 8-ml sample stained with Wright-Giemsa. Hemoglobin was determined in paraffin. Avidin binds strongly to the heparin-containing microplicae) was assessed by unbiased stereology (22). The volume fraction (which represents the fraction of the total volume of the cell occupied by granules) and the surface density (which is directly proportional to the membrane surface of the granules per unit of volume) were calculated with the point-counting method using a cycloid grid. The area of the cell profiles was measured using a point grid.

Fluorescence Microscopy—Ears from four mMCP-6+/+ mice and four mMCP-6−/− mice were excised, fixed overnight at 4 °C in 4% paraformaldehyde (pH 7.0), dehydrated, and embedded in paraffin. Avidin binds strongly to the heparin-containing microplicae) was assessed by unbiased stereology (22). The volume fraction (which represents the fraction of the total volume of the cell occupied by granules) and the surface density (which is directly proportional to the membrane surface of the granules per unit of volume) were calculated with the point-counting method using a cycloid grid. The area of the cell profiles was measured using a point grid.

In each experiment, 105 MCs were sensitized in medium containing 5 mMCP-6, 100 ng/ml DNP-conjugated human serum albumin (DNP-HSA; Sigma-Aldrich) for 3 h at 37 °C. After washing to eliminate excess antibody, cells were activated immunologically with 100 ng/ml DNP-conjugated human serum albumin (DNP-HSA; Sigma-Aldrich) at 37 °C. For histamine release, an equal amount of cells was lysed with 0.2% Triton X-100, and histamine was measured in each supernatant and lysate after 1 h. For TNF-α release, samples of the supernatants were taken at 0, 2, 4, 12, and 24 h. Histamine and TNF-α levels were measured by enzyme-linked immunosorbent assay (Oxford Biomedical Research).

Passive Cutaneous Anaphylaxis (PCA)—10–12-week-old mMCP-6+/+, mMCP-6−/−, mMCP-6−/−, and B6.Cg-KiW-sh/KitW-sh (W55/W55) mice were anesthetized with sodium 100 nm with an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a JEOL 200CX electron microscope. Cell profiles from each section were photographed using an unbiased random sampling technique. Twenty cell profiles from seven samples from each group were examined. The fraction of cell profiles identified morphologically as MCs (e.g. by the presence of their characteristic electron-dense granules, non-segmented nucleus, and surface microvilli) was assessed by unbiased stereology (22). The volume fraction (which represents the fraction of the total volume of the cell occupied by granules) and the surface density (which is directly proportional to the membrane surface of the granules per unit of volume) were calculated with the point-counting method using a cycloid grid. The area of the cell profiles was measured using a point grid.

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FIGURE 1. Generation of the mMCP-6−/− mouse strain by gene replacement. A, by homologous recombination of ES cells, the first three coding exons of the mMCP-6 gene were replaced with the Cre cDNA in the correct translational context. Neomycin resistance and thymidine kinase were used for positive and negative selection. B, a new BglI genomic fragment (4.8-kb) was detected with a radiolabeled DNA probe from a region outside the homology arms (Probe), confirming homologous recombination at the mMCP-6 locus in the cloned ES cell. C, multiplex PCR of genomic DNA using three primers (P1, P2, and P3) gives different products from the normal (wild type (WT), 250-bp) and mutated (knock-out (KO), 700-bp) mMCP-6 alleles, allowing the determination of zygosity for the mutation. D, immunoblotting of peritoneal MC lysates confirmed the absence of mMCP-6 protein in the mMCP-6−−/− mice (undetectable even when 5× more protein was loaded). Despite these data, the amounts of mMCP-4 and mMCP-5 protein stored in mMCP-6−−/− peritoneal MCs were not reduced (Fig. 1D). If anything, the levels of mMCP-5 protein increased in the peritoneal cavity MCs of these mice, presumably due to the availability of more serglycin/protoglycan-binding sites in the secretory granule. These MCs also contained normal levels of histamine (data not shown). Thus, the lack of mMCP-6 in a connective tissue-type MC does not adversely impact the storage of other mediators in the cell secretory granules.

Activated MCs from mMCP-6−/− Mice Release Appreciable Amounts of TNF-α and Histamine and Induce a Normal PCA Reaction—Isolated peritoneal MCs from our mMCP-6+/+ and mMCP-6−/− mice were sensitized with IgE and challenged with antigen in vitro. As shown in Fig. 3A, no significant difference in the secretion of TNF-α between the two groups was noted over the course of 24 h. Similarly, no difference in the exocytosis of histamine at 1 h was observed (Fig. 3B). MCs disruption of the mMCP-6 gene (Fig. 1, B and C). In agreement with the genotyping data, the levels of mMCP-6 protein were below detection in the MCs that resided in the peritoneal cavities of these transgenic mice (Fig. 1D). The antibody we used was raised against a unique 19-mer peptide (RKYHTGLYTGDDFPIVHDG) that corresponds to residues 160–178 in the mature domain of the protease (12). This amino acid sequence is encoded by the next to the last exon of the gene, which was not deleted by our targeting strategy. Given that no smaller protein bands were detected with this anti-mMCP-6 antibody in MC lysates from mMCP-6+/− and mMCP-6−/− mice (data not shown), we concluded that a truncated protein was not expressed.

Our mMCP-6−/− mice produced normal-sized litters, showed no obvious developmental abnormality, and had a normal lifespan when maintained in a pathogen-free animal facility. Histochemical analysis revealed normal numbers of MCs in the skin and peritoneal cavity (Fig. 2). The peritoneal MCs isolated from these mice also showed no substantial alteration in their size or shape or in the number, size, and density of their secretory granules (Fig. 2 and Table 1). Thus, even though mMCP-6 is expressed relatively early in MC development (5), this neutral protease is not essential for the migration, retention, and overall maturation of MC-committed progenitors in connective tissues.

Peritoneal MCs store substantial amounts of the chymotrypsin-like proteases mMCP-4 and mMCP-5 in their secretory granules in addition to the tryptase mMCP-6 (4). As assessed by SDS-PAGE immunoblot analysis, the amounts of mMCP-4 and mMCP-5 stored in the peritoneal MCs in our mMCP-6−/− mice were not decreased (Fig. 1D). If anything, the levels of mMCP-5 protein increased in the peritoneal cavity MCs of these mice, presumably due to the availability of more serglycin/protoglycan-binding sites in the secretory granule. These MCs also contained normal levels of histamine (data not shown). Thus, the lack of mMCP-6 in a connective tissue-type MC does not adversely impact the storage of other mediators in the cell secretory granules.

Innate Immunity Is Significantly Diminished in mMCP-6−/− Mice—Despite having normal MC numbers, morphology and secretory responses, and intact FcεRI-dependent responses, our mMCP-6-null mice exhibited the striking phenotype of a substantially reduced ability to combat K. pneumoniae infections of their peritoneal cavities. Only ~10% of our mMCP-6−/− mice survived 72 h after receiving just 1000 CFUs of K. pneumoniae (Fig. 4A), compared with ~40% of mMCP-6+/+ and ~80% of mMCP-6+/− mice. Confirming these results, no mMCP-6−/− mice survived a challenge with 10,000 CFUs of K. pneumoniae (Fig. 4B). This increased lethality was associated with a deficiency in the early extravasation of neutrophils into the peritoneal cavities of mMCP-6−/− mice (Fig. 5A). This deficit was independent of the number of circulating neutrophils, which were almost identical between mMCP-6+/+ and mMCP-6−−/− mice throughout the course of the infection (data not shown) but correlated with higher bacterial counts in the peri-
accumulated data suggest that enzymatically active mMCP-6 increase in the levels of interleukin 8 mRNA and protein (8), the assessed by transmission electron microscopy, the peritoneal MCs in 6 mMCP-6 phometry analysis can be found in Table 1.

Quantitation and morphometry of cutaneous and peritoneal MCs
Table 1

|               | mMCP-6+/+ | mMCP-6−/− |
|---------------|-----------|-----------|
| Skin MCs*     | 189 ± 8   | 183 ± 8   |
| (number/mm²)  |           |           |
| Peritoneal MCs*| 9.1 ± 1.2 | 9.3 ± 1.4 |
| (% (10⁷/ml)   | 171 ± 14  | 164 ± 12  |
| Morphometry*  | 0.35 ± 0.03 | 0.34 ± 0.03 |
| (Vg) (µm³)   | 19.65 ± 1.49 | 18.64 ± 1.77 |
| (Sg) (µm²)   | 44.01 ± 2.83 | 44.38 ± 2.55 |

*FITC-avidin cells with a Hoechst nuclear profile/mm² of dermis in random sections of ear (10 samples/animal).

**Neubauer chamber counts and Wright-Giemsa staining of cytospins of peritoneal lavages (3 samples/animal).

***Stereological analysis of randomly acquired electron microscopy images from peritoneal MCs. Vg = granule volume fraction; Sg = granule surface density; A = cell profile area. (20 cell profiles/sample, 7 samples/animal).

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We showed in previous studies that the viability of K. pneumoniae was not diminished by exposure to enzymatically active recombinant mMCP-6, thereby ruling out a direct bactericidal effect of this protease (8). In support of our new data, we also previously noted that the administration of just 0.3 nmol of recombinant mMCP-6 or its human ortholog hTryptase β1 into the peritoneal cavities or lungs of W/Wv mice results in a pronounced local neutrophilia (7, 8). Because exposure of cultured human endothelial cells to recombinant mMCP-6 or hTryptase β1 (but not their zymogens) results in a substantial increase in the levels of interleukin 8 mRNA and protein (8), the accumulated data suggest that enzymatically active mMCP-6 plays a beneficially indirect role in bacterial infections by inducing bystander cells in inflammatory sites to release substantial amounts of neutrophil-specific chemotactic factors that ultimately induce the extravasation of bacterial granulocytes into the site to control the infection. Nevertheless, the mechanism by which mMCP-6 activates these bystander cells remains to be determined.

In addition to the finding that W/Wv mice cannot efficiently combat a K. pneumoniae infection of their peritoneal cavities, Malaviya et al. (15) reported substantially lower levels of TNF-α and myeloperoxidase in the peritoneal cavities of these mice after infection. Another study notes a survival advantage in
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When activated, in vivo and in vitro differentiated mouse MCs transiently expressed numerous chemokines, cytokines (Fig. 3A), and arachidonic acid metabolites. However, none of these newly generated mediators were MC-restricted as were the preformed granule mediators of the cell. Although MCs contain appreciable amounts of histamine, mice deficient in histamine actually combat Escherichia coli infections better than wild-type mice (27). It is presumed that histamine plays an adverse role in this immunological response because of its vasopermeability properties, which allow the escape of bacteria and their harmful products from infected tissues. MCs also store large amounts of varied combinations of functionally distinct serine proteases in their secretory granules. The MCs in the peritoneal cavities of C57BL/6 mice lack mMCP-7 (23), and the corresponding MCs in BALB/c mice lack transmembrane trypsin/tryptase γ (28), yet these two mouse strains are not highly susceptible to sepsis. The other ten serine proteases found in mouse MCs (namely mMCP-1–5, mMCP-8–10, cathepsin G, and granzyme B) are members of the chromosome 14C1 family of chymotrypsin-like proteases. Unlike mMCP-6, the latter family of serine proteases require dipetidyl peptidase I for their activation (29). Surprisingly, dipetidyl peptidase I-null mice have decreased mortality in the caecal ligation and puncture (28), yet these two mouse strains are not highly susceptible to sepsis. The other ten serine proteases found in mouse MCs (namely mMCP-1–5, mMCP-8–10, cathepsin G, and granzyme B) are members of the chromosome 14C1 family of chymotrypsin-like proteases. Unlike mMCP-6, the latter family of serine proteases require dipetidyl peptidase I for their activation (29). Surprisingly, dipetidyl peptidase I-null mice have decreased mortality in the caecal ligation and puncture infection model relative to wild-type mice (26). It is not likely that any chromosome 14C1-derived protease plays a significant beneficial role in MC-dependent bacterial clearance. The accumulated data suggest that mMCP-6 is the primary preformed granule mediator that protects mice from acute bacterial infections. In support of this conclusion, transcription of the mMCP-6 gene in MCs is highly dependent on MITF (30), thereby explaining why tg/tg mice are so susceptible to bacterial infections.

The discovery that mMCP-6−/− mice have great difficulty in combating an acute K. pneumoniae infection highlights a critical role for this evolutionarily conserved serine protease in innate immunity. AIDS patients are highly susceptible to bacterial and other opportunistic infections. The observation that hTryptase β+ MCs and their progenitors can be infected with M-tropic strains of HIV-1 (31–33) coupled with the finding that AIDS patients often have reduced numbers of hTryptase β-expressing MCs in their gastrointestinal tracts (34) suggests that this protease plays a comparable beneficial immunoprotective role in humans. The fact that no human has been identified who lacks hTryptase β+ MCs is further evidence that these immune cells are essential to our survival. Finally, it needs to be pointed out that the pharmaceutical industry is presently developing hTryptase β-specific inhibitors in an attempt to dampen inflammation and connective tissue remodeling in asthma and other MC-dependent disorders. Our data raise the possibility that the chronic use of these hTryptase β-specific inhibitors in humans could result in a higher incidence of life-threatening bacterial infections.
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