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Selective inhibition of human mast cell tryptase by gabexate mesylate, an antiproteinase drug

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Abstract

Gabexate mesylate is a non-antigenic synthetic inhibitor of trypsin-like serine proteinases that is therapeutically used in the treatment of pancreatitis and disseminated intravascular coagulation and as a regional anticoagulant for hemodialysis. Considering the structural similarity between gabexate mesylate and arginine-based inhibitors of trypsin-like serine proteinases, the effect of gabexate mesylate on human and bovine mast cell tryptase action was investigated. Values of the inhibition constant (K_i) for gabexate mesylate binding to human and bovine tryptase were 3.4×10^{-9} M and 1.8×10^{-7} M (at pH 7.4 and 37.0°), respectively. Furthermore, gabexate mesylate inhibited the fibrinogenolytic activity of human tryptase. On the basis of the available x-ray crystal structure of human tryptase, the possible binding mode of gabexate mesylate to human and bovine tryptase was analyzed. Human tryptase inhibition by gabexate mesylate may account for the reported prevention of inflammation, erosion, and ulceration of skin and mucosae.

Keywords: Human tryptase; Bovine tryptase; Gabexate mesylate; Enzyme inhibition; Structural aspects

1. Introduction

Human mast cell tryptase (EC 3.4.21.59) is a structurally unique homotetrameric trypsin-like serine proteinase stored in the secretory granules of mammalian mast cells that has been suggested to play a central role in a number of mast cell-mediated allergic and inflammatory diseases, including rhinitis, conjunctivitis, and most notably, asthma. Moreover, human tryptase is implicated in certain gastrointestinal, dermatological, and cardiovascular disorders [1–3].

The physiological function of human tryptase is still unknown. However, *in vitro*, human tryptase induces mast cell degranulation as well as eosinophil and neutrophil migration. Moreover, this trypsin-like serine proteinase catalyzes the release of bradykinin from kininogens and the conversion of inactive prostromelysin to active stromelysin [1, 4]. Next, human tryptase degrades fibronectin, fibrinogen [5], as well as vasodilating and bronchorelaxing neuropeptides. Finally, it promotes the proliferation of fibroblasts [6], bronchial smooth muscle cells, and epithelial cells, and may function as a potent angiogenic factor [7]. There are no known endogenous protein serine proteinase inhibitors capable of modulating human tryptase activity: in the homotetrameric enzyme, the active center of each monomer faces the central pore and the dimension of the central cavity is not sufficient to allow access to most protein serine proteinase inhibitors [8].

The modulation of human tryptase activity appears to be related to the monomer–homotetramer equilibrium. In fact, monomeric human tryptase is inactive, the glycosaminoglycans-stabilized homotetramer representing the active enzyme form. Neutrophil lactoferrin and myeloperoxidase as well as antithrombin-III have been shown to modulate human tryptase *in vitro* by antagonizing the glycosaminoglycans (e.g. heparin and chondroitin sulfate) that stabilize the active homotetramer, thereby inducing the formation of the inactive monomer [9–11].

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Abbreviations: Boc-Phe-Ser-Arg-MCA, *N*-tert-butoxy-carbonyl-L-phenylalanyl-L-seryl-L-arginine 7-amido-4-methylcoumarin.

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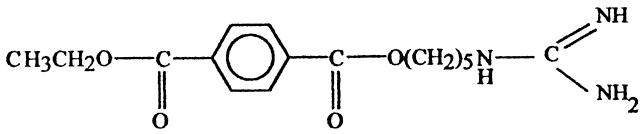

Fig. 1. The chemical structure of gabexate mesylate.

The identification of natural and synthetic inhibitors of human tryptase with appropriate potency and selectivity may be useful in the investigation of the role of tryptase *in vivo* as well as in providing opportunities for the discovery of drugs to be used for the treatment of tryptase-mediated diseases [2,9].

Gabexate mesylate, a non-antigenic synthetic inhibitor of trypsin-like serine proteinases [12,13], is therapeutically used in the treatment of pancreatitis and disseminated intravascular coagulation and as a regional anticoagulant for hemodialysis [14]. Moreover, gabexate mesylate prevents inflammation, erosion, and ulceration of the skin and mucosae [15]. Considering the structural similarity between gabexate mesylate (Fig. 1) and arginine-based inhibitors of trypsin-like serine proteinases, the inhibitory effect of this drug on human and bovine tryptase activity was investigated.

2. Materials and methods

Human tryptase was purified from routinely cultured HMC-1 cells (provided by Dr. J. H. Butterfield) [16]. Bovine tryptase was purified from the bovine liver capsule [17]. Gabexate mesylate (see Fig. 1) was a gift from Lepetit. Plasminogen-free human fibrinogen was purchased from Calbiochem. Heparin (from porcine intestinal mucosa, sodium salt) and Boc-Phe-Ser-Arg-MCA[1] were purchased from Sigma-Aldrich. All the other products were from Merck. All chemicals were of reagent or analytical grade and were used without further purification.

The values of the dissociation equilibrium inhibition constant K_i for gabexate mesylate binding to human and bovine tryptase were determined at pH 7.4 (5.0×10^{-2} M Tris buffer) and 37.0°. Values of K_i were obtained by following the inhibitory effect of gabexate mesylate on the human- and bovine tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA. Gabexate mesylate was not significantly hydrolyzed (<5%) during the assay time (~10 min). In a typical experiment, the inhibitory effect of gabexate mesylate on human and bovine tryptase activity was measured by adding 10 μL of the buffered enzyme solution to 2 mL of the buffered inhibitor solution. After incubation for 5 min, the enzyme activity was assayed by adding 5 μL of the dimethyl sulfoxide solution of Boc-Phe-Ser-Arg-MCA, and the fluorescence (370-nm excitation wavelength and 460-nm emission wavelength) was measured continuously over 5 min using a Kontron SFM25 spectrofluorimeter. The final human and bovine tryptase concentration ranged between 1.0×10^{-9} M and 5.0×10^{-9} M, the final gabexate mesylate concentration between 1.0×10^{-11} M and 1.0×10^{-4} M, and the final Boc-Phe-Ser-Arg-MCA concentration between 1.0×10^{-6} M and 2.5×10^{-5} M [18].

The values of the dissociation inhibition constant for gabexate mesylate binding to human and bovine tryptase (K_i) were obtained from the dependence of the molar fraction of the inhibited serine proteinase (Y) on the inhibitor concentration ([I], M) according to Eqn 1 [19]:

$$Y = [I]/([I] + (K_i \times (1 + [S]/K_m))) \quad (1)$$

where S is the substrate (i.e. Boc-Phe-Ser-Arg-MCA) and K_m is the Michaelis constant. The values of K_m for the human- and bovine tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA are 8.5×10^{-6} M and 7.0×10^{-6} M, respectively, at pH 7.4 and 37.0° (present study).

The inhibitory effect of gabexate mesylate on the human tryptase-catalyzed hydrolysis of the plasminogen-free human fibrinogen was followed at pH 7.4 (5.0×10^{-2} M Tris buffer) and 37.0°, in the presence of heparin (final concentration, 25 μg/mL). In a typical experiment, human tryptase (final concentration, 5.0×10^{-7} M) was preincubated with gabexate mesylate (with the final inhibitor concentration, 5.0×10^{-6} M, exceeding the K_i value) for 10 min. Then, plasminogen-free human fibrinogen (final concentration, 4.0×10^{-6} M) was added and incubated for 1 hr. The reaction was stopped by adding SDS–PAGE sample buffer. SDS–PAGE was performed under reducing conditions on a 12% mini-gel, which was then stained with Coomassie blue [20].

The molecular model of the human tryptase:gabexate mesylate complex was obtained from the structure of one subunit of human β-tryptase [8] (PDB ID code: 1A0L) and that of the bovine β-trypsin:gabexate mesylate adduct. The atomic coordinate data set of the bovine β-trypsin:gabexate mesylate complex was provided by Prof. M. Bolognesi. The two structures were superimposed with a final r.m.s.d. of 1.0 Å over 166 C_α atoms.

The gabexate mesylate molecule was manually docked to the human tryptase active center using the bovine β-trypsin:gabexate mesylate complex structure as the template. The human tryptase:gabexate mesylate interaction was then optimized by energy minimization run with Discover, a module of the InsightII program suite [21]. The consistent valence force field, a distance-dependent dielectric constant, charges on, no cross-term, and no Morse potential were used during energy minimization. An initial minimization was calculated fixing the gabexate mesylate molecule, while only human tryptase residues in contact with the inhibitor were free to move and subjected to a tethering force with a force constant of 100 kcal/Å. Minimization was run for 10 steps of steepest descent and conjugate gradient minimizers. Subsequently, all protein residues were released and subjected to a tethering force with a force constant equal to 100 kcal/Å except those of the catalytic triad (His57, Asp102,

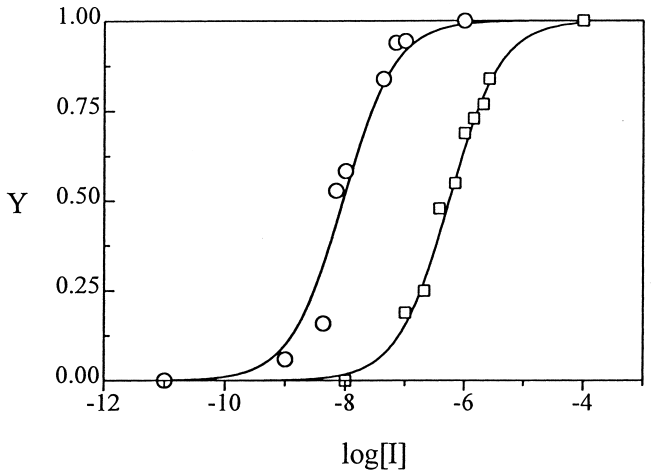

Fig. 2. Effect of the inhibitor concentration ([I], M) on the molar fraction of the inhibited enzyme (Y) for gabexate mesylate binding to human (circles) and bovine (squares) tryptase, at pH 7.4 and 37.0°. The continuous lines were calculated according to Eqn 1 with the following values of K_i: human tryptase, 3.4×10^{-9} M and bovine tryptase, 1.8×10^{-7} M. The substrate (Boc-Phe-Ser-Arg-MCA) concentration was 1.4×10^{-5} M. The values of K_m for the human- and bovine tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA were 8.5×10^{-6} M and 7.0×10^{-6} M, respectively. An average error value of ±8% was evaluated for K_i values. For further details, see text.

and Ser195), which were fixed. Then, a final minimization was run under the same conditions for 10 steps of steepest descent and conjugate gradients until the maximum derivative was less than 1.0 kcal/Å. The resulting structure was inspected for steric clashes and geometrical consistency.

The homology model of bovine tryptase [22] was constructed using Modeller 4.0 [23] in the InsightII suite [21] with human β-tryptase (PDB ID code: 1A0L) [8] and bovine α-chymotrypsin (PDB ID code: 4CHA) [24] as structural templates. Ten models were built at the highest refinement level, and the one with the best 'objective function' score and the Phe190 rotamer permitting gabexate mesylate binding was selected for further analysis. Docking of gabexate mesylate to bovine tryptase was performed as reported above for human tryptase. The model quality was assessed with the ProsaII [25] and Procheck [26] programs. The serine proteinase:gabexate mesylate interactions were analyzed with the LIGPLOT program [27].

3. Results

As shown in Fig. 2, gabexate mesylate inhibited the human- and bovine tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA, K_i values being 3.4×10^{-9} M and 1.8×10^{-7} M, respectively, at pH 7.4 and 37.0°. Under all the experimental conditions, human and bovine tryptase inhibition by gabexate mesylate occurred via a simple, competitive, and non-cooperative mechanism, K_i values being independent of the enzyme, substrate, and inhibitor concentration. Values of K_i indicated that the affinity of gabexate mesylate for human tryptase was the highest known among all the enzymes investigated [12–14,28,29] and that the drug behaves as a very selective inhibitor (see Table 1).

Moreover, gabexate mesylate inhibited the human tryptase-catalyzed cleavage of plasminogen-free human fi-

Table 1
Values of K_i for gabexate mesylate binding to serine proteinases, nitric oxide synthases, and copper amine oxidase[a]

Enzyme	K_i(M)	Reference	Selectivity factor[b]
Human tryptase[c]	3.4×10^{-9}	Present study	Reference enzyme
Bovine tryptase[c]	1.8×10^{-7}	Present study	53
Human thrombin[d]	5.0×10^{-7}	13	150
Bovine α-thrombin[e]	6.0×10^{-7}	12	180
Human urinary plasminogen activator[e]	1.3×10^{-6}	12	380
Human Lys77-plasmin[e]	1.5×10^{-6}	12	440
Porcine trypsin[f]	1.8×10^{-6}	13	530
Bovine Factor Xa[e]	2.2×10^{-6}	12	650
Bovine β-trypsin[e]	2.6×10^{-6}	12	760
Porcine pancreatic β-kallikrein-B[e]	5.0×10^{-4}	12	150 000
Human urinary kallikrein[e]	2.6×10^{-3}	12	760 000
Mouse nitric oxide synthase-I[g]	1.0×10^{-4}	29	29 000
Rat nitric oxide synthase-II[g]	5.0×10^{-3}	29	1 500 000
Swine kidney copper amine oxidase[h]	2.7×10^{-5}	28	7 900

[a] An average error value of about 8% was evaluated for K_i values.
[b] The selectivity factor indicates the ratio of K_i for each enzyme versus K_i for human tryptase, the enzyme showing the highest affinity for gabexate mesylate (i.e. the lowest K_i value).
[c] Values of K_i were obtained at pH 7.4 and 37.0°.
[d] The value of K_i was obtained at pH 6.8 and 20.0°.
[e] Values of K_i were obtained at pH 6.8 and 20.0°.
[f] The value of K_i was obtained at pH 7.8 and 20.0°.
[g] Values of K_i were obtained at pH 7.4 and 37.0°.
[h] The value of K_i was obtained at pH 7.0 and 25.0°.

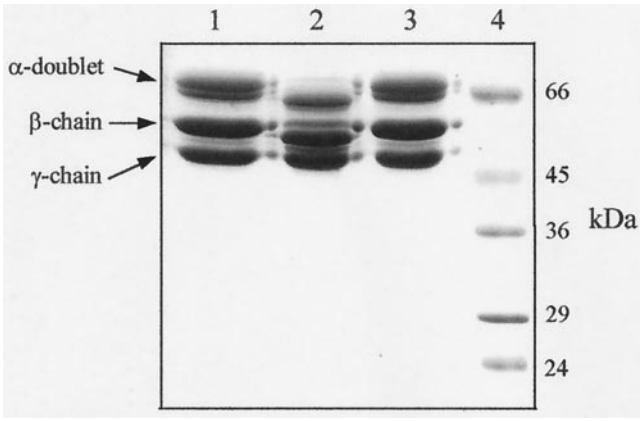

Fig. 3. Inhibitory effect of gabexate mesylate on the human tryptase-catalyzed cleavage of the plasminogen-free human fibrinogen, at pH 7.4 and 37.0°. Lane 1 shows the SDS–PAGE pattern of the plasminogen-free human fibrinogen, as control. Lane 2 shows the SDS–PAGE pattern of the plasminogen-free human fibrinogen upon cleavage by human tryptase. Lane 3 shows the SDS–PAGE pattern of the plasminogen-free human fibrinogen upon treatment with human tryptase inhibited by gabexate mesylate. Lane 4 shows the molecular weight markers. For further details, see text.

brinogen. As shown in Fig. 3, the SDS–PAGE pattern of the plasminogen-free human fibrinogen (characterized by the α-doublet and the β- and γ-chains) was unaffected by human tryptase in the presence of saturating amounts of gabexate mesylate. Indeed, human tryptase induced the collapse of the α-doublet of the plasminogen-free human fibrinogen to a single band and cleaved the β-chain to produce a β-fragment, as would be expected from the hydrolysis of the αArg572-αGly573 and βLys21-βLys22 bonds, respectively [5]. However, human tryptase did not affect the plasminogen-free human fibrinogen γ-chain [5].

Modelling studies (Fig. 4) indicated that gabexate mesylate bound to the primary specificity subsite S_1 of human and bovine tryptase. In particular, the positive charge of the ε-guanidino group of gabexate mesylate neutralized the negative charge of Asp189 (Fig. 4), the amino acid residue conferring cationic primary specificity to human tryptase. Moreover, the gabexate mesylate carbonyl group interacted

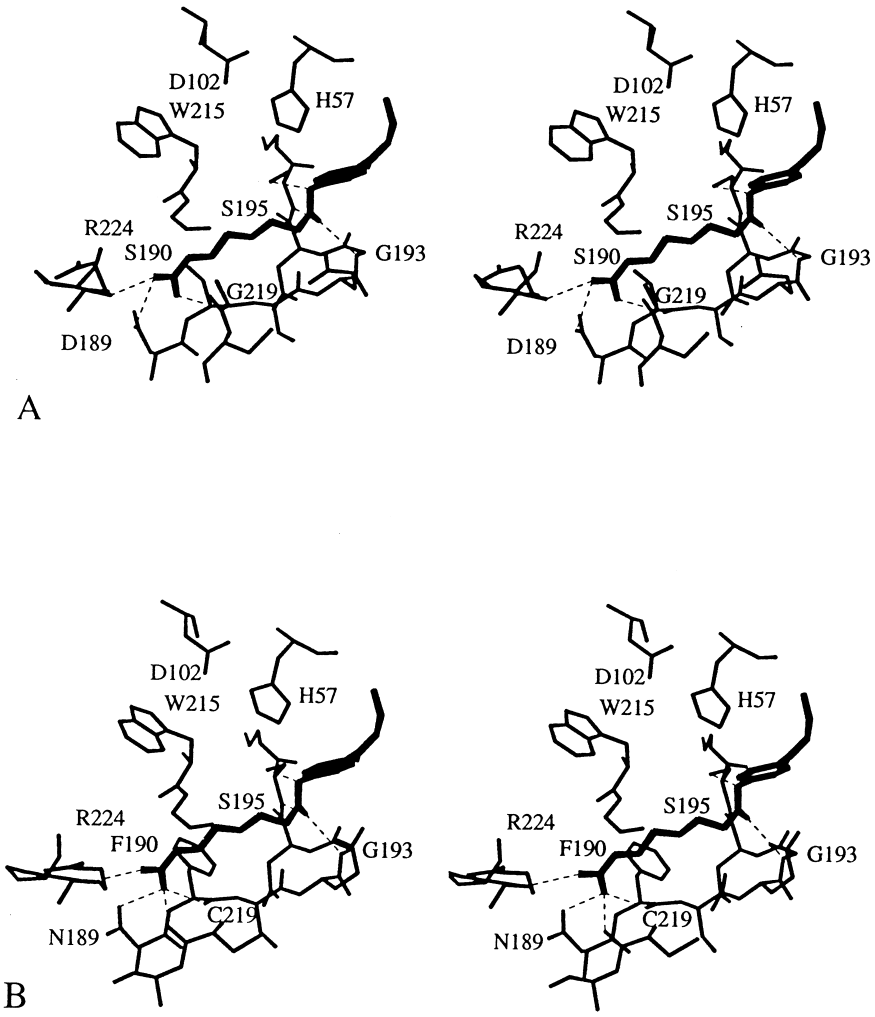

Fig. 4. Stereo view of the modeled human (panel A) and bovine (panel B) tryptase:gabexate mesylate complexes. The gabexate mesylate full structure and selected serine proteinase active center residues are shown. Gabexate mesylate is drawn with thick sticks. Dashed lines indicate hydrogen bonds. Enzyme residues are labeled. For further details, see text.

with the enzyme oxyanion binding hole, achieving hydrogen bonds to the peptide N atoms of Gly193 and Ser195.

The tight and selective binding of gabexate mesylate to human tryptase (Fig. 2 and Table 1) may be related to peculiar serine proteinase:inhibitor interactions. In particular, the carbonyl oxygen atoms of Ser190 and Arg224 of human tryptase may form hydrogen bonds with the ε-guanidino group of gabexate mesylate, thus contributing to the stability of the enzyme:inhibitor complex (Fig. 4).

The lower affinity of gabexate mesylate for bovine tryptase as compared to that for human tryptase (Fig. 2 and Table 1) may be ascribed to a weaker interaction of the inhibitor ε-guanidino group with the S_1 primary specificity pocket. Such a feature may reflect the presence of the Asn residue at position 189 in bovine tryptase, which resulted in a decreased negative charge at the bottom of the pocket with respect to human tryptase. Moreover, the Ser190 residue of human tryptase is replaced by Phe in bovine tryptase. The Phe190 side chain may be in steric clash with the ε-guanidino group of the incoming inhibitor, impairing bovine tryptase:gabexate mesylate complex formation. However, the carbonyl oxygen of the properly oriented Phe190 may form a hydrogen bond with the ε-guanidino group of gabexate mesylate. As observed in the human tryptase:inhibitor complex, the carbonyl oxygen atom of Arg224 of bovine tryptase may form a hydrogen bond with the ε-guanidino group of gabexate mesylate. Finally, a hydrogen bond may occur between the Cys219 carbonyl oxygen of bovine tryptase and the ε-guanidino group of gabexate mesylate (Fig. 4).

4. Discussion

Although the involvement of human tryptase in a number of mast cell-mediated diseases is now recognized, the pathophysiology of this unique serine proteinase is not completely understood. Thus, the search for and subsequent availability of new inhibitors, as well as knowledge of the biochemical bases of the inhibitory processes, may lead to a greater understanding of the physiological role of this enzyme and to the designing of new anti-inflammatory drugs. The tetrameric structure of human tryptase accounts for the restricted accessibility of most proteinaceous protease inhibitors to the four monomer active sites, which are directed toward the central cavity [3,8]; the only known exception is the leech-derived tryptase inhibitor (LDTI), isolated from the medical leech *Hirudo medicinalis* [30]. This small protein (46 aa) binds to two active sites of the tryptase tetramer with K_i ~1.4 nM, thus inhibiting 50% of the enzymatic activity at nanomolar concentrations. Total inhibition can be reached only at micromolar concentrations of the inhibitor [3]. In recent years, much effort has been directed toward the identification and synthesis of new low-molecular-weight compounds with potential inhibitory activity. Although selective active site-bridging non-peptidic inhibitors of human tryptase were reported to show subnanomolar inhibition constants, their high molecular weight made them fairly unattractive as drug discovery leads [31]. On the other hand, some inhibitors are not very selective toward human tryptase. For instance, BABIM, the human tryptase inhibitor bis-(5-amidino-2-benzimidazolyl)methane ($K_i = 1.8 \times 10^{-9}$ M) was only 10-fold less effective against trypsin [32]. Moreover, 1,5-bis-[4-[(3-carbamimidoyl-benzenesulfonylamino)-methyl]-phenoxy]-pentane (AMG-126737) inhibited human tryptase with low affinity ($K_i = 9.0 \times 10^{-8}$ M), the selectivity versus other serine proteinases being greater than 10- to 200-fold [33]. Furthermore, the human tryptase inhibitor *N*-(1-hydroxy-2-naphthoyl)-L-arginyl-L-prolinamide (APC-366), the first molecule that had advanced to clinical trials for the treatment of asthma, did show low activity against human tryptase ($K_i = 3.3 \times 10^{-7}$ M) at levels comparable to those seen with trypsin and thrombin [2]. Finally, the affinity of 4-amidinophenyl pyruvic acid for human tryptase ($K_i = 7.1 \times 10^{-7}$ M) was similar to that for trypsin [34].

The present results indicate that the synthetic drug gabexate mesylate is a potent ($K_i = 3.4 \times 10^{-9}$ M) and selective (selectivity factor > 50) inhibitor of human tryptase (see Table 1). The lower K_i for bovine tryptase, the second-best enzyme inhibited by gabexate mesylate among the proteases investigated, can be ascribed to the decreased negative charge in the primary specificity pocket of the bovine enzyme when compared to that of human tryptase (see Results). As a whole, the data reported here may represent the biochemical bases for the anti-inflammatory action of gabexate mesylate. Indeed, this drug prevents inflammation, erosion, and ulceration of skin and mucosae [15]. Moreover, gabexate mesylate, therapeutically used as an antiproteinase drug in the treatment of pancreatitis, may also inactivate human tryptase, which is released upon concomitant mast cell degranulation [35].

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