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## Mast cell proteases as pharmacological targets

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### Abstract

Mast cells are rich in proteases, which are the major proteins of intracellular granules and are released with histamine and heparin by activated cells. Most of these proteases are active in the granule as well outside of the mast cell when secreted, and can cleave targets near degranulating mast cells and in adjoining tissue compartments. Some proteases released from mast cells reach the bloodstream and may have far-reaching actions. In terms of relative amounts, the major mast cell proteases include the tryptases, chymases, cathepsin G, carboxypeptidase A3, dipeptidylpeptidase I/cathepsin C, and cathepsins L and S. Some mast cells also produce granzyme B, plasminogen activators, and matrix metalloproteinases. Tryptases and chymases are almost entirely mast cell-specific, whereas other proteases, such as cathepsins G, C, and L are expressed by a variety of inflammatory cells. Carboxypeptidase A3 expression is a property shared by basophils and mast cells. Other proteases, such as mastins, are largely basophil-specific, although human basophils are protease-deficient compared with their murine counterparts. The major classes of mast cell proteases have been targeted for development of therapeutic inhibitors. Also, a human  $\beta$ -tryptase has been proposed as a potential drug itself, to inactivate of snake venins. Diseases linked to mast cell proteases include allergic diseases, such as asthma, eczema, and anaphylaxis, but also include non-allergic diseases such inflammatory bowel disease, autoimmune arthritis, atherosclerosis, aortic aneurysms, hypertension, myocardial infarction, heart failure, pulmonary hypertension and scarring diseases of lungs and other organs. In some cases, studies performed in mouse models suggest protective or homeostatic roles for specific proteases (or groups of proteases) in infections by bacteria, worms and other parasites, and even in allergic inflammation. At the same time, a clearer picture has emerged of differences in the properties and patterns of expression of proteases expressed in human mast cell subsets, and in humans versus other mammals. These considerations are influencing prioritization of specific protease targets for therapeutic inhibition, as well as options of pre-clinical models, disease indications, and choice of topical versus systemic routes of inhibitor administration.

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## Index words

Mast cell; basophil; protease; tryptase; chymase; cathepsin G; carboxypeptidase A3; cathepsin C; dipeptidylpeptidase I; cathepsin L

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## 1. Introduction

A number of reviews published over the past decade have focused on mammalian mast cell and basophil proteases (Cairns, 2005; Caughey, 2007, 2011; Douaiher et al., 2014; Hallgren and Pejler, 2006; Harvima et al., 2014; Hellman and Thorpe, 2014; Pejler et al., 2010; Schwartz, 2006; Stevens and Adachi, 2007; Tojo and Urata, 2013; Trivedi and Caughey, 2010; Valent et al., 2012). These reviews emphasized major thrusts of research, including the use of these proteases in histochemical visualization and mast cell subsetting, their uses in clinical practice and research as biomarkers of mast cell activation, mastocytosis, and allergic disease phenotypes, their links to diseases not known to be associated with allergy and atopy, their sometimes unique properties as enzymes, their modes of activation, packing into granules, pathways of regulation and inactivation following release, and druggability, their genetic evolution in mammals, natural variation in mouse strains and human populations, their deficiency phenotypes, and, more recently, their suspected roles in homeostasis and host defense. The present review emphasizes recent developments and challenges in targeting mast cell granule-associated proteases for therapeutic inhibition. The primary focus is on human enzymes because these are presently the main targets of pharmaceutical development. The secondary focus is on mouse proteases because numerous genetically modified strains have been developed, including strains deficient in or over-expressing one or more mast cell proteases. Some of these strains have been offered as models of human disease. The differences between mouse and human mast cell and basophil proteases, in addition to their similarities, need to be appreciated to translate mouse genetic and pharmacological studies to human diseases and therapeutic responses.

This review concentrates on granule-associated proteases, especially those that are released outside of the cell in an active form with histamine following mast cell activation by allergen-bound IgE or other stimuli. However, it should be borne in mind that some secreted proteases, notably the soluble tryptases and the matrix metalloproteinase (MMP)9, are also shed in a non-regulated (i.e., constitutive) manner by unstimulated mast cells as pro-enzymes that may be activatable outside of the cell. The fate and roles, if any, of constitutively shed pro-tryptases are not yet clear. The granule-associated enzymes released in a regulated manner include serine-class proteases, such as soluble and transmembrane tryptases, chymases, cathepsin G, thiol (cysteine)-class proteases, such as dipeptidylpeptidase I (otherwise known as the exopeptidase cathepsin C), and the zinc metalloexopeptidase carboxypeptidase A3. Expression of the zinc metallo-endopeptidase MMP9 in mast cells may be regulated separately from the classic secretory granule serine proteases and may be released from separate structures, although this remains to be determined (Di Girolamo et al., 2006; Fang et al., 1997; Fang et al., 1996; Fang et al., 1999).

Mast cell proteases receive more attention than basophil proteases not only because we know more about the mast cell enzymes but because human basophils appear to have fewer and much smaller amounts of proteases compared with, for example, mouse basophils (Jogie-Brahim et al., 2004; Liu et al., 2012; Poorafshar et al., 2000; Raymond et al., 2005; Ugajin et al., 2009; Xia et al., 1995), and so have not been specifically targeted for therapeutic purposes. It is also worth stressing the general point that although mast cell proteases were at one time assumed to be destructive, inflammatory, and “bad” in the context of allergic disease, more recent evidence suggests that some of these proteases play homeostatic, protective, and even anti-inflammatory roles, with the present evidence being more definitive in mice than in humans (Balzar et al., 2005; Caughey et al., 1988; Dougherty et al., 2010; Mallen-St Clair et al., 2004; Maurer et al., 2004; Metz et al., 2006; Piliponsky et al., 2008; Piliponsky et al., 2012; Roy et al., 2014; Sugimoto et al., 2012; Thakurdas et al., 2007; Waern et al., 2013). In gauging the value of individual mast cell proteases as targets for therapeutic inhibition, one of course needs to consider what functions of general value may be lost to the host as a consequence of inhibition or depletion, in addition to impacts on a particular disease for which targeting of one or more mast cell proteases may be advantageous. This calculus is easier for some targets than others. And it should be noted that mast cell tryptases are considered not only targets for inactivation but also potentially as drugs themselves, for example to detoxify venins in human victims of envenomation by snakes.

## 2. Tryptases

### 2.1. A gene family

Tryptases occur in soluble and membrane anchored forms, which are related products of different genes (Caughey et al., 2000a; Hellman and Thorpe, 2014; McNeil et al., 2007; Miller et al., 1990; Pallaoro et al., 1999; Trivedi et al., 2007; Vanderslice et al., 1990; Wong et al., 1999). In mouse models of human disease, one or more tryptases have been implicated in a rather startling range of pathologies, including allergic airway inflammation (Oh et al., 2002), cigarette smoke-induced COPD (Beckett et al., 2013), itch (Ui et al., 2006); visceral pain (Cenac et al., 2007), aortic aneurysms (Zhang et al., 2011), colitis (Corvera et al., 1997; Hamilton et al., 2010; Jacob et al., 2005), and inflammatory arthritis (McNeil et al., 2008; Shin et al., 2009). In humans, measurements of soluble tryptases in the blood have become widely used as biomarkers of mastocytosis, anaphylaxis risk, and mast cell activation, as has been covered extensively in other reviews (Caughey, 2006, 2007; Hallgren and Pejler, 2006; Harvima et al., 2014; McNeil et al., 2007; Schwartz, 2006). Human mast cells are so abundantly endowed with tryptase transcripts and protein that these have emerged as perhaps the most sensitive and specific means of detecting mast cells in tissues and biopsies. For example, tryptase mRNAs are among the most abundant transcripts in epithelial brushings and biopsies in “Th2 high” asthma (Dougherty et al., 2010), allergic rhinitis (Takabayashi et al., 2012), and eosinophilic esophagitis (Abonia et al., 2010), even though mast cells are a small fraction of cells retrieved in such samples. Evidence of tryptase involvement in the gamut of pathologies suggested by the murine studies is less direct and less dramatic. The main focus to date has been on contributions of soluble tryptases to allergic inflammation and tissue remodeling in rhinitis and asthma. A second focus has been on intestinal

inflammation, especially colitis. Several early-phase trials involving tryptase inhibitors have shown efficacy in allergic asthma (Krishna et al., 2001), allergic rhinitis (Erin et al., 2006), and ulcerative colitis (Tremaine et al., 2002).

## 2.2. Soluble, tetramer-forming tryptases

Soluble tryptases are primarily restricted to mast cells and the classic and most extensively investigated members of the tryptase family. These enzymes are activated prior to storage in granules by removal of a unique pro-peptide (Hallgren et al., 2005; Le et al., 2011a; Le et al., 2011b; Sakai et al., 1996; Vanderslice et al., 1989; Wolters et al., 2001), and then are packed into the same secretory granules that contain other pre-formed mast cell products, most famously histamine. In human mast cells, especially, soluble tryptases can be highly concentrated, becoming incorporated into semi-solid lattices and crystalline forms (Craig et al., 1988). They achieve this by self-assembling into tetramers, which bind strongly to the granule's polyanionic heparin matrix. Even at high concentrations, they avoid damaging the cell because they are segregated into membrane-bound organelle (secretory granules) that maintain a low pH that suppresses enzyme activity, and by organizing into a toroidal oligomer with the active sites facing into a central pore, which restricts access to large substrates that a monomer would otherwise be able to cleave (Pereira et al., 1998). Tight packing within a macromolecular matrix of proteoglycan also restricts access of potential substrates.

Soluble tryptases retain their tetrameric configuration upon exocytosis of granule contents, and are thought to remain associated with heparin, even while diffusing away from the degranulated mast cell. How far tetrameric human tryptases travel as active, heparin-bound enzyme complexes after being ejected from mast cells is not clear. After all, these complexes can be very large and presumably sticky and would not be expected to diffuse readily out of tissues or across epithelial and endothelial barriers. What is clear is that immunoreactive tryptase appears in the bloodstream after major mast cell activation events, such as bee-sting anaphylaxis (Schwartz et al., 1987; Schwartz et al., 1989). How much, if any, of this tryptase is active is not known. It is likely that the remnants of exocytosed tryptase are mainly inactive monomers, which lost their association with heparin, dissociated from the tetramer (which is stabilized by heparin), and underwent spontaneous denaturation with resulting loss of activity (Schwartz and Bradford, 1986). Immunoreactive tryptases also are present in the bloodstream in the absence of systemic mast cell activation, but these proteins appear to be mainly inactive monomeric, unprocessed pro-enzymes that are shed constitutively by mast cells rather than being stored in secretory granules (Schwartz et al., 2003). Baseline levels of immature pro-tryptases in blood therefore are thought to reflect total body mast cell burden rather than activation per se, and are elevated in mastocytosis syndromes (Akin et al., 2007).

**2.2.1. General strategies for inactivating or preventing release of soluble tryptases**—The ability of human mast cells to store pre-activated tryptases with sterically restricted active sites at high concentration in a low-pH environment within a membrane-bound organelle presents challenges to those pondering pharmaceutical strategies to inactivate them. These challenges include identifying a tryptase-selective inhibitor that penetrates mast cell plasma and secretory granule lipid bilayers, passes into the interstices of

a semi-crystalline, polyanionic matrix, accesses the interior of the donut-shaped tryptase tetramer to reach the active sites sheltered within, and retains inhibitor properties following release of tryptase into the high-pH environment outside of the cell. This is a tall order for a drug. Recognizing these challenges, most pharmaceutical development of tryptase inhibitors has emphasized compounds that engage and inactivate tryptase not before but after release from a stimulated mast cell. This strategy, too, has its challenges. For one thing, because soluble tryptases are the major proteins of mast cells and nearly all of this protein can be released from a maximally stimulated mast cell in seconds to minutes, the concentration of tryptase in the immediate vicinity of a degranulating mast cell can be very high. This generates a requirement, if complete inhibition is to be achieved, of high local levels of inhibitor, very high inhibitor potency, or both. For some disease conditions in which tryptase has been implicated, high local levels are more likely to be achieved with topical rather than systemic inhibitors. These considerations also have led some to consider strategies other than those involving active-site inhibition to oppose unwanted effects of tryptases. Alternative strategies include preventing mast cells from degranulating in response to allergic stimuli by targeting IgE or IgE receptor, interfering with downstream degranulation signals, depleting mast cells themselves, or destabilizing released tryptases to promote tetramer dissolution and spontaneous inactivation.

**2.2.2. Insights from models of disease in mice and genetic variation of human tryptases**—Additional concerns influencing strategic approaches to development of inhibitors are raised by findings from mice lacking one or more tryptases. Although studies in genetically modified mice implicate soluble tryptases in several disease models, including immune arthritis, inflammatory bowel disease, and smoke-induced lung inflammation, mice lacking some tryptases are more susceptible to lethal peritonitis resulting from introduction of a gram-negative bacterium introduced into the abdominal cavity. These findings raise legitimate concerns that inhibition of mast cell tryptases may seriously impair host defense, especially if inhibition is systemic. It is presently unclear whether the findings in these mouse models can be freely extrapolated to human diseases and host defense. However, several differences between mice and human tryptases suggest reasons to be cautious in this regard. For one thing, human mast cells appear to make more tryptase protein than mouse mast cells do, and humans have more soluble mast cell tryptase genes. The mouse tryptase gene most similar to the classic human  $\beta$ -tryptases (which are expressed at two adjacent but separate loci) in form and biophysical properties is mast cell protease 6 (product of the *Mcpt6* gene). A second mouse tryptase (mast cell protease 7, product of *Mcpt7*), appears to be most closely related in a phylogenetic sense to a different human tryptase,  $\delta$ , which is produced by *TPSD1* gene (Min et al., 2001; Pallaoro et al., 1999; Trivedi et al., 2008). However, human  $\delta$  tryptase is severely truncated and its enzymatic activity is either severely reduced or absent (Trivedi et al., 2008; Wang et al., 2002). Furthermore, some mouse strains, including commonly used mice of the C57BL/6 background, natively do not express mast cell protease 7 (Hunt et al., 1996), though mice of other backgrounds, such as BALB/c, do express this enzyme, which is more independent of heparin than is the human enzyme, and can appear in active form in mice with mastocytosis (Ghildyal et al., 1996). Thus, differences between humans and mice, and between strains of mice with different disease phenotypes and genetic backgrounds, need to be considered when weighing implications of

mouse findings in strategies for developing anti-tryptases. Murine mast cells and basophils also have a tryptase-like mastin (mast cell protease 11, see below) that humans lack because the human mastin gene is a pseudogene (Raymond et al., 2005; Raymond et al., 1995; Reimer et al., 2010; Ugajin et al., 2009; Wong et al., 2004). These differences warrant consideration in weighing relative contributions of mast cell and basophil tryptases in allergic inflammation and other phenotypes.

Human populations manifest surprising degrees of variation in the numbers and types of tryptases inherited by individuals (Akin et al., 2007; Soto et al., 2002; Trivedi et al., 2008; Trivedi et al., 2009; Trivedi et al., 2007). Additional diversity arises from size isoforms translated from mRNA splice variants (Jackson et al., 2008). These variations potentially influence the relative importance of contributions of the inherited complement of soluble tryptases to diseases and to host defense functions. They also may lead to variations in inhibitor response, and so are summarized here. Inheritance of loss-of-function tryptase alleles (in addition to the universally defective  $\delta$  genes) is common (Soto et al., 2002; Trivedi et al., 2009). Loss-of-function genes include  $\alpha$ -tryptase and frame-shifted  $\beta$ III-tryptase (Trivedi et al., 2009). Individuals with 4 active  $\beta$  genes lack  $\alpha$  genes altogether, a situation that arises because  $\alpha$  genes in most cases are alleles at a locus that also accepts  $\beta$  genes. Some individuals also may have duplicated loci containing  $\alpha$  genes (Abdelmotleb et al., 2014). Among active  $\beta$  tryptases, one of the common variations is loss of an N-linked glycosylation site in  $\beta$ II tryptase, compared to the classical  $\beta$ I form, which may alter stability. The implications of these genetic variations for diseases susceptibility and host defense capabilities are presently not well understood but are under investigation. Inherited differences in circulating levels of immunoreactive tryptase, the genetic basis of which remains to be explained, potentially relate to differences in inheritance of tryptase alleles (Lyons et al., 2014; Sabato et al., 2014).

**2.2.3. Inhibitors of  $\beta$ -tryptase: the challenge of generating selectivity and potency**—Earlier efforts to develop selective inhibitors of soluble mast cell tryptases were reviewed previously (Cairns, 2005; Caughey, 2007, 2011). In general, the sheltering of  $\beta$ -tryptase active sites in the interior of a donut-shaped tetramer (Pereira et al., 1998) confers resistance to larger, proteinaceous serine protease inhibitors, including all of the many general anti-peptidases circulating in human blood and extravascular fluids. This resistance is a property of the tryptase tetramer, not the monomer, which is inhibitor-sensitive (Fajardo and Pejler, 2003; Fukuoka and Schwartz, 2004). There is presently no strong evidence that the catalytically active monomer, although it can be created in the laboratory using highly specific conditions, exists in vivo. To a small molecule, active sites in the tryptase tetramer are readily accessible. The maximum size of an inhibitor that can be accommodated in the active sites of tetrameric tryptases varies. Human  $\beta$ -tryptases are more restrictive in this regard than certain other tryptases, for example resisting aprotinin (a compact ~6-kDa protein of that has been used as an anti-protease drug in humans), whereas dog tryptase is sensitive to aprotinin (Raymond et al., 2005). Potent natural inhibitors of tryptase have been identified in leeches (Raymond et al., 2005; Sommerhoff et al., 1994; Stubbs et al., 1997) and ticks (Paesen et al., 2007), which may use these inhibitors to minimize tryptase-associated inflammation and itching linked to mast cell degranulation in response to

skin penetration by parasites seeking a blood meal. Other compact proteins, notably a cyclic cystine-knot miniprotein from seeds of *Momordica cochinchinensis* (gac) (Sommerhoff et al., 2010), have been modified to produce potent inhibitors of additional potential pharmaceutical interest. The stoichiometry of inactivation of active sites in the trypsin tetramer may be less than one and still achieve effective inhibition, especially towards large substrates, due to steric blockage of the activesite donut hole (Raymond et al., 2005).

The lion's share of effort in developing inhibitors of human  $\beta$ -trypsin has been devoted to identifying small, high-potency compounds that are selective for trypsin and occupy active sites reversibly, while meeting pharmacodynamic and pharmacokinetic goals, such as oral absorbability. Achieving selectivity and avoiding off-target effects is challenging, because there are dozens of tryptic serine proteases involved in essential processes such as food digestion, fibrinolysis, hemostasis, complement activation, cellular signaling and growth, and control of ion flux. To small molecules, many of these proteases have active sites that look similar to those of trypsin. The first high-potency  $\beta$ -trypsin inhibitors to be reported were aromatic diamidines (Caughey et al., 1993a), including bis (5-amidino-2-benzimidazolyl) methane, which blocked aeroallergen-induced bronchoconstriction in a sheep model of asthma (Clark et al., 1995). These inhibitors were not highly selective for  $\beta$ -trypsin versus pancreatic trypsin, but helped pave the way for more selective amidino compounds (Burgess et al., 1999; Oh et al., 2002; Ono et al., 1999; Wright et al., 1999), including elongated, bivalent compounds designed to bridge the central pore of trypsin tetramer by engaging two active sites (Schaschke et al., 2002; Selwood et al., 2003). A related compound, nafamostat, that was already in use as a drug in humans but not for the purpose of targeting trypsin, was discovered to be an usually potent inhibitor of  $\beta$ -trypsin (Mori et al., 2003), raising the question of whether some of the drug's effects are due to trypsin inactivation. Nafamostat differs from some other dibasic aromatic protease inhibitors in that can form long-lasting, covalent adducts with the active site serine (Ramjee et al., 2000), and thus is not a typical competitive inhibitor. Inhibition of trypsin is proposed to be the basis of protection from lung dysfunction in a rat model of iodinated contrast agent allergy (Sendo et al., 2003), protection from colitis in rats (Isozaki et al., 2006), and allergic airway inflammation in mice (Chen et al., 2006), all of which may involve mast cell inactivation and trypsin release. Additional de novo pharmaceutical development of trypsin inhibitors, encouraged by efficacy in human allergic rhinitis of a topical dual inhibitor of trypsin and trypsin (Erin et al., 2006), identified more selective inhibitors of trypsin with oral activity in pre-clinical models of asthma in guinea pigs and sheep (Costanzo et al., 2008).

### 2.3. Transmembrane $\gamma$ -trypsin

$\gamma$ -Trypsin differ from the soluble trypsin in being I transmembrane proteins with a short, C-terminal extension of the catalytic domain. The C-terminal peptide is just long enough to span a lipid bilayer (Caughey et al., 2000a). Although several related type I transmembrane peptidases, such as prostasin, exchange the membrane-spanning peptide for a lipid (glycosylphosphatidylinositol) anchor, this does not appear to be the case for  $\gamma$ -trypsin (Verghese et al., 2006). Although being a transmembrane protein on first inspection would seem to place  $\gamma$ -trypsin into a class of peptidases not closely related to soluble trypsin,

phylogenetic analysis suggests that soluble mast cell tryptases evolved from  $\gamma$ -like ancestral transmembrane proteins early in mammalian evolution (Trivedi et al., 2007). In further support of such a transition, the example of marapsin suggests a rather facile, one-step evolutionary pathway for converting a type I transmembrane tryptic protease to its soluble homologue (Raman et al., 2013). The standard topology of type I proteins predicts that  $\gamma$ -tryptase is anchored to the inside of the secretory granule lipid bilayer (Caughey et al., 2000a). Thus it is unlikely to be able to achieve the concentrations inside the granule reached by the soluble tryptases, which can be packed into the interior. Upon degranulation,  $\gamma$ -tryptase catalytic domains are expected to decorate the outer surface of the mast cell as the membranes of exocytosing granules fuse with the plasma membrane. In the human and mouse genomes, the  $\gamma$ -tryptase gene *TPSG1/Tpsg1* anchors one end of the multi-gene locus containing soluble tryptase genes. It should be noted that many mammals lack the *TPSG1* gene entirely (Trivedi et al., 2007). When the catalytic domain of human  $\gamma$ -tryptase is expressed as a recombinant, soluble enzyme, unlike  $\beta$ -tryptases it does not oligomerize and it is susceptible to inhibition by endogenous anti-proteases (Wong et al., 2002). However, there is no evidence that  $\gamma$ -tryptase is shed from the membrane by proteolysis or exists in vivo as a soluble enzyme (Verghese et al., 2006). Nonetheless, the little that is known of the enzymatic properties of  $\gamma$ -tryptase comes from studies of the recombinant soluble enzyme (Wong et al., 2002; Yuan et al., 2006), the properties of which differ from those of human  $\beta$ 1-tryptase both in terms of substrates preferred in a library of peptidic substrates and in comparative susceptibility to inactivation by a library of druglike inhibitors. It is possible that the substrate preferences and inhibitor susceptibility of  $\gamma$ -tryptase in its membrane-anchored form differ from those of its soluble forms. Although, knowledge of its enzymatic properties in vivo is limited and the absence of the *TPSG1* gene suggests that the enzyme is expendable in many mammals, several lines of evidence suggest that it has the potential to influence pathological phenomena in mice and humans. The first evidence of this came from the demonstration of inflammation and airway hyperresponsiveness in mice into which recombinant human  $\gamma$ -tryptase was introduced to the respiratory tract (Wong et al., 2002). More recently, mice with inactivated  $\gamma$ -tryptase gene *Tpsg1* in mice were shown to resist development of inflammation in models of colitis and cigarette-induced lung inflammation (Hansbro et al., 2014), thus suggesting roles in pathogenesis of inflammation not redundant with soluble tryptases, the absence of which is associated with similar phenotypes (Beckett et al., 2013; Hansbro et al., 2014).

#### 2.4. Mastins

Mastins are oligomerizing, tryptase-like serine proteases that are not expressed in humans because of mutations in a non-transcribed pseudogene located in the tryptase gene cluster on chromosome 16p13.3. From the perspective of developing drugs to treat human diseases, mastin's importance lies in its presence in mammals that serve as pre-clinical models, where mastin activity may lead to results that are confusing to translate to humans. Mastin in dogs was the first tryptase-like protease of any kind to be cloned and sequenced as a cDNA (Vanderslice et al., 1989). Initially it was labeled "mastocytoma protease" because it was identified in mast cell tumors, from which it was cloned and then purified as a highly active peptidase separate from canine tryptase, with unique properties (Raymond et al., 2005; Raymond et al., 1995). Canine mastin forms disulfide-linked multimers and is even more



inhibitor-resistant than either canine or human tryptase—resisting leech-derived tryptase inhibitor, for example, thereby suggesting highly protected active sites. Apart from mastocytomas, canine mast cells and a subset of circulating polymorphonuclear cells (that may be basophils) express mastin. Subsequently, mastin was identified in porcine lungs (as influenza A-activating enzyme “tryptase TC30”) and in mice as “mast cell protease 11”, which is transcribed from the *Prss34* gene. Phylogenetic surveys of other mammalian genomes suggest that mastins are a distinct branch of tryptase-related enzymes, and that several other mammals have intact mastin genes, including cattle, which may have two (Trivedi et al., 2007). Mouse mastin is expressed by mast cells and basophils, is catalytically active as a tryptic protease, and appears to be a major protein of basophil granules (Ugajin et al., 2009; Wong et al., 2004). Thus, mastin expression is a major phenotypic difference between human and mouse basophils. In mouse basophils, mastin may be the protease responsible for degradation of chaperone gp96 (Liu et al., 2012), which is a phenomenon not seen in human basophils.

### 3. Chymase-related proteases

#### 3.1. Chymases with chymotryptic activity

Human genomes possess a single chymase gene (*CMA1*) encoding a serine-class endoprotease that is primarily chymotryptic, which is to say that it cleaves peptides after aromatic amino acids, especially phenylalanine and tyrosine (Caughey et al., 1993b; Caughey et al., 1991). This tendency is especially pronounced when preferences are profiled using small (peptidic) substrates (Andersson et al., 2009; Powers et al., 1985; Raymond et al., 2003). However, when confronting globular proteins, which tend to bury aromatic residues in hydrophobic interiors where they are inaccessible to hydrolytic attack, chymase can cleave after leucine (Caughey et al., 2008; Raymond et al., 2006). In vitro, human chymase is not highly selective, and is capable of cleaving a variety of peptide and protein targets, both endogenous and exogenous (as from pathogens) (Powers et al., 1985). Given this broad capability, it is likely that some chymase functions are related to general peptidase activity rather than to hydrolysis of one or a few selected targets. Nonetheless, human chymase, among mammalian chymases, is exceptionally active in hydrolyzing angiotensin I at a specific site to its active form, angiotensin II (Caughey et al., 2000b; Kinoshita et al., 1991; Muilenburg et al., 2002; Reilly et al., 1982; Wintroub et al., 1984). This cleavage not only exhibits favorable kinetic attributes (perhaps more favorable than for angiotensin converting enzyme itself) but is highly selective for angiotensin Phe<sup>8</sup>, rather than the Tyr<sup>4</sup> that is attracted to other chymotryptic enzymes, including some rodent chymases, which thereby destroy angiotensin. Moreover, human chymase has the unusual capability of activating angiotensin while bound to circulating alpha-2-macroglobulin (Raymond et al., 2009), which is proposed to allow human chymase to generate angiotensin II systemically, perhaps supporting blood pressure during anaphylaxis, influencing recovery from ischemia, or affecting vascular and myocardial remodeling. Indeed, transgenic mice expressing human chymase are hypertensive (Koga et al., 2003), as are transgenic mice expressing rat vascular chymase, which is not found in humans but is similar to human chymase in its ability to generate angiotensin II (Ju et al., 2001). It remains to be established whether angiotensin II generated by human chymase is good, bad, or indifferent (or perhaps more than one of these

possibilities depending on context). Nonetheless, attention in the arena of pharmaceutical development of chymase inhibitors primarily has focused on cardiovascular indications (Dell'Italia and Husain, 2002; Miyazaki et al., 2006; Nishimoto et al., 2001; Pat et al., 2010; Zheng et al., 2014). Studies in mice suggest that mast cell chymases are the main source of angiotensin-converting enzyme-independent generation of angiotensin II and are responsible for the portion of angiotensin-dependent blood that resists angiotensin-converting enzyme inhibitors while responding to angiotensin receptor blockers (Li et al., 2004).

In contrast to human genomes, mouse and rat genomes contain several genes encoding chymase-like proteins (Gallwitz and Hellman, 2006; Gallwitz et al., 2006; Lutzelschwab et al., 1997). Several aspects of this multiplicity are challenging to relate to the sole human chymase gene and the properties and functions of its product. First of all, all or nearly all mouse and rat mast cells express one or more chymotryptic chymases (Reynolds et al., 1990; Stevens et al., 1994), whereas chymase expression in human mast cells is confined to subsets of cells, which are present in many organs but are especially abundant and a very high fraction of total mast cells in the dermis of skin (Irani et al., 1989). Compared to humans, mice and rats express chymases in a higher fraction of mast cells residing in mucosal locations. Most mouse mucosal mast cells, for example, express mast cell protease (MCP)-1, which is an active, chymotryptic enzyme with no phylogenetic or functional equivalent in humans (Caughey, 2004, 2007; Gallwitz et al., 2006). MCP-1 is the product of the *Mcpt1* gene. Although the biological targets of MCP-1 remain to be identified, studies in mice lacking MCP-1 suggest that this enzyme helps to defend against certain intestinal parasites (Knight et al., 2000) and may influence bronchial responses to allergic inflammation (Sugimoto et al., 2012). The mouse enzyme that is the phylogenetic equivalent of human chymase is MCP-5 (product of the *Cma1* gene) (Caughey et al., 2008; Gallwitz et al., 2006; Gurish et al., 1993; Huang et al., 1991; McNeil et al., 1991) and is expressed in similar locations in mice and humans. However, mutations in the active site introduced during rodent evolution changed specificity so that it is no longer chymotryptic (see additional discussion below) (Kunori et al., 2002).

In mice, the second major chymase with chymotryptic activity is MCP-4 (product of the *Mcpt4* gene). Although this enzyme has no phylogenetic equivalent in human or other primate genomes, its patterns of expression in mast subsets—as well as certain biophysical and functional properties, including angiotensin II-generating and MMP-9-activating activity—resemble those of human chymase (Caughey et al., 2000b; Lundquist et al., 2004; Tchougounova et al., 2005; Tchougounova et al., 2003). Therefore, mouse MCP-4 in mice often is considered to be the “functional equivalent” of human chymase. With respect to bronchial reactivity in allergic inflammation, the actions of MCP-1 and MCP-4 appear to be opposed (Sugimoto et al., 2012). Indeed, selective absence of MCP-4 in mice appears to enhance allergic inflammation (Waern et al., 2009; Waern et al., 2013), which is consistent with the absence of chymase in human mast cells infiltrating the lower and upper airway in asthma (Dougherty et al., 2010), allergic rhinitis (Takabayashi et al., 2012), and eosinophilic esophagitis (Abonia et al., 2010), and with the correlation of chymase-positive mast cells in small airways with better lung function in severe asthma (Balzar et al., 2005; Balzar et al., 2010). Although MCP-4's apparent opposition to allergic inflammation may suggest that chymase is an unsuitable target for pharmaceutical inhibition to relieve this condition,

human chymase's roles in this regard remain to be established. MCP-4 also may play protective roles in protection from the lethal effects of sepsis (by cleaving TNF $\alpha$  (Piliponsky et al., 2012) and alarmins (Roy et al., 2014)), and of toxins (for example, Gila monster venom (Akahoshi et al., 2011)), although, again, human chymase's in these phenomena is uncertain.

### 3.2. Chymases with elastolytic, leu-ase or absent catalytic activity

Mouse chymase-like genes cluster on chromosome 14 (Gurish et al., 1993). Even more such genes are in the rat genome (Hellman and Thorpe, 2014; Lutzelschwab et al., 1997; Puente and Lopez-Otin, 2004). *Mcpt1* and *Mcpt4*, respectively encode the chymotryptic proteases MCP-1 and MCP-4 noted above. The other mouse "chymases" appear to lack chymotryptic activity due to mutations affecting amino acids near the active site. For example, MCP-2, which is co-expressed with MCP-1 in mucosal mast cells, appears to be enzymatically inactive, has no known function, and lacks a human counterpart (Caughey, 2011). MCP-5, like orthologs in rats and hamsters, is elastolytic (able on to hydrolyze peptides after small neutral amino acids) (Karlson et al., 2003; Kervinen et al., 2008; Kunori et al., 2002), although its nucleotide and amino acid sequence overall are most similar to those of the sole human mast cell chymase, which is not an elastase and has almost no overlap in the types of substrates it can hydrolyze. Therefore, MCP-5 is not an enzyme that is an attractive target in efforts to model the roles of human chymase in host defense and pathobiology, although in mice it appears to contribute to ischemia-reperfusion injury in skeletal muscle (Abonia et al., 2005) and to inflammation after burn injury to the skin (Younan et al., 2010). Human mast cells do not produce or secrete an elastase per se, although human cathepsin G's activity profile overlaps to a minor extent with that of elastases (Raymond et al., 2010). The major chymase-related enzyme in guinea pigs appears to have diverged even more, for this enzyme is neither primarily chymotryptic nor elastolytic, but a leu-ase (Caughey et al., 2008). The differences in mast cell subset-specific expression, primary specificity, and target preferences among mammalian chymases present a challenge in selecting appropriate pre-clinical models in which to explore the roles of human chymase in disease (Kervinen et al., 2010), and also illustrate that it is comparatively easy in the evolutionary sense to change "chymase" specificity by changing key residues in the active site. Potential solutions to using mice as a pre-clinical model of chymase function in humans include 1) focusing on a particular mouse chymase, MCP-4, which has properties similar but not identical to the human enzyme (Tchougounova et al., 2005; Waern et al., 2009; Waern et al., 2013), 2) studying transgenic mice expressing human chymase (Chen et al., 2002; Rafiq et al., 2014), and 3) choosing another model mammal, such as dogs, which express a single chymase with properties highly similar to those of the human enzyme (Caughey et al., 1991; Fang et al., 1997; Muilenburg et al., 2002; Zheng et al., 2014).

### 3.3. Cathepsin G

Cathepsin G is different from classic lysosomal cathepsins in that it is a serine-class protease. It deserves attention in discussions of mast cell chymotryptic proteases because it is related to chymases and is expressed by mast cells, but also by other myeloid cell types. It is especially abundant in neutrophils, from which it is released to the cell surface and is a component of neutrophil extracellular traps. In humans, cathepsin G is the closest relative of

chymase and its gene *CTSG* is next door to chymase gene *CMAI* on human chromosome 14 (Caughey et al., 1993b; Hohn et al., 1989). In human mast cells, cathepsin G is expressed in the same subsets of cells that express chymase and in similar amounts (Schechter et al., 1990), and, like chymase, is activated by cathepsin C, which removes its pro-dipeptide (Adkison et al., 2002). Human cathepsin G shares some functional properties with human chymase, such as the ability to cleave angiotensin I at a selective site to generate angiotensin II (Raymond et al., 2010; Tonnesen et al., 1982). However, it is a weaker general endopeptidase, with broader specificity, including the unusual ability to cleave targets at tryptic as well as chymotryptic sites (Raymond et al., 2010). In mast cells, its functions relative to those of chymase are not known. Although it is thought to be secreted with chymase from activated human mast cells, its fate after release may differ, for it is more likely to be irreversibly inactivated by serpins (Travis et al., 1978), and less likely to be captured by macroglobulin (Raymond et al., 2009). The broad specificity of human cathepsin G appears to be anomalous among mammalian cathepsin Gs, including mouse, which is a highly active though more narrowly chymotryptic enzyme (Kalupov et al., 2009; Raymond et al., 2010). The difference in substrate profile between the human and mouse enzymes is due largely to a single mutation near the substrate binding site that appeared in a recent primate ancestor of humans and closely related great apes (Raymond et al., 2010). In mice, cathepsin G appears to contribute to effective host defense against certain bacterial infections (Hahn et al., 2011; Raptis et al., 2005; Steinwede et al., 2012; Woloszynek et al., 2012), but it is not known whether its expression in neutrophils, other myeloid cells, or mast cells (or combinations of these cells) is important in this regard. In humans, its contributions to host defense remain to be demonstrated. Given the major, recent changes in enzymatic properties late in primate evolution, it is speculated that cathepsin G in humans is more destructive and less helpful to host defense than its murine ortholog (Raymond et al., 2010). Because human chymase and cathepsin G have similar active sites and share some functions as well as the attribute of mast cell expression, one pharmaceutical strategy has been to develop dual chymase-cathepsin G inhibitors (de Garavilla et al., 2005). The use of mice as pre-clinical models of cathepsin G function in human disease is complicated by potentially redundant function with murine chymases and by the differences in activity and substrate preferences of the human and mouse versions of cathepsin G (Kalupov et al., 2009; Raymond et al., 2010).

#### 4. Carboxypeptidase A3

At one time carboxypeptidase A3 was termed mast cell carboxypeptidase (Irani et al., 1991; Pejler et al., 2009). However, as transcripts encoding this enzyme were discovered in basophils, its name was changed to reflect broader expression. The carboxypeptidase A3 gene *CPA3* also may be transcribed in other cell types, although there is little information concerning its functions and potential for storage in non-mast cells. In basophils, there is scant evidence that it can be stored and accumulated in granules as it is in mast cells, in which carboxypeptidase A3 seems to be codependent on the presence of chymase and heparin proteoglycan (Feyerabend et al., 2005; Goldstein et al., 1992; Grujic et al., 2013; Humphries et al., 1999). Lack of chymase in basophils and/or low levels of heparin may explain weak storage of carboxypeptidase A despite the presence of transcripts. This co-

dependence makes functional sense, for carboxypeptidase A3 can remove aromatic amino acids at the neo-C-termini of peptides and proteins attacked by chymase. Thus, these endo- and ecto-proteases can act in tandem and may serve host defense and homeostatic functions as a team. On the other hand, a new human mast cell subtype appearing in mucosa of tissues involved with allergic inflammation appears to have high levels of carboxypeptidase and tryptase but little chymase. This cell has been reported in “Th2-high” asthma (Dougherty et al., 2010), allergic rhinitis (Takabayashi et al., 2012), and eosinophilic esophagitis (Abonia et al., 2010). In these tissues, the CPA3 transcript is highly upregulated and readily detected in luminal brushings and biopsies, making it a useful biomarker of allergic inflammation. Whether carboxypeptidase A3 expressed in these cells augments or potentially opposes inflammation in these tissues is unknown at this point. Initially, the functions of carboxypeptidase A3 were hard to determine from *Cpa3* knockout mice because of disturbances involving other mediators and the very structure of the mast cell secretory granule (Feyerabend et al., 2005). More recently, using a selective knockin mutation rendering the carboxypeptidase inactive but still present as a “placeholder” and allowing preservation of granule structure, evidence was found that active carboxypeptidase A3 protects from toxic effects of endogenous endothelin and from endothelin-like sarafotoxin class of snake venins (Schneider et al., 2007). Similarly, chymase protects from toxic effects of Gila monster and scorpion venoms (Akahoshi et al., 2011). These studies suggest roles carboxypeptidase and chymase in detoxification, acting individually or in tandem. It is also notable that the *Cpa3* promoter has found use in generating mice lacking mast cells and with reduced or absent basophils (Feyerabend et al., 2011; Lilla et al., 2011). However, at this point it is an open question whether pharmaceutical targeting of carboxypeptidase A3 for inhibition would have therapeutic benefits, or would perhaps interrupt an important homeostatic mechanism for limiting dangerous effects of internal and external toxins.

## 5. Cathepsin C/Dipeptidylpeptidase I

Cathepsin C (gene name *CTSC*), also known as dipeptidylpeptidase I, is a cysteine exopeptidase that is expressed in many cells but is especially abundant in mast cells (Wolters et al., 2000). It is a potential pharmaceutical target because of its role as an upstream activator of tryptases, chymases, and cathepsin G, from which it removes N-terminal pro-peptides from the zymogen forms of these proteases. It plays a similar role for other immune cell proteases, including neutrophil elastase and many granzymes of cytotoxic lymphocytes and natural killer cells (Adkison et al., 2002; McGuire et al., 1993; Pagano et al., 2007; Pham and Ley, 1999). These activation events occur inside the cell. Although cathepsin C is found in mast cell granules and can be secreted, its activity outside of cells is limited by its restricted endopeptidase activity and susceptibility to inactivation by cystatins (Wolters et al., 2000; Wolters et al., 1998). As a potential assist to structure-based design of inhibitors, crystal-derived structures of cathepsin C, reveal a tetramer with restricted active sites compared to related cysteine cathepsins (Turk et al., 2001; Turk et al., 2012). Studies in cathepsin C knockout mice suggest that chymases, including mast cell protease 4, are present as proenzyme zymogens in mast cells but are almost completely inactive in these mice (Wolters et al., 2001). Levels of active tryptase mast cell protease 6 are reduced but not absent, suggesting the presence alternative pathways for activating pro-tryptases in mice. In

human mast cells, alternative tryptase activation pathways include the classic lysosomal cysteine protease cathepsin L (Le et al., 2011a; Le et al., 2011b). Notwithstanding multiple deficits in immune cell proteases, cathepsin C-null mice are viable and fertile when raised in barrier conditions. Indeed, in some models, as in septic peritonitis (Mallen-St Clair et al., 2004) and gram-negative pneumonia (Sutherland et al., 2014), the lack of cathepsin C confers a surprising survival advantage, possibly related to higher local levels of interleukin-6 and surfactant collectin proteins that would otherwise be degraded directly by cathepsin C or indirectly by one or more of the proteases activated by cathepsin C. These findings provide further incentive for considering therapeutic inhibition of cathepsin C. However, the nature and significance of the host defense deficits in cathepsin C-null mice remain to be fully explored. In humans, mutations of the *CTSC* gene can produce Papillon-Lefevre syndrome, which includes severe periodontitis (Pham et al., 2004), but is overall perhaps surprisingly well tolerated. It is not yet clear if the involvement of cathepsin C in activating human mast cell and other inflammatory cell proteases is as important as it appears to be in mice.

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**Table 1**

## Disease associations

**Tryptases**

Anaphylaxis (Caughey, 2006; Schwartz, 2006)  
 Asthma (Cairns, 2005; Chen et al., 2006; Costanzo et al., 2008; Krishna et al., 2001)  
 Allergic rhinitis (Erin et al., 2006; Takabayashi et al., 2012)  
 Eosinophilic esophagitis (Abonia et al., 2010)  
 Inflammatory bowel disease (Hamilton et al., 2010; Hansbro et al., 2014; Isozaki et al., 2006; Tremaine et al., 2002)  
 Arthritis (Shin et al., 2009)  
 Cigarette-associated lung and airway disease (Beckett et al., 2013; Hansbro et al., 2014)  
 Allergic skin disease (Järvikallio et al., 1997)  
 Bacterial infection (protective) (Thakurdas et al., 2007)  
 Aortic aneurysm (Zhang et al., 2011)

**Chymases**

Systemic arterial hypertension (Ju et al., 2001; Koga et al., 2003; Wei et al., 2010)  
 Ischemia-reperfusion injury (Abonia et al., 2005; Jin et al., 2001; Morikawa et al., 2005; Pat et al., 2010)  
 Fibrosis: skin, lung, kidney, heart, liver (Cha et al., 2012; Shiota et al., 1997; Tchougounova et al., 2005)  
 Pulmonary artery hypertension (Kosanovic et al., 2014; Wang et al., 2010)  
 Asthma (protective) (Balzar et al., 2005; Sugimoto et al., 2012; Waern et al., 2009)  
 Envenomation (protective) (Akahoshi et al., 2011)  
 Intestinal parasitosis (protective) (Knight et al., 2000)  
 Skin malignancy (Coussens et al., 1999)  
 Aortic aneurysm (Furubayashi et al., 2008; Inoue et al., 2009; Sun et al., 2009)

**Carboxypeptidase A3**

Envenomation (protective) (Metz et al., 2006)

**Cathepsin C/Dipeptidylpeptidase I**

Septic peritonitis (Mallen-St Clair et al., 2004)  
 Gram-negative pneumonia (Sutherland et al., 2014)  
 Periodontitis/Papillon-Lefevre syndrome (Pham et al., 2004)

Table 2

Cell-specific expression of mast cell-associated proteases<sup>a</sup>

	Mast Cell				
	Mucosal	Connective tissue	Basophil	Neutrophil	Eosinophil
Trypsinases					
Human	+++	+++	+	-	-
Mouse	+++	+++	MCP-11/mastin	-	?
Chymase					
Human	-	+++	-	-	-
Mouse	+++	+++	-	-	?
Cathepsin G					
Human	+	+++	-	+++	-
Mouse	+	++	?	+++	?
Carboxypeptidase A3					
Human	++	+++	+++	-	-
Mouse	+/-	+++	+++	?	-
Cathepsin C/Dipeptidylpeptidase I					
Human	+++	+++	?	++	?
Mouse	++	++	?	++	?

<sup>a</sup>Data derived from some of the following references: (Feyerabend et al., 2011; Irani et al., 1989; Irani et al., 1991; Lilla et al., 2011; Saito et al., 2006; Schechter et al., 1990; Takabayashi et al., 2012; Voehringer et al., 2004; Waern et al., 2009; Xing et al., 2011)

Table 3

Structural/biochemical features of human mast cell proteases

Protease	Gene	Class	Activity	Structure	Inhibition
$\beta$ -Tryptases	<i>TPSAB1</i> and <i>TPSB2</i>	Serine	Tryptic	Inactive pro-monomer or heparin-bound, active tetramer	Serpin-resistant
$\alpha$ -Tryptase	<i>TPSAB1</i>	Serine	Tryptic (weak)	Inactive pro-monomer or heparin-bound tetramer	Serpin-resistant
$\delta$ -Tryptase	<i>TPSD1</i>	Serine	Probably inactive	Truncated; likely monomeric	Not known
$\gamma$ -Tryptase	<i>TPSG1</i>	Serine	Tryptic	Membrane-bound	Serpin-sensitive in soluble form; unknown in transmembrane form
Mastin	Pseudo-gene	Serine	-	-	-
Chymase	<i>CMA1</i>	Serine	Chymotryptic, leu-ase	Monomeric, heparin-binding	Remains active bound to $\alpha_2$ -macroglobulin
Cathepsin G	<i>CTSG</i>	Serine	Chymotryptic, tryptic, leu-ase, met-ase	Monomeric, heparin-binding	Inactivated by serpins
Cathepsin C	<i>CTSC</i>	Thiol/cysteine	Dipeptidyl amino-peptidase	Tetrameric	Sensitive to cystatins
Carboxy-peptidase A3	<i>CPA3</i>	Zn <sup>++</sup> metallo	Exopeptidase (aromatic, neutral)	Can complex with heparin and chymase	?