

# Inhibition of tryptase release from human colon mast cells by protease inhibitors

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## Abstract

**AIM:** To investigate the ability of protease inhibitors to modulate tryptase release from human colon mast cells.

**METHODS:** Enzymatically dispersed cells from human colon were challenged with anti-IgE or calcium ionophore A23187 in the absence or presence of tryptase and chymase inhibitors, and tryptase release was determined.

**RESULTS:** IgE dependent tryptase release from colon mast cells was inhibited by up to approximately 37%, 40% and 36.6% by chymase inhibitors Z-Ile-Glu-Pro-Phe-CO<sub>2</sub>Me (ZIGPFM), *N*-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK), and  $\alpha_1$ -antitrypsin, respectively. Similarly, the inhibitors of tryptase leupeptin, *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and lactoferrin were also able to inhibit anti-IgE induced tryptase release by a maximum of 39.4%, 47.6% and 36.6%, respectively. The inhibitory actions of chymase inhibitors, but not tryptase inhibitors on colon mast cells were enhanced by preincubation of them with cells for 20 min before challenged with anti-IgE. At a concentration of 10  $\mu$ g/mL, protamine was able to inhibit anti-IgE and calcium ionophore induced tryptase release. However, at 100  $\mu$ g/mL, protamine elevated tryptase levels in supernatants. A specific inhibitor of aminopeptidase amastatin had no effect on anti-IgE induced tryptase release. The significant inhibition of calcium ionophore induced tryptase release was also observed with the inhibitors of tryptase and chymase examined. The inhibitors tested by themselves did not stimulate tryptase release from colon mast cells.

**CONCLUSION:** It was demonstrated for the first time that both tryptase and chymase inhibitors could inhibit IgE dependent and calcium ionophore induced tryptase release from dispersed colon mast cells in a concentration dependent manner, which suggest that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic of colitis in man.

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## INTRODUCTION

It has been reported that increased number of mast cells and mast cell degranulation are closely associated with a number of gastrointestinal diseases including idiopathic inflammatory bowel disease<sup>[1]</sup>, chronic ulcerative colitis<sup>[2]</sup>, Crohn's disease<sup>[3-5]</sup>, gastritis<sup>[5]</sup> and collagenous colitis<sup>[6,7]</sup>, irritable bowel syndrome<sup>[8,9]</sup> and chronic inflammatory duodenal bowel disorders<sup>[10]</sup>. Through releasing their proinflammatory mediators including histamine, tryptase, chymase, heparin and some cytokines<sup>[11]</sup>, mast cells actively participate in the pathogenesis of these intestinal diseases.

Tryptase is a tetrameric serine proteinase that constitutes about 20% of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells<sup>[12]</sup> in a catalytically active form<sup>[13]</sup>. Relatively higher secretion of tryptase has been detected in ulcerative colitis<sup>[14]</sup>, implicating that this mediator is involved in the pathogenesis of intestinal diseases. Evidence is emerging that tryptase may be a key mediator of allergic inflammation and a promising target for therapeutic intervention<sup>[15]</sup> as it has been found to be able to induce microvascular leakage in the skin of guinea pig<sup>[16]</sup>, bronchoconstriction<sup>[17]</sup> in allergic sheep airways, inflammatory cell accumulation in peritoneum of mouse<sup>[18]</sup> and release of IL-8 from epithelial cells<sup>[19]</sup>. Moreover, tryptase has been proved to be a unique marker of mast cell degranulation *in vitro* as it is more selective than histamine to mast cells<sup>[20]</sup>.

In recent years, inhibitors of tryptase<sup>[21,22]</sup> and chymase<sup>[23]</sup> were discovered to possess the ability to inhibit histamine release from human skin, tonsil, synovial<sup>[24]</sup> and colon mast cells<sup>[25]</sup>, suggesting these inhibitors are likely to be developed as a novel class of mast cell stabilizers. However, little is known of the actions of tryptase and chymase inhibitors on tryptase release from human colon mast cells. We therefore investigated the effects of these two groups of inhibitors on IgE dependent or independent tryptase release from human colon mast cells in the current study.

## MATERIALS AND METHODS

### *Dispersion of mast cells*

Human colon tissue was obtained from patients with carcinoma of colon at colectomy. Only macroscopically normal tissue was used for the study. After removal of fat, tissue was washed and chopped finely with scissors into fragments of 0.5-2.0 mm<sup>3</sup>, and then incubated with 1.5 mg/mL collagenase (Sigma) and 0.75 mg/mL hyaluronidase (Sigma) in minimum essential medium (MEM) containing 2% fetal calf serum (1 g colon/10 mL buffer) for 70 min at 37 °C. Dispersed cells were separated from undigested tissue by filtration through nylon gauze (pore size 100  $\mu$ m in diameter), washed and maintained in MEM (Gibco) (containing 10% FCS, 200 U/mL penicillin, 200  $\mu$ g/mL streptomycin) on a roller overnight at room temperature. Mast cell purity, as determined by light microscopy after stained by alcian blue, ranged from 3.5% to 5.4%.

### *Mast cell challenge*

Dispersed cells were resuspended in HEPES buffered salt solution (HBSS, pH 7.4) with CaCl<sub>2</sub> and MgCl<sub>2</sub> (complete HBSS), and 100  $\mu$ L aliquots containing 4-6 $\times$ 10<sup>3</sup> mast cells

were added to a 50  $\mu$ L anti-IgE (Serotec, UK), calcium ionophore (Sigma), or inhibitor in complete HBSS and incubated for 15 min at 37 °C. The reaction was terminated by addition of 150  $\mu$ L ice cold incomplete HBSS and the tubes were centrifuged immediately (500 g, 10 min, 4 °C). All experiments were performed in duplicate. Supernatants were stored at -20 °C until tryptase concentrations were determined.

### Inhibition of release of tryptase

For some experiments, protease inhibitor was preincubated with cells for 20 min before anti-IgE or calcium ionophore was added. Protease inhibitor and anti-IgE or calcium ionophore were also added to cells at the same time (no preincubation period). Data were expressed as the percentage inhibition of tryptase release, taking into account tryptase release in the presence and absence of the inhibitor. As for our previous experiments, the optimal tryptase release from colon mast cells was induced by 10  $\mu$ g/mL anti-IgE or 1  $\mu$ g/mL calcium ionophore<sup>[20]</sup>, and therefore they were chosen as standard concentrations throughout the study.

### Tryptase measurement

Tryptase concentrations were measured with a sandwich ELISA procedure with a specific polyclonal antibody against human tryptase as the capture antibody and AA5 a monoclonal antibody specific for human tryptase as the detecting antibody<sup>[26]</sup>.

### Statistical analyses

Statistical analyses were performed with SPSS software. Data were expressed as mean $\pm$ SEM. Analysis of variance indicated significant differences between groups with ANOVA. For the preplanned comparison of interest, Student's *t* test was applied. For all analyses,  $P < 0.05$  was taken as statistically significant.

## RESULTS

### Effects of secretagogues and inhibitors on tryptase release from mast cells

At 15 min following incubation, anti-IgE at 10  $\mu$ g/mL and calcium ionophore at 1  $\mu$ g/mL were able to induce 41.6 $\pm$ 4.3 ng/mL and 38.8 $\pm$ 3.0 ng/mL tryptase release from colon mast cells, respectively, whereas at the same time point spontaneous tryptase release (buffer alone) was 22.4 $\pm$ 3.2 ng/mL. The same concentrations of anti-IgE and calcium ionophore were also able to provoke a significant tryptase release from colon mast cells following a 35 min incubation period (Table 1). All protease inhibitors tested had no stimulatory effect on colon mast cells following a 15 min or a 35 min incubation period (data not shown).

**Table 1** Spontaneous and anti-IgE or calcium ionophore induced tryptase release from human colon mast cells

Compound	Tryptase released (ng/mL)	
	15 min	35 min
Buffer alone	22.4 $\pm$ 3.2	20.5 $\pm$ 2.8
Anti-IgE 10 $\mu$ g/mL	41.6 $\pm$ 4.3 <sup>a</sup>	37.6 $\pm$ 2.6 <sup>a</sup>
CI 1.0 $\mu$ g/mL	38.8 $\pm$ 3.0 <sup>a</sup>	38.4 $\pm$ 3.6 <sup>a</sup>

The values shown are mean $\pm$ SEM for six separate experiments. <sup>a</sup> $P < 0.05$  compared with buffer alone control (Student's *t* test).

### Inhibition of anti-IgE induced tryptase release from mast cells

The concentration dependent inhibition of anti-IgE induced release of tryptase from colon mast cells was observed when anti-IgE and various concentrations of chymase inhibitors ZIGPFM, TPCK, and  $\alpha_1$ -antitrypsin were added to cells at the same time. Up to approximately 37%, 40% and 36.6%

inhibition of IgE dependent tryptase release were achieved with ZIGPFM, TPCK, and  $\alpha_1$ -antitrypsin, respectively (Figure 1). As little as 10 ng/mL ZIGPFM was able to significantly inhibit IgE dependent tryptase release. Preincubation of ZIGPFM and TPCK with cells for 20 min before challenged with anti-IgE was able to moderately enhance their inhibitory actions on cells (Figure 2).

The inhibitors of tryptase leupeptin, TLCK and lactoferrin were also able to inhibit anti-IgE induced tryptase release in a concentration dependent manner, and a maximum of some 39.4%, 47.6%, and 36.6% of inhibition was achieved with 200  $\mu$ mol/mL leupeptin, 100  $\mu$ mol/mL TLCK, and 30  $\mu$ mol/mL lactoferrin, respectively (Figure 1). Preincubation of inhibitors of tryptase with cells for 20 min before anti-IgE was added had little effect on their abilities to inhibit anti-IgE induced tryptase release (Figure 2). A specific inhibitor of aminopeptidase, amastatin had no effect on anti-IgE induced tryptase release. When 10  $\mu$ g/mL protamine was added to cells at the same time with anti-IgE, or when 1.0  $\mu$ g/mL protamine was preincubated with cells before addition of the stimulus, anti-IgE induced tryptase release was significantly inhibited. However, when 100  $\mu$ g/mL protamine and anti-IgE was added to cells at the same time, tryptase concentrations measured in cell supernatants were much higher than those induced by anti-IgE alone (Table 2).

**Table 2** Effect of protamine on anti-IgE or calcium ionophore (CI) induced tryptase release from human colon mast cells

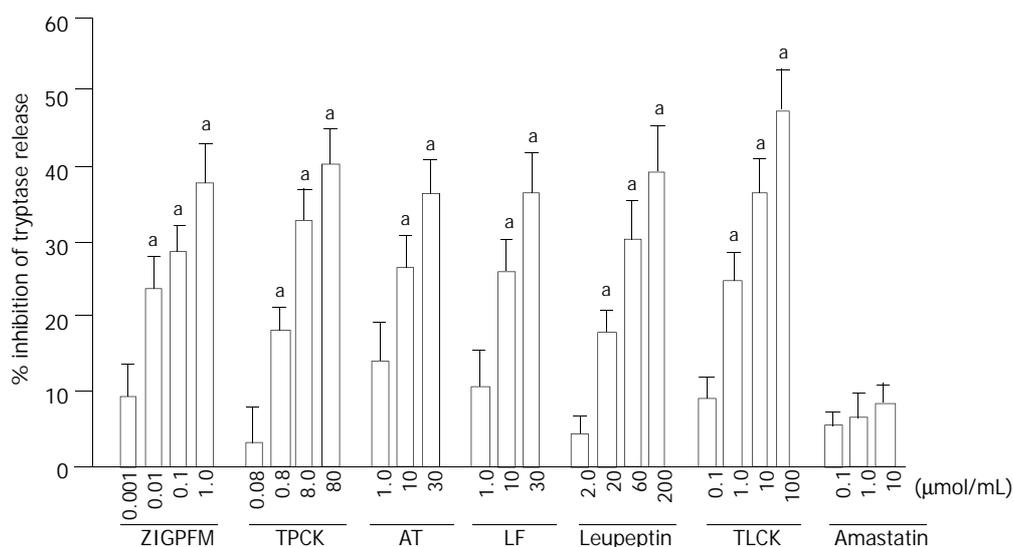
Protamine concentration ( $\mu$ g/mL)	% inhibition of tryptase release			
	No preincubation		20 min preincubation	
	Anti-IgE	CI	Anti-IgE	CI
0.1	4.0 $\pm$ 2.3	5.1 $\pm$ 4.5	nd	nd
1.0	17.3 $\pm$ 5.2	16.7 $\pm$ 4.6	21.4 $\pm$ 3.8 <sup>a</sup>	19.5 $\pm$ 2.9 <sup>a</sup>
10	20.9 $\pm$ 4.3 <sup>a</sup>	17.1 $\pm$ 3.8	-0.7 $\pm$ 1.8	-9.7 $\pm$ 11
100	-54.7 $\pm$ 13 <sup>b</sup>	-24.9 $\pm$ 8.9 <sup>b</sup>	nd	nd

The values shown are mean $\pm$ SEM for six separate experiments. Protamine was either added to cells at the same time with anti-IgE or CI, or preincubated with cells for 20 min before challenged with anti-IgE or CI. <sup>a</sup>levels of tryptase reduced in comparison with the uninhibited control ( $P < 0.05$ , Student's *t* test). <sup>b</sup>levels of tryptase elevated in comparison with the uninhibited control ( $P < 0.05$ , Student's *t* test). nd=not done.

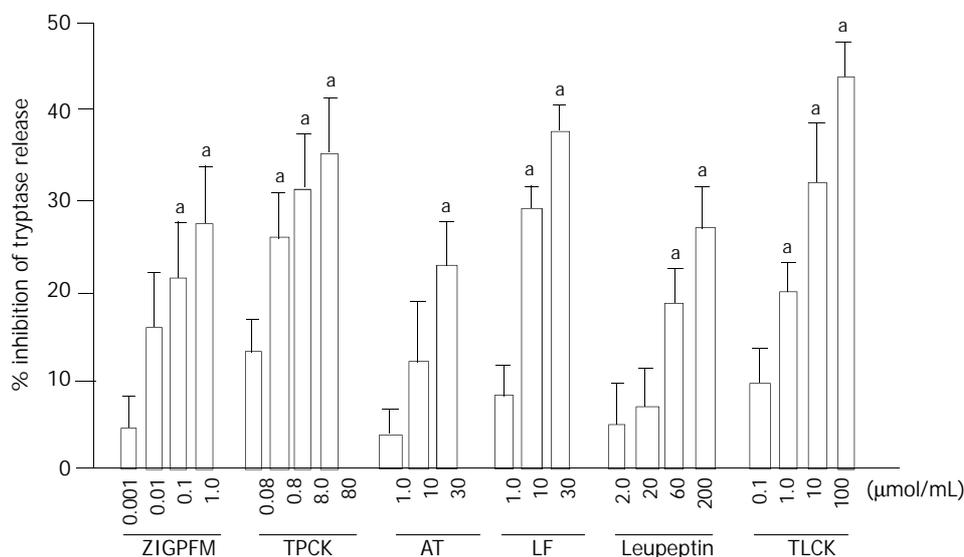
### Inhibition of calcium ionophore induced tryptase release from mast cells

The concentration dependent inhibition of calcium ionophore induced tryptase release from colon mast cells was observed when calcium ionophore and various concentrations of chymase inhibitors ZIGPFM, TPCK, and  $\alpha_1$ -antitrypsin were added to cells at the same time. Up to approximately 27.6%, 35.3% and 23% inhibition of IgE dependent tryptase release were achieved with ZIGPFM, TPCK, and  $\alpha_1$ -antitrypsin, respectively (Figure 3). Preincubation of the inhibitors with cells for 20 min before challenged with calcium ionophore enhanced the inhibitory ability of ZIGPFM, but not TPCK (Figure 4).

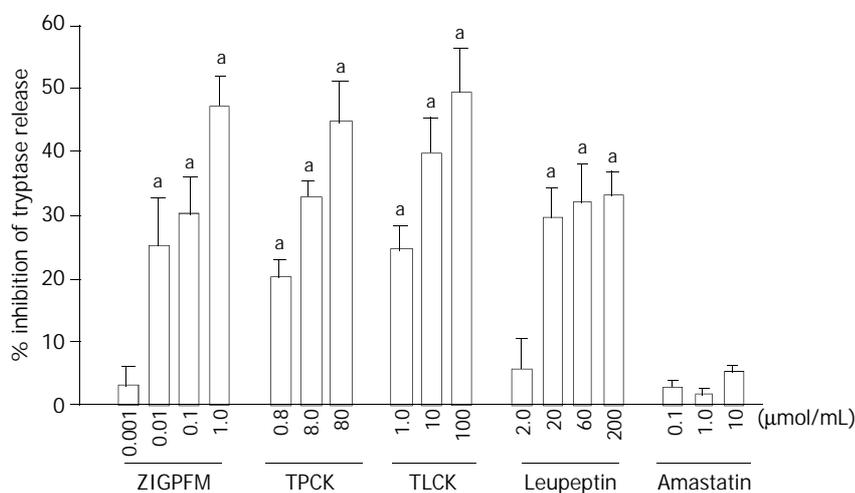
Calcium ionophore stimulated tryptase release was also reduced by addition of the various concentrations of inhibitors of tryptase to cells. Leupeptin, TLCK and lactoferrin were able to inhibit calcium ionophore stimulated tryptase release by up to approximately 27.1%, 44.1% and 38.2% respectively, when they were added to cells together with calcium ionophore (Figure 3). The extent of inhibition by leupeptin and TLCK was not affected by preincubation of them with cells for 20 min before calcium ionophore was added (Figure 4). Protamine at a concentration of 1  $\mu$ g/mL was also able to inhibit calcium ionophore induced tryptase release (Table 2).



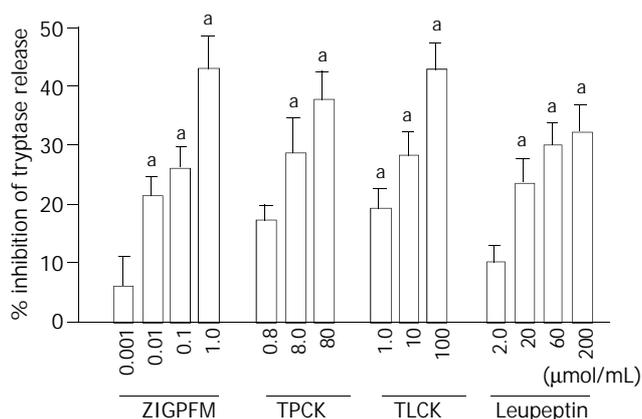
**Figure 1** Inhibition of anti-IgE (10 µg/mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. <sup>a</sup>*P*<0.05 compared with the responses with uninhibited controls. AT=α<sub>1</sub>-antitrypsin; LF=lactoferrin.



**Figure 2** Inhibition of anti-IgE (10 µg/mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors were preincubated with cells for 20 min before anti-IgE was added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. <sup>a</sup>*P*<0.05 compared with the responses of uninhibited controls.



**Figure 3** Inhibition of calcium ionophore (1 µg/mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors and anti-IgE were added to cells at the same time (no preincubation). Data are presented as mean±SEM for four to six separate experiments performed in duplicate. <sup>a</sup>*P*<0.05 compared with the responses with uninhibited controls. AT=α<sub>1</sub>-antitrypsin; LF=lactoferrin.



**Figure 4** Inhibition of calcium ionophore (1 µg/mL) induced tryptase release from dispersed colon mast cells by protease inhibitors. The inhibitors were preincubated with cells for 20 min before calcium ionophore was added. Data are presented as mean±SEM for four to six separate experiments performed in duplicate. <sup>a</sup>*P*<0.05 compared with the responses of uninhibited controls.

## DISCUSSION

We have found for the first time that inhibitors of tryptase and chymase were able to inhibit anti-IgE and calcium ionophore induced tryptase release from dispersed human colon mast cells, which may indicate a potential of a novel therapy for inflammatory bowel disease or other mast cell related intestinal diseases.

Up to approximately 40% inhibition of IgE dependent tryptase release from colon mast cells was observed with inhibitors of chymase, indicating that a chymase activity was involved in the process of IgE dependent gut mast cell degranulation. This was consistent with our previous finding that chymase inhibitors inhibited IgE dependent histamine release, which has indirectly proved that tryptase and histamine are likely to share a similar degranulation process. Unlike inhibition of histamine release, preincubation of ZIGPFM and TPCK with cells for 20 min before challenged with anti-IgE appeared to further reduce the quantity of tryptase released from mast cells, implicating that there may be some difference between tryptase and histamine release processes. Similar to chymase inhibitors, tryptase inhibitors inhibited up to some 47.6% anti-IgE induced tryptase release from colon mast cells, which implicated that a tryptase activity was likely to be involved in the process of colon mast cell degranulation. Once again, this was consistent with our previous finding that tryptase inhibitors were able to inhibit IgE dependent histamine release.

Since the majority of these inhibitors at the concentrations used in the current study were able to inhibit more than 95% tryptase or chymase activity in enzyme assays<sup>[27]</sup>, the incomplete inhibition of tryptase release from mast cells may suggest that some pathways other than tryptase and chymase pathways are involved in the anti-IgE induced degranulation of gut mast cells. A specific inhibitor of aminopeptidase amastatin, which did not inhibit chymotrypsin and trypsin activities<sup>[28]</sup>, was used as an irrelevant protease inhibitor control. It had no significant effects on anti-IgE induced tryptase release from colon mast cells, which proved the specificity of actions of tryptase and chymase inhibitors on tryptase release from mast cells.

Calcium ionophore is a calcium carrier that could help to elevate the calcium concentration in cytoplasm of mast cells<sup>[29]</sup>, and therefore acts on the downstream site of the process of mast cell degranulation. The inhibition of calcium ionophore induced tryptase release by the inhibitors of tryptase and chymase in the current study might also suggest the

involvement of tryptase and chymase activities in mast cell degranulation process was at the downstream site and most likely after influx of calcium ions into mast cells. The evidence that tryptase and chymase are sited in the granules of mast cells in their fully active form supports further the likelihood that these two mast cell serine proteases are involved in IgE dependent activation of colon mast cells. The results that tryptase levels were elevated when 100 µg/mL protamine was added to cells at the same time with unexpected anti-IgE. This was most likely due to the tetrameric structure of tryptase being dissociated by protamine<sup>[30]</sup>, thus more tryptase monomers existed in supernatants, and were recognized by AA5 as an intact tryptase molecule.

Some of the latest reports on tryptase inhibitors demonstrated the importance of these potential anti-inflammatory drugs. Inhaled APC366 was able to attenuate allergen-induced late-phase airway obstruction in asthma<sup>[31]</sup>, and APC2059 could improve the symptomatic scores of patients with mildly to moderately active ulcerative colitis in an open-label pilot study<sup>[32]</sup>. Our findings in the current study may at least partially explain why tryptase inhibitors could treat these diseases. Moreover, the successful treatment of acute ulcerative colitis<sup>[33]</sup> and Crohn's disease<sup>[34]</sup> with mast cell stabilizer ketotifen further strongly suggests that inhibitors of tryptase and chymase are likely to become a novel class of anti-inflammatory drugs with their anti-inflammatory actions and mast cell stabilizing properties.

In conclusion, the inhibitors of both tryptase and chymase are able to inhibit anti-IgE dependent and calcium ionophore induced tryptase release from colon mast cells, indicating that they are likely to be developed as a novel class of anti-inflammatory drugs to treat chronic colitis in man.

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