

## Short Communication

# Nafamostat Mesilate Is an Extremely Potent Inhibitor of Human Tryptase

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**Abstract.** Previously, nafamostat mesilate was found to be a potent inhibitor of human tryptase. In present study, we performed a kinetic study to determine its  $K_i$  value for tryptase and compared it with that of gabexate mesilate. Nafamostat mesilate inhibited human tryptase in a competitive manner. The apparent  $K_i$  value was estimated to be 95.3 pM, which was 1,000 times lower than that of gabexate mesilate (95.1 nM). These results strongly indicated that nafamostat mesilate is an extremely potent inhibitor of tryptase and suggested that some of its beneficial effects in the treatment of clinical status may be due to tryptase inhibition.

**Keywords:** nafamostat mesilate, tryptase, inhibition

Although nafamostat mesilate (6-amidino-2-naphthyl *p*-guanidinobenzoate dimethane sulfonate) was originally developed as an inhibitor of complements (1), it is also capable of inhibiting a variety of serine proteases including trypsin and some proteases in the coagulation cascade (2–5). Based on these inhibitory profiles, this drug has been widely used for the treatment of acute pancreatitis and disseminated intravascular coagulation (DIC) and for exocorporeal circulation in Japan (6, 7).

Recently, the protease-activated receptor (PAR) family was identified as one branch of the G protein-coupled receptor superfamily (8). The mechanism for the activation of PAR is unique and distinct from those of other receptors for amines and peptide neurotransmitters. Serine proteinases capable of activating PAR cleave the N-terminal extracellular portion of PAR, producing the new N-terminal sequence. The newly exposed N-terminal sequence in turn can bind to a yet undefined binding site of the receptor, leading to G protein coupled signal transduction such as the activation of phosphatidyl inositol phospholipase C, the production of inositol trisphosphate, and the activation of protein kinase C. Similarly, a five to six amino acids tethered peptide ligand can also bind to PAR, stimulat-

ing the G protein coupled signal transduction. Inhibition of a serine protease by a specific inhibitor abolished the receptor activating activity of the serine protease, and the mutant receptors that have resistant sequences to agonist protease could not be activated by the protease. These findings support the unique mechanism of PAR activation by serine protease.

Tryptase, one of the mast cell proteases, was demonstrated to be capable of activating PAR-2 in the sensory nerve endings, vascular endothelial cells, and airway epithelial cells (8). We recently reported that nafamostat mesilate potently inhibits the activity of purified human lung tryptase (9) as well as the tryptase-induced increase in the permeability of cultured endothelial cells (10), although the precise mode of tryptase inhibition by this compound is still unknown. We also found that the protein extravasation induced in rat lungs by an intravascular injection of ioxaglate, a radiographic contrast medium with a potent mast cell-degranulating action (11), is abolished by the systemic injection of nafamostat mesilate (9). These results strongly indicated that tryptase may participate in the pathogenesis of a number of mast cell-mediated inflammatory processes and prompted us to verify that some beneficial effects of nafamostat mesilate may be based on its inhibitory action on tryptase activity. Determination of the  $K_i$  value

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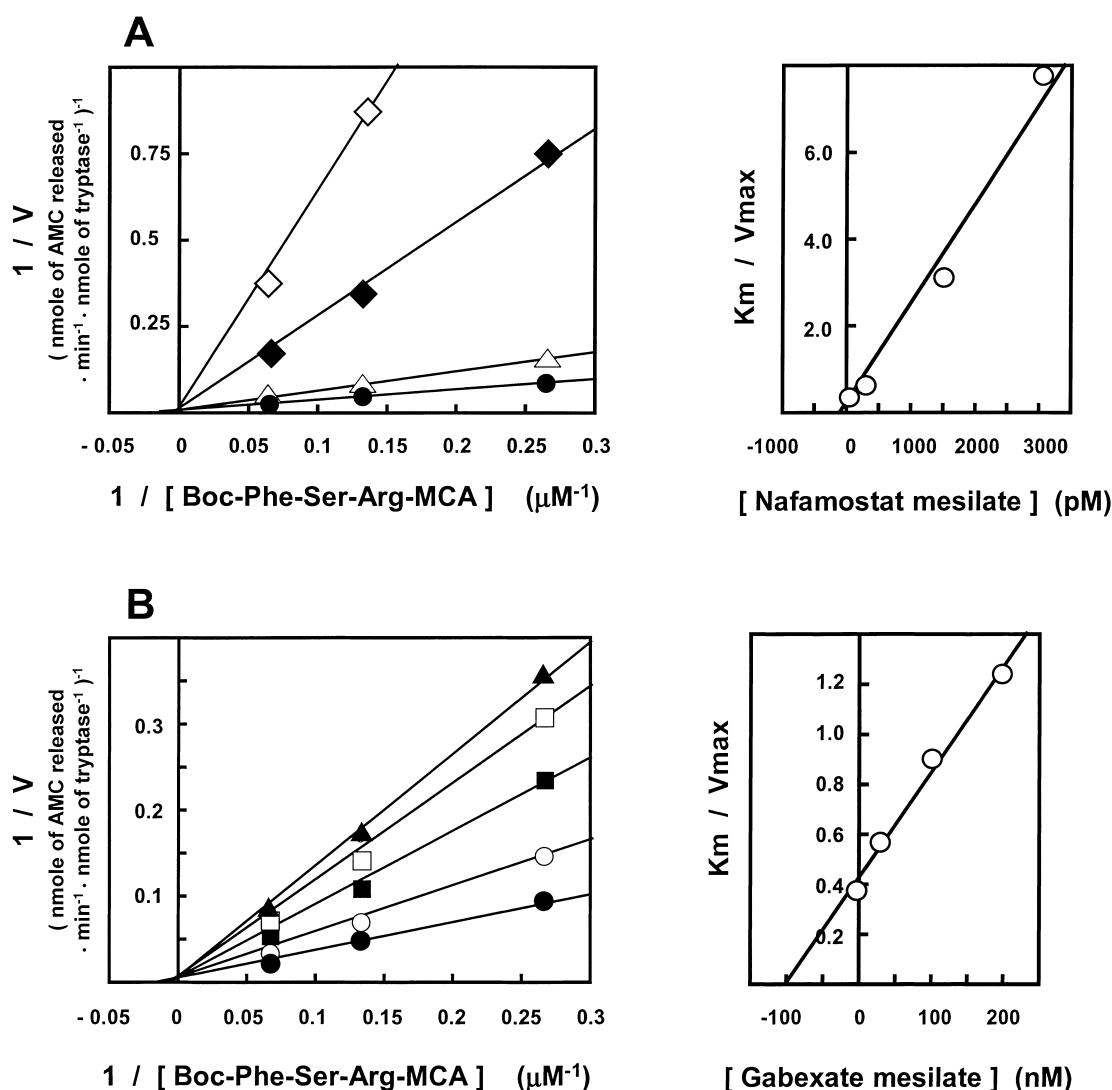


**Fig. 1.** Effect of nafamostat mesilate (A) or gabexate mesilate (B) on human trypsin activity. A, left: Lineweaver-Burk plot in the presence of nafamostat mesilate at concentrations of 0 (closed circle), 0.3 (open triangle), 1.5 (closed diamond), and 3 nM (open diamond). Trypsin activity was assayed using the synthetic peptide substrate as described in the text. A, Right: Secondary plot of the Lineweaver-Burk plot. The slopes were plotted against nafamostat mesilate concentration. The intercept on the x-axis gives an estimate of  $K_i$ . B, left: Lineweaver-Burk plot in the presence of gabexate mesilate at concentrations of 0 (closed circle), 30 (open circle), 100 (closed square), 200 (open square), and 300 nM (closed triangle). Trypsin activity was assayed using the synthetic peptide substrate as described in the text. B, Right: Secondary plot of the Lineweaver-Burk plot. The slopes were plotted against gabexate mesilate concentration. The intercept on the x-axis gives an estimate of  $K_i$ .

of nafamostat mesilate enables us to draw the precise inhibition profile of nafamostat mesilate for different kinds of serine proteinases, and it was necessary for the comprehensive understanding of the mechanism of its therapeutic effect. Therefore, in the present study, we performed a kinetic study on the inhibition by nafamostat mesilate of human trypsin, and we found that this drug is an extremely potent inhibitor of human trypsin with the apparent  $K_i$  value of 95.3 pM.

Nafamostat mesilate was a gift from Torii Pharmaceutical Co., Ltd. (Tokyo). Gabexate mesilate was

obtained from Ono Pharmaceutical Co., Ltd. (Osaka). Trypsin from human lung was purchased from Calbiochem (San Diego, CA, USA). Boc-Phe-Ser-Arg-MCA was from Peptide Institute, Inc. (Osaka). The trypsin activity was measured as described previously (9, 10). In brief, the human trypsin (1 nM) was incubated for 5 min at 37°C with various concentrations of Boc-Phe-Ser-Arg-MCA (0, 3.75, 7.5, and 15  $\mu$ M) in 300  $\mu$ l of 100 mM Tris-HCl (pH 7.8) containing 1 M glycerol, 0.1 mg/ml bovine serum albumin, and 20  $\mu$ g/ml heparin in the presence of nafamostat mesilate or gabexate mesi-

late. The reaction was terminated by heating for 30 s at 95°C. Then, the increase in fluorescence intensity was measured fluorometrically at the excitation wavelength of 370 nm and the emission wavelength of 460 nm using a Hitachi 650-10S fluorescence spectrophotometer (Tokyo). Tryptase activity was expressed as the amounts of AMC released  $\cdot \text{min}^{-1} \cdot \text{nanomole of tryptase}^{-1}$ . The final concentration of inhibitors ranged from 0.3 to 3 nM for nafamostat mesilate and from 30 to 200 nM for gabexate mesilate. The  $K_i$  values of inhibitors for human tryptase were estimated from the secondary plot (slope vs inhibitor concentration) of Lineweaver-Burk plots.

As shown in Fig. 1A, nafamostat mesilate competitively inhibited tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA in a dose-dependent manner, and its apparent  $K_i$  value was estimated to be 95.3 pM. Gabexate mesilate similarly inhibited the tryptase activity (Fig. 1B), but its potency was observed to be 1,000 times lower ( $K_i = 95.1$  nM) than that of nafamostat mesilate. Table 1 summarizes the  $K_i$  values of nafamostat mesilate for various serine proteases and other enzymes thus far examined. To our knowledge, the affinity of nafamostat mesilate for human tryptase is the highest among all enzymes examined (Table 1). Thus, nafamostat mesilate is an extremely potent inhibitor and selective (selectivity factor >100) for human tryptase when used at relatively low concentration.

Tryptase released from mast cells can catalyze the conversion of PAR-2 to the active form on the vascular endothelial cells, leading to an increase in the vascular permeability due to the contraction of endothelial cells, the upregulation of tissue factor expression on vascular endothelial cells (12) associated with the enhancement of extrinsic coagulation cascade through TF-VIIa-Xa complex (13), and the secretion of proinflammatory

cytokines such as IL-6 and IL-8 from endothelial cells (14). Therefore, the inhibition of tryptase by nafamostat mesilate is likely to contribute to the prevention of the pre-DIC state. Furthermore, recent studies demonstrated that PAR-2 stimulation by tryptase releases substance P and calcitonin gene-related peptide from nerve terminals of primary sensory neurons, suggesting the involvement of PAR-2 in nociception and neurogenic inflammation (8, 15). Nafamostat mesilate may alleviate tissue edema as observed in ioxaglate-induced lung edema in rats (9).

The *in vivo* pharmacokinetics study in patients with DIC revealed that the concentration of nafamostat mesilate in blood reached to  $0.9 - 2.4 \times 10^{-7}$  M when infused continuously at the dose of  $0.2 \text{ mg} \cdot \text{kg}^{-1} \text{ h}^{-1}$  (6). Thus, it is likely that nafamostat mesilate actually inhibits the tryptase activity *in vivo* because its  $K_i$  value (95.3 pM) was much lower than the therapeutic blood concentration. Tryptase seems to be the primary target of nafamostat mesilate judging from selective factors of other serine proteases, and it is possible that the reduced dosing of nafamostat mesilate can lead to the selective inhibition of tryptase. These findings suggest that nafamostat mesilate may be applicable for the treatment of microcirculatory dysfunction and inflammatory processes mediated by tryptase.

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**Table 1.**  $K_i$  values of nafamostat mesilate for some serine proteases and other enzymes

| Enzyme                             | $K_i$ (M)              | Selective factor*    | Reference     |
|------------------------------------|------------------------|----------------------|---------------|
| Human tryptase                     | $9.53 \times 10^{-11}$ | Reference enzyme (1) | Present study |
| Bovine trypsin                     | $1.5 \times 10^{-8}$   | 157                  | 1             |
| Human thrombin                     | $8.4 \times 10^{-7}$   | 8,814                | 1             |
| Human thrombin                     | $1.3 \times 10^{-6}$   | 13,600               | 3             |
| Human C1r                          | $4.4 \times 10^{-7}$   | 4,617                | 4             |
| Human C1r                          | $1.4 \times 10^{-8}$   | 147                  | 1             |
| Human C1s                          | $3.8 \times 10^{-8}$   | 399                  | 4             |
| Human C1 esterase                  | $3.8 \times 10^{-8}$   | 399                  | 1             |
| Human TF-VIIa complex              | $2.0 \times 10^{-7}$   | 2,099                | 5             |
| Human factor B                     | $6.0 \times 10^{-5}$   | 630,000              | 2             |
| Cobra venom factor-human factor Bb | $4.0 \times 10^{-5}$   | 420,000              | 2             |
| Human factor Xa                    | $4.1 \times 10^{-6}$   | 43,000               | 3             |

\*Selective factor indicates the ratio of  $K_i$  for each enzyme versus  $K_i$  for human tryptase.

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