Selective Inhibition of Trypsin by (2R,4R)-4-Phenyl-1-[N\(^\alpha\)-(7-methoxy-2-naphthalenesulfonyl)-l-arginyl]-2-piperidinecarboxylic Acid* 

Evidence is accumulating indicating that trypsin stimulates divergent cellular reactions through the protease-activated receptor, in addition to its role as the digestive enzyme. In this report, we introduce (2R,4R)-4-phenyl-1-[N\(^\alpha\)-(7-methoxy-2-naphthalenesulfonyl)-l-arginyl]-2-piperidinecarboxylic acid as a potent and selective trypsin inhibitor. The agent inhibited trypsin competitively with the \( K_i \) value of 0.1 \( \mu \)M. It inhibited thrombin weakly \( (K_i = 2 \mu M) \) and did not inhibit plasmin, plasma kallikrein, urokinase, and mast cell trypsinase \( (K_i \) values for these enzymes are >60 \( \mu \)M). Comparative studies with several established protease inhibitors revealed that the compound was the first small molecular weight trypsin inhibitor without trypside inhibitory activity.

A docking study has provided a plausible explanation for the molecular mechanism of the selective inhibition showing that the agent fits into the active site of trypsin without any severe collision but that it comes into clash at the 4-phenyl group of piperidine ring against the “60-insertion loop” of thrombin and at the 7-methoxy-2-naphthalenesulfonyl group against Gln\(^\alpha\) of trypside.

We have been searching for selective inhibitors of serine enzymes, because potent inhibitors selective to respective enzyme are beneficial tools to elucidate the roles of the enzyme concerned in pathophysiological conditions. Starting from L-arginine, we obtained number 205 (DAPA)\(^1\) as one of the representatives of our series of thrombin inhibitors (1, 2). The structure-activity relationship studies on the substitution at the 2- and 4-positions in the piperidine ring provided us crucial information for designing inhibitors selective to thrombin and trypside. The introduction of 2-COOH into the piperidine ring caused a marked decrease in acute toxicity, whereas the thrombine inhibitory activity remained unchanged (3, 4). This led us to the finding of the potent, selective and low toxic thrombin inhibitor, argatroban (MQPA) (5, 6). In addition, the 4-phenyl introduction into the piperidine ring decreased the thrombin inhibitory activity dramatically, whereas such modification did not reduce or even increased the inhibitory activity for trypsin (4, 7).

Based on the different effects of 4-phenyl introduction into the piperidine ring on the inhibitory activity for thrombin and trypsin, we searched for selective trypsin inhibitor. Although many synthetic inhibitors have been reported to exhibit trypsin inhibitory activity, few of them can discriminate trypsin from other trypsin-like enzymes, especially from trypside. This report introduces a new synthetic trypsin inhibitor, MNAPPA, which to our knowledge is the first small molecular weight inhibitor that discriminates trypsin from trypside. In addition, a computer-aided modeling study was attempted, and it provided new insight into the molecular mechanism to explain the selective inhibition of MNAPPA for trypsin.

Experimental Procedures

Materials—MQPA was synthesized as reported previously (5). Nafamostat mesilate (6-amido-2-naphthyl \( p \)-guanidinobenzoate dimethanesulfonate) was purchased from Torii Pharmaceutical Co. (Tokyo, Japan). Soybean trypsin inhibitor (SBTI), leupeptin, and antipain were from Sigma. Enzymes used were as follows: trypsin (bovine pancreas, Sigma), thrombin (\( \alpha \)-thrombin, human, American Diagnostica Co., CA), SBTI (100 \( \mu \)M) for the determination of \( K_i \) of the inhibitors, Mochida Pharmaceutical Co., Tokyo, Japan) for the determination of IC\(_{50}\) plasma (human, Kabi Diagnostica, Stockholm, Sweden), plasma kallikrein (human, Kabi Diagnostica), urokinase (human, Mochida Pharmaceutical), and trypside (human lung mast cell, Cortrex Biochem, San Leandro, CA).

Synthesis of MNAPPA—\( N^-\{\text{tert-Butyloxycarbonyl}\}-N^-\{\text{nitro-l-arginyl}\}-4\text{-phenyl-2-piperidinecarboxylic acid} \) (1.96 g) dissolved in tetrahydrofuran (100 ml) was cooled to –25 to –20 \( ^\circ \)C and isobutylchloroformate (1.22 g) was added. After 30 min, a solution of optically active methyl 4-phenyl-2-piperidinecarboxylate (1ml\(^{27}\) = 61.0° (c 0.66, MeOH)) (1.96 g) dissolved in tetrahydrofuran (5 ml) was added dropwise and stirred at –25 to –20 \( ^\circ \)C for 1 h. The solution was washed successively with water, 10% aqueous solution of citric acid, saturated aqueous solution of NaHCO\(_3\) and water, and dried over Na\(_2\)SO\(_4\). After purification by column chromatography on silica gel using CHCl\(_3\)-MeOH (97:3) as eluent, the product was obtained as white amorphous solid.

The solution of optically active methyl 1-[N\(^\alpha\)-\{tert-butylxocarbonyl\}-N\(^\alpha\)-nitro-l-arginyl]-4-phenyl-2-piperidinecarboxylate was obtained in the form of amorphous solid.

The abbreviations used are: DAPA, 4-ethyl-1-[N\(^\alpha\)-\{3-dimethylamino-1-naphthalenesulfonyl\}-l-arginyl]-2-piperidinecarboxylic acid; MQPA, (2R,4R)-4-ethyl-1-[\{3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl\}-l-arginyl]-2-piperidinecarboxylic acid; MNAPPA, (2R,4R)-4-phenyl-1-[N\(^\alpha\)-(7-methoxy-2-naphthalenesulfonyl)-l-arginyl]-2-piperidinecarboxylic acid; SBTI, soybean trypsin inhibitor; APA, 4-amidinophenylpyruvic acid.

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Potent and Selective Trypsin Inhibitor

The chemical structure of MNAPPA is shown in Fig. 1 (left panel). Its skeleton is L-arginine, and it bears 2-piperidinecarboxylic acid with 4-phenyl group at the C terminus. It inhibited trypsin competitively with the $K_i$ value of 0.1 $\mu M$ as shown in Fig. 2. MNAPPA inhibited thrombin weakly ($K_i = 2 \mu M$) and did not inhibit plasmin, plasma kallikrein, and urokinase ($K_i$ for these enzymes > 60 $\mu M$) (Table I). Noteworthy was that MNAPPA did not inhibit tryptase ($K_i > 60 \mu M$).

MNAPPA is a homologue of MQPA, the selective thrombin inhibitor (Fig. 1, right panel). The structural change from MQPA to MNAPPA resulted in a marked decrease in the thrombin inhibitory activity (Table I). The $K_i$ value of MNAPPA was 100 times larger than that of MQPA. On the other hand, the structural change from MQPA to MNAPPA rather increased the trypsin inhibitory activity. Thus, the potent trypsin inhibitor MQPA was converted to a potent trypsin inhibitor by changing the $N$ and $C$ termini of L-arginine skeleton.

In addition to our previous results that MQPA inhibited thrombin potently, inhibited trypsin only weakly and actually did not inhibit other serine proteinases (5, 6), MQPA was studied to compare its inhibitory activity for human mast cell tryptase with MNAPPA. The result shown in Table I that MQPA as well as MNAPPA did not inhibit tryptase up to the concentration of 60 $\mu M$ provided additional clear evidence indicating their high selectivity for thrombin and trypsin, respectively.

The IC$_{50}$ values of MNAPPA for trypsin, thrombin, plasmin, and tryptase were compared with several established trypsin inhibitors in Table II. All of the trypsin inhibitors tested except SBTI inhibited both of trypsin and trypstatin. Although SBTI discriminated trypsin from trypstatin, it inhibited plasmin rather strongly. Consequently, MNAPPA was a highly selective trypsin inhibitor among those inhibitors tested. In particular, MNAPPA was the first small molecular weight inhibitor that discriminated trypsin from trypstatin precisely.
Docking Study of Enzyme-Inhibitor Complexes

We attempted to get insight into the molecular mechanism of trypsin-specific inhibition of MNAPPA with a computer-aided modeling technique. Because MQPA has been reported to bind the active site of trypsin and thrombin in nearly identical conformation (18, 19), we made the following docking study on the supposition that MNAPPA will bind the active sites of enzymes holding the same conformation as MQPA in 1DWC.

Reason Why the Thrombin Inhibitory Activity of MNAPPA Is Weak—The crystallographic evidence has revealed that MQPA fits well into the active site of thrombin through its arginine side chain, quinolinesulfonyl group, and methylpiperidine group to the S1 specificity pocket, aryl binding pocket, and S2 cavity, respectively (10, 19). Our experimental results indicated that arginine derivatives lost their thrombin inhibitory activity but reserved trypsin inhibitory activity when a phenyl group was introduced into the 4-position of the piperidine ring (4, 7 and this report). To know the binding geometry of the 4-phenyl group, we visualized the C36 atom at the para-position of 4-phenyl group attached to the piperidine ring of MQPA.

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in 1DWC. As shown in Fig. 3A, C36 introduced into the MQPA molecule revealed severe steric clash against Tyr60A of the “60-insertion loop” of thrombin. When MQPAC36 was superposed on other thrombin coordinates such as 1DWB, 1AHT, and 1FPC, every docking experiment resulted in similar steric collisions between C36 and Tyr60A.

Fig. 3A also shows trypsin superposed on MQPAC36-thrombin. In the case of trypsin, the 60-insertion loop is not present (Fig. 4), and, therefore, there is no projecting obstacle but a sufficient space at the site. Consequently, C36 did not cause any clash against trypsin. Neither tetrahydro-8-quinolinesulfonyl group nor 7-oxy-2-naphthalenesulfonyl group collided with the enzyme. When we used other trypsin coordinates such as 2TBS, 1TPP, and 1PPC, similar results were obtainable showing no collision between ligand and enzyme.

**Reason Why the Tryptase Inhibitory Activity of MNAPPA/MQPA Is Weak—**Fig. 3B shows the MQPAC36-thrombin complex superposed on trypsin monomer A. Although trypsin has a 60-insertion loop, it does not project as actively as in thrombin, and, therefore, C36 did not collide with trypsin. However, the tetrahydro-8-quinolinesulfonyl group or 7-oxy-2-naphthalenesulfonyl group at the N terminus of the ligands collided with Gln98 of trypsin. Similar results were obtained when the ligands were docked into the other monomers of trypsin (monomer B, C, and D).

Our docking study suggests the following plausible explanation; the inhibitory effect of MNAPPA on thrombin is weak because the 4-phenyl group of MNAPPA collides with the 60-insertion loop of thrombin, and MNAPPA and MQPA do not inhibit trypsin because the 7-oxy-2-naphthalenesulfonyl and tetrahydro-8-quinolinesulfonyl group collide with trypsin. Consequently, MNAPPA has acquired the trypsin-selective inhibitory activity.

**DISCUSSION**

In our previous reports, we described the strategies to obtain inhibitors selective to thrombin and those selective to trypsin respectively, among the derivatives of L-arginine (7, 20). The selective thrombin inhibitor MQPA is the representative of the former. In this report, we introduced MNAPPA as one of the representatives of the latter. The enzymes tested were trypsin, thrombin, plasmin, plasma kallikrein, urokinase, and trypase, all of which are the key enzymes in the fields of digestion, coagulation, fibrinolysis, kinin formation, and mast cell diseases. The enzyme inhibitory activities of MNAPPA as shown in Table I revealed that MNAPPA was a potent and selective trypsin inhibitor.

Tryptase is a trypsin-like serine proteinase from mast cells. It hydrolyzes peptide bonds next to arginine and lysine residue (21). Recently, Pereira et al. (17) revealed the x-ray crystallography of the complex of APA and tetrameric human trypase; the specificity pocket of trypase is virtually identical to that of trypsin to accommodate arginine and lysine side chain, the trypase monomers are positioned at each corner of the tetramer, the active sites are directed to the center of the tetramer, and macromolecule substrates or inhibitors are restricted to access to the active site by the neighboring monomers. Therefore, the S1 site occupying compounds of small molecular weight such as benzamidine, APA, and N\(^\text{\textsuperscript{N}}\)-tosyl-L-lysine chloromethyl ketone inhibit trypase and trypsin as well (22, 23). Furthermore, studies on benzamidine and bis-benzamidine derivatives revealed that the affinities of these compounds to trypsin and trypase are closely correlated (22). None of the small molecular weight compounds had been reported to discriminate trypsin from trypase precisely.

Because the molecular weight of MNAPPA is small and comparable with those of antipain ($M_r = 605$) and leupeptin ($M_r = 427$), both of which inhibit trypsin and trypase (Refs. 23 and 24 and this report), and because the arginine side chain of MNAPPA is assumed to be accommodated in the specificity pocket of trypase, we expected that MNAPPA could access to the active site of trypase to inhibit it. But it did not inhibit trypase as shown in Table I. After we compared the enzyme inhibitory spectrum of MNAPPA with those of the established trypsin inhibitors such as leupeptin, antipain, and nafamostat mesilate as shown in Table II, we came to a conclusion that MNAPPA was the most selective trypsin inhibitor of small molecular weight ever reported.

rapidly advanced protein chemistry in recent years has provided much knowledge of three-dimensional structures of enzymes, allowing our understanding of enzyme functions at the molecular level. Selective proteinase inhibitors have been useful in the experimental procedures of crystallographic analysis of enzymes, and moreover, it is very helpful for visualizing the active sites of enzymes. Considering the steric hindrance between ligand and enzyme, we obtained visual models that agree with the experimental results of inhibition studies (Fig. 3). The steric collision of 4-phenylpiperidine with 60-insertion loop especially seems to be a satisfactory explanation for the loss of thrombin inhibitory activity of MNAPPA, because the conformation of 60-insertion loop appeared rigid, and the same severe clash was also observed in other docking experiments using the coordinates of thrombin complexed with benzamidine, APA, and DAPA. Furthermore, steric hindrance between N terminus of the ligands and trypase seemed probable, because the tilting of Gln98 might be restricted in tetrameric trypase, because the active sites of monomer A and D (and those of B and C) are spatially close to each other, and Gln98 of monomer A and Tyr95 of monomer D are neighboring (17).

It has been reported that certain enzymes of trypsin families such as trypsin, thrombin, and trypase cleave proteinase-activated receptors on the cell surface and trigger signals that regulate multiple cellular reactions (see review in Ref. 25). Proteinase-activated receptor 2, the receptor for trypsin, is widely distributed in the body tissues such as the gastrointestinal tract, pancreas, kidney, liver, airway, and prostate and in endothelial cells, smooth muscle cells, and certain tumor cell lines. In addition, endothelial cells and some cancer cells express trypsinogen, and gene expression of trypsinogen was increased in vascular endothelial cells around gastric tumors and in patients with disseminated intravascular coagulation (26). Furthermore, trypsin may escape into the circulation during pancreatitis. The selective trypsin inhibitor MNAPPA may be a useful tool to elucidate the roles of trypsin in the pathogenesis of various diseases.

**REFERENCES**


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