Inhibition of Trypsin, Plasmin, Thrombin and Kallikrein by Various Esters of Guanidino- and Aminido-Acids

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Received August 9, 1993; accepted October 27, 1993

Inhibitory effects of various phenolic esters of trans-4-guanidinomethylcyclohexanecarboxylic acid, amidinopiperidine-4-alkanoic acids or trans-4-amidinocyclohexane-4-alkanoic acids on trypsin, thrombin, plasmin and pancreatic kallikrein were examined. Their inhibitory effects were strongly affected by the acid portion and phenolic group constituting the esters. The effects of the acidic portion and phenolic group on the inhibitory effect varied with each protease; they were most effective on thrombin and plasmin and least effective on kallikrein. The inhibitory effect of these esters on trypsin was affected mainly by acid portion.

Keywords inhibitor; trypsin; plasmin; thrombin; pancreatic kallikrein

Recent studies on proteolytic enzymes strongly indicate the participation of proteases in the regulation of various biological events in organisms, and the role of proteolysis has been comprehensively reviewed by many authors. Therefore, the search for a suitable and specific protease inhibitor must yield a useful probe for investigating the regulatory mechanism of biological events which involve protease(s).

Muramatu and Fuji reported that \(\omega\)-amino and \(\omega\)-guanidino acid esters strongly inhibit trypsin-like proteases, such as trypsin, plasmin, thrombin and plasma kallikrein. Tamura et al. and Fuji and Hitomi reported strong inhibitory effects of various esters of p'-guanidinobenzoic acid on trypsin, plasmin, plasma kallikrein, thrombin, \(c_3\) and \(c_4\) esterase.

So far, we have reported the effects of various esters of guanidino- and amino-acids on some interesting cellular events, i.e. the suppression of histamine release from mast cells, elongation of the cell cycle and blocking of cell cycle progression of HeLa cells, and inhibition of bacterial cell growth. However, we have never noted their inhibitory effects on trypsin or on well-known trypsin-like proteases except for the inhibitory effects of some esters on trypsin. Therefore, it seems to be very important to examine the effects of these inhibitors on well-known proteases, trypsin, plasmin, pancreatic kallikrein and thrombin.

In this paper, we reported the inhibitory effects of various phenolic esters of trans-4-guanidinomethylcyclohexanecarboxylic acid (GMCHA), amidinopiperidine-4-alkanoic acids and trans-4-amidinocyclohexane-4-alkanoic acids on trypsin, plasmin, pancreatic kallikrein and thrombin.

MATERIALS AND METHODS

Materials N\(^\text{\textsuperscript{o}}\)-Benzoyl-DL-arginine \(\pi\)-nitroanilide hydrochloride (Bz-Arg-pNA), 4-methylcoumarin-7-amides of tert-butylxycarbonyl-L-valyl-L-prolyl-L-arginine (Boc-Val-Pro-Arg-NH-Mec), tert-butylxycarbonyl-L-valyl-L-leucyl-L-lysine (Boc-Val-Leu-Lys-NH-Mec) and L-prolyl-L-phenylalaninyl-L-arginine (Pro-Phe-Arg-NH-Mec) were from the Protein Research Foundation, Osaka. Various esters of GMCHA, amidinopiperidine-4-carboxylic acid (APCA), amidinopiperidine-4-propionic acid (APPA), amidinopiperidine-4-acetic acid (APAA) and amidinopiperidine-4-butyric acid (APBA) were from Nippon Chemiphar Co., Ltd., Tokyo, as hydrochlorides. Various esters of trans-4-amidinocyclohexanecarboxylic acid (ACHCA) and trans-4-amidinocyclohexanepronic acid (ACHPA) were from Teikoku Kagaku Sangyo Co., Ltd., Osaka, as hydrochlorides. Thrombin (type III), plasminogen (human) and pancreatic kallikrein were from Sigma Chem. Co., St. Louis. Thrombin was from Mochida Pharm. Co., Ltd., Tokyo, and streptokinase (validase), plasminogen activator, was from Lederle (Japan), Ltd., Tokyo.

Inhibition of Trypsin Activity The inhibitory effects of various esters of guanidino- and amino-acids on trypsin activity were examined with Bz-Arg-pNA as the substrate, and \(K_s\) was calculated from Lineweaver-Burk plots as described previously.

Inhibition of Thrombin Activity Thrombin (500 units) was dissolved in 5 ml of 0.1 M borate buffer, pH 8.0, containing 10% glycerol and stored at \(-20^\circ\)C. The stored thrombin solution was diluted 400-fold with the same buffer. Boc-Val-Pro-Arg-NH-Mec was dissolved in dimethylsulfoxide to 10 mM concentration, diluted with the same buffer and used as the substrate. Mixtures of 0.98 ml of 0.1 M borate buffer solution containing various concentrations of the inhibitor and 0.5 ml of the substrate solution were mixed with 20 ml of the diluted thrombin solution and incubated at 37 \(^\circ\)C. After 10 min, 1 ml of 30% acetic acid was added to the mixtures and the released 7-amino-4-methylcoumarin was determined as described by Kanaoka et al. \(K_s\) was calculated as described above.

Inhibition of Plasmin Activity Human plasminogen was dissolved in 0.1 M borate buffer, pH 8.0, containing 0.15 M NaCl to 0.025 unit/ml. Streptokinase (1 mg) was dissolved in 10 ml of the same buffer. Boc-Val-Leu-Lys-NH-Mec was dissolved in the same buffer and used as the substrate. A mixture of 0.1 ml of plasminogen solution and 0.1 ml streptokinase solution was incubated
at 25°C for 10 min, and then 1.3 ml of the same buffer containing various concentrations of the inhibitor and Boc-Val-Leu-Lys-NH-Mec was added and incubated at 37°C. After 30 min, 1 ml of 30% acetic acid was added, and the released 7-amino-4-methylcoumarin was determined as described above. $K_I$'s were calculated as described above.

**Inhibition of Pancreatic Kallikrein Activity** Pancreatic kallikrein was dissolved in 0.1 M borate buffer, pH 8.7, to 1.25 munit/ml. Pro-Phe-Arg-NH-Mec was dissolved in the same buffer and used as the substrate. Mixtures of 0.9 ml of various concentrations of the inhibitor dissolved in the same buffer and 1 ml of the substrate solution was incubated with 0.1 ml pancreatic kallikrein solution at 37°C. After 20 min, 1 ml of 30% acetic acid was added to the mixtures and the released 7-amino-4-methylcoumarin was determined as described above. $K_I$'s were calculated as described above.

**RESULTS AND DISCUSSION**

**Inhibitory Effects on Trypsin Activity** Trypsin was competitively inhibited by various esters of guanidino- and amido-amino-acid esters, and their concentration for 50% inhibition ($IC_{50}$) and $K_I$'s for trypsin are shown in Table I. The results indicate that when the phenolic portion is the same, the inhibitory effect of guanidino- and amido-amino-acid esters on trypsin is strongly affected by the acid portion. The ratio of $K_I$ for the 4-tert-butylyphenyl ester of APPA (APPA-OH/Bu), which was the most effective ester, to that for APPA-OH/Bu, which was the least effective ester, was about 1:440. When the acid portion is the same, such as in the GMCHA esters and APPA esters, the inhibitory effects of the esters was not as affected by the phenolic group.

**Inhibitory Effects on Thrombin** Thrombin was competitively inhibited by various esters of guanidino- and amido-amino-acid esters, and their $IC_{50}$ and $K_I$'s for thrombin are shown in Table I. In contrast to the inhibitory effects of these esters on trypsin, the inhibitory effects on thrombin were greatly affected by both the acid portion and phenolic group: among the esters possessing the same phenolic group, the ratio of $K_I$ for APPA-OH/Bu, which was the most effective inhibitor, to that for ACHCA-OH/Bu, which was least effective, was about 1:280. Among the GMCHA esters, the ratio of $K_I$ for GMCHA-OH/Bu, the most effective inhibitor, to that for 2-methoxyphenyl ester of GMCHA (GMCHA-2MetPh), the least effective, was about 1:40, and among APPA esters, the ratio of $K_I$ for APPA-OH/Bu to that of the 2,4-dimethylphenyl ester of APPA (APPA-DMePh) was about 1:120. Therefore, among the esters examined, the ratio of $K_I$ for the most effective inhibitor to the least effective one was about 1:5500.

**Inhibitory Effects on Plasmin** Plasmin was competitively inhibited by various esters of guanidino- and amido-amino-acid esters, and their $IC_{50}$ and $K_I$'s for plasmin are shown in Table I. The inhibitory effect of these esters on plasmin also seems to be strongly affected by both the acid portion and phenolic group. Among the esters possessing the same phenolic group, the ratio of $K_I$ for the most effective inhibitor, APPA-OH/Bu, to that for the least effective one, APPA-OH/Bu, was about 1:170. Among GMCHA esters, the most effective ester was 4-tert-butylyphenyl ester and the least effective one was 4-methoxyphenyl ester; the ratio of $K_I$ for both was 1:60. Among APPA esters, the most effective ester was biphenyl ester and the least was 2,4-dimethylphenyl ester; the ratio of $K_I$ for both was about 1:50. Among the inhibitors examined, the most effective was the biphenyl ester of APPA (APPA-OH/Ph) and the least was the 4-methoxyphenyl ester of GMCHA (GMCHA-4MetPh); the ratio of their $K_I$ was about 1:7000.

**Inhibitory Effects on Pancreatic Kallikrein** Pancreatic kallikrein was competitively inhibited by various esters of guanidino- and amido-amino-acid esters, and their $IC_{50}$ and

**Table I. IC$_{50}$'s and $K_I$ Values of Various Phenolic Esters of Guanidino- and Amido-Acids on Trypsin, Thrombin, Plasmin and Pancreatic Kallikrein**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Trypsin</th>
<th>Thrombin</th>
<th>Plasmin</th>
<th>Kallikrein</th>
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<tr>
<td></td>
<td>$IC_{50}$</td>
<td>$K_I$</td>
<td>$IC_{50}$</td>
<td>$K_I$</td>
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<tr>
<td>GMCHA-2MetPh</td>
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<td>112</td>
<td>237</td>
<td>117</td>
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<td>36</td>
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<td>7.5</td>
<td>3.1</td>
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<td>APPA-OH/Ph/Bu</td>
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<tr>
<td>ACHCHA-OH/Ph</td>
<td>0.6</td>
<td>0.74</td>
<td>0.91</td>
<td>1.6</td>
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$IC_{50}$'s were measured in 0.1 M borate buffer, pH 8.0, at 37°C as described in the text. Bz-Arg-pNA (1 mM) was used as the substrate for trypsin, Boc-Val-Pro-Arg-NH-Mec (10 mM) for thrombin, Boc-Val-Leu-Lys-NH-Mec (20 mM) for plasmin and Pro-Phe-Arg-NH-Mec (20 mM) for pancreatic kallikrein. $K_I$ values were calculated from the Lineweaver–Burk plots. Abbreviations used: 1NPh, 1-naphthyl ester; MAPH, 2-methoxy-4 allylphenyl ester.
$K_i$'s for pancreatic kallikrein are shown in Table I. Among the esters possessing the same phenolic group, the ratio of $K_i$ for the most effective inhibitor, GMCHA-O Ph'Bu, to that for the least effective inhibitor, ACHCA-O Ph'Bu, was about 1:40. Among GMCHA esters, the ratio of $K_i$ for the most effective ester, 4-tert-butylyphenyl ester, to the least effective one, 4-methoxyphenyl ester, was about 1:40. And also among APPA esters, the ratio of $K_i$ for the most effective ester, 4-tert-butylyphenyl ester, to the least, 2,4-dimethylphenyl ester was about 1:30. The ratio of $K_i$ for the most effective inhibitor, GMCHA-O Ph'Bu, to that for the least effective was about 1:190.

In this paper, we reported the inhibitory effects of various phenolic esters of guanidino- and amidino-acids on trypsin, thrombin, plasmin and kallikrein. Their inhibitory effects were strongly affected by the acid portion and phenolic group constituting the esters. The effects of the acidic portion and phenolic group on the inhibitory effect varied with each protease, and were most effective on thrombin and plasmin and least on kallikrein. The inhibitory effect of these esters on trypsin was mainly affected by the acidic portion. Among the various esters examined, APPA-O Ph'Bu strongly inhibited trypsin, thrombin and plasmin, their $K_i$'s being $10^{-7}$ to $10^{-8}$ M. Its inhibitory effect on kallikrein was less effective ($10^{-6}$ M). Although APCA-O Ph'Bu strongly inhibited thrombin and plasmin (their $K_i$'s were $10^{-7}$ to $10^{-8}$ M), its $K_i$'s for trypsin and kallikrein were $10^{-6}$ M.

Thus, various esters of guanidino- and amidino-acids, which represented specific suppression effects on the cell cycle of HeLa cells synchronized by a double-thymidine block,\textsuperscript{14-17} also strongly inhibited well-known trypsin-like proteases such as trypsin, thrombin, plasmin and pancreatic kallikrein.

REFERENCES