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

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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.^{1–5)} 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 Fujii reported that ω -amino and ω -guanidino acid esters strongly inhibit trypsin-like proteases, such as trypsin, plasmin, thrombin and plasma kallikrein.^{6,7)} Tamura *et al.*⁸⁾ and Fujii and Hitomi⁹⁾ reported strong inhibitory effects of various esters of *p*'-guanidinobenzoic acid on trypsin, plasmin, plasma kallikrein, thrombin, $c_1\bar{r}$ and c_1 esterase.

So far, we have reported the effects of various esters of guanidino- and amidino-acids on some interesting cellular events, *i.e.* the suppression of histamine release from mast cells,^{10–13)} elongation of the cell cycle and blocking of cell cycle progression of HeLa cells,^{14–17)} and inhibition of bacterial cell growth.^{18–22)} However, we have never noted their inhibitory effects on trypsin or on well-known trypsin-like proteinases except for the inhibitory effects of some esters on trypsin.²³⁾ 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*²-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Bz-Arg-*p*NA), 4-methylcoumarin-7-amides of *tert*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine (Boc-Val-Pro-Arg-NH-Mec), *tert*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine (Boc-Val-Leu-Lys-NH-Mec) and L-prolyl-L-phenylalanyl-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-amidinocyclohexanepropionic acid (ACHPA) were from Teikoku Kagaku Sangyo Co., Ltd., Osaka, as hydrochlorides. Trypsin (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 amidino-acids on trypsin activity were examined with Bz-Arg-*p*NA as the substrate, and K_i 's were calculated from Lineweaver-Burk plots as described previously.²⁴⁾

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°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 μl of the diluted thrombin solution and incubated at 37°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.*²⁵⁾ K_i 's were 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 amidino-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 amidino-acid esters on trypsin is strongly affected by the acid portion. The ratio of K_i for the 4-*tert*-butylphenyl ester of APPA (APPA-OPh'Bu), which was the most effective ester, to that for APBA-OPh'Bu, which was the least effective one, 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 amidino-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-OPh'Bu, which was the most effective inhibitor, to that for ACHCA-OPh'Bu, which was least effective, was about 1:280. Among the GMCHA esters, the ratio of K_i for GMCHA-OPh'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-OPh'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 amidino-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, APCA-OPh'Bu, to that for the least effective one, APBA-OPh'Bu, was about 1:170. Among GMCHA esters, the most effective ester was 4-*tert*-butylphenyl 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 esters was about 1:50. Among the inhibitors examined, the most effective was the biphenyl ester of APPA (APPA-OPhPh) 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 amidino-acid esters, and their IC_{50} and

TABLE I. IC_{50} 's and K_i Values of Various Phenolic Esters of Guanidino- and Amidino-Acids on Trypsin, Thrombin, Plasmin and Pancreatic Kallikrein

Inhibitor	Trypsin		Thrombin		Plasmin		Kallikrein	
	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)
GMCHA-2MetPh	236	112	237	117	30	40	175	130
GMCHA-4MetPh	95	101	25	36	1140	1500	100	150
GMCHA-OPhPh	50	54	4	4.1	80	37	21	37
GMCHA-OPh'Bu	33	38	7.5	3.1	50	25	7	3.5
APCA-OPh'Bu	7.1	5.4	0.041	0.056	0.2	0.25	3.5	4
APPA-OPh'Bu	0.47	0.5	0.044	0.047	0.8	0.91	5	3.8
APPA-1NPh	0.48	0.56	0.15	0.22	—	—	—	—
APPA-DMePh	2.77	2.13	3	5.6	10	11	180	130
APPA-OPhPh	0.65	0.53	0.2	0.3	0.2	0.22	6.5	9.5
APPA-MAPh	0.86	0.68	1.8	5	1.2	1	27	36
APAA-OPh'Bu	6.1	6.9	0.21	0.15	5	4.2	120	150
APBA-OPh'Bu	188	220	3	1.8	28	43	130	150
ACHCA-OPh'Bu	38	55	28	13	1.1	1.2	170	150
ACHCA-DMePh	98	83	256	257	75	83	770	650
ACHPA-OPh'Bu	0.6	0.74	0.91	1.6	1	0.75	15	23.3

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 μ M) for thrombin, Boc-Val-Leu-Lys-NH-Mec (20 μ M) for plasmin and Pro-Phe-Arg-NH-Mec (20 μ M) 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-OPh'Bu, to that for the least effective inhibitor, ACHCA-OPh'Bu, was about 1:40. Among GMCHA esters, the ratio of K_i for the most effective ester, 4-*tert*-butylphenyl 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*-butylphenyl ester, to the least, 2,4-dimethylphenyl ester was about 1:30. The ratio of K_i for the most effective inhibitor, GMCHA-OPh'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-OPh'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-OPh'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,¹⁴⁻¹⁷⁾ also strongly inhibited well-known trypsin-like proteases such as trypsin, thrombin, plasmin and pancreatic kallikrein.

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