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A NOVEL SERIES OF SYNTHETIC THROMBIN-INHIBITORS HAVING EXTREMELY POTENT AND HIGHLY SELECTIVE ACTION

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Indexing Words

thrombin; thrombin-inhibitor; synthetic inhibitor; trypsin; plasmin; reptilase; arginine derivative

Shosuke OKAMOTO, Akiko HIJIKATA, Kiyokatsu KINJO, Ryoji KIKUMOTO, Kazuo OHKUBO, Shinji TONOMURA and Yoshikuni TAMAO. *A Novel Series of Synthetic Thrombin-Inhibitors Having Extremely Potent and Highly Selective Action.* Kobe J. Med. Sci. 21, 43-51, June 1975——For the purpose of searching for potent and highly selective synthetic inhibitors to thrombin, ca. 360 chemical compounds were designed and synthesized by the authors, starting with the simple arginine derivatives having weaker anti-thrombin activity, in such a way as increasing the anti-thrombin activity by stepwise chemical modifications.

Fortunately, these studies led the authors to find a new series of the thrombin-inhibitors which belongs to N^{α}-naphthalenesulfonyl-L-arginine derivatives; values of I_{50} of these potent inhibitors are found in the range from 0.03 μM to 2.0 μM, when fibrinogen (3 μM) or N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginine p-nitroanilide (100 μM) is used as substrate. However, the values of I_{50} to plasmin, reptilase and trypsin are far larger (hundreds or thousands times), indicating very high selectivity of these inhibitors to thrombin. Mode of inhibition of these inhibitors is found kinetically competitive, when N^{α}-benzoyl-DL-arginine p-nitroanilide is used as substrate. These potent thrombin-inhibitors are tentatively called OM-inhibitors according to the code name in our laboratories. Further extensive studies are now proceeding.

INTRODUCTION

Thrombin is characterized by inherent ability to select fibrinogen as the corresponding natural substrate with high accuracy. More precisely speaking, thrombin splits certain arginyl bonds of fibrinogen molecule with higher velocity and affinity

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as well, while the same enzyme does not split any lysine bonds of fibrinogen molecule.[1,2] It is also known that certain arginine esters such as N^{α}-tosyl-L-arginine methyl ester (TAMe) inhibit thrombin activity for clotting fibrinogen.[14] These facts tempted several groups of workers to investigate different arginine derivatives such as N^{α}-tosyl-L-arginyl-sarcosine methyl ester (TASMe)[16] and other guanidino or amidino-compounds,[5, 6, 9] for obtaining proper thrombin-inhibitors; though the compounds so far known seemed to be unsatisfactory in their selective inhibitory activity to thrombin.

The present studies by the authors were started with TAMe, I_{50} of which was ca. 1,000 μM when fibrinogen (ca. 3 μM) was used as substrate. However, TAMe was easily split by thrombin, liberating methyl alcohol and losing its anti-thrombin activity. In the earlier studies by the authors, substitution of methyl group of the tosyl-side of TAMe by methoxy group caused a remarkable decrease in the rate of splitting methyl ester, its anti-thrombin activity being remained unchanged.[11] Encouraged by this result, the authors performed chemical modifications of TAMe homologs in a broad sense: The potent and selective inhibitors——called OM-inhibitors in our laboratories——fortunately obtained are reported here in brief. The other results concerned will appear elsewhere.[8, 13]

MATERIALS

Thrombin (bovine, topical): Mochida Pharm. Co. Ltd., Tokyo.
Purified thrombin (bovine): Kindly supplied by Dr. S. Iwanaga, Osaka Univ., Osaka.
Reptilase-R: Pentapharm. Ltd., Basel.
Trypsin: 194 u/mg, Worthington Biochemical Co., Freehold, New Jersey.
Urokinase: Green Cross Co. Lts., Osaka.
Plasminogen-free fibrinogen (bovine): Daiichi Pure Chemicals Co. Ltd., Tokyo.
Fibrinogen (bovine): Poviet Production N.V., Amsterdam.
N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginine p-nitroanilide. HCl (Bz-Phe-Val-Arg-pNA): AB Bofors, Nobel Division, Mölndal.
N^{α}-benzoyl-DL-arginine p-nitroanilide • HCl (BANA): BDH Chemicals Ltd., Poole.
4-aminomethylcyclohexanecarboxylic acid (t-AMCHA): Daiichi Pharm. Co. Ltd., Tokyo.
Lysine-sepharose: Kindly supplied by Dr. U. Okamoto, Kobe-Gakuin Univ., Kobe.
Borate-saline buffer, pH 7.4 and 7.8: Principally according to Norman,[10] 0.05 M sodium borate was mixed with 0.2 M boric acid salt solution until the desired pH (7.4 or 7.8) was obtained by glass electrode.
Tris-imidazole buffer, pH 8.3: Prepared according to Svendsen et al.[15]
Plasminogen: Obtained from human dry plasma by lysine-sepharose affinity chromatography. Elution was made with 0.01 M t-AMCHA in borate-saline buffer (pH 7.4), and then the plasminogen rich fraction was separated from t-AMCHA by Sephadex G-25 gel filtration.
Plasmin: 1.2 ml of ca. 2 CTA units/ml plasminogen, 0.7 ml of borate-saline buffer (pH 7.4) and 0.1 ml of 2,000 ploug units/ml urokinase were incubated at 37°C for 15 minutes and the reaction mixture thus obtained was used as plasmin solution.
OM-inhibitors: TAMe (N^{α}-tosyl-L-arginine methyl ester • HCl), OM-27 (N^{α}-dansyl-

L-arginine methyl ester • 2HCl • H_2O), OM-46 (N^{α}-dansyl-L-arginine-n-butyl ester • 2TsOH) and OM-205 (N^{α}-dansyl-L-arginine-4-ethylpiperidine amide•2CH_3COOH) were synthesized by the authors.

METHODS

Clotting of fibrinogen.

Thrombin: To the mixture of 0.8 ml of 0.12% (clottable) fibrinogen borate-saline buffer (pH 7.4) solution and 0.1 ml of various concentrations of the inhibitor to be tested, 0.1 ml of 5 NIH units/ml thrombin was added and the time required for clotting was estimated at 25°C.

Reptilase: To the same mixture of the fibrinogen and inhibitor above mentioned, 0.1 ml of 1 NIH unit/ml reptilase-R was added and the time required for clotting was measured at 37°C.

Fibrinolysis: 0.3 ml of 0.78% (clottable) plasminogen-free fibrinogen, 0.1 ml of plasmin, 0.1 ml of the inhibitor to be tested and 0.4 ml of borate-saline buffer (pH 7.4) were mixed and 0.1 ml of 3.5 NIH units/ml reptilase-R was added at 37°C when the stop watch was started. The fibrin formation occurred within 2 minutes and the time required for the clot resolution was measured.

Hydrolysis of Bz-Phe-Val-Arg-pNA.

According to Svendsen et al.[15] principally, the rate of releasing p-nitroaniline was measured with the increase of OD_{405} using Hitachi UV-VIS spectrophotometer Type 139 and Hitachi recorder Type 056. The assay systems of the enzymes were as follows:

Thrombin: To the mixture of 0.25 ml of 10^{-3} M Bz-Phe-Val-Arg-pNA, 0.25 ml of the inhibitor to be tested and 1.90 ml of tris-imidazole buffer (pH 8.3), 0.10 ml of 10 NIH units/ml thrombin was added and the increase of OD_{405} was measured at 25°C.

Trypsin: To the same mixture of the substrate, inhibitor and buffer above mentioned, 0.10 ml of 0.01 mg/ml trypsin was added and the increase of OD_{405} was measured at 37°C.

Reptilase: To the mixture of 0.25 ml of 10^{-3} M Bz-Phe-Val-Arg-pNA, 0.25 ml of the inhibitor to be tested and 1.7 ml borate-saline buffer (pH 7.8), 0.3 ml of 3.5 NIH units/ml reptilase-R was added and the increase of OD_{405} was measured at 37°C.

Plasmin: To the mixture of 1.00 ml of plasmin, 1.00 ml of borate-saline buffer and 0.25 ml of the inhibitor, 0.25 ml of 10^{-3} M Bz-Phe-Val-Arg-pNA was added and the increase of OD_{405} was measured at 37°C.

Hydrolysis of BANA.

To the mixture of 0.30 ml of 1 M tris-HCl buffer, pH 8.0, and 0.40 ml of BANA solution, various concentrations of the inhibitor to be tested and distilled water were added to make the total volume 2.90 ml. Then, 0.10 ml of 1,000 NIH units/ml

thrombin was added and the rate of releasing p-nitroaniline at 37°C was measured with the increase of OD_{410}.

RESULTS

A) *Chemical structures of the OM-inhibitors and their anti-thrombin activities for clotting fibrinogen.*

Results obtained by the stepwise chemical modifications of TAMe indicated that substitution of a tosyl radical of TAMe by a dansyl radical (dimethylaminonaphthalenesulfonyl radical) increased the anti-thrombin activity by 50 times approximately. In fact, I_{50} of TAMe was ca. 1,000 μM, while I_{50} of N^{α}-dansyl-L-arginine methyl ester (OM-27) was found ca. 20 μM. Furthermore, it was also indicated that substitution of methyl ester of OM-27 by another radical was able to increase the anti-thrombin activity by 200 times indeed (Table 1).

Table 1

Code or abbreviation	Chemical Name	I_{50} for thrombin (M)
TAMe	N^{α}-tosyl-L-arginine-methyl ester	1×10^{-3}
OM-27	N^{α}-dansyl-L-arginine-mehyl ester	2×10^{-5}
OM-85	N^{α}-dansyl-L-arginine-n-butyl ester	2×10^{-6}
OM-46	N^{α}-dansyl-L-arginine-n-butyl amide	3×10^{-6}
OM-205	N^{α}-dansyl-L-arginine-4-ethylpiperidine amide	9×10^{-8}

Results shown in Table 1 indicated that I_{50} values of OM-85, OM-46 and OM-205 were 2 μM, 3 μM and 0.09 μM respectively. Particularly noteworthy was that ethylpiperidine compound called OM-205 was extremely potent in its inhibitory action on fibrinogen-fibrin conversion by thrombin, and that, as 3 μM fibrinogen was used in assaying system, I_{50} value of OM-205 was calculated to be about one thirtieth of the substrate concentration. The chemical structure of OM-205 is referred in Fig. 1.

B) *Selectivity of the inhibitory action of the OM-inhibitors.*

The very potent anti-thrombin activity of the OM-inhibitors would raise a question whether or not these inhibitors exhibit high selectivity to thrombin. Comparative studies were therefore made on plasmin, trypsin and reptilase. Results obtained are shown in Fig. 2 and summarized in Table 2. In this study OM-205 was used as the inhibitor.

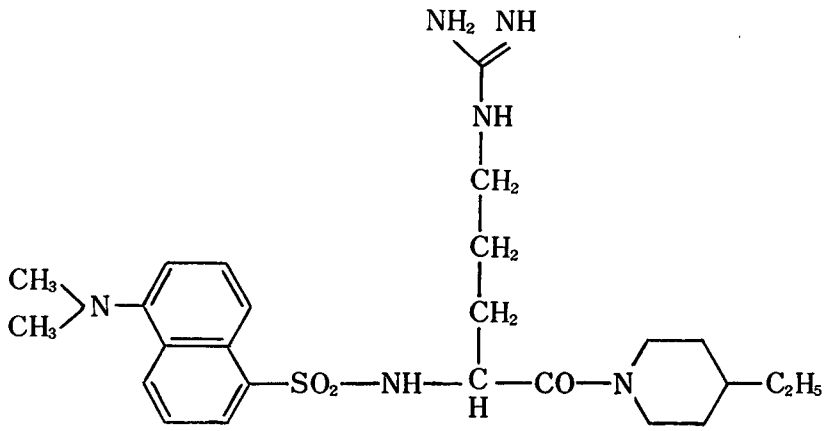

Fig. 1 Chemical structure of OM-205.

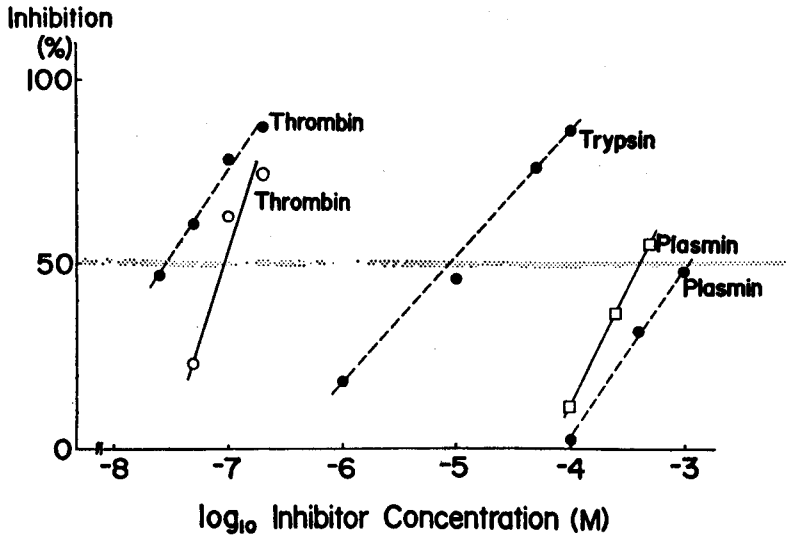

Fig. 2 Action of the inhibitor to proteolytic enzymes. The ordinate indicates inhibition rate in percentage; the abscissa, concentration of OM-205 in log scale. Solid lines are the results with natural substrate; fibrinogen for thrombin (○—○) and a kind of fibrin (formed by reptilase) for plasmin (□—□). Dotted lines are the results with synthetic peptide substrate (Benz-Phe-Val-Arg-pNA: 100 μM).

Table 2 Selectivity of OM-205.

Substrate	Enzyme	I_{50} (M)
Benz-Phe-Val-Arg-pNA	Thrombin	3×10^{-8}
ibid.	Trypsin	9×10^{-6}
ibid.	Plasmin	1×10^{-3}
ibid.	Reptilase	$> 1\times10^{-3}$
Fibrinogen	Thrombin	9×10^{-8}
Fibrin (R)	Plasmin	4×10^{-4}
Fibrinogen	Reptilase	$> 5\times10^{-4}$

1) When a kind of fibrin (formed by reptilase from fibrinogen) was used as substrate, I_{50} of OM-205 to plasmin was found 4×10^{-4} M (400 μM), while I_{50} of OM-205 to thrombin was 9×10^{-8} M (0.09 μM); that is, the I_{50} to plasmin was more than 4,000 times larger than that to thrombin. When synthetic substrate (Bz-Phe-Val-Arg-pNA) was used as substrate, I_{50} of OM-205 to plasmin was 1×10^{-3} M (1,000 μM), while the I_{50} to thrombin was 3×10^{-8} M (0.03 μM), the former being more than 30,000 times larger than the latter. These results clearly indicated the very high selectivity of OM-205 to thrombin when compared with plasmin.

2) When Bz-Phe-Val-Arg-pNA (100 μM) was used as substrate, I_{50} of OM-205 to trypsin was 9×10^{-6} M (9 μM), which was ca. 300 times of that to thrombin.

3) More interesting would be the action of OM-205 to reptilase converting fibrinogen to fibrin-like gel. That is, I_{50} values of OM-205 to reptilase for splitting Bz-Phe-Val-Arg-pNA and for converting fibrinogen to gel were found more than $1\times$

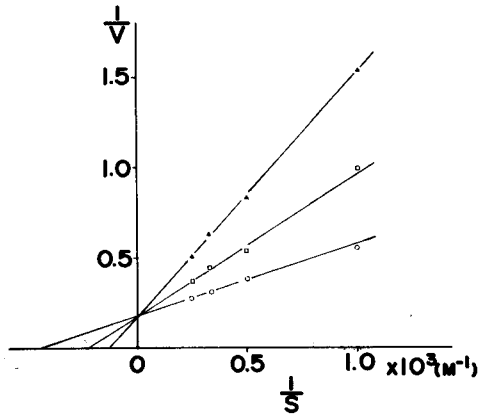

Fig. 3 Mode of Action of OM-205. Substrate: BANA, Enzyme: thrombin. ○—○; no inhibitor, □—□; OM-205, 1×10^{-6} M, ▲—▲; OM-205, 2×10^{-6} M.

10^{-3} M (1,000 μM) and 5×10^{-4} M (500 μM) respectively, indicating that OM-205 exhibits little inhibitory action to reptilase.

C) *Mode of inhibition of OM-205.*

Extremely potent action of OM-205 to thrombin led the authors to the investigation of the mode of action of the inhibitor, though the reversible inhibition would be expected from the time course of inhibition. The study was made by the plots according to Lineweaver and Burk. The substrate used was BANA, and the enzyme, thrombin. Results shown in Fig. 3 indicated that the thrombin inhibition by OM-205 was competitive, when BANA was used as substrate.

DISCUSSION

The present paper is to deal with the first but brief communication of a new series of the synthetic thrombin-inhibitors (tentatively called OM-inhibitors in our laboratories). Results obtained indicated that the OM-inhibitors exhibit very potent and selective inhibitory action to thrombin. It should be noted that OM-85, OM-46 and OM-205, assuming the same basic structure chemically, are also characterized by the tri-pod structure as a whole; the first pod bears a positively charged guanidino-group, the second, a strongly aromatic group, and the third, a hydrophobic group of a certain size as typically shown in Fig. 1. The L-arginine skeleton of the inhibitor can not be replaced by D-arginine or L-lysine without decreasing its anti-thrombin activity. The α-NH_2 of arginine is covered with naphthalenesulfonyl group; the COOH of arginine, with the ester or the amide having a certain number of carbon atoms.

The readers who notice the amidino-group of the OM-inhibitors would compare the inhibitors with p-amidinophenylpyruvic acid (APPA) which was first noticed by Geratz[4] as an enterokinase-inhibitor and further studied by Markwaldt et al.[9] as a promising thrombin-inhibitor. However, I_{50} of APPA to plasmin was found within the same order of the I_{50} to thrombin.[9] Compared with APPA, the OM-inhibitors are much characterized by the high selectivity to thrombin; in fact, I_{50} of OM-205 to plasmin was 4,000-30,000 times larger than the I_{50} to thrombin, with the natural substrate or Bz-Phe-Val-Arg-pNA. The other OM-inhibitors also indicated high selectivity to thrombin,[11] though these results have not been introduced in this brief paper.

In a historical aspect, substituted arginine and lysine methyl esters were comparatively studied by Sherry et al. 1965.[14] This earlier study indicated that protection of α-NH_2 of arginine by a tosyl or benzoyl group increased substrate sensibility. On the clotting by thrombin, TAMe and N^{α}-benzoyl-L-arginine methyl ester (BAMe) exhibited the strongest inhibition among the esters examined. It was also noticed that TAMe was more potent than N^{ϵ}-tosyl-L-lysine methyl ester (TLMe) in delaying the clotting by thrombin. TAMe as well as the OM-inhibitors has the aromatic group; however, the potent OM-inhibitons are much characterized by the specific aromatic group, e. g. dansyl radical which presumably elevates the affinity to thrombin.

Later Weinstein and Doolittle reported that N^{α}-tosyl-L-arginyl-sarcosine methyl ester (TASMe) which was stable for enzymatic splitting was ca. 80% as effective as TAMe in delaying of the clot formation by thrombin. I_{50} of TASMe would be over 1,200 μM, which may be more than 10,000 times larger than I_{50} of OM-205 to thrombin.

Theoretically more valuable contribution has been made by Shaw and his group on the synthetic inhibitors of the trypsin-like proteinases such as thrombin. Their studies on TLCK-type inhibitors and other amidino-compounds would remind the workers of the interest in the active-site-directed inhibitors.[6] Among their inhibitors, nitrophenyl p-amidinophenylmethanesulfonate should be referred here,[17] because the compound irreversibly inactivated thrombin alone, though it inhibited the other trypsin-like enzymes only competitively. Thus, it was suggested by them that the selectivity would be possible with active-site-directed reagents even among enzymes with homologous active centers, though the value of Ki of the compound to thrombin determined from presteady state kinetics was within the same range as that to trypsin.

In the case of OM-205, however, the mode of action was reversible and competitive. Besides, very high selectivity of OM-205 to thrombin was demonstrated. Thus, OM-205 seems to be much characterized by its reversible inhibition and high selectivity, when compared with the TLCK-type inhibitors as well as nitrophenyl p-amidinophenylmethanesulfonate. Therefore, theoretically or practically speaking, OM-205 would provide a unique tool for the further studies on thrombin.

Another problem, also significant theoretically, would be a chemical "code", as it were, of the part of fibrinogen to be "read" by thrombin.[12] It is known that the fibrinopeptide A, which contains arginine and phenylalanine, exhibits anti-thrombin activity.[7] As suggested from fibrinopeptide A, a series of peptides having arginine and phenylalanine was synthesized by Blombäck et al. and anti-thrombin activity of some of these peptides was reported by the same authors.[3] At present, however, it would be quite difficult to compare these thrombin-inhibitors of peptide nature with the OM-inhibitors, chemically. Further accumulation of the knowledge would perhaps permit us to discuss the "gross" similarity between the OM-inhibitors and the peptide-inhibitors (or the code of fibrinogen to be read by thrombin).

In closing, compared with different kinds of synthetic thrombin-inhibitors so far reported, it would be said that the OM-inhibitors can be a novel series of the competitive thrombin-inhibitors having extremely potent and highly selective action.

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