A NOVEL SERIES OF SYNTHETIC THROMBIN-INHIBITORS : II. RELATIONSHIPS BETWEEN STRUCTURE OF MODIFIED OM-INHIBITORS AND THROMBIN INHIBITORY EFFECT

TONOMURA, Shinji ; KIKUMOTO, Ryoji ; TAMAO, Yoshikuni ; OHKUBO, Kazuo ; OKAMOT, Shosuke ; KINJO, Kiyokatsu ; HIJIKATA, Akiko

(Citation)
The Kobe journal of the medical sciences,26(1):1-9

(Issue Date)
1980-03

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100488854

Kobe J. Med. Sci. 26, 1 - 9 , March 1980

A NOVEL SERIES OF SYNTHETIC THROMBIN-INHIBITORS II. RELATIONSHIPS BETWEEN STRUCTURE OF MODIFIED OM-INHIBITORS AND THROMBIN INHIBITORY EFFECT#

Shinji TONOMURA*, Ryoji KIKUMOTO*, Yoshikuni TAMAO*,
Kazuo OHKUBO*, Shosuke OKAMOTO**,
Kiyokatsu KINJO** and Akiko HIJIKATA**

*Central Research Laboratories
Mitsubishi Chemical Industries Ltd.

**1st Department of Physiology
Kobe University School of Medicine

INDEXING WORDS

synthetic thrombin-inhibitor; arginine derivative; chemical modification; structure-activity relationship

SYNOPSIS

The chemical modification of OM-inhibitors, which we previously reported as synthetic thrombin-inhibitors having extremely potent and high selective action, was carried out. The conversion of L-arginine part of OM-inhibitors, which belongs to N^{α}-naphthalenesulfonyl-L-arginine derivatives, to other homologous amino acids or guanidino compounds such as D-arginine, L-homoarginine, L-lysine, L-ornithine, L-citrulline, L-argininic acid, agmatine and ω-guanidino pentanoic acid decreased the thrombin inhibitory effect. The protection of guanidino group or α-amino group also lowered the inhibitory potency. The relationship between the structure of these inhibitors and the thrombin inhibitory effect was investigated.

This will be a part of the dissertation of Shinji TONOMURA submitted to Kobe University School of Medicine for the requirement of Doctor of Medical Sciences.

Received for publication October 12, 1979

Authors' names in Japanese: 殿村信二　菊本亮二　玉尾嘉邦　大窪和夫
岡本彰祐　金城清勝　土方明子

INTRODUCTION

In the blood clotting process, thrombin transforms fibrinogen into fibrin monomer,[14] which subsequently forms the fibrin clot. The action of thrombin on fibrinogen is particularly remarkable because of its high specificity. Only four peptide bonds in fibrinogen are rapidly hydrolyzed by thrombin.[1,2] Many attempts to obtain the specific inhibitor of thrombin have been made, but these compounds such as N^{α}-tosyl-L-arginyl sarcosine methyl ester[16] and other guanidino or amidino compounds[3,4,9] seem to be unsatisfactory in their magnitude of inhibitory effect and selectivity to thrombin.

However, in the previous paper[11] we reported N^{α}-dansyl-L-arginine esters and amides as inhibitors of thrombin. Values of I_{50} of these inhibitors (we called OM-inhibitors) were found in the range between 0.03 μM and 20 μM, when fibrinogen (3 μM) or N^{α}-benzoyl-L-phenylalanyl-L-valyl-L-arginine p-nitroanilide (100 μM) was used as substrate. In contrast, the values of I_{50} for plasmin, reptilase and trypsin were far larger (hundreds or thousands times). Mode of inhibitory action of OM-inhibitors were found kinetically competitive.

In this paper, we synthesized the compounds in which the L-arginine part of OM-inhibitors was converted to various homologous amino acids, and measured the inhibitory effect on thrombin to investigate the relationship between the structure of OM-inhibitors and the anti-thrombin activity. The chemical modifications of other main functional groups (dansyl part and alkyl amides or alkyl esters part) and their anti-thrombin activity were described elsewhere.[6,7,12]

MATERIALS

Thrombin (bovine, topical): Mochida Pharm. Co. Ltd., Tokyo.
Fibrinogen (bovine): Poviet Production N.V., Amsterdam.
Borate-saline buffer, pH 7.4: 0.05 M sodium borate was mixed with 0.2 M boric acid salt solution until the desired pH (7.4) was obtained by glass electrode, principally according to the method of Norman.[10]
Compound 1~16: These compounds were synthesized by the authors as shown below.

N^{α}-dansyl-L-arginine, which was prepared from L-arginine and dansyl chloride, was esterified with appropriate alcohol to give compound 1 (OM-27) and compound 2 (OM-85). Likewise, compound 5, 11 and 16 were synthesized from D-arginine, L-citrulline, which was derived from L-ornithine,[8] and L-argininic acid, which was derived from L-arginine,[5] respectively.

The reaction of 1 with excess n-butyl amine at room temperature gave compound 3 (OM-46). Compound 8 was prepared by the same method from L-homoarginine, which was derived from L-lysine.[15] Dansylation of agmatine gave compound 6. Esterification of ω-guanidino pentanoic acid gave 7.

The reaction of 1 with excess 4-ethyl piperidine failed; therefore, N^{α}-tert-butyloxycarbonyl-N^{G}-nitro-L-arginine was condensed with 4-ethyl piperidine by mixed anhydride method using isobutyl chloroformate to give 4-ethyl-1-(N^{α}-tert-butyloxycarbonyl-N^{G}-nitro-L-arginyl) piperidine, then removal of tert-butyloxycarbonyl group (AcOEt-HCl) followed by dansylation gave compound 12. Hydrogenolysis (Pd-C) of 12 afforded compound 4 (OM-205).

Compound 9 was synthesized from N^{ω}-carbobenzyloxy-L-lysine by N^{α}-dansylation, successive esterification and hydrogenolysis (Pd-C). Compound 10 was synthesized from N^{α}-tert-butyloxycarbonyl-N^{ω}-carbobenzyloxy-L-ornithine by condensation with 4-ethyl piperidine (mixed anhydride method), removal of N^{α}-tert-butyloxycarbonyl group (AcOEt-HCl), N^{α}-dansylation (dansyl chloride) and hydrogenolysis (Pd-C).

Compound 13 and 14 were prepared by the same procedure as mentioned in the case of 1 from L-ornithine, which was reacted with N-alkyl-O-methyl isourea in alkaline medium to give corresponding N^{G}-alkyl-L-arginine. N^{α}-methyl-L-arginine, from which compound 15 was synthesized by the same method mentioned above, was derived from N^{G}-nitro-L-arginine by successive N^{α}-benzylation, N^{α}-methylation and removal of N^{G}-nitro and N^{α}-benzyl groups by hydrogenolysis.[13]

METHODS

Inhibition Studies of Clotting Activity of Thrombin.

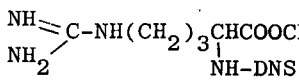
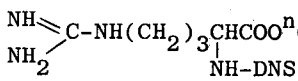
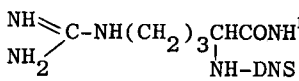
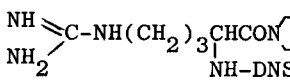
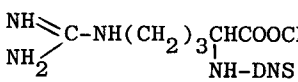
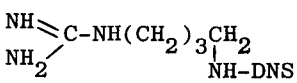
To the mixture of 0.8 ml of 0.12% fibrinogen dissolved in borate saline buffer (pH 7.4) and 0.1 ml of various concentrations

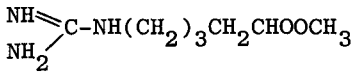
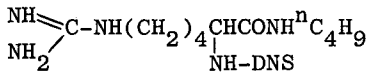
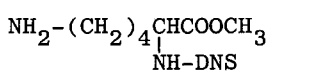
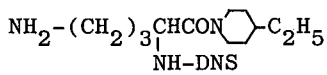
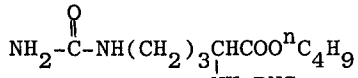
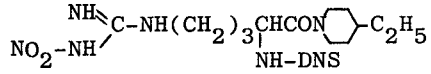
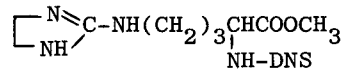
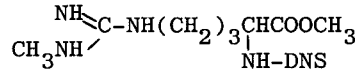
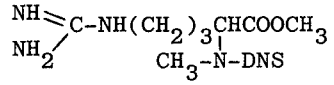
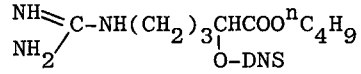
of the inhibitor to be tested was added 0.1 ml of 5 units/ml bovine thrombin. The assay was carried out at 25°C, and the time from the addition of thrombin to the formation of a clot was recorded.

RESULTS

Table 1 shows the chemical structure and I_{50} values for thrombin of compound 1 ~ 16. I_{50} value of D-arginine derivative (5) was 100 times that of the corresponding OM-27 (1). L-Homo-arginine derivative (8), L-lysine derivative (9), L-ornithine derivative (10) and L-citrulline derivative (11) were far less inhibitory than corresponding L-arginine derivatives (1, 2, 3 and 4). Chemical modifications of guanidino part (12, 13 and 14) or α-amino part (15 and 16) decreased the inhibitory effect.

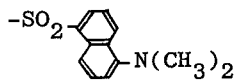
Table 1

Compd. No.	Chemical Structure[a, b]	I_{50} for thrombin (M)
1 (OM-27)		2.0×10^{-5}
2 (OM-85)		2.0×10^{-6}
3 (OM-46)		3.0×10^{-6}
4 (OM-205)		9.0×10^{-8}
5	 (D-Arg. deriv.)	2.0×10^{-3}
6		$>2.0 \times 10^{-3}$

7		$>1.0 \times 10^{-2}$
8		$>1.0 \times 10^{-4}$
9		$>2.0 \times 10^{-3}$
10		3.0×10^{-4}
11		$>1.0 \times 10^{-4}$
12		$>1.0 \times 10^{-4}$
13		$>1.0 \times 10^{-3}$
14		$>1.0 \times 10^{-3}$
15		$>1.0 \times 10^{-3}$
16		$>1.0 \times 10^{-4}$

a All amino acids had L configuration except those mentioned.

b DNS = 5-dimethylamino-1-naphthalenesulfonyl



DISCUSSION

It is interesting from the viewpoint of chemistry that OM-inhibitors consist of three parts, namely,positively charged guanidino group, hydrophobic aromatic group and ester or amide group of a certain size as typically shown in OM-205. These three parts are attached to arginine skeletone, which has L configuration, and build up the molecule of tri-pod structure. In thrombin molecule, there would be three binding sites corresponding to these parts, namely, negatively charged binding site, lipophilic binding site and hydrophobic binding site of a certain size near the active site. These binding sites probably exist on the surface of the large protein molecule of thrombin at a certain distance and at a certain place. Therefore, modification of L-arginine part of OM-inhibitor not only to D-arginine (5) but also to L-homoarginine (8), which has one more carbon chain, decreased the inhibitory effect. Dansyl agmatine (6), which loses one of three parts for binding, exhibited the low affinity. By the same reason, compound 7 did not inhibit thrombin.

L-Lysine derivative (9) and L-ornithine derivative (10), which have amino group instead of guanidino group, exhibit the lower effect (100 times and 3,000 times respectively) as compared with the corresponding L-arginine derivatives (1 and 4). This fact shows that amino group, which has weaker basicity than guanidino group, is not sufficient to interact with the negatively charged binding site of thrombin. It could be also said that the length of the carbon chain, even in the case of lysine, is insufficient and that the ability of hydrogen bonding is not so effective as that of guanidino group.

Compound 11 has urea group instead of guanidino group. Though the bulkiness of urea group may be equal to that of guanidino group, the lack of basicity is the major reason for the low effect. Because nitro guanidino group has no basic nature, compound 12 also exhibits the low inhibitory effect.

Compound 13 and 14 have modified guanidino groups, which have the same basicity as the guanidino group. However, the effect of these compounds was also low. In compound 13 or 14, since the ability of electrostatic bonding might mostly remain, the steric hindrance of relatively small methyl or ethylene group is considered

to cause the large decrease in the effect.

In compound 15 and 16, the role of the α-amino proton was shown. The lower inhibitory effect of compound 15 as compared with OM-27 (1) would be mainly due to the loss of the ability of the hydrogen bonding. The steric hindrance could also play a partial role for decrease in the inhibitory potency. Compound 16 which can never act as hydrogen donor at α-oxygen, exhibits the lower effect too. Therefore, in thrombin there would be one more binding site (fourth binding site), at which the α-amino proton of OM-inhibitor was accepted. But in compound 16, the change of the bond angle might influence the inhibitory effect.

Our results that small change of OM-inhibitor caused the decrease or the disappearance of the inhibitory effect indicate that the location of the binding sites on thrombin is settled more accurately than ever expected before. It is strongly suggested that the compounds (5 ~ 16) we reported in this paper, though their effect was far less as compared with OM-inhibitors (1 ~ 4), are full of suggestions for design of serine protease inhibitors.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Dr. S. Hattori, the general manager of the Bioscience Laboratory, Central Research Laboratories, Mitsubishi Chemical Industries Ltd., for valuable discussions and encouragement.

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