IN VITRO AND *IN VIVO* PROPERTIES OF SYNTHETIC INHIBITORS OF THROMBIN: RECENT ADVANCES

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The purpose of our study of synthetic inhibitors of thrombin was to find out novel compounds which inhibit thrombin greatly and selectively and to establish their structural features. In the process of the research, we recognized that synthetic inhibitors of thrombin should have additional properties, such that the acute and subacute toxicities are low, and that the Vd (volume of distribution) is small. We considered that our thrombin inhibitor should meet these requirements, in perspective of using it in animal experiments and further even in clinical trials.

Chemical Modification of TAMe

Nα-tosyl-L-arginine methyl ester (TAMe)[1] was reported by Sherry et al to be a substrate of and, at the same time, an inhibitor of thrombin, although TAMe had a very low antithrombin effect and high susceptibility to hydrolysis by thrombin. On the other hand, Nα-4-methoxy-benzene-sulfonyl-L-arginine methyl ester[2] and Nα-tosyl-L-arginine sarcosine methyl ester[3], which are structurally analogous to TAMe, had inhibitory effects similar to that of TAMe on thrombin but markedly reduced susceptibility to hydrolysis by thrombin (Figure 1). These results suggested that thrombin inhibitors which are undegradable with thrombin can be obtained by chemically modifying the alpha-NH_2 and alpha-COOH groups of the arginine.

TAMe, as a basic structure, was modified systemically at alpha-NH_2 and alpha-COOH groups of the arginine (Figure 2)[4-6]. Substituting dansyl group for tosyl group of TAMe markedly elevated the antithrombin activity (I_{50}, $2x10^{-5}$M). Moreover, when n-butylamide[5] was substituted for its methyl ester, the antithrombin activity was further elevated (I_{50}, $2x10^{-6}$M). This n-butylamide was replaced with 4-ethylpiperidine, and the

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cyclic structure resulted in 20 times more potent antithrombin activity (I_{50}, $1x10^{-7}$M; Ki, $3.7x10^{-8}$M). The compound, designated as No.205: Nα-dansyl-L-arginine 4-ethyl-piperidine amide, inhibited other analogous enzymes (plasmin, trypsin, Xa and urokinase) relatively weakly (Ki value, 10^{-4}~10^{-5}M), which showed its high selectivity[7]. No.205 has the structural features of thrombin inhibitor, which was characterized as a tripod structure. It has (i) a positive charge of L-arginine (D-arginine has no antithrombin activity) at its centre, (ii) an aromatic structure at its N-terminus, and (iii) a hydrophobic structure of a certain size at its C-terminus.

Reduction in Acute and Subacute Toxicities and in Volume of Distribution

No.205 had a potent antithrombin activity and high selectivity. However, this compound showed high toxicity (LD_{50}, 80mg/kg IP in mice) and high Vd (volume of distribution in the body). These properties of this compound required a deliberate protocol when it was used in animal experiments.

In the studies of No.205 and homologous compounds, we found that there was a correlation between the acute toxicity and the anti-pseudocholinesterase action. To exclude this anti-pseudocholinesterase action, -CH_2COOH group was introduced into the carboxyl-

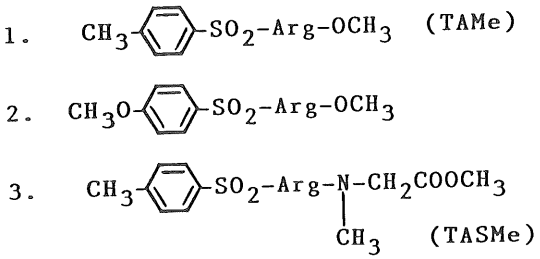


Figure 1 TAMe and its homologues.

protecting group, and No.407, Nα-6,7-dimethoxynapthalene-2-sulfonyl-L-arginyl-N-methoxy-ethylglycine[7] was synthesized (Figure 2). In the step of chemical modification, we recognized that, in No.407, its anti-pseudocholinesterase activity was so weak as to be negligible, although that antithrombin activity was maintained high. The acute toxicity was greatly reduced and Vd was also reduced[8]. Replacement of the N-methoxyethylglycine of No.407 with 4-methylpiperidine with -COOH at position 2 yielded a compound with almost the same properties. Thus the introduction of a carboxyl group in the carboxyl-protecting group of arginine was important in reducing toxicity. After a series of pharmacological and adverse effect studies of synthetic inhibitors, we finally obtained No.805: (2R,4R)-4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidinecarboxylic acid, which had a potent antithrombin activity, high selectivity and safety (Figure 3)[9,10]. This compound was then designated as argatroban (previously reported as No.805, MD-805 or MCI-9038).

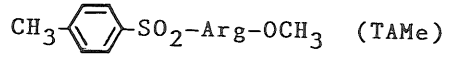
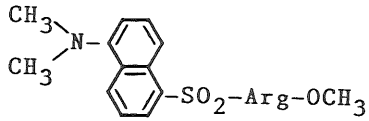
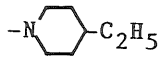
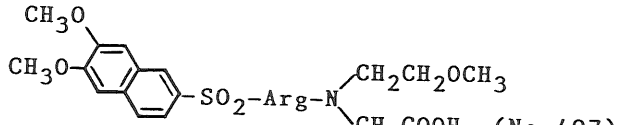
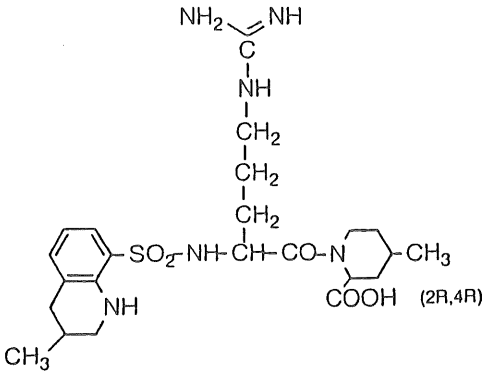
	Structure	I_{50}(μM)
1.	 (TAMe)	1000
4.		20
5.	$-NHCH_2CH_2CH_2CH_3$	2
6.	 (No.205)	0.1
7.		0.3

Figure 2 Structure-function relation of arginine derivatives.



K_i (μM)				
Thrombin	Trypsin	Plasmin	Factor Xa	Plasma Kallikrein
0.019 (Bovine) 0.038 (Human)	5.0	800	210	1,500

Figure 3 Nature of argatroban.

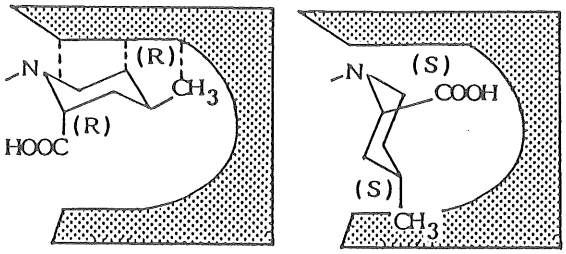
Decoding Mechanism of Thrombin, and Thrombin Inhibitor as Noise

Thrombin hydrolyzes the fibrinogen Aα chain at the site of Arg^{16}-Gly^{17} and fibrinogen Bß at Arg^{14}-Gly^{15}, with the release of fibrinopeptides A and B, forming fibrin. In view of the fact that various kinds of animals have the similar amino acid sequence of fibrinopeptide A, Blombäck et al synthesized thrombin inhibitors which consisted of peptides, and demonstrated that Arg and the Phe at its N-terminus play an important role in the interaction between thrombin and fibrinogen[11]. The above suggested that in its active centre thrombin has a portion that has a mechanism of recognizing Arg and the aromatic structure bound to its N-terminus. Our studies also showed that Arg and the aromatic structure at its N-terminus are important in the interaction between thrombin and its inhibitors.

The 4-methyl-2-piperidinecarboxylic acid portion includes 4 types of stereoisomers[10]. When the 4-methyl-2-piperidinecarboxylic acid took the configuration of 2R and 4R, the most potent antithrombin activity (Ki, 0.019μM) was exerted. When this portion took the configuration of 2S and 4S, in which the lowest antithrombin activity was exerted, Ki value was 280 μM. The former was Ca. 10,000 times more potent than the latter (Figure 4). These results show that the active site is able to distinguish precisely the stereoisomers at the C-terminus of arginine.

Recent X-ray analysis of the complex of argatroban with thrombin revealed the mode of binding of argatroban to the active site of thrombin[12]. It was found that two hydrophobic pockets (P-pocket and D-pocket) are present near the active centre of

	Ki for thrombin (μM)
2R , 4R	0.019
2R , 4S	0.24
2S , 4R	1.9
2S , 4S	280



(2R, 4R) (2S, 4S)

Figure 4 Stereo-isomers of piperidine derivatives and Ki values for thrombin.

thrombin and that a portion of 4-methylpiperidine of argatroban fits into the shallow pocket (P-pocket), whereas R-COOH at position 2 is located externally, thus preventing steric hindrance. The positively charged guanidino group of arginine binds to negatively charged Asp^{199} in the active centre and 1,2,3,4-tetrahydroquinoline ring at the N-terminus of arginine fits into the D-pocket that contains of Trp^{227}, thus inducing hydrophobic interaction. This confirmed that argatroban binds directly to the active centre of the enzyme, and the finding from the structure-activity relationship studies contributed to determining the stereostructure of the active centre of the enzyme.

Antithrombotic Action of Argatroban

Thrombin plays the leading part of blood coagulation. Argatroban exerts an antithrombosis action by strongly suppressing thrombin action: i.e. (1) conversion of fibrinogen to fibrin[10], (2) activation of factor XIII[13] and (3) platelet aggregation[14]. Unlike heparin, (4) argatroban requires no cofactor, i.e. antithrombin III (ATIII), for its action[15]. Therefore, argatroban is expected to exhibit less individual variation in its efficacy in the patient. In fact, argatroban reveals an antithrombosis action regardless of the amount of ATIII, providing a stable pharmacological effect[16].

(A) Chronic Arterial Occlusion

Argatroban was approved as a new drug by the Japanese Government in 1990. It is now clinically used as a remedy for chronic arterial occlusion. This disease is a chronic condition that is often associated with ulcer or necrosis in the lower extremities. The

characteristics of the disease are intravascular thrombus formation, and repeated aggravation and recurrences. Argatroban inhibited the progress of the disease significantly in rat model for peripheral arterial occlusion[17]. Clinical trials of argatroban were conducted in patients with chronic arterial occlusion. The administration of argatroban (20 mg daily in two divided doses by continuous intravenous infusion for 4 weeks) markedly improved ischaemic ulcer, pain and cold sensation, and the drug was confirmed to be useful clinically[18,19]. These results suggest that thrombin is involved in the mechanism of aggravation of chronic arterial occlusion.

(B) Cerebral Thrombosis

It has been reported that increased fibrinopeptide A (FPA) and thrombin-antithrombin III complex (TAT) occur in acute-phase cerebral thrombosis[20,21]. The increased levels of FPA are maintained over several weeks after onset of the disease. This suggests that thrombin continues to be active after onset of the disease, thus providing a state of accelerated blood coagulation. Since argatroban is powerful in antithrombin properties *in vitro* and demonstrated an antithrombosis action in animal experiments, its efficacy on thrombosis such as cerebral thrombosis is now being evaluated in clinical trials. Results obtained are distinctly promising. Tanaka et al reported that FPA was significantly reduced by administration of argatroban in patients with acute-phase cerebral thrombosis[22], and Imiya et al reported that TAT was reduced by administration of argatroban in patients with subacute cerebral thrombosis[23]. Kobayashi et al and Yonekawa et al also reported that this drug was useful in acute-phase cerebral thrombosis[24,25], which indicated that thrombin continues to exert an active role in the progress of acute-phase cerebral thrombosis. Thrombin has the action of vasoconstriction in the basilar artery[26] and stimulates endotheline release from vascular endothelial cells[27]. Argatroban greatly inhibits these actions of thrombin. This fact has aroused the keen interest of a number of investigators.

(C) Myocardial Infarction

Thrombolytic therapy has recently been used extensively as one of the therapeutic approaches to myocardial infarction. Although this treatment provides high rates of restored patency of arteries in patients with arterial occlusion, reocclusion is a new problem of this approach. Gulba et al reported apparently increased TAT level in patients with myocardial infarction who had reocclusion after receiving thrombolytic therapy[28], and Owen et al also reported increased FPA levels in patients who underwent thrombolytic therapy[29]. Argatroban apparently inhibited reocclusion occurring after thrombolytic treatment in an experimental arterial thrombosis model[30,31]. These results suggested strongly that thrombin continued to be formed or released during thrombolytic treatment and that thrombin is involved in the mechanism of reocclusion.

Argatroban was reported to have another action, by which it accelerates thrombolysis due to thrombolytic drugs (u-PA and t-PA). In Table 1, heparin and argatroban, each amount of which was adjusted to exert a similar anticoagulant action, were compared for their effects on the time of thrombolysis induced with t-PA. As a result, argatroban clearly shortened the time of thrombolysis, whereas heparin had no such effect[13]. Argatroban accelerated thrombolysis more markedly than heparin in the model for experimental arterial thrombosis[13,30,31].

The fact that argatroban accelerated the thrombolysis induced with the thrombolytic drugs and that it inhibited reocclusion after thrombolytic treatment suggest that this drug is useful in establishing a new thrombolytic therapy.

Table 1 Effect of argatroban on clot lysis by t-PA.

Compound	Conc.	Clot lysis induced by t-PA	
		Lysis time (min)	Clotting time (min)
Argatroban	0 μM	78.3±13.8	3.8±0.2
	0.01	57.2± 8.8	4.4±0.2*
	0.03	46.4± 7.5	4.9±0.3*
	0.10	33.1± 6.5**	6.7±0.4***
	0.30	25.1± 2.4**	9.0±0.4***
Heparin	0 IU/ml	84.9± 9.4	3.5±0.2
	0.01	76.8±10.8	4.6±0.3*
	0.02	56.5± 4.2*	6.8±0.4***
	0.03	65.8± 5.7	9.0±0.6***

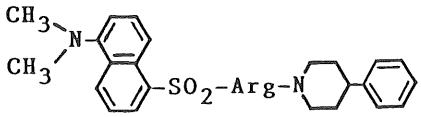
*($p<0.05$), **($p<0.01$), ***($p<0.001$)

Development of Other Proteinase-Selective Inhibitors

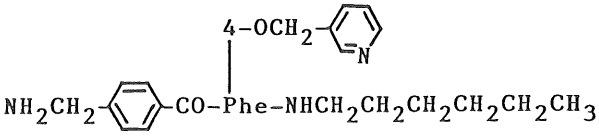
We searched for the agent that has high affinity and selectivity to thrombin, and developed successfully a small molecular inhibitor of thrombin, argatroban. The chemical structure of this inhibitor suggested that thrombin has the portion within about 15 Å in diameter in its active centre, which controls the specificity of the enzyme.

On the assumption that each of the other related enzymes also has its own decoding mechanism which is possibly inhibited by noise, i.e. chemical inhibitor[32], we searched for a selective inhibitor against each of the enzymes. Very fortunately, we were able to find a series of synthetic selective inhibitors against pseudocholinesterase, plasmin and plasma kallikrein, respectively (Figure 5).

Pseudocholinesterase inhibitor



Plasmin inhibitor (OS-535)



Plasma kallikrein inhibitor (PKSI-527)

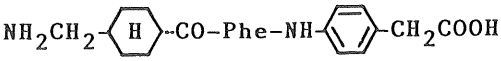


Figure 5 Synthetic selective inhibitors on pseudocholinesterase, plasmin and plasma kallikrein.

(A) Pseudocholinesterase

The search for thrombin inhibitors also gave us a series of anti-pseudocholinesterase. No.205 exhibited a strong antithrombin activity and anti-pseudocholinesterase activity as well. Antithrombin activity was removed by introduction of phenyl group into piperidine ring of No.205 without any decrease of anti-pseudocholinesterase activity. Thus a selective anti-pseudocholinesterase agent, Nα-dansyl-L-arginine-4-phenylpiperidine amide (Ki, 1.6×10^{-8}M) was successfully obtained[33].

(B) Plasmin

With respect to antiplasmin agents, epsilon-aminocaproic acid (EACA) and tranexamic acid (t-AMCHA) are known. However, these antiplasmin agents bind to lysine binding site apart from the active centre of plasmin to exert its antiplasmin action, which is different from argatroban that acts on the active centre of thrombin. Therefore, these antiplasmin agents inhibited only fibrin degradation due to plasmin (I_{50}, $6x10^{-5}M$) and very rarely inhibited hydrolysis of synthetic substrates and fibrinogen and kinin formation induced with plasmin[34].

After the search for antiplasmin agents that act on the active centre and selectively inhibit plasmin, we finally found out OS-535: Nα-(4-aminomethylbenzoyl)-4-(3-picolyloxy)-L-phenylalanine-n-hexylamide[35]. OS-535 inhibited not only degradation of fibrin by plasmin (I_{50}, $2.9x10^{-6}M$) but also other actions of plasmin, which could not be inhibited with t-AMCHA and other plasmin inhibitors: OS-535 at a dose of approximately 1/40 of that of t-AMCHA inhibited experimental ascites tumour growth, which was accompanied with increased plasmin activity in ascites[36]. Results obtained indicated that OS-535 is considered promising as an antiplasmin agent.

(C) Plasma Kallikrein

Aprotinin is well known as an antikallikrein agent. This drug, however, more strongly inhibits glandular kallikrein and plasmin rather than plasma kallikrein[37]. Our study of potent and selective inhibitor of plasma kallikrein finally reached to PKSI-527: Nα-(trans-4-aminomethyl-cyclohexylcarbonyl)-L-phenylalanine 4-carboxymethylanilide. PKSI-527 greatly inhibited plasma kallikrein (Ki, $8.6x10^{-7}M$) but only weakly inhibited other related enzymes in the blood, and thereby this compound was considered to be a selective inhibitor of plasma kallikrein[38]. PKSI-527 inhibited blood coagulation, fibrinolysis and kinin formation through activation of factor XII, thus indicating the important role of plasma kallikrein in the mechanism involving factor XII[39]. How far plasma kallikrein is implicated in various diseases is a problem remaining unsolved, but PSKI-527 may be useful in providing insights into the problem.

CONCLUSION

We studied compounds, each of which inhibits a certain enzyme greatly and selectively, and determined their structural formulas. The structural formula of thrombin inhibitor suggested that a proteinase has a portion within 15 Å of its active centre, where structural information is precisely decoded, although its mechanism and the structure of the portion probably differ from enzyme to enzyme, and also that a small molecular selective inhibitor against each enzyme can be present. Moreover, these results suggested that such synthetic selective inhibitors of enzymes are useful in elucidating the physiological and pathological roles of each enzyme and providing clinically useful remedies.

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