Anticoagulant Activity of Original Synthetic Peptide Derivatives

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Original synthetic peptide derivatives exhibit anticoagulant activity *in vitro* and *in vivo*. They delayed fibrin clot formation from human blood plasma in tests for the intrinsic coagulation pathway (activated partial thromboplastin time) and final stage of plasma coagulation (thrombin time) and inhibited amidolytic activity of thrombin. We determined the minimum effective dose of the most active compound providing a 2-fold lengthening of blood clotting time (activated partial thromboplastin time test and thrombin time test), which persisted for 2-3 h.

Key Words: *synthetic peptide derivatives; anticoagulant activity; thrombin inhibition; pharmacodynamics*

Vascular thromboses often cause disability and death. Thrombin, the key enzyme of the blood coagulation cascade, which plays the major role in physiological (hemostasis) and pathological (thrombosis) processes, contributes to transformation of plasma-soluble fibrinogen into plasma-insoluble fibrin, and activates blood coagulation factors V, VIII, XI, and XIII and proteinase-activated platelet receptors [1, 10]. Apart from standard anticoagulant heparin and warfarin, direct thrombin inhibitor proteins (recombinant hirudins and synthetic hirudin analogues capable of binding to the active site of thrombin and fibrinogen-binding site) [3-8] and analogues of amino acids and short peptides (agmatine, argatroban, and melagatran) are used in clinical practice for the prevention and therapy of thromboses.

This work was designed to perform a search for direct thrombin inhibitors among synthetic peptide derivatives with different structure.

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MATERIALS AND METHODS

Peptide derivatives were synthesized at the Laboratory of Peptide Chemistry (Federal State Unitary Enterprise "State Research Institute for Genetics and Selection of Industrial Microorganisms"). Experiments were performed with ZAla-Ala-Arg-*Pip**TFA (peptide 1, molecular weight 632 Da), ZAla-Ala-Arg-Mf*HBr (peptide 2, molecular weight 601 Da), As-Trp-Arg-Mf*HCl (peptide 3, molecular weight 508 Da), Fta-Gly-Arg-Pip*TFA (peptide 4, molecular weight 560 Da), and As-Trp-Arg-Pip*TFA (peptide 5, molecular weight 584 Da).

The ability of synthetic peptide derivatives 1, 2, and 3 to inhibit fibrin clot formation from human blood plasma was estimated in the activated partial thromboplastin time (APTT) test and thrombin time (TT) test [1]. The blood was collected into plastic tubes with 0.11 M $C_6H_5O_7Na$ in the 9:1 ratio. The blood was centrifuged at 1400*g* for 20 min to obtain platelet-depleted plasma. The optimal concentrations were estimated from inhibitory activity and 2-3-fold increase in the plasma clotting time in the presence of an inhibitor (compared to that in the

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absence of the inhibitor). The concentrations (31-570 μM) were selected experimentally.

The inhibition of thrombin amidolytic activity by synthetic peptides 1, 2, 3, 4, and 5 was evaluated to select the most potent inhibitors. We studied the effect of these peptides on hydrolysis of the chromogenic thrombin substrate [9]. Variations in the rate of hydrolysis were recorded spectrophotometrically at 405 nm. The concentration of inhibitors that decreased the amidolytic reaction rate by 2 times (IC_{50}) was calculated as described elsewhere [2].

The effect of most potent peptide 4 (1, 2, and 3 mg/kg intravenously) on anticoagulant activity of blood plasma from male Wistar rats weighing 280-340 g was studied in the APTT test and TT test [1]. The animals were intraperitoneally anesthetized with nembutal in a dose of 60 mg/kg. The jugular vein blood was put into plastic tubes with 0.11 M $C_6H_5O_7Na$ in the 9:1 ratio. The blood was centrifuged at 1400*g* for 20 min to obtain platelet-depleted plasma.

RESULTS

Peptides 1, 2 (analogues of the chromogenic thrombin substrate), and 3 (structural analogue of argatroban) modified human plasma clotting time in the APTT test. Addition of peptides 1 and 3 in concentrations of 31-380 μM to the plasma was followed by a significant dose-dependent increase in the blood clotting time (Table 1). Peptide 3 in a concentration of 71 μM increased the blood clotting time by 2 times (from 31.4 to 72.8 sec). The same effect was observed after addition of peptide 1 in a concentration of 380 μM. Peptide 2 did not inhibit clot formation in this test. An increase in the human plasma clotting time to 100 sec (APTT test) was observed after treatment with commercial preparations of direct thrombin inhibitors bivalirudin, argatroban, and lepirudin in final concentrations of 2-4 μM [10].

The effect of peptides 1, 2, and 3 on the human blood plasma clotting time was studied in the TT test. Addition of peptides 1 and 3 in concentrations of 48-570 μM to the plasma was followed by a dose-dependent increase in the plasma clotting time (Table 1). The clotting time increased by 2 times (from 9.4 to 18.8 sec) after treatment with peptides 3 and 1 in concentrations of 370 and 520 μM, respectively. Peptide 2 was ineffective in this test. The inhibition of thrombin amidolytic activity (0.006 μg, 0.015 U) was studied after addition of peptides 1, 2, 3, 4, and 5 in final concentrations of 41-1791 μM (Fig. 1). Peptide 2 had the lowest inhibitory activity. The inhibitory effect of peptide 3 was higher than that of peptide 1. Peptides 4 and 5 were most potent in inhibiting the enzyme. The formulas of these compounds were estimated by a comparative study of the relationship between structural characteristics and anticoagulant activity of peptides 1, 2, and 3. IC_{50} for peptides 1 and 2 was more

TABLE 1. *In Vitro* Clotting Time of Human Blood Plasma in the APTT Test and TT Test after Addition of Peptide Thrombin Inhibitors (sec, $X_m \pm m$)

Final concentration of synthetic peptide derivatives, μM	Peptide 1		Peptide 2		Peptide 3	
	APTT	TT	APTT	TT	APTT	TT
0	31.4±0.4	9.4±0.2	31.4±0.4	9.9±0.2	31.4±0.4	9.4±0.2
31	31.7±0.3	9.3±0.2	31.0±0.3	9.4±0.5	38.6±0.4**	11.5±1.2
48	32.3±0.5	9.7±0.4	31.5±0.2	9.4±0.3	45.2±1.3**	13.9±0.3**
63	32.0±0.4	9.9±0.5	31.4±0.7	9.3±0.6	52.9±1.7***	20.0±0.9**
75	32.8±0.9	10.3±1.2	31.4±0.4	9.5±0.5	71.8±1.1***	20.3±2.0**
95	32.7±0.5	10.5±0.9	31.7±0.5	9.4±0.4	>100[x]	25.3±0.6***
126	33.1±0.6	10.4±0.7	31.6±0.3	9.6±0.2		41.7±3.5***
143	35.0±1.5	10.6±0.5	32.0±0.3	9.9±0.7		56±2.8***
190	37.3±2.6	10.6±0.8	32.1±0.4	9.8±0.8	>100[x]	—
253	40.4±2.8**	11.3±2.2	32.5±0.5	10±1.0		
380	58±4.3***	11.7±1.3**	33.0±1.6	10.0±1.5		
570	>100[x]	23.1±1.5***	33.0±1.4	9.9±1.3		

Note. n=5; *p<0.05, **p<0.01, ***p<0.001 compared to data obtained in the absence of peptides (final concentration 0 M). *When clotting time exceeded 100 sec, higher concentration were not tested.

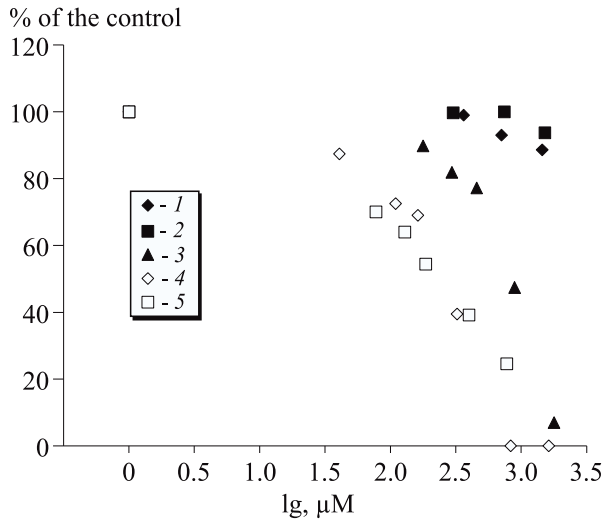

Fig. 1. Inhibition of thrombin amidolytic activity. Abscissa, peptide concentration. Peptide thrombin inhibitors 1 (*1*), 2 (*2*), 3 (*3*), 4 (*4*), and 5 (*5*).

than 1000 μM. IC_{50} for peptides 3, 5, and 4 were 794±33, 316±21, and 281±15 μM, respectively. Peptide 4 exhibited the highest inhibitory activity. The inhibition constant (Ki) for peptide 4 was 270 μM, which exceeded that for commercial thrombin inhibitors by 2-3 orders of magnitude. For example, Ki for bivalent thrombin inhibitors are 1.9-2.3 nM (lepirudin) [7], 200 fM (desirudin) [8], and 60 fM (bivalirudin) [6], Ki for univalent thrombin inhibitors are 5 nM (argatroban) [3,5] and 2 nM (ximelagatran) [4].

Intravenous injection of the most potent synthetic derivative (peptide 4) to Wistar rats was followed by a dose-dependent increase in the blood plasma clotting time in the APTT test and TT test (Table 2). The blood plasma clotting time in the APTT test increased by 1.5-2 times under the influence of peptide 4 in doses of 2 mg/kg (15, 30, 60, and 90 min after treatment) and 3 mg/kg (over 2 h post-treatment). The blood plasma clotting time in the TT test increased by 1.5-2 times under the influence of peptide 4 in doses of 2 mg/kg (5 and 15 min after treatment) and 3 mg/kg (over 3 h post-treatment).

Our results indicate that the human blood plasma clotting time in the APTT test and TT test increases, while amidolytic activity of thrombin decreases with increasing the concentration of synthetic peptide derivatives (except for peptide 2). Hence, these substances have anticoagulant activity. The minimum effective dose of the most potent synthetic derivative (peptide 4) inducing a 2-fold increase in the rat plasma clotting time in the APTT test (15, 30, and 60 min after addition) and TT test (5 min after addition) was 3 mg/kg. Anticoagulant activity of rat blood plasma reached maximum by the 15th minute after intravenous injection of the peptide (APTT test). It can be hypothesized that this peptide produces a modulatory effect on other blood coagulation factors. Probably, the dose of this peptide should be increased to prolong the effect.

TABLE 2. Clotting Time of Rat Plasma in the APTT Test and TT Test after Intravenous Injection of Peptide Thrombin Inhibitor 4 (sec, X_m±*SEM*)

Time after injection, min	Dose of peptide 4, mg/kg					
	1 mg/kg		2 mg/kg		3 mg/kg	
	APTT	TT	APTT	TT	APTT	TT
0	23±2	10.4±0.5	23±2	10.4±0.5	23±2	10.4±0.5
5	29±4*	12.3±0.8	26±2	14.5±2.0*	35±5*	21.0±1.1*
15	31±4*	13.7±1.3	40±8*	14.8±1.7*	53±6*	16.4±1.0*
30	29±6*	12.3±1.5	35±9*	13.2±1.0	50±5*	15.5±2.0*
60	23±1	12.7±1.2	32±7*	13.5±1.8	55±8*	15.0±1.2*
90	23±3	13.2±1.3	28±5*	12.4±0.6	42±4*	14.7±0.8*
120	21±1	11.0±0.9	23±2	12.0±0.7	28±3*	13.3±0.9*
150	21±2	12.6±1.6	24±2	11.5±0.6	25±3	13.1±0.5*
180	N.d.	11.9±1.0	22±3	11.0±0.6	26±3	13.6±0.7*
210	N.d.	N.d.	N.d.	N.d.	22±1	12.0±0.7

Note. n=10; *p<0.05 compared to the pre-injection parameter (0 min after injection). N.d., not determined. The anticoagulant effect persisted for 60, 90, and 150 min after injection of the peptide in doses of 1, 2, and 3 mg/kg, respectively (APTT test). Anticoagulant activity of the plasma was not observed after injection of the peptide in a dose of 1 mg/kg (TT test). The effect persisted for 90 min after injection of the peptide in a dose of 2 mg/kg (TT test).

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