ELSEVIER

European Journal of Pharmacology 406 (2000) 181–189

ejp

www.elsevier.nl/locate/ejphar

Inhibitory effect of various thrombin inhibitors on shear-induced platelet function and dynamic coagulation

Tomomi Taka [a,b], Yasuo Konishi [c], Jacek Slon-Usakiewicz [c], Viacheslav Medvedkin [c], Yuko Tsuda [b,d], Yoshio Okada [b,d], Junji Seki [e], Junichiro Yamamoto [a,b,*]

[a] *Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe 651-2180, Japan*
[b] *High Technology Research Center, Kobe Gakuin University, Kobe, Japan*
[c] *Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada*
[d] *Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, Japan*
[e] *Department of Biomedical Engineering, National Cardiovascular Center Research Institute, Osaka, Japan*

Received 15 August 2000; accepted 22 August 2000

Abstract

We assessed the effects of active site-directed, fibrinogen recognition exosite (FRE)-directed and bifunctional thrombin inhibitors, on shear-induced platelet reactivity (adhesion/aggregation) and dynamic coagulation (coagulation of flowing blood). An in vitro test for shear-induced haemostatic plug formation and dynamic coagulation (haemostatometry) was employed using non-anticoagulated rat blood. The active site-directed inhibitors (argatroban, P891, P899) caused inhibition of platelet reactivity and coagulation at 1-, 100- and 100-μM concentrations, respectively. Bifunctional inhibitors (P553, P1053) exerted inhibitory effects at 0.1 μM. A dimeric bifunctional inhibitor P824 caused significant inhibition at 1 μM. The FRE-directed inhibitor (P960) inhibited shear-induced platelet reactivity at 10 μM but the dynamic coagulation at 1 μM. Combination of active site-directed argatroban and FRE-directed P960 did not show any synergistic effect. The most potent inhibition was observed in monomeric bifunctional inhibitors. The inhibitory effects were compared with the K_i values against human thrombin and with the IC_{50} values against fibrin clot formation. The minimum effective concentrations on shear-induced platelet reactivity and dynamic coagulation were comparable with the IC_{50} values, but not with the K_i values.

Keywords: Thrombin; Platelet aggregation; Coagulation; Shear; Bifunctional inhibitor

1. Introduction

Arterial thrombi causing myocardial infarction and stroke are formed at atherosclerotic and/or stenosed sites and are triggered by platelet activation due to high shear forces and/or lysophosphatidic acid. Exposure of subendothelial collagen is certainly important for primary haemostasis, but not for platelet activation following plaque rupture. Recent studies have shown that the lipid-rich core of atherosclerotic lesions, which contains the platelet-activating lipid lysophosphatidic acid, and not the exposed collagen is thrombogenic (Fernandez-Ortiz et al., 1994; Siess et al., 1999). Platelet-rich thrombus is stabilised by fibrin, which is converted from fibrinogen by a serine protease thrombin (Sixma, 1994). Thrombin is also the most potent physiological activator of platelet aggregation. Experiments using animal models of thrombosis have suggested that suppression of an enhanced thrombin activity may be a very promising way of preventing arterial thrombosis (Badimon et al., 1991; Sasaki et al., 1993; Harker et al., 1995; Umemura et al., 1995; Yamashita et al., 1998). Indeed, in clinical trials, thrombin inhibitors prevented myocardial infarction and ischemic heart disease (Antman, 1994; GUSTO IIa Investigators, 1994; GUSTO IIb Investigators, 1996; Matsuo et al., 1997).

Thrombin binds to G-protein-coupled thrombin receptor of platelet to split peptide bond at N-terminus. The newly formed N-terminus (tethered ligand) binds to the specific site of the receptor to transmit signals for platelet activation (Vu et al., 1991). The effect of thrombin on platelets can be inhibited by inactivation of its enzymatic activity or by hampering the binding of tethered ligand to the recep-

* Corresponding author. Tel.: +81-78-974-1551; fax: +81-78-974-5689.
E-mail address: yamamoto@nutr.kobegakuin.ac.jp (J. Yamamoto).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00655-5

tors. The latter can be performed by small molecular mimetics of the tethered ligands. However, this approach was not successful, and this might be due to species differences of tethered ligand receptors and the presence of different thrombin receptors (Vu et al., 1991; Connolly et al., 1994; Kahn et al., 1998). In addition, the external mimetics could not compete with the high concentration of the intramolecular tethered ligand.

The development of argatroban, a highly specific active site-directed thrombin inhibitor, was based on the fibrinopeptide A sequence (Kikumoto et al., 1984). A naturally occurring potent thrombin inhibitor, hirudin, was purified from medicinal leech Hirudo *medicinalis* (Markwardt, 1991). Three types of thrombin inhibitors have been developed from studies on the interaction between thrombin and hirudin, i.e. active site-directed, fibrinogen recognition exosite (FRE)-directed and bifunctional (active site-directed plus FRE directed) inhibitors (Rydel et al., 1990; Krstenansky et al., 1990; Maraganore et al., 1990; DiMaio et al., 1990; Markwardt 1991; Szewczuk et al., 1992; Tsuda et al., 1994).

The agonist-induced platelet aggregometry has been widely used to assess platelet reactivity (Born, 1962). However, recent studies suggest that shear-induced platelet function tests are superior to platelet aggregometry in reflecting platelet reactivity in vivo (Peters et al., 1989; Gorog and Kovacs, 1990, 1995; Ratnatunga et al., 1992; Uchiyama et al., 1994; Cattaneo et al., 1995; Moake, 1995; Ikarugi et al., 1999; Goto et al., 1999). Further, it has been demonstrated that calcium ion plays important role in shear-induced platelet aggregation (Sakariassen et al., 1984; Chow et al., 1992; Ikeda et al., 1993) suggesting that a test, which uses non-anticoagulated blood is physiologically more relevant (Gorog and Kovacs, 1990, 1995; Peters et al., 1989; Ratnatunga et al., 1992; Ikarugi et al., 1999).

The aim of the present study was to assess the effects of various types of synthesized thrombin inhibitors on platelet reactivity and dynamic coagulation. This was achieved by using a unique test for the measurement of haemostatic plug formation and dynamic coagulation, which allowed comparing the effects on platelet reactivity and coagulation with the K_i and IC_{50} values. The tests were carried out using blood from rat because the need of repeated withdrawals of fresh blood samples excluded the use of human blood.

2. Materials and methods

2.1. Agents

Argatroban (Novastan, Mitsubishi–Tokyo Pharmaceuticals, Tokyo, Japan) was diluted in saline. Three types of thrombin inhibitors (Table 1) were synthesized by using conventional Fmoc-strategy of solid phase peptide synthesis. All peptides showed >98 % purity based on the HPLC absorption profile at 210 nm. They were dissolved and diluted in saline containing 5% Tween 80. Factor Xa inhibitor, DX9065a, (donated from Daiichi Pharmaceutical, Tokyo, Japan) was dissolved and diluted in saline. Human fibrinogen (Grade L, Kabi Vitrum, Stockholm, Sweden) was dissolved (0.6%) in borate-buffered saline (pH 7.8; 0.01 M $Na_2BO_4O_7 \cdot 10H_2O$, 0.16 M H_3BO_3, 0.04 M NaCl) and stored at -80°C until use. Bovine α-thrombin (5000 unit/vial; Mochida Pharmaceutical, Tokyo, Japan) was dissolved in distilled water (500 unit/ml), diluted in borate-buffered saline to 100 unit/ml and stored at -80°C until use. The stock solutions were diluted to the desired concentrations with borate-buffered saline just before the measurement.

Table 1
Sequences of the synthetic thrombin inhibitors

Sequences		References
Active site-directed inhibitors		
P891	Bbs-R-dPip-Nle-amide-AcOH	
P899	Bbs-R-dPip-Thi-amide-AcOH	
FRE-directed inhibitor		
P960	Ac-DYEPIPEEA-Cha-dGlu-OH	Krstenansky et al., 1990
Bifunctional inhibitors		
P553	Bbs-R-dPip-Ada-Abu-DYEPIPEEA-Cha-dGlu-OH	Tsuda et al., 1994
P1053	Bbs-R-dPip-Ada-Abu-DFEEIPEEg-Nle-LQ-OH · AcOH	
P824	(Bbs-R-dPip-Ada-Abu-DFEPIPEEA-Cha-C-OH · AcOH)$_2$	

Bbs, 4-*tert*-Butylbenzenesulfonyl; dPip, D-pipecolic acid; Thl, β-(2-thienyl)-alani Ada, 12-aminododecanoic acid; Abu, 4-aminobutyric acid; Cha, 2-aminocyclohexane proplonic acid.

2.2. Animals

Male Wistar ST rats aged 10–11 weeks (Japan, Hamamatsu, Japan) were housed in compliance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" (published by the Physiological Society of Japan).

2.3. Haemostatometer

A three-channel haemostatometer (Ratnatunga et al., 1992; Yamamoto et al., 1999), was purpose built in the Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University in order to measure shear-induced platelet reactivity and dynamic coagulation. The principle of the measurement is shown in Fig. 1. The haemostatometer has three parts: (A) holds the syringe with non-anticoagulated blood at 37°C. Thrombin inhibitors or vehicles (control) were added to the blood in 1% or 10% volume. Paraffin liquid was infused at a constant flow of 0.057 ml/min into the syringe to drive the blood to parts (B) and (C). Part (B) is a polyethylene tube (outer diameter is 1.00 ± 0.02 mm; inner diameter is 0.50 ± 0.01 mm; 52.5-

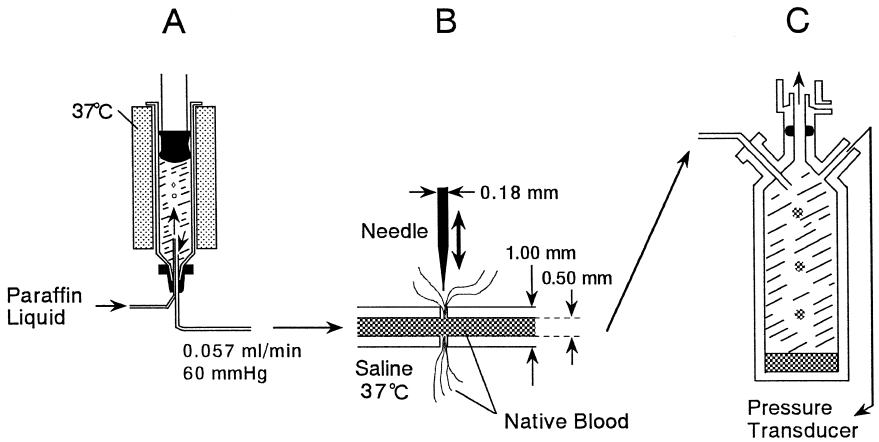

Fig. 1. Principle of haemostatometry. Haemostatometer is composed of three parts, (A), (B) and (C). Non-anticoagulated blood in a syringe is kept vertically in a holder at 37°C (A). Non-anticoagulated blood is displaced by paraffin liquid at 0.057 ml/min, resulting in blood flow in the other polyethylene tubing (o.d., 1.00 ± 0.02 mm; i.d., 0.05 ± 0.01 mm). Bleeding in the surrounding saline at 37°C is caused by punching with a needle, 0.18 mm in diameter, followed by haemostasis, shear-induced platelet-rich plug formation (B). Polyethylene tubing is connected to a blood waste reservoir under 60 mm Hg and changes in pressure are recorded (C).

cm long) surrounded by saline at 37°C. Blood flows to part (C) through the tube. Part (C) is a reservoir connected to the pressure transducer to monitor pressure changes in the system.

2.4. Shear-induced platelet-rich plug formation and dynamic coagulation

The three-channel haemostatometer was designed to measure shear-induced platelet-rich plug formation and dynamic coagulation from three blood samples concurrently. Blood was perfused through the tube by paraffin liquid displacement. The pressure in the perfusion system was stabilised at 60 mm Hg. Then, two through-holes were made by piercing the tube with a 0.18-mm diameter needle at 150 s after withdrawal of blood. This led to 'bleeding' and resulted in an immediate drop of the pressure. Thereafter, the perfusion pressure was gradually recovered to the pre-punch 60 mm Hg as platelet-rich haemostatic plugs were formed in the holes. The initial shear stress in each hole was calculated to be 375 dyn/cm^2. After haemostasis, the pressure stayed at 60 mm Hg until it dropped gradually or suddenly to zero. This was because of the clot formation in the tube causing cessation of flow. The pressure changes were defined as four parameters: H1, H2, CT1 and CT2. The initial haemostatic reaction (H1) was defined in the pressure curve, as the area of 30% pressure recovery. The completed haemostasis (H2) was defined as the area of 90% pressure recovery. Commencement of coagulation (CT1) was defined as the time from the start of the test until the pressure drops $\geq$ 10 mm Hg from the 60-mm Hg value. The completed coagulation (CT2) was defined as the time when pressure drops below 10 mm Hg.

2.5. Typical haemostatogram

A typical recording in Fig. 2 shows the effect of thrombin inhibitors. When they were effective, thrombin inhibitors delayed recovery of pressure after the puncture-induced sharp pressure drop, and also inhibited the dynamic coagulation.

2.6. Measurements of platelet reactivity and dynamic coagulation

Blood withdrawn from the abdominal aorta of rat (anaesthetised with nembutal, 60 mg/kg, i.m.) was dispensed to 1.5 ml in syringes (part A) containing 15 or 150 μl of thrombin inhibitor or vehicle. After mixing the inhibitor with the blood by several inversions, perfusion of the blood was started by pumping paraffin liquid into the blood sample at a constant rate of 0.057 ml/min. When the pressure was stabilised at 60 mm Hg, holes were made by punching the tube with a needle at 150 s after withdrawal of blood. Bleeding stopped by the formation of platelet-rich haemostatic plugs in the holes, and subsequently coagulation occurred, resulting in the arrest of blood flow. Both phenomena were monitored by the pressure changes.

2.7. K_i measurement

K_i values of inhibitors against thrombin were measured spectrophotometrically by using fluorescent substrate Tos-Gly-Pro-Arg-AMC in Tris–HCl buffered saline (pH 7.8; 0.1 M NaCl, 0.05 M tris containing 0.1% polyethylene glycol 8000) at room temperature, as described elsewhere (Szewczuk et al., 1992).

2.8. IC_{50} measurement

A 170-μl of 0.6% fibrinogen or citrated rat blood (blood: 3.2% sodium citrate = 9:1) was mixed with 30 μl of inhibitor or vehicle and the mixture was incubated at 37°C for 120 s. Coagulation was induced by 100 μl of

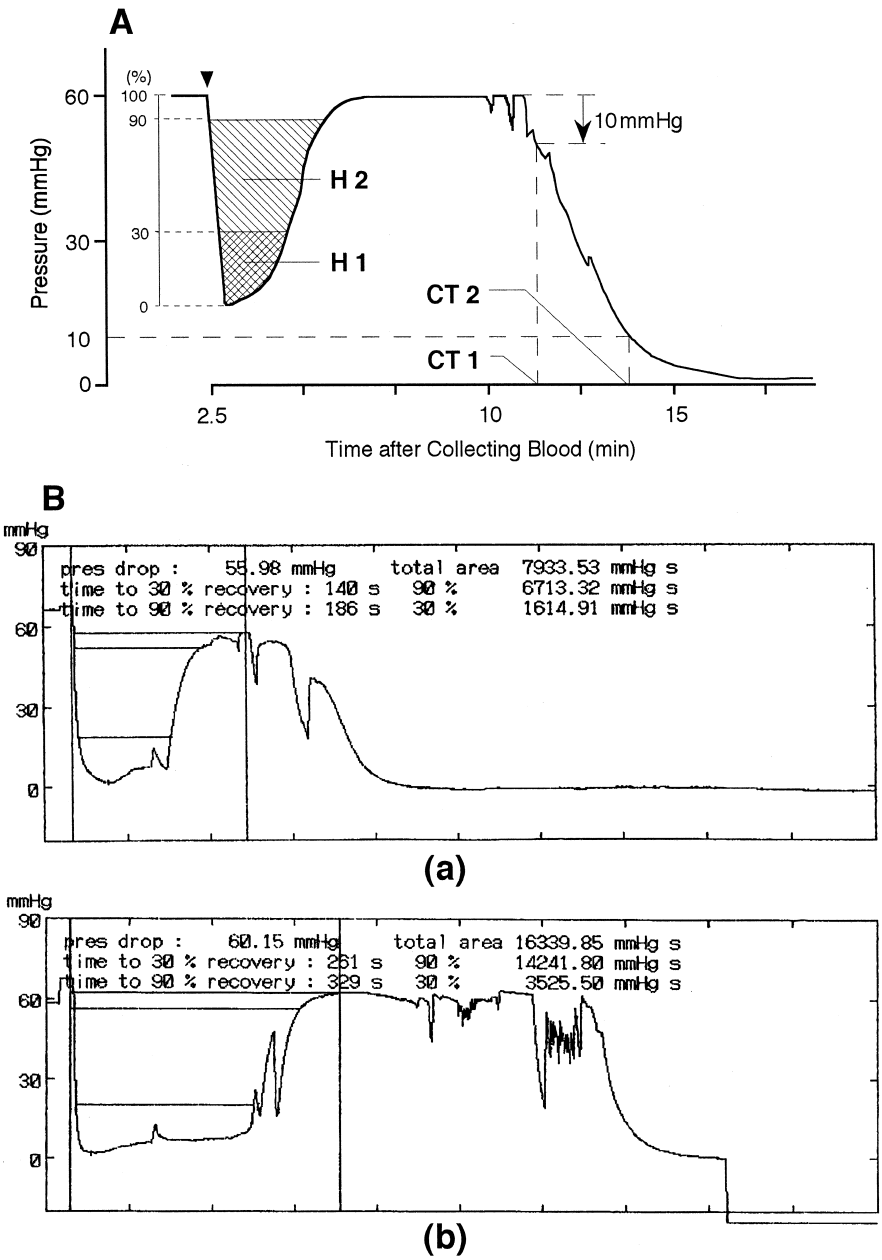

Fig. 2. (A) A typical haemostatogram. (▼) punching. H1, the area until 30% pressure recovery; H2, the area until 90% pressure recovery; CT1, the time until a pressure drop of 10 mm Hg from baseline, 60 mm Hg, by clotting; CT2, the time to reach 10 mm Hg and to keep below 10 mm Hg for 1 min. (B) The haemostatogram in the absence and presence of thrombin inhibitor using the rat blood. (a) Saline; (b) argatroban (final concentration 1 μM).

bovine thrombin solution (final concentration 0.83 unit/ml). Clotting time was measured by a Coagulometer KC1A (Amelung, Lehbrinksweg, Germany). The IC_{50} value was defined as the inhibitor concentration, which caused prolongation of the clotting time to that induced by thrombin at a final concentration of 0.415 unit/ml.

2.9. Statistical analysis

Results obtained by haemostatometry are expressed as means ± S.E.M. H1 and H2 data were converted to logarithmic before statistical analysis by paired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of thrombin inhibitors on platelet reactivity and dynamic coagulation

Argatroban significantly inhibited H1 and H2 (indices of platelet reactivity) at final concentration of 1 μM. CT1

Table 2
Effects of the active site-directed thrombin inhibitors on platelet reactivity and dynamic coagulation

		Platelet reactivity		Dynamic coagulation	
		H1 (mm Hg · s)	H2 (mm Hg · s)	CT1 (min)	CT2 (min)
Argatroban	control	2174 ± 383	8444 ± 1369	8.26 ± 0.63	10.60 ± 0.83
	0.1 μM	2217 ± 342	8931 ± 1038	9.27 ± 0.83	11.86 ± 0.95*
	control	1933 ± 450	7532 ± 1439	9.00 ± 0.80	10.77 ± 1.19
	1 μM	3423 ± 679*	12781 ± 2140*	14.65 ± 1.12**	18.36 ± 1.25**
P891	control	3491 ± 597	13863 ± 1644	10.26 ± 0.25	12.06 ± 0.33
	10 μM	3730 ± 182	13924 ± 1001	9.75 ± 0.55	11.49 ± 0.53
	control	3491 ± 597	13863 ± 1644	10.26 ± 0.25	12.06 ± 0.75
	100 μM	7268 ± 482**	26313 ± 1802**	14.26 ± 0.18**	18.09 ± 0.57*
P899	control	4812 ± 248	16499 ± 839	9.88 ± 0.46	12.63 ± 0.90
	10 μM	4146 ± 338	15177 ± 1110	10.05 ± 0.39	11.67 ± 0.62
	control	4812 ± 218	16499 ± 839	9.88 ± 0.46	12.63 ± 0.90
	100 μM	8719 ± 1094*	28681 ± 2561**	15.29 ± 1.08*	18.39 ± 0.90*

Values are means ± S.E.M. $n = 5–8$ in each group.
* $p < 0.05$.
** $p < 0.01$ vs. control.

and CT2 (indices of coagulation) were significantly inhibited at 0.1 μM. P891 and P899 significantly inhibited both of platelet reactivity and coagulation at 100 μM (Table 2).

The FRE-directed inhibitor, P960, significantly inhibited platelet reactivity at 10 μM and coagulation at 1 μM (Table 3).

Table 3
Effects on an FRE-directed thrombin inhibitor on platelet reactivity and dynamic coagulation

		Platelet reactivity		Dynamic coagulation	
		H1 (mm Hg · s)	H2 (mm Hg · s)	CT1 (min)	CT2 (min)
P960	control	1502 ± 145	6116 ± 369	8.62 ± 0.33	10.93 ± 0.49
	1 μM	1606 ± 325	6982 ± 1118	10.44 ± 0.36**	14.11 ± 0.57**
	control	1375 ± 164	5714 ± 644	9.20 ± 0.37	12.21 ± 0.61
	10 μM	3504 ± 821*	14436 ± 2611**	16.41 ± 0.68**	24.92 ± 2.45**

Values are means ± S.E.M. $n = 6–7$ in each group.
* $p < 0.05$.
** $p < 0.01$ vs. control.

Table 4
Effects of bifunctional thrombin inhibitors on platelet reactivity and dynamic coagulation

		Platelet reactivity		Dynamic coagulation	
		H1 (mm Hg · s)	H2 (mm Hg · s)	CT1 (min)	CT2 (min)
P553	control	1703 ± 158	2312 ± 640	9.12 ± 0.45	12.64 ± 1.04
	0.01 μM	1769 ± 274	7179 ± 861	8.98 ± 0.48	12.78 ± 1.02
	control	1680 ± 158	7327 ± 758	9.84 ± 0.46	3.62 ± 0.70
	0.1 μM	2312 ± 289*	7292 ± 791*	12.02 ± 0.60**	16.88 ± 1.58*
P1053	control	2398 ± 190	9582 ± 582	11.08 ± 0.42	14.51 ± 0.70
	0.01 μM	2378 ± 251	9272 ± 767	12.24 ± 0.88	15.99 ± 0.86
	control	2558 ± 187	9834 ± 597	10.94 ± 0.37	14.49 ± 0.62
	0.1 μM	3750 ± 358*	13902 ± 1265*	14.55 ± 0.83**	19.76 ± 1.07**
P824	control	3168 ± 554	12120 ± 1776	10.68 ± 0.66	13.06 ± 0.74
	0.1 μM	3230 ± 610	12527 ± 2044	11.44 ± 1.11	15.48 ± 1.02**
	control	2391 ± 497	9699 ± 1579	9.79 ± 0.46	11.83 ± 0.45
	1 μM	3689 ± 679*	14541 ± 1989*	12.76 ± 1.04*	17.24 ± 1.40**

Values are means ± S.E.M. $n = 6–11$ in each group.
* $p < 0.05$.
** $p < 0.01$ vs. control.

Table 5
Effects of a factor Xa inhibitor on platelet reactivity and dynamic coagulation

		Platelet reactivity		Dynamic coagulation	
		H1 (mm Hg · s)	H2 (mm Hg · s)	CT1 (min)	CT2 (min)
DX9065a	control	1478 ± 68	5897 ± 241	7.97 ± 0.35	10.21 ± 0.45
	1 μM	1315 ± 173	6222 ± 752	8.92 ± 0.31	11.74 ± 0.54**
	control	1339 ± 118	5359 ± 491	7.65 ± 0.52	9.85 ± 0.69
	10 μM	2988 ± 706*	12778 ± 1375**	12.02 ± 0.73**	15.14 ± 0.41**

Values are means ± S.E.M. $n = 5–7$ in each group.
* $p < 0.05$.
** $p < 0.01$ vs. control.

Bifunctional inhibitors, P553 and P1053, significantly inhibited both platelet reactivity and coagulation at 0.1 μM. A dimeric bifunctional inhibitor, P824, inhibited platelet reactivity at 1 μM and coagulation at 0.1 μM (Table 4).

3.2. Effects of factor Xa inhibitor

An inhibitor of the enzyme, which converts prothrombin to thrombin, DX9065a, significantly inhibited platelet reactivity at 10 μM and coagulation at 1μM (Table 5).

3.3. K_i, IC_{50} and haemostatometric parameters

The minimum concentration of inhibitors necessary for significant inhibition of platelet reactivity and coagulation are shown in Table 6 together with their IC_{50}, K_i and molecular weights.

4. Discussion

Platelet aggregation and stabilization of such primary thrombus by fibrin strings play a central role in arterial thrombogenesis. Thrombin is bifunctional both by activating platelets and converting fibrinogen to fibrin. Thus, control of thrombin is very important and can be achieved by two ways: (1) inhibiting its enzymatic activity and (2) inhibiting the effect of thrombin on platelets. In the present study, the former was assessed by K_i values using synthetic substrate Tos-Gly-Pro-Arg-AMC, IC_{50} values using fibrinogen and citrated blood and blood clotting time under flow (dynamic coagulation). Small molecular synthetic substrates are often used in enzyme kinetic studies but as shown in the present study are not suitable for assessment of FRE-directed inhibitors.

IC_{50} values obtained using fibrinogen and citrated blood showed good correlation with the minimum effective concentrations on dynamic coagulation (fibrinogen: IC_{50} vs.

Table 6
Comparison of K_i, IC_{50} values with haemostatometric parameters

	MW	K_i ($\times 10^{-8}$ M)	IC_{50} ($\times 10^{-8}$ M)		Minimum effective final concentration ($\times 10^{-8}$ M)	
			Fg	Blood	Platelet reactivity	Coagulation
Active site-directed inhibitor						
Argatroban	526	3.9[a]	1.5	3.0	100	100 (CT1), 10 (CT2)
P891	655	2.4	1000	690	10000	10000
P899	695	0.53	750	700	10000	10000
FRE-directed inhibitor						
P960	1386	5000	75	25	1000	100
Bifunctional inhibitor						
P553	2150	0.0002[b]	0.20	0.48	10	10
P1053	2169	0.0003	0.50	0.55	10	10
P824	4215	0.0004	0.45	0.78	100	100 (CT1), 10 (CT2)
Factor Xa inhibitor						
DX9065a	571	4.1[c]			1000	1000 (CT1), 100 (CT2)

K_i values.
[a] Kikumoto et al., 1984.
[b] Tsuda et al., 1994.
[c] Hara et al., 1994 (K_i value against factor Xa).

CT1, $r = 0.984$, $p < 0.0001$; IC_{50} vs. CT2, $r = 0.984$, $p < 0.0001$; citrated blood: IC_{50} vs. CT1, $r = 1.000$, $p < 0.0001$; IC_{50} vs. CT2, $r = 1.000$, $p < 0.0001$). On the other hand, K_i had no correlation with CT1 and CT2 (K_i vs. CT1, $r = -0.255$, $p = 0.602$, K_i vs. CT2, $r = -0.252$, $p = 0.607$). Correlations of IC_{50} values with dynamic coagulation were slightly higher in citrated blood than in fibrinogen, and this may be due to physical contribution of blood cells to coagulation under flow. This suggests that for developing antithrombotic agents fibrinogen is a better substrate than the small molecular weight synthetic substrates.

The minimum effective doses of thrombin inhibitors on dynamic coagulation are about 3- to 130-fold higher than IC_{50} values obtained in citrated rat blood. This difference may be due to differences in thrombin concentrations in the two systems. As haemostatometry uses non-anticoagulated blood, thrombin formed under flow may induce further thrombin generation via intrinsic coagulation factor XI activation (Gailani and Broze, 1991; Oliver et al., 1999) and for this reason higher concentration of thrombin inhibitor was needed. On the contrary, in the measurement of IC_{50} using citrated blood, thrombin generation by the positive feedback may not work because of calcium ion chelation accordingly lower concentration of thrombin inhibitors were sufficient to inhibit the exogenous thrombin.

Growing evidence suggests that shear-induced platelet aggregation tests reflect platelet reactivity in vivo more truly than agonist-induced platelet aggregometry (Peters et al., 1989; Gorog and Kovacs, 1990, 1995; Ratnatunga et al., 1992; Uchiyama et al., 1994; Cattaneo et al., 1995; Moake, 1995; Ikarugi et al., 1999; Goto et al., 1999). Shear-induced platelet function test using non-anticoagulated blood is ever more physiological (Peters et al., 1989; Gorog and Kovacs, 1990, 1995; Ratnatunga et al., 1992; Ikarugi et al., 1999), because physiological calcium ion concentration and involvement of erythrocytes and leucocytes are important in shear-induced platelet aggregation (Sakariassen et al., 1984; Chow et al., 1992; Ikeda et al., 1993; Hamburger and McEver, 1990; Valles et al., 1991; Lopez-Farre et al., 1996).

During haemostatic platelet plug-formation, which occurs in the haemostatometer, platelets are activated by high shear forces in the punched holes. The initial shear stress of 375 dyn/cm^2 is varied with the time as the hole becomes occluded. Morphometric analysis of the haemostatic plug has indicated that haemostatic plug formation is a reliable index of platelet reactivity (Yamamoto et al., 1999). Platelet reactivity is inhibited by heparin, argatroban, calcium chelating agents such as citrate and EDTA, stable prostacyclin, thromboxan A_2 receptor antagonist, stable prostaglandin E_1 and aspirin (Yamamoto et al., 1999). Antiplatelet agents inhibit platelet reactivity (H1 and H2), but not coagulation (CT1 and CT2). Platelet reactivity as measured by haemostatometry can detect prothrombotic state (Peters et al., 1989; Gorog and Kovacs, 1990, 1995; Ratnatunga et al., 1992; Ikarugi et al., 1999). Further, this method is highly sensitive: in contrast to platelet aggregometry, which uses micromolar concentrations of catecholamines (Mustonen and Lassila, 1996), haemostatometry can detect the effect of catecholamines in physiological (nanomolar) concentrations (Ikarugi et al., 1999).

Active site-directed inhibitor, P891, is homologous to argatroban, but its binding is extended to S1′ subsite by adding the P1′ residue L-Nle at the C-terminal. This inhibitor was investigated to study the effect of the P1′ residue. Since the molecular weight and K_i value of P891 are similar to that of argatroban, it was expected to inhibit platelet reactivity and coagulation comparable to argatroban. However, the obtained IC_{50} values and the minimum effective concentrations by haemostatometry were several hundred times higher than that of argatroban. P899 is homologous to P891 except L-Thi was used as a P1′ residue and its K_i value is smaller than that of argatroban. This inhibitor was added to show that the low potency of P891 was not due to the property of L-Nle. Inhibitory potency of P899 was also lower than argatroban in IC_{50} value and by haemostatometry. Thus, weak potency of P891 and P899 is a general feature of this class of inhibitors. These findings suggest that the affinity to thrombin in an in vitro purified system using synthetic substrate do not correlate with the in vivo situation reflected by the haemostatometry.

P553 is a potent synthetic thrombin inhibitor with a K_i value of 2.1 pM. Its high affinity comes from its simultaneous binding to the active site and the FRE of thrombin. P1053 uses the sequence of hirudin from leech except L-Nle instead of L-Tyr of hirudin sequence in order to minimize the antigenicity of the inhibitor, i.e., hirudin sequence has very low antigenic property. Since the K_i value of P1053 is comparable to that of P553, it was expected to have comparable haemostatometric effect with improved antigenic property and this was indeed demonstrated in the present study.

P824 is homologous to P553, although it is dimerised through the C-terminal cystine residue. The dimer slows down the off-rate of thrombin from the inhibitor in case of excess amount of the inhibitor because thrombin has a high chance to re-bind to another inhibitor segment of the dimer before it is released to the solution. The effect of the dimerisation (or slow off-rate) was studied by haemostatometry. Contrary to our expectation, the efficacy of dimeric P824 was limited and this may be due to the limited access to fibrinogen and platelets by the steric hindrance.

The combination of active site-directed inhibitor, argatroban, and FRE-directed inhibitor, P960, had no additive or synergetic inhibition on either platelet reactivity or coagulation. The potent inhibitory effect of the bifunctional inhibitors could be due to an increased inhibitor concentration on the thrombin molecule. Inhibiting throm-

bin by the potent FRE-directed inhibitors may be useful in various clinical thrombotic conditions.

In conclusion, the effects of active site-directed inhibitors, argatroban, P891 and P899, FRE-directed inhibitor P960, monomeric bifunctional inhibitors P553 and P1053 and dimeric bifunctional inhibitor P824 on shear-induced platelet aggregation and dynamic coagulation were assessed. The most potent inhibitory effects were demonstrated by the monomeric bifunctional inhibitors. This suggests that these compounds can be exploited as potent antithrombotic agents. Thrombin inhibitors equally suppressed platelet reactivity and coagulation. The present findings suggest that these thrombin inhibitors may be useful in the prevention and treatment of arterial thrombosis.

Acknowledgements

The authors would like to express thanks to Drs. T. Yamashita and Y. Sasaki for the valuable discussions. This work was supported in part by a grant from the Science Research Promotion Fund of the Japanese Private School Promotion Foundation.

References

Antman, E.M., 1994. Hirudin in acute myocardial infarction: safety report from the thrombolysis and thrombin inhibition in myocardial infarction (TIMI) 9A trial. Circulation 90, 1624–1630, for the TIMI 9A Investigators.

Badimon, L., Badimon, J.J., Lassila, R., Heras, M., Chesebro, J.H., Fuster, V., 1991. Thrombin regulation of platelet interaction with damaged vessel wall and isolated collagen type I at arterial flow conditions in a porcine model: effects of hirudins, heparin, and calcium chelation. Blood 78, 423–434.

Born, G.V.R., 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194, 927–929.

Cattaneo, M., Pareti, F.I., Zighetti, M.L., Lecchi, A., Lombardi, R., Mannucci, P.M., 1995. Platelet aggregation at high shear is impaired in patients with congenital defects of platelet secretion and is corrected by DDAVP: correlation with the bleeding time. J. Lab. Clin. Med. 125, 540–547.

Chow, T.W., Hellums, J.D., Moake, J.L., Kroll, M.H., 1992. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. Blood 80, 113–120.

Connolly, T.M., Condra, C., Feng, D.M., Cook, J.J., Stranieri, M.T., Reilly, C.F., Nutt, R.F., Gould, R.J., 1994. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. Thromb. Haemostasis 72, 627–633.

DiMaio, J., Gibbs, B., Munn, D., Lefebvre, J., Ni, F., Konishi, Y., 1990. Bifunctional thrombin inhibitors based on the sequence of hirudin$^{45-65}$. J. Biol. Chem. 265, 21698–21703.

Fernandez-Ortiz, A., Badimon, J.J., Falk, E., Fuster, V., Meyer, B., Mailhac, A., Weng, D., Shah, P.K., Badimon, L., 1994. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. JACC 23, 1562–1569.

Gailani, D., Broze, G.J. Jr., 1991. Factor XI activation in a revised model of blood coagulation. Science 253, 909–912.

Gorog, D.A., Kovacs, I.B., 1995. Thrombotic status analyser: measurement of platelet-rich thrombus formation and lysis in native blood. Thromb. Haemostasis 73, 514–520.

Gorog, P., Kovacs, I.B., 1990. Modelling coronary thrombosis from nonanticoagulated human blood in vitro. Hematol. Pathol. 4, 43–52.

Goto, S., Sakai, H., Goto, M., Ono, M., Ikeda, Y., Handa, S., Ruggeri, Z.M., 1999. Enhanced shear-induced platelet aggregation in acute myocardial infarction. Circulation 99, 608–613.

GUSTO IIa Investigators, 1994. Randomized trial of intravenous heparin versus recombinant hirudin for acute coronary syndromes. Circulation 90, 1631–1637.

GUSTO IIb Investigators, 1996. A comparison of recombinant hirudin with heparin for the treatment of acute coronary syndromes. N. Engl. J. Med. 335, 775–782.

Hamburger, S.A., McEver, R.P., 1990. GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood 75, 550–554.

Hara, T., Yokoyama, A., Ishihara, H., Yokoyama, Y., Nagahara, T., Iwamoto, M., 1994. DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. Thromb. Haemostasis 71, 314–319.

Harker, L.A., Hanson, S.R., Runge, M.S., 1995. Thrombin hypothesis of thrombus generation and vascular lesion formation. Am. J. Cardiol. 75, 12B–17B.

Ikarugi, H., Taka, T., Nakajima, S., Noguchi, T., Watanabe, S., Sasaki, Y., Haga, S., Ueda, T., Seki, J., Yamamoto, J., 1999. Norepinephrine, but not epinephrine, enhances platelet reactivity and coagulation after exercise in humans. J. Appl. Physiol. 86, 133–138.

Ikeda, Y., Handa, M., Kamata, T., Kawano, K., Kawai, Y., Watanabe, K., Kawakami, K., Sakai, K., Fukuyama, M., Itagaki, I., Yoshioka, A., Ruggeri, Z.M., 1993. Transmembrane calcium influx associated with von Willebrand factor binding to GP Ib in the initiation of shear-induced platelet aggregation. Thromb. Haemostasis 69, 496–502.

Kahn, M.L., Zheng, Y.W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R.V., Tam, C., Coughlin, S.R., 1998. A dual thrombin receptor system for platelet activation. Nature 394, 690–694.

Kikumoto, R., Tamao, Y., Tezuka, T., Tonomura, S., Hara, H., Ninomiya, K., Hijikata, A., Okamoto, S., 1984. Selective inhibition of thrombin by (2*R*, 4*R*)-4-methyl-1-[*N*2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl)]-2-piperidinecarboxylic acid. Biochemistry 23, 85–90.

Krstenansky, J.L., Broersma, R.J., Owen, T.J., Payne, M.H., Yates, M.T., Mao, S.J.T., 1990. Development of MDL 28,050, a small stable antithrombin agent based on a functional domain of the leech protein, hirudin. Thromb. Haemostasis 63, 208–214.

Lopez-Farre, A., Riesco, A., Digiuni, E., Mosquera, J.R., Caramelo, C., Miguel, L.S., Millas, I., Frutos, T., Cernadas, M.R., Monton, M., Alonso, J., Casado, S., 1996. Aspirin-stimulated nitric oxide production by neutrophils after acute myocardial ischemia in rabbits. Circulation 94, 83–87.

Maraganore, J.M., Bourdon, P., Jablonski, J., Ramachandran, K.L., Fenton, J.W. II, 1990. Design and characterization of hirulogs: a novel class of bivalent peptide inhibitors of thrombin. Biochemistry 29, 7095–7101.

Markwardt, F., 1991. Hirudin and derivatives as anticoagulant agents. Thromb. Haemostasis 66, 141–152.

Matsuo, T., Koide, M., Kario, K., 1997. Development of argatroban, a direct thrombin inhibitor, and its clinical application. Semin. Thromb. Hemostasis 23, 517–522.

Moake, J.L., 1995. Thrombotic thrombocytopenic purpura. Thromb. Haemostasis 74, 240–245.

Mustonen, P., Lassila, R., 1996. Epinephrine augments platelet recruitment to immobilized collagen in flowing blood-evidence for a von Willebrand factor-mediated mechanism. Thromb. Haemostasis 75, 175–181.

Oliver, J.A., Monroe, D.M., Roberts, H.R., Hoffman, M., 1999. Throm-

bin activates factor XI on activated platelets in the absence of factor XII. Arterioscler., Thromb. Vasc. Biol. 19, 170–177.

Peters, N.S., Abrams, L.S.M., Dymond, D.S., Kovacs, I.B., 1989. Platelet hyperreactivity and inefficient spontaneous thrombolysis in patients at high risk from an acute coronary event. Cardiovasc. Res. 23, 567–572.

Ratnatunga, C.P., Edmondson, S.F., Rees, G.M., Kovacs, I.B., 1992. High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation. Circulation 85, 1077–1082.

Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., Fenton, J.W. II, 1990. The structure of a complex of recombinant hirudin and human α-thrombin. Science 249, 277–280.

Sakariassen, K.S., Ottenhof-Rovers, M., Sixma, J.J., 1984. Factor VIII-von Willebrand factor requires calcium for facilitation of platelet adherence. Blood 63, 996–1003.

Sasaki, Y., Morii, S., Yamashita, T., Yamamoto, J., 1993. Antithrombotic effect of argatroban on the pial vessels of the rat: a study with He–Ne laser-induced thrombus formation. Haemostasis 23, 104–111.

Siess, W., Zangl, K.J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G., Aefelbacher, M., 1999. Lysophosphatidic acid mediates the rapid acivaiton of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc. Natl. Acad. Sci. U. S. A. 96, 6931–6936.

Sixma, J.J., 1994. Interaction of blood platelets with the vessel wall. In: Blood, A.S., Forbes, C.D., Thomas, D.P., Tuddenham, E.G.D. (Eds.), Haemostasis and Thrombosis. Livingstone, London, pp. 259–285.

Szewczuk, Z., Gibbs, B.F., Yue, S.Y., Purisima, E.O., Konishi, Y., 1992. Conformationally restricted thrombin inhibitors resistant to proteolytic digestion. Biochemistry 31, 9132–9140.

Tsuda, Y., Cygler, M., Gibbs, B.F., Pedyczak, A., Fethiere, J., Yue, S.Y., Konishi, Y., 1994. Design of potent bivalent thrombin inhibitors based on hirudin sequence: incorporation of nonsubstrate-type active site inhibitors. Biochemistry 33, 14443–14451.

Uchiyama, S., Yamazaki, M., Maruyama, S., Handa, M., Ikeda, Y., Fukuyama, M., Itagaki, I., 1994. Shear-induced platelet aggregation in cerebral ischemia. Stroke 25, 1547–1551.

Umemura, K., Kawai, H., Ishihara, H., Nakashima, M., 1995. Inhibitory effect of clopidogrel, vapiprost and argatroban on the middle cerebral artery thrombosis in the rat. Jpn. J. Pharmacol. 67, 253–258.

Valles, J., Santos, M.T., Aznar, J., Marcus, A.J., Martinez-Sales, V., Portoles, M., Broekman, M.J., Safier, L.B., 1991. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, adenosine diphosphate release, and recruitment. Blood 78, 154–162.

Vu, T.K.H., Hung, D.T., Wheaton, V.I., Coughlin, S.R., 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64, 1057–1068.

Yamamoto, J., Taka, T., Nakajima, S., Ueda, M., Sugimoto, E., Sasaki, Y., Muraki, T., Seki, J., Watanabe, S., 1999. A shear-induced in vitro platelet function test can assess clinically relevant anti-thrombotic effects. Platelets 10, 178–184.

Yamashita, T., Tsuda, Y., Konishi, Y., Okada, Y., Matsuoka, A., Giddings, J.C., Yamamoto, J., 1998. The antithrombotic effect of potent bifunctional thrombin inhibitors based on hirudin sequence, P551 and P532, on He–Ne laser-induced thrombosis in rat mesenteric microvessels. Thromb. Res. 90, 199–206.