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YM-60828, a novel factor Xa inhibitor: Separation of its antithrombotic effects from its prolongation of bleeding time

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Abstract

The antithrombotic effects of intravenous infusions of YM-60828 ([*N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]acetic acid dihydrochloride), a novel factor Xa inhibitor, argatroban, heparin and dalteparin in an arterio–venous shunt model were studied in comparison with their effects on template bleeding time. In an arterio–venous shunt model, all agents exerted antithrombotic effects in a dose-dependent manner. ID_{50} values of YM-60828, argatroban, heparin and dalteparin were 0.0087 mg/kg/h, 0.027 mg/kg/h, 22 IU/kg/h and 11 IU/kg/h, respectively. In bleeding time studies, all agents prolonged bleeding time in a dose-dependent manner. Doses (ED_2) of YM-60828, argatroban, heparin and dalteparin, which caused 2-fold prolongation of bleeding time in the saline group, were 3.0 mg/kg/h, 0.25 mg/kg/h, 18 IU/kg/h and 26 IU/kg/h, respectively. The risk-benefit ratio (ED_2/ID_{50}) of YM-60828 was much greater than that of the other agents. These data suggest that the antithrombotic effect of YM-60828 is separate from its prolongation of bleeding time and that YM-60828 is much safer than conventional anticoagulant agents. © 1997 Elsevier Science B.V.

Keywords: YM-60828; Argatroban; Heparin; Dalteparin; Arterio–venous shunt; Bleeding time

1. Introduction

Heparin (Damus et al., 1973) and warfarin (Hirsh, 1991) have been widely used in anticoagulant therapy. Despite their long history of use, their use is associated with problems such as difficulty in controlling their anticoagulant activity and their adverse effect on bleeding. Since thrombin is the final product in the coagulation cascade (Rosenberg et al., 1975), inhibition of thrombin could lead to efficient anticoagulant activity. In fact, potent anticoagulant agents that specifically inhibit thrombin have recently been developed (Lefkovits and Topol, 1994). However, since thrombin-induced platelet activation and aggregation is crucial for normal hemostasis (Vu et al., 1991; Verstraete and Zoldhelyi, 1995), bleeding is the major problem of the thrombin inhibitors in clinical use (Maffrand, 1992). Therefore, agents which can be easily controlled and have little effect on bleeding are now being sought for clinical use.

The activated serine-protease factor Xa (FXa) is the key enzyme at the convergent point of the intrinsic and extrinsic coagulant pathways. It forms a prothrombinase complex with factor Va, calcium and phospholipid to produce thrombin (Rosenberg et al., 1975). Therefore it is thought that anticoagulant effects can be more efficiently exerted by inhibiting FXa rather than thrombin. Moreover, because FXa inhibitors affect coagulation specifically, but not platelet function, this mechanism should notably decrease the tendency to bleed. FXa-inhibiting peptides derived from natural products, as well as DX-9065a, a synthetic and selective FXa inhibitor, have been reported to exert antithrombotic effects in various thrombosis models (Neeper et al., 1990; Schaffer et al., 1993; Yamazaki et al., 1994; Herbert et al., 1996). Hara reported that DX-9065a inhibited thrombosis without affecting the bleeding time (Hara et al., 1995).

Recently, YM-60828, a potent and selective FXa inhibitor, has been synthesized in our laboratory. This compound inhibits human FXa with a K_i value of 1.3 nM (Taniuchi et al., submitted). In this study, the antithrombotic effects of intravenous infusions of YM-60828 and the currently used anticoagulant agents, argatroban (a spe-

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cific thrombin inhibitor, Okamoto et al., 1981), heparin and dalteparin (a low molecular weight heparin, Hamano et al., 1992) in an arterio–venous shunt model were studied in comparison with their effects on template bleeding time.

2. Materials and methods

2.1. Materials

YM-60828 ([*N*-[4-[(1-acetimidoyl-4-piperidyl)-oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]-acetic acid dihydrochloride) was synthesized at Yamanouchi Pharmaceutical Co. Heparin sodium, dalteparin sodium and argatroban were purchased from Takeda Chemical (Shimizu®, Osaka), Kissei Pharmaceutical (Fragmin®, Nagano) and Tokyo-Tanabe Pharmaceutical (Novastan®, Tokyo), respectively. YM-60828 was dissolved in saline before use. Heparin, dalteparin and argatroban were diluted with saline.

2.2. Arterio–venous shunt model in rats

Non-fasted male Sprague–Dawley rats (300–330 g, Japan SLC, Hamamatsu) were anesthetized by intraperitoneal injection of urethane (0.96 g/kg). The left jugular vein and the right carotid artery were cannulated with a 12 cm long polyethylene tube (o.d. 0.965 mm, PE-50, Clay Adams, NJ, USA). These catheters were connected to the ends of a 10 cm long polyethylene tube (o.d. 1.52 mm, PE-100, Clay Adams, NJ, USA) containing a 2 cm long copper wire (o.d. 0.3 mm). All agents were administered via the femoral vein by infusion 2 h before blood circulation in the shunt. Ten minutes after blood circulation started, the copper wire was gently removed and the thrombus attached to the wire was dissolved in 2 ml of 0.5 N NaOH. The thrombus protein content was measured by photometry, using a dye-binding assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a protein standard.

After blood circulation, a 1 ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior vena cava. Platelet-poor plasma was immediately prepared by centrifugation ($9510G \times 10$ min, TCF-12, Iwaki, Japan) at room temperature. Anticoagulant activity was measured with a coagulometer (KC-10, Amelung, FRG). To measure prothrombin time, 50 μl of platelet-poor plasma was incubated for 1 min at 37°C. Coagulation was induced by the introduction of 50 μl of prothrombin time reagent (Ortho-Clinical Diagnostic K.K., Tokyo). To measure activated partial thromboplastin time, 50 μl of Platelet-poor plasma and activated partial thromboplastin time reagent (Ortho-Clinical Diagnostic K.K., Tokyo) were mixed and incubated for 3 min at 37°C. Coagulation was induced by the introduction of 50 μl of 20 mM $CaCl_2$ solution. Anticoagulant activity was expressed as the relative increase in coagulation time compared with that in the saline group.

2.3. Template bleeding time in rats

Non-fasted male Sprague–Dawley rats (290–310 g, Japan SLC, Hamamatsu) were anesthetized by intraperitoneal injection of urethane (0.96 g/kg). All agents were administered via the femoral vein by infusion 2 h before the right planta was incised. A template bleeding device (Simplate®, Organon Teknika, Tokyo) was placed on the right planta and triggered. Blood flowing from the incision was gently wiped away with filter paper every 30 s. Bleeding time was measured as time elapsed until bleeding stopped. When template bleeding time was prolonged beyond 30 min, measurement was stopped and the bleeding time was recorded as 30 min.

After measurement of bleeding time, a 1 ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior vena cava. Coagulation time was measured by the methods described above.

2.4. Statistical analysis

All data represent the means ± S.E.M. Statistical analysis was performed by using Dunnett's multiple comparison test for coagulation time or Steel's test for the arterio–venous shunt model and template bleeding time, compared with the saline group. A P value of less than 0.05 was considered significant.

2.5. Ethical considerations

All experiments were performed in accordance with the regulations of the Animal Ethical Committee of Yamanouchi Pharmaceutical.

3. Results

3.1. Arterio–venous shunt model in rats

Fig. 1 represents the inhibition ratio of the anticoagulant agents relative to saline (2.27 ± 0.198 mg, $n = 20$). All agents exerted antithrombotic effects in a dose-dependent manner ($n = 6$). YM-60828 and argatroban significantly inhibited thrombus formation at doses of 0.01 mg/kg/h and 0.03 mg/kg/h, respectively. Heparin and dalteparin exerted significant antithrombotic effects at doses of 30 IU/kg/h and 10 IU/kg/h, respectively. Table 1 shows approximate ID_{50} values estimated from the dose-inhibition curves. In the arterio–venous shunt model, ID_{50} values of YM-60828, argatroban, heparin and dalteparin were 0.0087 mg/kg/h, 0.027 mg/kg/h, 22 IU/kg/h and 11 IU/kg/h, respectively. The antithrombotic effect of YM-

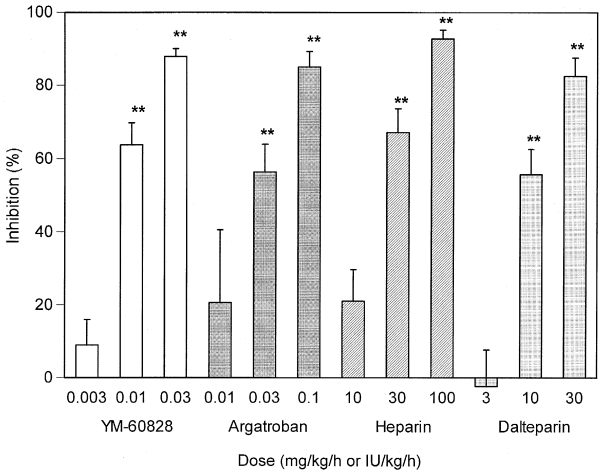

Fig. 1. Antithrombotic effects of YM-60828 (open columns), argatroban (closed columns), heparin (hatched columns) and dalteparin (stippled columns) after intravenous infusion in the rat arterio–venous shunt model. The agents were administered 2 h before blood circulation in the shunt. Data represent the percent inhibition compared with the thrombus protein content in the saline group and are expressed as means ± S.E.M. ($n = 6$). Statistical analysis was performed by using Steel's test. $^{**}P < 0.01$ compared with the saline group.

60828 was about 3-fold more potent than that of argatroban in the arterio–venous shunt model.

Table 2 shows the effects of the anticoagulant agents on coagulation time in the arterio–venous shunt model. YM-60828 and argatroban did not prolong coagulation time even at doses which completely inhibited thrombus formation. Heparin prolonged activated partial thromboplastin time in a dose-dependent manner. The antithrombotic effect correlated closely with the prolongation of activated partial thromboplastin time. Dalteparin prolonged activated partial thromboplastin time significantly at a dose of 30 IU/kg/h, which completely inhibited thrombus formation, but the prolongation of coagulation time was far shorter than that caused by heparin.

3.2. Template bleeding time in rats

Fig. 2 represents the effects of the anticoagulant agents on template bleeding time. Template bleeding time in the saline group was 3.3 ± 0.26 min ($n = 12$). All agents prolonged template bleeding time in a dose-dependent manner ($n = 6$). YM-60828 and argatroban prolonged bleeding time significantly at doses of 3 mg/kg/h and 0.3 mg/kg/h, respectively. Both heparin and dalteparin prolonged bleeding time significantly at doses of 30 IU/kg. However, heparin greatly prolonged bleeding time at a dose of 100 IU/kg/h. Table 1 shows ED_2, the dosage causing a 2-fold prolongation of bleeding time in the saline group, values estimated from the dose–response curves. ED_2 values of YM-60828, argatroban, heparin and dalteparin were 3.0 mg/kg/h, 0.25 mg/kg/h, 18 IU/kg/h and 26 IU/kg, respectively. YM-60828 was more than

Table 1
Risk–benefit ratio of the anticoagulant agents

	YM-60828 (mg/kg/h)	Argatroban (mg/kg/h)	Heparin (IU/kg/h)	Dalteparin (IU/kg/h)
ID_{50}	0.0087	0.027	22	11
ED_2	3.0	0.25	18	26
ED_2/ID_{50}	340	9.3	0.82	2.4

ID_{50}: dose causing 50% inhibition in the arterio–venous shunt model.
ED_2: dose causing 2-fold prolongation of bleeding time in the saline group.

Table 2
The effects of the anticoagulant agents on coagulation time in the arterio–venous shunt model

		Prothrombin time	Activated partial thromboplastin time
YM-60828 (mg/kg/h)	0.003	1.0 ± 0.0	1.0 ± 0.1
	0.01	1.0 ± 0.1	1.0 ± 0.1
	0.03	1.0 ± 0.0	1.0 ± 0.0
Argatroban (mg/kg/h)	0.01	1.1 ± 0.0	1.1 ± 0.0
	0.03	1.1 ± 0.1	1.0 ± 0.0
	0.1	1.1 ± 0.0	1.1 ± 0.0
Heparin (IU/kg/h)	10	1.0 ± 0.1	1.1 ± 0.0
	30	1.2 ± 0.0	2.1 ± 0.1[a]
	100	2.0 ± 0.1[a]	> 10[a]
Dalteparin (IU/kg/h)	3	1.0 ± 0.1	1.0 ± 0.0
	10	1.1 ± 0.1	1.1 ± 0.0
	30	1.0 ± 0.0	1.3 ± 0.1[a]

Data represent the relative increase in coagulation time compared with that of the saline group (prothrombin time: 25 ± 0.82 s, activated partial thromboplastin time: 38 ± 1.3 s) and are expressed as means ± S.E.M. ($n = 6$). The infusions of the agents were started 2 h before blood sampling. Statistical analysis was performed by Dunnett's multiple comparison test. [a] $P < 0.01$ compared with the saline group.

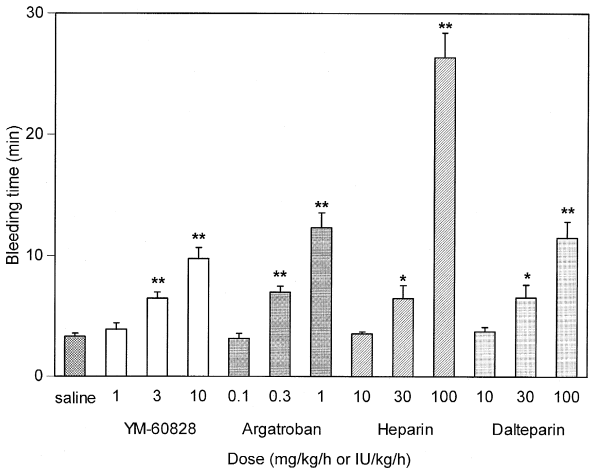

Fig. 2. Effects of YM-60828 (open columns), argatroban (closed columns), heparin (hatched columns) and dalteparin (stippled columns) on rat template bleeding time after intravenous infusion. The agents were administered 2 h before incision of the planta. Data are expressed as mean ± S.E.M. ($n = 6$–12) bleeding times. Statistical analysis was performed by using Steel's test. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the saline group.

Table 3
The effects of the anticoagulant agents on coagulation time in the study of bleeding time

		Prothrombin time	Activated partial thromboplastin time
YM-60828 (mg/kg/h)	1	1.8 ± 0.1^a	1.2 ± 0.0
	3	3.3 ± 0.1^a	1.7 ± 0.1^a
	10	4.8 ± 0.1^a	2.6 ± 0.1^a
Argatroban (mg/kg/h)	0.1	1.1 ± 0.1	1.0 ± 0.0
	0.3	1.3 ± 0.0^a	1.5 ± 0.1^a
	1	1.8 ± 0.1^a	1.9 ± 0.0^a
Heparin (mg/kg/h)	10	1.0 ± 0.1	1.1 ± 0.1
	30	1.1 ± 0.1^a	2.2 ± 0.2^a
	100	2.1 ± 0.2^a	$> 10^a$
Dalteparin (mg/kg/h)	10	1.1 ± 0.0	1.1 ± 0.1
	30	1.1 ± 0.0	1.4 ± 0.1^a
	100	1.2 ± 0.1^a	4.0 ± 0.2^a

Data represent the relative increase in coagulation time compared with that of the saline group (prothrombin time: 24 ± 0.58 s, activated partial thromboplastin time: 26 ± 0.53 s) and are expressed as means $\pm$ S.E.M. ($n = 6$). The infusions of the agents were started 2 h before blood sampling. Statistical analysis was performed by Dunnett's multiple comparison test. [a] $P < 0.01$ compared with the saline group.

10-fold less potent than argatroban in prolonging the bleeding time.

Table 3 shows the effects of the anticoagulant agents on coagulation time in the study of bleeding time. All agents prolonged coagulation time in a dose-dependent manner and all prolonged activated partial thromboplastin time significantly at doses which significantly prolonged bleeding time. The prolongation of bleeding time correlated closely with that of activated partial thromboplastin time.

3.3. Risk-benefit ratio of the anticoagulant agents

Table 1 shows ED_2/ID_{50} values of the anticoagulant agents as the risk-benefit ratio in terms of therapeutic windows. The ratio of YM-60828 was 30-fold greater than that of argatroban, 140-fold greater than that of dalteparin, and more than 400-fold greater than that of heparin.

4. Discussion

In this study, the antithrombotic effects of intravenous infusion of YM-60828, a new, potent and selective FXa inhibitor, and of the currently used anticoagulant agents, argatroban, heparin and dalteparin in a rat arterio–venous shunt model were studied in comparison with their effects on template bleeding time. The antithrombotic effect of YM-60828 was markedly different from its effect on prolongation of the bleeding time compared with that of the other anticoagulant agents.

This study used an arterio–venous shunt model with a copper wire to induce thrombogenesis. In our previous study, the antithrombotic potency of the anticoagulant agents in this model was the same as that in the well-established stasis-induced venous thrombosis model (Sato et al., submitted). The arterio–venous shunt model produces a mixed thrombus of fibrin and platelets, with the total size of the thrombus depending on the formation of a fibrin thrombus (Peters et al., 1991). Therefore, this model is thought to be suitable for the evaluation of the antithrombotic activity of anticoagulant agents.

According to our previous study, the agents used in this study had different in vivo durations of activity (data not shown). Therefore, to estimate the effects of these agents accurately, the agents were administered by intravenous infusion. Since the anticoagulant activity (the prolongation of prothrombin time and activated partial thromboplastin time) of all agents used in this study became constant 2 h after infusion (data not shown), measurements were taken 2 h after the intravenous infusion started.

It is important to note that while YM-60828 exerted potent antithrombotic effects, it had little effect on the bleeding time. The risk-benefit ratio of YM-60828 was 30-fold greater than that of argatroban, 140-fold greater than that of dalteparin, and more than 400-fold greater than that of heparin. Thrombin not only cleaves fibrinogen, but also potently activates platelets (Lefkovits and Topol, 1994). Its affinity for platelets is 10,000-fold higher than that for fibrinogen (Higgins et al., 1983; Berndt et al., 1986). Since YM-60828 is a competitive FXa inhibitor, YM-60828 cannot suppress the production of thrombin completely. Therefore the minimal amount of thrombin sufficient to activate and aggregate platelets for hemostasis is produced, although it is not enough to cleave fibrinogen. In fact, DX-9065a, another competitive FXa inhibitor, also exerts its antithrombotic effect without prolonging the bleeding time (Hara et al., 1995; Herbert et al., 1996).

YM-60828 potently inhibited FXa with a K_i value of 1.3 nM, but did not affect thrombin ($K_i = 100$ μM) in human plasma (Taniuchi et al., submitted). In human plasma, YM-60828 doubled the prothrombin time and the activated partial thromboplastin time at 0.21 and 0.24 μM, respectively. It is well known that there are considerable species differences in regard to the sensitivity of anticoagulant agents. In fact, in rat plasma, the concentrations of YM-60828 needed to double the prothrombin time and activated partial thromboplastin time were 1.7 and 1.3 μM, and were more than 5-fold higher than those in human plasma. In humans, YM-60828 may exert antithrombotic effects at the lower dose than in rats.

Dalteparin exerted antithrombotic effects that were about 3-fold more potent than those of heparin, although it did not prolong bleeding time as greatly as heparin did. Heparin is a glycosaminoglycan with an average molecular weight of 10,000–15,000 (Andersson et al., 1979). Heparin forms a complex with antithrombin III, which exerts antithrombotic activity (Rosenberg and Damus, 1973). Since heparin shows not only anti-FXa activity but also antithrombin activity, use of heparin involves some prob-

lems such as an increased bleeding tendency. Low molecular weight heparins with a molecular weight of 2000–9000 have been reported to show antithrombotic properties linked to FXa inhibition rather than antithrombin activity (Holmer et al., 1982; Carter et al., 1982). Dalteparin is a low molecular weight heparin and is expected to decrease the adverse effect on bleeding because it exerts anti-FXa activity comparable to that of normal heparin, but does not have the antithrombin activity of normal heparin (Hamano et al., 1992). The results of the present study support these previous reports. However, the risk–benefit ratio of dalteparin is only about 3-fold greater than that of heparin, which means that the safety of dalteparin is similar to that of heparin.

Thrombin is the final product of the coagulation cascade (Rosenberg et al., 1975). Therefore, agents that specifically inhibit thrombin are thought to have potent anticoagulant activity. Recently such agents have been vigorously developed (Lefkovits and Topol, 1994). Among these, a specific thrombin inhibitor, argatroban, has been shown to have potent antithrombotic effects (Imura et al., 1992; Berry et al., 1994a). In this study, argatroban exerted potent antithrombotic effects, but argatroban also prolonged the bleeding time at a dose 10-fold lower than that of YM-60828. Since argatroban shows potent antiplatelet activity (Hara et al., 1986) in addition to its potent antithrombotic effect, it may increase the bleeding tendency by inhibiting the platelet aggregation induced by thrombin, which plays a crucial role in hemostasis. However, the risk–benefit ratio of argatroban is more than 10-fold higher than that of heparin. One possible explanation for this is that argatroban may exert its antithrombotic effect at a comparatively low dose compared with that of heparin, because the heparin–antithrombin III complex is inaccessible to clot-bound thrombin (Mirshahi et al., 1989; Weitz et al., 1990), while argatroban is a potent inhibitor of clot-associated thrombin (Berry et al., 1994b).

YM-60828, argatroban and dalteparin exerted significant antithrombotic effects even at doses which only slightly prolonged prothrombin time and activated partial thromboplastin time compared with the effect of heparin. It seems that prothrombin time and activated partial thromboplastin time may not be the most suitable parameters for monitoring the antithrombotic effect of the agents used in this study. More appropriate parameters (for example: Thrombin Time for argatroban, Xa clotting time for YM-60828, etc.) which correlate more closely with their antithrombotic effects should be measured. In contrast, the prolongation of activated partial thromboplastin time correlated closely with the prolongation of bleeding time for all agents used in this study. The details of the mechanism have yet to be defined, but thrombin produced via the intrinsic pathway of the coagulation cascade may play an important role in hemostasis.

In conclusion, the antithrombotic effects of intravenous infusions of YM-60828, a new, potent and selective FXa inhibitor, and argatroban, heparin and dalteparin in an arterio–venous shunt model were studied in comparison with their effects on template bleeding time. The antithrombotic effects of YM-60828 were different from its effects on the prolongation of bleeding time, more than those of the other agents. Therefore YM-60828 may prove to be a much safer agent for clinical use than conventional anticoagulant agents.

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