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Antithrombotic effect of YM-75466 is separated from its effect on bleeding time and coagulation time

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Abstract

The antithrombotic effects of YM-75466 ([*N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]acetic acid monomethane sulfonate), a novel orally-active factor Xa inhibitor, and its effects on bleeding time and coagulation time were studied in rats and compared with those of warfarin. Both agents were orally administered. In the venous thrombosis model, YM-75466 and warfarin inhibited thrombus formation dose-dependently, with ID_{50} values of 3.3 and 0.56 mg/kg, respectively. Ex vivo study showed that both YM-75466 and warfarin prolonged prothrombin time dose-dependently, with doses, causing a two-fold prolongation of prothrombin time in the control group, of 89 and 0.38 mg/kg, respectively. In bleeding time studies, YM-75466 and warfarin prolonged bleeding time dose-dependently, with doses, causing a two-fold prolongation of bleeding time in the control group, of >100 and 0.43 mg/kg, respectively. These results show that the antithrombotic effects of YM-75466 are markedly separate from its effects on bleeding time and coagulation time compared with warfarin.

Keywords: YM-75466; Warfarin; Oral anticoagulant; Factor Xa inhibitor; Venous thrombosis; Bleeding time

1. Introduction

Warfarin has been widely used as an oral anticoagulant agent. Warfarin exerts its potent anticoagulant effects by inhibiting biosynthesis of vitamin K-dependent coagulant factors (factor II, VII, IX, X, etc.). However, due to its mode of action, warfarin has many clinical problems such as slow onset of action, narrow therapeutic range, adverse effects on bleeding and interaction with many drugs and foods (Harder and Thurmann, 1996; Hirsh, 1991; Wells et al., 1994). Moreover, administration of warfarin must be strictly controlled in clinical use due to fear of inducing severe bleeding. Therefore, novel oral anticoagulant agents are required which have new mechanisms of action and are much safer and easier to use than warfarin.

The activated serine-protease factor X (FXa) is the key enzyme at the convergent point of the intrinsic and extrinsic coagulation pathways. It forms a prothrombinase complex with factor Va, Ca^{2+} and phospholipid to produce thrombin (Rosenberg et al., 1975) and it has been demonstrated that one molecule of FXa can generate 138 molecules of thrombin in 1 min (Elódi and Varadi, 1979). Therefore, anticoagulant effects may be more efficiently exerted by inhibiting FXa rather than thrombin. Moreover, because FXa inhibitors specifically affect the plasma coagulation system, but not platelet function, this mechanism should notably decrease the increased bleeding tendency seen in warfarin therapy. FXa-inhibiting peptides derived from natural products and DX-9065a, a synthetic and selective FXa inhibitor, have been reported to exert antithrombotic effects in various thrombosis models (Schaffer et al., 1992; Neeper et al., 1990; Herbert et al., 1996). Moreover, DX-9065a inhibited thrombosis without affecting bleeding time (Hara et al., 1995). Thus, inhibition of FXa is a promising target for the treatment of thrombosis.

YM-75466 is a newly synthesized, potent and selective FXa inhibitor. This compound specifically inhibits human FXa with a K_i value of 1.3 nM (Taniuchi et al., 1998). Our previous studies show that intravenous administration

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of YM-75466 exerts profound antithrombotic effects without prolonging bleeding time and coagulation time compared to heparin, a low molecular weight heparin, dalteparin, and a thrombin inhibitor, argatroban (Sato et al., 1997, 1998). Additionally, YM-75466 can exert its anticoagulant effects also when administered orally (Taniuchi et al., 1998). Therefore, it is crucial to investigate whether, by oral administration, YM-75466 still exerts its antithrombotic effects without prolonging bleeding time and coagulation time.

To clarify whether YM-75466 is easier to use than warfarin, the antithrombotic effects of YM-75466 and its effects on bleeding time and coagulation time were studied in rats and compared with those of warfarin.

2. Materials and methods

2.1. Materials

YM-75466 (Fig. 1, [*N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]acetic acid monomethanesulfonate) was synthesized at Yamanouchi Pharmaceutical. Warfarin sodium was purchased from Sigma (USA). YM-75466 was dissolved in 0.05 N HCl before use. Warfarin was dissolved in distilled water.

2.2. Ex vivo studies

YM-75466 and warfarin were orally administered to male Sprague–Dawley rats (280–320 g, Japan, Hamamatsu, Japan) which had fasted more than 12 h. A 2 ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior vena cava of rats anesthetized by intraperitoneal injection of urethane (0.96 g/kg). In dose-dependency studies, YM-75466 was administered 0.5 h and warfarin 18 h before blood sampling. Platelet-poor plasma was immediately prepared by centrifugation ($1870 \times g$; 10 min; PR05-22, Hitachi, Japan) at 4°C. 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 adding 50 μl of prothrombin reagent (Ortho-Clinical Diagnostic, Tokyo, Japan). To measure activated partial thromboplastin time, 50 μl of platelet-poor plasma and activated partial thromboplastin time reagent (Ortho-Clinical Diagnostic) were mixed and incubated for 3 min at 37°C. Coagulation was induced by adding 50 μl of 20 mM $CaCl_2$. Anticoagulant activity was expressed as the relative increase in coagulation time compared with that before the administration of the agent or that in the control group.

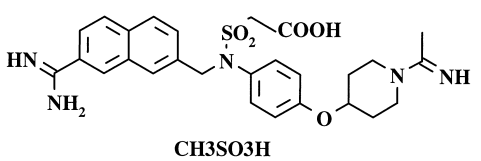

Fig. 1. Structure of YM-75466. *N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]acetic acid monomethanesulfonate.

2.3. Thromboplastin-induced venous thrombosis model in rats

Thrombus formation was induced using the method of Herbert (Herbert et al., 1992). Male Sprague–Dawley rats (290–320 g, Japan, Hamamatsu, Japan) which had fasted more than 12 h were anesthetized by intraperitoneal injection of urethane (0.96 g/kg). The abdomen was surgically opened and the inferior vena cava was isolated. Venous thrombosis was induced by injection of thromboplastin (25 μg/kg, Ortho-Clinical Diagnostic, Tokyo, Japan) via the femoral vein as a bolus. One min after injection of thromboplastin, two tight ligations 1.0 cm apart with cotton thread were made on the inferior vena cava just below the left renal venous branch. YM-75466 was orally administered 0.5 h and warfarin 18 h before injection of thromboplastin. Ten min after ligation, the vascular segment between the ligations was longitudinally opened by incision. The thrombus was gently removed and dissolved in 2 ml of 0.5 N NaOH. The protein content of the thrombus was measured by a photometric method using a dye-binding assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA) as a protein standard.

2.4. Template bleeding time in rats

Male Sprague–Dawley rats (280–320 g, Japan) which had fasted more than 12 h were anesthetized by intraperitoneal injection of urethane (0.96 g/kg). YM-75466 was orally administered 0.5 h and warfarin 18 h before the right planta was incised. A template bleeding device (Simplate®, Organon Teknika, Tokyo, Japan) 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.

2.5. Statistical analysis

All data represent the mean ± S.E.M.. Statistical analysis was performed by using Steel's test compared with the control group. A P value of less than 0.05 was considered significant.

2.6. Ethical considerations

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

3. Results

3.1. Ex vivo studies

Oral administration of 30 mg/kg YM-75466 maximally prolonged both prothrombin time (1.6 ± 0.1 times) and activated partial thromboplastin time (1.2 ± 0.0 times) 0.5 h after administration. Anticoagulant activity almost completely disappeared 4 h after administration (Fig. 2a, b). In contrast, oral administration of 0.5 mg/kg warfarin maximally prolonged both prothrombin time (2.1 ± 0.1 times) and activated partial thromboplastin time (1.5 ± 0.1 times) 18 h after administration. Anticoagulant activity almost completely disappeared 48 h after administration (Fig. 2c, d). Therefore, the following experiments were performed 0.5 h after oral administration of YM-75466 and 18 h after oral administration of warfarin.

Both YM-75466 and warfarin prolonged both prothrombin time and activated partial thromboplastin time in a dose-dependent manner either 0.5 or 18 h after their respective oral administrations (Table 1). The dose-dependency of YM-75466 was not steep, while that of warfarin for prothrombin time was markedly steep. Table 2 shows the CT_2 values of YM-75466 and warfarin, which were the doses causing a two-fold prolongation of prothrombin time in the control group and estimated from the dose–response curves. Warfarin prolonged prothrombin time at a greater than 200-fold smaller dose than YM-75466.

Table 1
Anticoagulant effects of YM-75466 and warfarin

		Prothrombin time (relative increase)	Activated partial thromboplastin time (relative increase)
YM-75466	25 mg/kg	1.5 ± 0.0	1.2 ± 0.0
	50 mg/kg	1.6 ± 0.1	1.3 ± 0.0
	100 mg/kg	2.1 ± 0.1	1.5 ± 0.1
Warfarin	0.25 mg/kg	1.0 ± 0.0	1.0 ± 0.0
	0.5 mg/kg	1.9 ± 0.1	1.6 ± 0.1
	1 mg/kg	5.9 ± 0.1	1.8 ± 0.1

Data represent the relative increase in coagulation time compared with that in the control group and are expressed as mean ± S.E.M. ($n = 6$).
Agents were orally administrated 0.5 h (YM-75466) or 18 h (warfarin) before blood sampling.
Prothrombin time in the control group: 21 ± 0.36 s (0.5 h) and 21 ± 0.31 s (18 h).
Activated partial thromboplastin time in the control group: 32 ± 0.59 s (0.5 h) and 30 ± 0.56 s (18 h).

3.2. Thromboplastin-induced venous thrombosis model in rats

Both YM-75466 and warfarin exerted antithrombotic effects in a dose-dependent manner ($n = 6$, Fig. 3). YM-

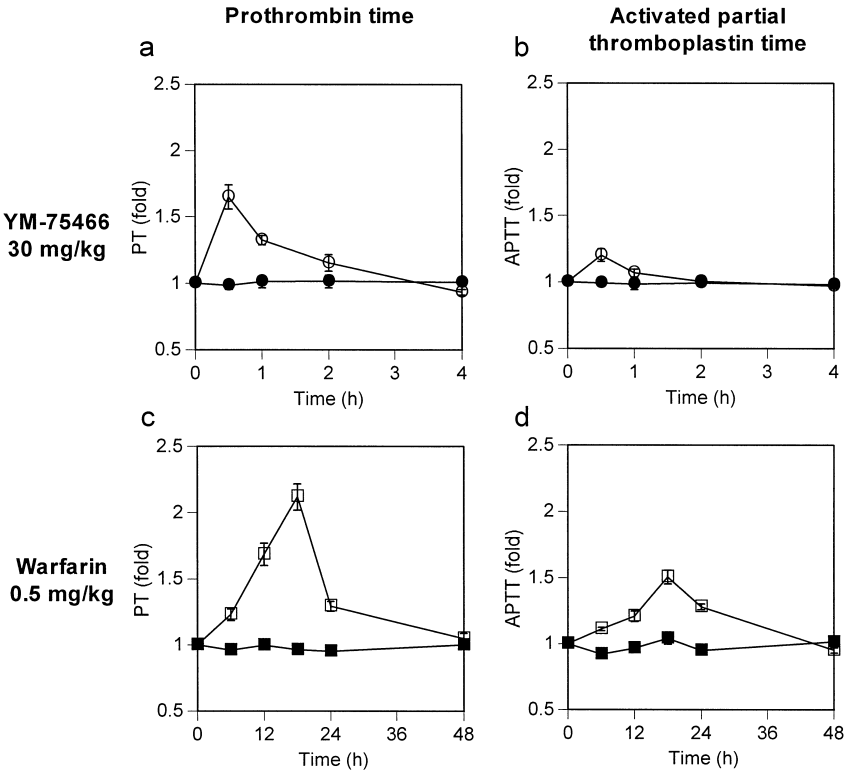

Fig. 2. Time course of the anticoagulant effects of YM-75466 and warfarin on prothrombin time (PT, panel a, c) and activated partial thromboplastin time (APTT, panel b, d) in rats. YM-75466 30 mg/kg (open circles), warfarin 0.5 mg/kg (open squares) and the vehicle (closed circles and squares) were orally administered. Data represent the relative increase in coagulation time compared with that before the administration of the agents (prothrombin time: 21 ± 0.43 s; activated partial thromboplastin time: 30 ± 0.47 s) as the mean ± S.E.M. ($n = 6$).

Table 2
Separation of antithrombotic effects from prolongation of bleeding time or coagulation time

	YM-75466 (mg/kg)	Warfarin (mg/kg)
ID_{50}	3.3	0.56
ED_2	> 100	0.43
CT_2	89	0.38

ID_{50}: dose causing 50% inhibition in the venous thrombosis model.
ED_2: dose causing two-fold prolongation of bleeding time in the control group.
CT_2: dose causing two-fold prolongation of prothrombin time in the control group.

75466 and warfarin significantly inhibited thrombus formation at doses of 2.5 and 0.5 mg/kg, respectively, compared with each control group (2.57 ± 0.134 and 2.52 ± 0.103 mg). Table 2 shows the ID_{50} values of YM-75466 and warfarin, which were estimated from the dose–inhibition curve. Warfarin inhibited thrombus formation at a greater than five-fold smaller dose than YM-75466.

3.3. Template bleeding time in rats

Both YM-75466 and warfarin prolonged template bleeding time in a dose-dependent manner ($n = 6$, Fig. 4). YM-75466 and warfarin prolonged bleeding time significantly at doses of 100 and 0.5 mg/kg, respectively, compared with each control group (3.5 ± 0.32 and 3.3 ± 0.21 min). Table 2 shows the ED_2 values of YM-75466 and warfarin, which were the doses causing a two-fold prolongation of bleeding time in the control group and estimated from the dose–response curves. Although YM-75466 did not exert two-fold prolongation of bleeding time even at a dose of 100 mg/kg, higher doses could not be investigated due to its insolubility at greater concentrations. Warfarin prolonged bleeding time at a greater than 200-fold smaller dose than YM-75466.

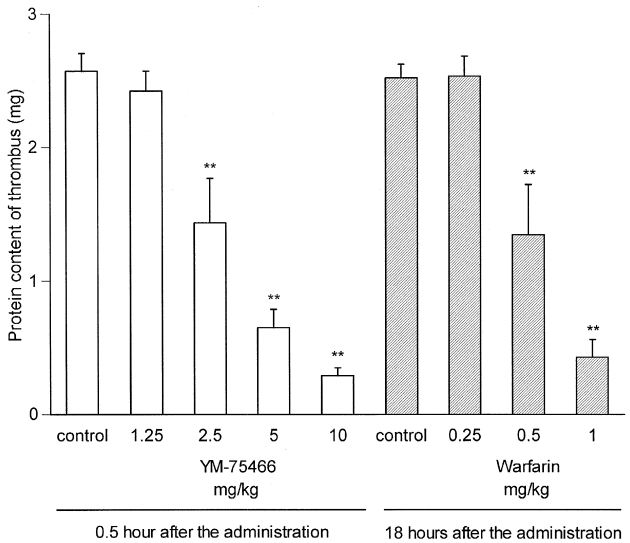

Fig. 3. Antithrombotic effects of YM-75466 (open columns) and warfarin (hatched columns) in the thromboplastin-induced venous thrombosis model in rats. YM-75466 was orally administered 0.5 h and warfarin 18 h before the injection of 25 μg/kg thromboplastin. Data are expressed as mean ± S.E.M. ($n = 6$). Statistical analysis was performed by using Steel's test. ** $P < 0.01$ compared with the control group.

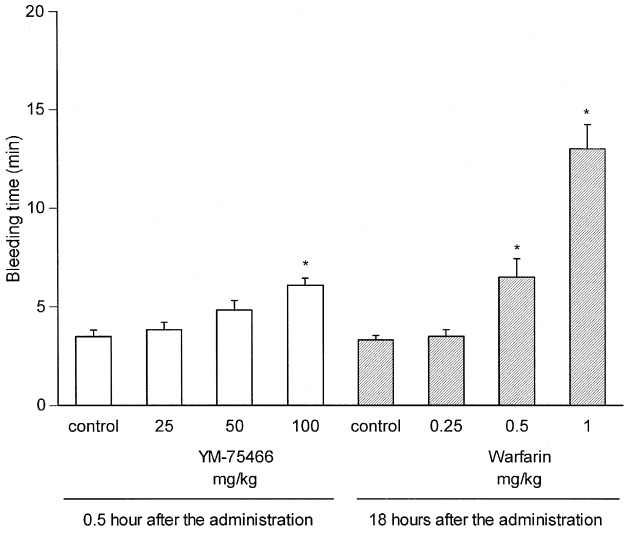

Fig. 4. Effects of YM-75466 (open columns) and warfarin (hatched columns) on template bleeding time in rats. YM-75466 was orally administered 0.5 h and warfarin 18 h before incision of the planta. Data are expressed as mean ± S.E.M. ($n = 6$). Statistical analysis was performed by using Steel's test. * $P < 0.05$ compared with the control group.

4. Discussion

In this study, the antithrombotic effects of YM-75466, a novel orally-active FXa inhibitor, and its effects on bleeding time and coagulation time were studied in rats and compared with those of warfarin. The antithrombotic effects of YM-75466 were markedly separate from its prolongation of bleeding time and coagulation time compared to those of warfarin.

Ex vivo studies showed that YM-75466 exerted its anticoagulant effects much faster than warfarin. Warfarin exerts its anticoagulant effects by inhibiting biosynthesis of vitamin K-dependent coagulant factors (Hirsh, 1991). In contrast, YM-75466 exerts its anticoagulant effects through direct inhibition of FXa (Taniuchi et al., 1998). Therefore, it is thought that YM-75466 acts much more rapidly than warfarin due to these different mechanisms of inhibition. Although these two agents, YM-75466 and warfarin, have quite different modes of action, it is valid to compare their pharmacological profiles if the study is performed at the anticoagulant activity peak of each agent. The thromboplastin-induced venous thrombosis model employed in this study is generally and widely used to estimate antithrombotic activity of anticoagulant agents (Talbot et al., 1989; Freund et al., 1990). Also, in this study, both YM-75466 and warfarin exerted dose-dependent antithrombotic activ-

ity, suggesting that this model is suitable to estimate antithrombotic activity of different anticoagulant agents even if they have different modes of action.

YM-75466 exerted potent antithrombotic effects with little prolongation of bleeding time. 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). Therefore, the minimal amount of thrombin sufficient to activate and aggregate platelets for hemostasis is likely to be produced, although it is not enough to cleave fibrinogen, which leads to fibrin clot formation. Furthermore, the antithrombotic effects of YM-75466 were markedly separate from its prolongation of coagulation time. The exact mechanism of how YM-75466 exerts its antithrombotic effect at a dose which hardly prolongs coagulation time has yet to be clarified in detail. However, it has been demonstrated that DX-9065a, a synthetic FXa inhibitor, can inhibit clot-bound FXa which is much more active than free FXa in plasma (Hérault et al., 1997). Therefore, YM-75466 may exert efficient antithrombotic activity through inhibition of FXa specifically on the thrombus at a dose which has little effect on coagulation time of peripheral blood. The lack of the need to monitor coagulation time when using YM-75466 would, if confirmed in human, seem to be of profound clinical merit.

In contrast, the antithrombotic effects of warfarin were closely correlated with its prolongation of bleeding time and coagulation time. In the ex vivo studies, the dose-dependency of warfarin for prothrombin time was much steeper than that of YM-75466. Although it is said that the therapeutic dose of warfarin prolongs prothrombin time by 1.5 to 2.5 times, it seems to be a difficult practical matter to control prothrombin time in this range due to its steep dose-dependency. This difficulty in controlling the dose of warfarin may be one reason which gives rise to problems such as severe bleeding, drug interaction, and the need for close dose control by monitoring peripheral blood.

5. Conclusion

In conclusion, this study clearly demonstrates that the antithrombotic effects of YM-75466 are markedly separate from its effects on bleeding time and coagulation time compared with warfarin. These results suggest that YM-75466 may prove to be an oral anticoagulant agent which is much easier to use than warfarin.

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