Comparison of the Anticoagulant and Antithrombotic Effects of YM-75466, a Novel Orally-Active Factor Xa Inhibitor, and Warfarin in Mice

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Received April 10, 1998 Accepted July 21, 1998

ABSTRACT—The anticoagulant and antithrombotic effects of YM-75466 (N-[4-[(1-acetimidoyl-4-piperidyl) oxy]phenyl]-N-[7-amidino-2-naphthyl)methyl]sulfamoyl acetic acid monomethanesulfonate), a novel orally-active factor Xa (FXa) inhibitor, and warfarin were compared in mice. Both agents were orally administered in all studies. In ex vivo studies, the peak effects of YM-75466 occurred 1 hr after administration while the peak of warfarin activity occurred 18 hr after administration. At each peak, both YM-75466 and warfarin prolonged coagulation time dose-dependently. The dose response curve of warfarin for prothrombin time was steeper than that of YM-75466. In a thromboplastin-induced thromboembolism model, administration of 30 mg/kg YM-75466 or 3 mg/kg warfarin significantly improved the lethality ratio. In blood loss studies, YM-75466 did not increase blood loss from the tail even at 30 mg/kg, while warfarin markedly increased blood loss at 3 mg/kg. Agents that interfere with warfarin action did not interfere with YM-75466 action. In conclusion, this study shows that YM-75466 has advantages over warfarin: i) rapid onset of anticoagulant activity, ii) wide therapeutic range, iii) little effect on bleeding and iv) lack of drug interaction with agents that interfere with warfarin. These results suggest that YM-75466 may be promising as a novel oral anticoagulant agent.

Keywords: YM-75466, Warfarin, Oral anticoagulant, Factor Xa inhibitor

Warfarin has been widely used as an oral agent in anticoagulant therapy. Warfarin exerts its potent anticoagulant effects by inhibiting biosynthesis of the vitamin K-dependent coagulant factor. However, due to its mode of action, the use of 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 (1–3). Therefore, novel oral anticoagulant agents having new mechanisms of action have been actively sought.

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, Ca²⁺ and phospholipid to produce thrombin (4). Therefore it is thought that anticoagulant effects can be exerted more efficiently by inhibiting FXa than thrombin. Moreover, it is thought that the risk of uncontrollable bleeding will decrease because FXa inhibitors specifically affect the plasma coagulation system but not platelet function, which plays a crucial role in normal hemostasis. Thus, inhibition of FXa is a promising target for the treatment of thrombosis.

Previous studies indicated that intravenous administration of YM-60828 (N-[4-[(1-acetimidoyl-4-piperidyl) oxy]phenyl]-N-[7-amidino-2-naphthyl)methyl]sulfamoyl acetic acid dihydrochloride), a FXa inhibitor that selectively inhibits human FXa with a Kᵢ value of 1.3 nM (5), exerts profound antithrombotic effects without prolonging the bleeding time or coagulation time compared with heparin; dalteparin, a low molecular weight heparin; and argatroban, a thrombin inhibitor (6, 7). However, YM-60828 was unsuitable for the oral administration study due to its chemical instability. Therefore, YM-75466 (N-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-N-[7-amidino-2-naphthyl)methyl]sulfamoyl acetic acid monomethanesulfonate), which has the same chemical structure as YM-60828 but is a methanesulfonate salt compound, was newly synthesized. It is crucial to study the effects of orally-administered YM-75466 and clarify whether YM-75466 can solve the problems in warfarin use.
In this study, the anticoagulant and antithrombotic effects of YM-75466, a novel orally-active FXa inhibitor, were studied in mice and compared to those of warfarin.

MATERIALS AND METHODS

Ex vivo studies

YM-75466 and warfarin were administered orally (10 ml/kg) to male ICR mice (26–33 g; Japan SLC, Hamamatsu) which had fasted for more than 12 hr. Blood samples, 1 ml each, with citrate as the anticoagulant (1:10 dilution, 3.8% sodium citrate), were collected from the inferior vena cava of mice after anesthetization with ether. Platelet-poor plasma (PPP) was immediately prepared by centrifugation at 4320 x g for 10 min (CF-15D2; Hitachi, Tokyo) at 4°C. Anticoagulant activity was measured with a coagulometer (KC-10; Amelung, Lebriningsweg, FRG). To measure prothrombin time (PT), 50 μl of PPP was incubated for 1 min at 37°C. Coagulation was induced by adding 50 μl of PT reagent (Ortho-Clinical Diagnostics K.K., Tokyo). To measure activated partial thromboplastin time (APTT), 50 μl of PPP and APTT reagent (Ortho-Clinical Diagnostics K.K.) were mixed and incubated for 3 min at 37°C. Coagulation was induced by adding 50 μl of 20 mM CaCl₂. Six mice were used for each treatment.

Thromboplastin-induced thromboembolism model in mice

Male ICR mice (28–32 g, Japan SLC) that had fasted more than 12 hr were orally given (10 ml/kg) either YM-75466 1 hr or warfarin 18 hr before the experiment began. Ten mice were used in each treatment group. The mice were injected with 25 mg/kg thromboplastin (dissolved in saline, Ortho-Clinical Diagnostics K.K.) into the tail vein. Survival of the mice for 10 min after thromboplastin injection was recorded and used as the index for the antithrombotic effects of the agents.

Blood loss in mice

Male ICR mice (28–32 g, Japan SLC) that had fasted for more than 12 hr were orally given (10 ml/kg) either YM-75466 1 hr or warfarin 18 hr before the experiment began. Eight mice were used in each treatment group. The mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Tail tips, 5 mm from their distal ends, were surgically transected with a surgical blade (No. 22; Futaba, Tokyo). Immediately after transection, the tails were immersed in 10 ml of saline at 37°C for 20 min. The collected blood was hemolyzed by the addition of 1 ml of Triton X-100 (25% dilution in water), and the optical density was measured photometrically at 546 nm. A standard curve was constructed with pooled blood collected from three intact mice. The amount of blood lost was deduced from the standard curve.

Drug interaction in mice

Cimetidine (100 mg/kg), carbamazepine (30 mg/kg), rifampicin (30 mg/kg), erythromycin (100 mg/kg), phenytoin (30 mg/kg) or phytomadione (10 mg/kg) were orally administered (10 ml/kg) to non-fasted male ICR mice (31–42 g, Japan SLC) twice daily (at 9:00 and 19:00) for five days. Each agent was used at a dose lower than its maximal pharmacological activity, which did not affect coagulation time in the control group. From the third day, YM-75466 (200 mg/kg) and warfarin (1.5 mg/kg) were orally administered (10 ml/kg) once daily (at 13:00) for three days.

At 15:00 on Day 5, a 600-μl citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior vena cava of mice after anesthetization with ether. Prothrombin time was measured by the methods described previously. When PT was prolonged beyond 120 sec, measurement was stopped and PT was recorded as 120 sec. Eight mice were used in each treatment group.

Drugs

YM-75466 (Fig. 1) was synthesized at Yamanouchi Pharmaceutical Co. Warfarin potassium, cimetidine, carbamazepine, rifampicin, erythromycin stearate, phenytoin and phytomadione were purchased from Eisai Co. (Warfarin®, Tokyo), Fujisawa Pharmaceutical Co. (Tagamet®, Osaka), Ciba-Geigy Japan (Tegretol®, Takarazuka), Daichi Pharmaceutical Co. (Rifadin®, Tokyo), Dainippon Pharmaceutical Co. (Erythrocin®, Osaka), Dainippon Pharmaceutical Co. (Aleviatin®) and Eisai Co. (Kaywan®), respectively. All agents were suspended in a 0.5% methylcellulose (MC) solution.

Statistical analyses

All data except the lethality ratio in the thromboplastin-induced thromboembolism model represent the
mean±S.E.M. Statistical analysis was performed by Steel's test for blood loss and drug interaction and by the $\chi^2$ test for the thromboplastin-induced thromboembolism model for comparisons with the control group. A P value of less than 0.05 was considered significant.

**Ethical considerations**

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

**RESULTS**

**Ex vivo studies**

Oral administration of 100 mg/kg YM-75466 maximally prolonged both PT and APTT 1 hr after administration. The anticoagulant activity completely disappeared 4 hr after administration. In contrast, oral administration of 3 mg/kg warfarin maximally prolonged both PT and APTT maximally 18 hr after the administration. The anticoagulant activity completely disappeared 36 hr after administration (Fig. 2). Therefore, the following experiments were performed 1 hr after oral administration of

![Fig. 2](image-url)  
**Fig. 2.** Time course of the anticoagulant effects of YM-75466 and warfarin on prothrombin time (PT, panel A) and activated partial thromboplastin time (APTT, panel B). YM-75466 at 100 mg/kg (○), warfarin at 3 mg/kg (▲) and 0.5% MC solution (■) were orally administered. Data are expressed as the mean±S.E.M. (n=6).

![Fig. 3](image-url)  
**Fig. 3.** Dose-dependency of the anticoagulant effects of YM-75466 and warfarin on prothrombin time (PT, panel A) and activated partial thromboplastin time (APTT, panel B). YM-75466 (○) and warfarin (▲) were orally administered either 1 or 18 hr, respectively, before blood sampling. Data are expressed as the mean±S.E.M. (n=6).
YM-75466 and 18 hr after oral administration of warfarin.

Both YM-75466 and warfarin dose-dependently prolonged both PT and APTT either 1 hr (control PT: 12.3±0.202 sec, control APTT: 32.3±0.634 sec) or 18 hr (control PT: 12.0±0.141 sec, control APTT: 32.4±0.387 sec) after oral administration (Fig. 3). The PT dose response curve of warfarin was steeper than that of YM-75466.

**Thromboplastin-induced thromboembolism model in mice**

The lethality ratios in the MC control group were 90% at both 1 and 18 hr after administration in mice. YM-75466 dose-dependently improved the lethality ratio and exerted significant antithrombotic effects at 30 mg/kg. Warfarin dose-dependently improved the lethality ratio and exerted significant antithrombotic effects at 3 mg/kg (Fig. 4).

**Blood loss in mice**

The volumes of blood lost in the 20 min following tail transection in the MC control group were 15.6±11.5 and 16.4±5.43 μl 1 and 18 hr after oral administration, respectively. YM-75466 did not significantly increase the volume of blood lost at 30 mg/kg, a dose at which it exerted significant antithrombotic effects in the thromboplastin-induced thromboembolism model. In contrast, warfarin markedly increase the volume of blood lost at 3 mg/kg, at dose at which it exerted significant antithrombotic effects (Fig. 5).

**Drug interaction in mice**

Prothrombin times in the MC chronic administration group administered with 0.5% MC solution, YM-75466 or warfarin were 11.9±0.261, 24.6±2.25 and 28.4±4.55 sec, respectively. Prothrombin time in the control group was not affected by chronic administration of the tested agents. The anticoagulant activity of warfarin was significantly enhanced in the cimetidine and erythromycin chronic administration groups, while it was significantly attenuated in the carbamazepine, rifampicin, phenytoin and phytonadione chronic administration groups. In contrast, the anticoagulant activity of YM-75466 was not affected by chronic administration of any agent (Fig. 6).

![Graph showing lethality ratio (%) against various doses of YM-75466 and warfarin](image)

**Fig. 4.** Protective effects of YM-75466 and warfarin on the thromboplastin-induced thromboembolism in mice. YM-75466 was orally administered 1 hr and warfarin 18 hr before injection of 25 mg/kg thromboplastin. Data represent the lethality ratio in mice (n=10). Statistical analysis was performed by the χ² test. **P<0.01, compared with the MC control group.
Comparison of YM-75466 and Warfarin

Fig. 5. Effects of YM-75466 and warfarin on blood loss in mice. YM-75466 was orally administered 1 hr and warfarin 18 hr before tail transection. Data represent the volume of blood lost for 20 min as the mean ± S.E.M. (n = 8). Statistical analyses was performed by Steel’s test. **P < 0.01, compared with the 0.5% MC control group.

Fig. 6. Lack of drug interaction between YM-75466 and the agents (0.5% MC: □, cimetidine; □, carbamazepine; □, rifampicin: □, erythromycin; □, phenytoin: □, phytonadione: □) that interfere with the anticoagulant effects of warfarin. Data represent the prothrombin time as the mean ± S.E.M. (n = 8). Statistical analysis was performed by Steel’s test. *P < 0.05, **P < 0.01, compared with the MC chronic administration group.
DISCUSSION

In this study, to clarify whether YM-75466 can solve the problems in warfarin use, the anticoagulant and antithrombotic effects of YM-75466, a novel orally-active FXa inhibitor, were studied in mice and compared to those of warfarin.

An ex vivo study showed that YM-75466 exerted its anticoagulant effect more rapidly than warfarin. Warfarin exerts its anticoagulant effects by inhibiting biosynthesis of the vitamin K-dependent coagulant factor (2). In contrast, YM-75466 exerts its anticoagulant effects through direct inhibition of FXa (5). Therefore, it is thought that YM-75466 acted more rapidly than warfarin. Moreover, the duration of the anticoagulant effects of YM-75466 was shorter than that of warfarin. This result suggests that YM-75466 is easier to manage than warfarin in the sense that in the event of hemorrhage, hemostatic function could be more rapidly restored after the end of administration of YM-75466 than that of warfarin. Additionally, the PT dose-dependent curve of warfarin was steeper than that of YM-75466. This suggests that the therapeutic range of YM-75466 is wide and therefore YM-75466 has a greater safety margin than warfarin. Considering that the clinically effective dose of warfarin exerts a 1.5- to 2.5-fold prolongation of PT, warfarin is an agent whose anticoagulant effects are difficult to control. This narrow therapeutic range may be one reason for problems associated with warfarin use such as bleeding, drug interaction, and the need for close dose control by monitoring peripheral blood.

The thromboplastin-induced thromboembolism model used in this study leads to death from pulmonary thromboembolism due to fibrin clot induced by thromboplastin injection (8). Since the thrombus formed is composed of fibrin, this model is suitable and widely used for evaluating the antithrombotic activity of anticoagulant agents (8, 9). In this model, YM-75466 significantly improved the lethality ratio of the mice at 30 mg/kg. At this dose, YM-75466 slightly prolonged PT by 1.3-fold. In contrast, warfarin prolonged PT by 1.5-fold at 1 mg/kg, but at this dose, warfarin did not significantly improve lethality ratio. These results suggest that YM-75466 exerts its antithrombotic effects more efficiently than warfarin in light of their effects on the plasma coagulant system. Although the mechanism of this has yet to be clarified in detail, it has been demonstrated that DX-9065a, another synthetic FXa inhibitor, can inhibit clot-bound FXa, which is much more active than free FXa in plasma (10). Similarly, YM-75466 may exert efficient antithrombotic activity through inhibition of FXa specifically on the thrombus at a dose that has little effect on the coagulation time of peripheral blood. Moreover, YM-75466 did not increase the volume of blood lost at the dose that exerted significant antithrombotic effects. YM-75466 may exert its antithrombotic effects without affecting bleeding, because YM-75466 affects specifically coagulation but not platelet function, which plays a crucial role in primary hemostasis. Warfarin significantly improved the lethality ratio at 3 mg/kg. However, at this dose, warfarin significantly increased the volume of blood lost and prolonged PT by fivefold. Although the therapeutic dose of warfarin prolongs PT by 1.5- to 2.5-fold, it is difficult to control PT in this range due to its steep dose-dependency. This difficulty in controlling the dose of warfarin may lead to increased blood loss.

Warfarin is prone to life-threatening drug interactions due to its unfavorable properties such as high protein binding, cytochrome-P450-dependent metabolism and a narrow therapeutic range (1). In this study, the co-administration of cimetidine, carbamazepine, rifampicin, erythromycin and phenytoin interfered with the anticoagulant activity of warfarin. It has been reported that these agents augment or attenuate the action of warfarin through affecting the metabolic enzyme of warfarin in the liver (11–16). It is suggested that the metabolism of YM-75466 is not influenced by these agents. Since warfarin exerts its anticoagulant effects through the inhibition of vitamin K, the anticoagulant effects of warfarin are inhibited by vitamin K or high vitamin K content foods (17–19). In this study, the co-administration of phytonadione (vitamin K3) inhibited the anticoagulant effects of warfarin. Since the anticoagulant effects of YM-75466 were not affected by phytonadione, it is also expected that YM-75466 would not be influenced by high vitamin K content foods.

In conclusion, this study showed that YM-75466 has many advantages over warfarin: i) rapid onset of anticoagulant activity, ii) wide therapeutic range, iii) little effect on bleeding and iv) lack of drug interactions with agents which interfere with warfarin. These results suggest that YM-75466 may be promising as a novel oral anticoagulant agent.

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