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Short communication

Antithrombotic effects of YM-60828 in three thrombosis models in guinea pigs

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Abstract

The antithrombotic effects of a novel factor Xa inhibitor, YM-60828 ([*N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl]acetic acid dihydrochloride), in three thrombosis models in guinea pigs were studied in comparison with its effect on bleeding time. The antithrombotic effects of YM-60828 were most pronounced in the venous thrombosis and the arterio-venous shunt models but YM-60828 showed 10-fold weaker effects in the carotid thrombosis model. However, YM-60828 prolonged bleeding time at a much higher dose than that required in all thrombosis models. In conclusion, YM-60828 exerted its antithrombotic effects without prolonging bleeding time in all thrombosis models and may be of clinical value not only in venous thrombosis but also in arterial thrombosis.

Keywords: YM-60828; Factor Xa inhibitor; Venous thrombosis; Arterial thrombosis; Bleeding time

1. Introduction

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 in the previous study, it has been demonstrated that one molecule of FXa can generate 138 molecules of thrombin in 1 min (Elódi and Varadi, 1979). Therefore, it is thought that the anticoagulant effects can be exerted more efficiently by inhibiting FXa than thrombin. Moreover, the risk of bleeding is expected to decrease because FXa inhibitors affect coagulation specifically but not platelet function. FXa inhibiting peptides and a synthetic FXa inhibitor, DX-9065a, have been reported to exert antithrombotic effects in various thrombosis models (Neeper et al., 1990; Mellott et al., 1992; Herbert et al., 1996). Furthermore, DX-9065a exerted its antithrombotic effects without affecting bleeding time (Hara et al., 1995). Thus, inhibition of FXa is a promising target for the treatment of thrombosis. YM-60828 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) and exerts potent antithrombotic effects in rat thrombosis models (Sato et al., 1997, 1998). In this study, the antithrombotic effects of a novel FXa inhibitor, YM-60828, in three thrombosis models in guinea pigs: a venous thrombosis model, an arterio-venous shunt model and a carotid thrombosis model, were studied in comparison with its effect on 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 Ya-

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manouchi Pharmaceutical. YM-60828 was dissolved in saline.

2.2. Ex vivo studies

Non-fasted male Hartley guinea pigs (230–300 g, Japan SLC, Hamamatsu, Japan) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg). YM-60828 was administered via the femoral vein as a bolus. A 4 ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior vena cava 1 min after drug administration. Platelet-poor plasma was immediately prepared by centrifugation at room temperature. Anticoagulant activity was measured with a coagulometer (KC-10, Amelung, Germany). To measure prothrombin time, platelet-poor plasma and the drug solution were mixed and incubated for 1 min at 37°C. Coagulation was induced by the addition of the prothrombin time reagent (Ortho-Clinical Diagnostic K.K., Tokyo, Japan). To measure activated partial thromboplastin time, platelet-poor plasma, the drug solution and the activated partial thromboplastin time reagent (Ortho-Clinical Diagnostic K.K.) were mixed and incubated for 3 min at 37°C. Coagulation was induced by the addition of a 20 mM $CaCl_2$ solution.

2.3. Venous thrombosis model in guinea pigs

Thrombus formation was induced using the method of Reyers et al. (1980). Non-fasted male Hartley guinea pigs (310–410 g, Japan SLC) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg). The abdomen was surgically opened and the inferior vena cava was isolated. Venous thrombosis was induced by tight ligation of the inferior vena cava just below the left renal venous branch using a cotton thread. YM-60828 was administered via the femoral vein as a bolus 1 min before the ligation. About 2 h after the ligation, the vena cava was clamped about 2 cm below the ligation and the vascular segment between the ligation and the clamp was longitudinally opened. The thrombus was gently removed and dissolved in 2 ml of 0.5 M NaOH. The thrombus protein content was measured by photometry using a dye binding assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA) as a protein standard.

2.4. Arterio-venous shunt model in guinea pigs

Non-fasted male Hartley guinea pigs (270–450 g, Japan SLC) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/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) containing a 2 cm long copper wire (o.d. 0.3 mm). YM-60828 was administered via the femoral vein as a bolus 1 min before blood circulation in the shunt. About 10 min 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 M NaOH. The thrombus protein content was measured by the methods described above.

2.5. Carotid thrombosis model in guinea pigs

Experiments were performed modifying the method of Roux et al. (1994). Non-fasted male Hartley guinea pigs (340–510 g, Japan SLC) were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/kg, Parke Davis, Berlin, Germany) and xylazine (10 mg/kg, Bayer, Leverkusen, Germany). The left carotid artery was carefully dissected free and a 1 mm diameter doppler probe (DBF10R, Primetech, Tokyo, Japan) was put to monitor the blood flow. The carotid blood flow was recorded on a polygraph (WI-681G, Nihon Kohden, Tokyo, Japan). YM-60828 was administered via the femoral vein as a bolus. A distance of 2 mm from the probe, damage to the subendothelium was induced by pinching the carotid artery with surgical forceps (A-14, Natumeseisakujo, Tokyo, Japan) 1 min after the administration of the agents. The pinching was performed 5 times at 1-s intervals. Following thrombus formation, blood flow started to decline and stop. When the flow stopped, gentle shaking of the carotid artery made the occlusive thrombus release and the flow resume. Every time the flow was occluded, restoration of the flow was performed. The frequency of occlusion for 20 min was measured as the index of thrombus formation.

2.6. Template bleeding time in guinea pigs

Template bleeding time was measured using the method of MacDonald et al. (1994). Non-fasted male Hartley guinea pigs (180–360 g, Japan SLC) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg). YM-60828 was administered via the femoral vein as a bolus 1 min before incising the ear. A template bleeding device (Simplate®, Oragnon Teknika, Tokyo, Japan) was placed on the dorsal surface of the auricle and triggered. Blood flowing from the incision was gently wiped away with a filter paper every 30 s. Time elapsed until bleeding stopped was measured as the bleeding time.

2.7. Statistical analysis

All data represent the mean ± S.E.M. Statistical analysis was performed by Dunnett's multiple comparison test for the venous thrombosis and the carotid thrombosis models 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.

2.8. 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

YM-60828 prolonged both prothrombin time and activated partial thromboplastin time in a dose-dependent manner (Table 1). The CT_2 values, which were the doses required to double coagulation time in the saline group, were 0.75 and 0.88 mg/kg for prothrombin time and activated partial thromboplastin time, respectively.

3.2. Venous thrombosis model in guinea pigs

Fig. 1 represents percentage of inhibition of YM-60828 compared with the saline group (2.29 ± 0.26 mg, $n = 18$). YM-60828 exerted antithrombotic effects in a dose-dependent manner ($n = 6$) and significantly inhibited thrombus formation at 0.03 mg/kg. The ID_{50} value of YM-60828 was 0.012 mg/kg in the venous thrombosis model.

3.3. Arterio-venous shunt model in guinea pigs

Fig. 1 represents percentage of inhibition of YM-60828 compared with the saline group (1.13 ± 0.06 mg, $n = 22$). YM-60828 exerted antithrombotic effects in a dose-dependent manner ($n = 6$) and significantly inhibited thrombus formation at 0.01 mg/kg. The ID_{50} value of YM-60828 was 0.0094 mg/kg in the arterio-venous shunt model.

3.4. Carotid thrombosis model in guinea pigs

Fig. 1 represents percentage of inhibition of YM-60828 compared with the saline group (7.73 ± 0.56 times, $n = 15$). YM-60828 exerted antithrombotic effects in a dose-dependent manner ($n = 6$) and significantly inhibited thrombus formation at 0.3 mg/kg. The ID_{50} value of YM-60828 was 0.14 mg/kg in the carotid thrombosis model.

Table 1
Anticoagulant effects of YM-60828

YM-60828 (mg/kg)	Prothrombin time (relative increase)	Activated partial thromboplastin time (relative increase)
0.1	1.1 ± 0.0	1.1 ± 0.0
0.3	1.5 ± 0.1	1.5 ± 0.1
1	1.8 ± 0.1	1.8 ± 0.1
3	3.2 ± 0.1	2.9 ± 0.2

Data represent the relative increase in coagulation time compared with that in the saline group and are expressed as mean ± S.E.M. ($n = 3$).
YM-60828 was intravenously administrated as a bolus 1 min before blood sampling.
Prothrombin time in the control group: 38 ± 3.5 s.
Activated partial thromboplastin time in the control group: 24 ± 1.5 s.

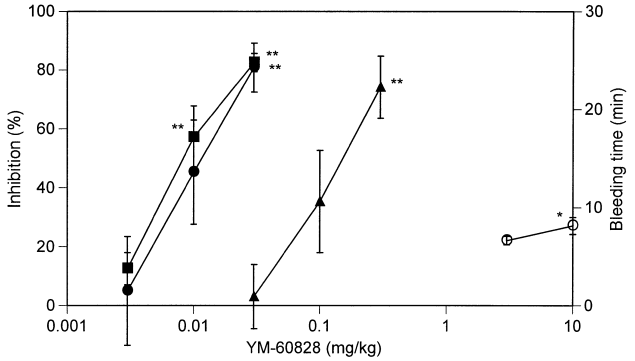

Fig. 1. Antithrombotic effects of YM-60828 in the venous thrombosis (closed circles), the arterio-venous shunt (closed squares) and the carotid thrombosis (closed triangles) models and its effects on template bleeding time (open circles) in guinea pigs. YM-60828 was intravenously administered as a bolus 1 min before each study. Data represent percentage of inhibition compared with the saline group or bleeding time as the mean ± S.E.M. Statistical analysis was performed by Dunnett's multiple comparison test for the venous thrombosis and the carotid thrombosis models or Steel's test for the arterio-venous shunt model and bleeding time compared with the saline group. $* P < 0.05$, $** P < 0.01$ compared with the saline group.

3.5. Template bleeding time in guinea pigs

Fig. 1 represents the effect of YM-60828 on template bleeding time. The bleeding time in the saline group was 5.14 ± 0.52 min ($n = 14$). YM-60828 prolonged bleeding time slightly but significantly at a dose of 10 mg/kg.

4. Discussion

In this study, the antithrombotic effects of a novel FXa inhibitor, YM-60828, were studied in three thrombosis models in guinea pigs: the venous thrombosis model, the arterio-venous shunt model and the carotid thrombosis model, in comparison with its effect on template bleeding time. YM-60828 exerted its antithrombotic effects without prolonging bleeding time in all thrombosis models.

FXa inhibitors have already been described as being effective not only in venous thrombosis but also in arterial thrombosis models (Neeper et al., 1990; Mellott et al., 1992; Herbert et al., 1996). However, to the best of our knowledge, this is the first report directly comparing the antithrombotic effects of a FXa inhibitor in different types of thrombosis model in the same species. This study showed that the antithrombotic effects of YM-60828 were most pronounced in the venous thrombosis model, in

which the thrombus formed is made almost entirely of fibrin and contained few platelets (Reyers et al., 1980), and the arterio-venous shunt model, in which that is made of both platelet and fibrin (Peters et al., 1991). This results support the previous report that although the arterio-venous shunt model is a mixed thrombus model of fibrin and platelets, the total size of the thrombus depends on the formation of a fibrin thrombus (Peters et al., 1991). In contrast, YM-60828 showed a 10-fold weaker effect in the carotid thrombosis model. It is likely that since the thrombus formed in this model is made mainly of platelets (Roux et al., 1994), YM-60828 is effective at a much higher dose than that required in the venous thrombosis or the arterio-venous shunt models. The prolongation of bleeding time was not observed until a dose of 10 mg/kg, while the dose necessary for its antithrombotic effect was more than 30-fold less, greatly separating its efficacy from its prolongation of bleeding time. Thrombin not only cleaves fibrinogen but is also a potent activator of platelets (Lefkovits and Topol, 1994). The affinity of thrombin to platelets is 10 000-fold higher than that to fibrinogen (Higgins et al., 1983; Berndt et al., 1986). Therefore, it seems that even at the effective dose of YM-60828, enough thrombin to activate and aggregate platelets for primary hemostasis is produced, this concentration of thrombin is too low to cleave fibrinogen and lead to secondary hemostasis. Moreover, in the venous thrombosis and arterio-venous shunt models, YM-60828 exerted significant antithrombotic effects even at a dose which prolonged ex vivo coagulation time only slightly. Clinically, administration of agents such as warfarin and heparin must be strictly controlled for fear of bleeding. Although the mechanism by which YM-60828 exerts its antithrombotic effect without prolonging coagulation time has yet to be clarified in detail, the lack of need to monitor coagulation time would, if confirmed in human, seem to be of profound clinical merit.

In this study, YM-60828 was intravenously administered as a bolus. Because the periods required in the thrombosis models used in this study were different from each other, all data may be able to be explained by the clearance rate of YM-60828. However, according to our previous study, YM-60828 (1 mg/kg i.v.) prolonged prothrombin time by 1.7 and 1.6 times at 1 and 30 min after the administration, respectively. This means that the anticoagulant effects of YM-60828 were almost maintained at least 30 min after the bolus administration. Moreover, in the venous thrombosis model, the level of YM-60828 in the vena cava should be maintained during the study because it is thought that, by the ligation, the blood flow is stopped and YM-60828 in the vena cava cannot be metabolized or excreted. Therefore, it is unlikely that all data can be explained by differences in its clearance rate.

In conclusion, YM-60828 exerted its antithrombotic effect without its prolongation of bleeding time in all thrombosis models. Consequently, YM-60828 may prove to be of clinical value not only in venous thrombosis but also in arterial thrombosis.

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