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Relationship between the antithrombotic effect of YM-75466, a novel factor Xa inhibitor, and coagulation parameters in rats

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Abstract

The relationship between the antithrombotic effects of intravenous infusions of YM-75466 [*N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl] sulfamoyl]acetic acid monomethanesulfonate), a novel factor Xa (FXa) inhibitor, and various coagulation parameters (prothrombin time, activated partial thromboplastin time, thrombin–antithrombin III complex (TAT), anti-FXa activity and anti-thrombin activity) in rats was studied and compared with results for heparin. In the arterio–venous shunt model, both agents exerted antithrombotic effects in a dose-dependent manner. Coagulation parameters were studied simultaneously with antithrombotic effects. YM-75466 did not prolong coagulation time even at the dose which exerted significant antithrombotic effects, while it decreased TAT level in plasma in a dose-dependent manner. YM-75466 exerted anti-FXa activity but not anti-thrombin activity. In contrast, heparin prolonged activated partial thromboplastin time in a dose-dependent manner and decreased TAT level in plasma with increasing inhibition of thrombus formation. Heparin exerted both anti-FXa and anti-thrombin activity in a dose-dependent manner. These results suggest that TAT is a suitable parameter for monitoring the antithrombotic effect of YM-75466 in the arterio–venous shunt model in rats and that YM-75466, unlike heparin, exerts its antithrombotic effect through specific inhibition of FXa without any effect on thrombin. © 1998 Elsevier Science B.V.

Keywords: YM-75466; Heparin; Arterio–venous shunt; Thrombin–antithrombin III complex; Factor Xa inhibitor

1. Introduction

The activated serine–protease factor Xa (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). 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 bleeding tendency.

A potent and selective FXa inhibitor, YM-75466, which has been recently synthesized in our laboratory, exerts its antithrombotic effect without prolonging bleeding time in comparison with heparin, a low-molecular-weight heparin and a thrombin inhibitor (Sato et al., 1997, 1998). Moreover, even at a dose which exerts significant antithrombotic effects, YM-75466 insignificantly prolonged both prothrombin time and activated partial thromboplastin time, which have been used for monitoring the antithrombotic effects of conventional anticoagulant agents, such as heparin or warfarin. Similar results were obtained in studies of other FXa inhibitors (Sitko et al., 1992; Hara et al., 1995; Wong et al., 1996). However, a suitable parameter for monitoring the antithrombotic effects of FXa inhibitors, including YM-75466, has not been demonstrated. Recently, sensitive parameters for indicating thrombin generation, such as thrombin–antithrombin III complex (TAT) or prothrombin fragment 1 + 2, have become available for clinical use (Pelzer et al., 1988, 1991; Boneu et al., 1991; Estivals et al., 1991; Deguchi et al., 1991; Kario et al., 1992, 1996). It has been shown that TAT level in rat plasma can be detected by a commercially available human ELISA kit (Ravanat et al., 1995, 1996; Dickneite et

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al., 1994). Therefore, TAT may be an appropriate parameter for indicating the antithrombotic effects of FXa inhibitor in rats, because TAT is a sensitive indicator of thrombin production. Moreover, although YM-75466 has been reported to specifically inhibit FXa with a K_i value of 1.3 nM, but not thrombin ($K_i > 100$ μM) in vitro (Taniuchi et al., 1998), it is necessary to clarify whether YM-75466 exerts its antithrombotic effect by specific inhibition of FXa in vivo.

In this study, the relationship between the antithrombotic effects of YM-75466 and various coagulation parameters (prothrombin time, activated partial thromboplastin time, TAT, anti-FXa activity, anti-thrombin activity) in rats was studied and compared with results for heparin.

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. Heparin sodium was purchased from Takeda Chemical Industries (Shimizu®, Osaka, Japan). YM-75466 was dissolved in saline before use. Heparin was diluted with saline.

2.2. In vitro studies

A 5-ml citrated (1:10 dilution, 3.8% sodium citrate) blood sample was collected from the inferior aorta of male Sprague–Dawley rats (310–320 g, Japan SLC, Hamamatsu, Japan) anesthetized by intraperitoneal injection of urethane (0.96 g kg^{-1}). Platelet-poor plasma was immediately prepared by centrifugation (1870 g; 10 min; PR05-22, HITACHI, Japan) at 4°C. Anticoagulant activity was measured with a coagulometer (KC-10, Amelung, Germany). To measure prothrombin time, platelet-poor plasma and the drug solutions were mixed and incubated for 1 min at 37°C. Coagulation was induced by the addition of the prothrombin time reagent (Ortho-Clinical Diagnostic, Tokyo, Japan). To measure activated partial thromboplastin time, platelet-poor plasma, the drug solutions and the activated partial thromboplastin time reagent (Ortho-Clinical Diagnostic K.K., Tokyo, Japan) were mixed and incubated for 3 min at 37°C. Coagulation was induced by the addition of a 20 mM $CaCl_2$ solution. Each experiment was performed six times.

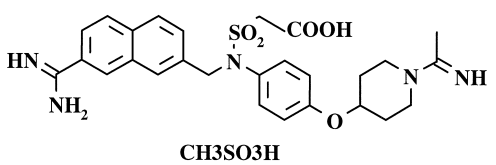

Fig. 1. Structure of YM-75466. *N*-[4-[(1-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl)methyl]sulfamoyl acetic acid monomethanesulfonate.

2.3. Arterio–venous shunt model in rats

Non-fasted male Sprague–Dawley rats (310–340 g, Japan SLC, Hamamatsu, Japan) were anesthetized by intraperitoneal injection of urethane (0.96 g kg^{-1}). 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 30 min 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 M NaOH. The protein content of thrombus was measured by photometry, using a dye-binding assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a protein standard.

2.4. Measurement of coagulation parameters

After blood circulation in the shunt, a 5-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 (1870 g; 10 min; PR05-22, HITACHI, Japan) at 4°C.

Coagulation time was measured by the methods described above.

The levels of TAT in plasma were measured with the ELISA immunoassay Enzygnost® TAT micro (Behringwerke, Germany) using standards of human origin (2 to 60 μg l^{-1} TAT) for calibration.

Anti-FXa activity of plasma from animals after administration of YM-75466 or heparin was measured using a chromogenic substrate, S-2222 (Kabi Vitrum, Sweden), and human FXa (Enzyme Research Laboratories, USA). 7.5 μl of platelet-poor plasma and 30 μl of 2 mM S-2222 were mixed with 87.5 μl of 0.1 M Tris–0.2 M NaCl buffer (pH 8.4). The reaction was started with the addition of 25 μl of 0.05 U ml^{-1} human FXa solution and the mixture was incubated for 30 min at 37°C. The reaction was terminated with the addition of 100 μl of 60% acetic acid and the absorbance was measured at 405 nM. The anti-FXa activity (inhibition %) was calculated as follows; Inhibition % = (1 − O.D. of the inhibitor/O.D. of the saline control) × 100.

Anti-thrombin activity of plasma from animals after administration of YM-75466 or heparin was measured using a chromogenic substrate, S-2238 (Kabi Vitrum, Sweden), and human thrombin (SIGMA, USA). 7.5 μl of

Table 1
The CT_2 values of YM-75466 and heparin in vitro

CT_2	YM-75466 (μM)	Heparin (IU/ml)
PT	1.6	1.5
APTT	2.5	0.073

PT: prothrombin time.
APTT: activated partial thromboplastin time.
CT_2: dose required to double PT and APTT of the control.

platelet-poor plasma and 30 μl of 1 mM S-2238 were mixed with 87.5 μl of 0.1 M Tris–0.2 M NaCl buffer (pH 8.4). The reaction was started with the addition of 25 μl of 0.1 U ml^{-1} human thrombin solution and the mixture was incubated for 45 min at 37°C. The reaction was terminated with the addition of 100 μl of 60% acetic acid and the absorbance was measured at 405 nm. The anti-thrombin activity (inhibition %) was calculated as follows: Inhibition % = (1 − O.D. of the inhibitor/O.D. of the saline control) × 100.

2.5. Statistical analysis

All data represent the means ± S.E.M. Statistical analysis was performed by using Dunnett's multiple comparison test for coagulation parameters or Steel's test for the arterio–venous shunt model compared with the saline 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. In vitro anticoagulant effects

Both YM-75466 and heparin prolonged prothrombin time and activated partial thromboplastin time in a concentration-dependent manner. Table 1 shows the CT_2 values which were the concentrations required to double coagulation time in the saline group and estimated from the concentration response curves. YM-75466 prolonged prothrombin time and activated partial thromboplastin time to the same extent, while heparin doubled activated partial thromboplastin time at about 20-fold lower dose than prothrombin time.

3.2. Arterio–venous shunt model in rats

Both YM-75466 and heparin exerted antithrombotic effects in a dose-dependent manner ($n = 10$, Fig. 2). YM-

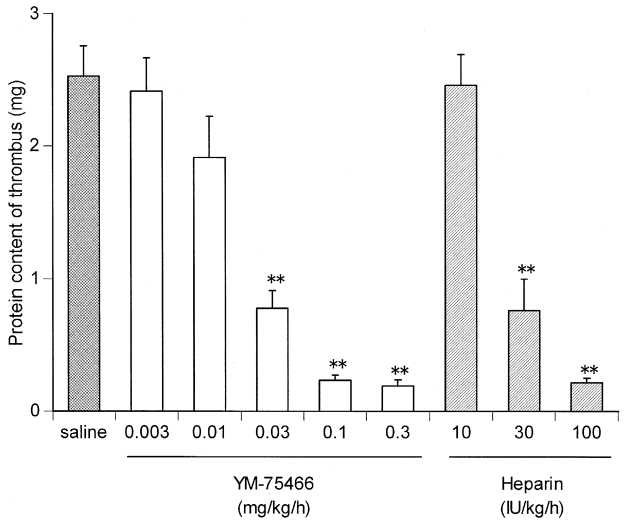

Fig. 2. Antithrombotic effects of YM-75466 (open columns) and heparin (hatched column) after intravenous infusion in the rat arterio–venous shunt model. The agents were administered 30 min before blood circulation in the shunt. Data are expressed as means ± S.E.M. ($n = 10$). Statistical analysis was performed by using Steel's test. ** $P < 0.01$ compared with the saline group.

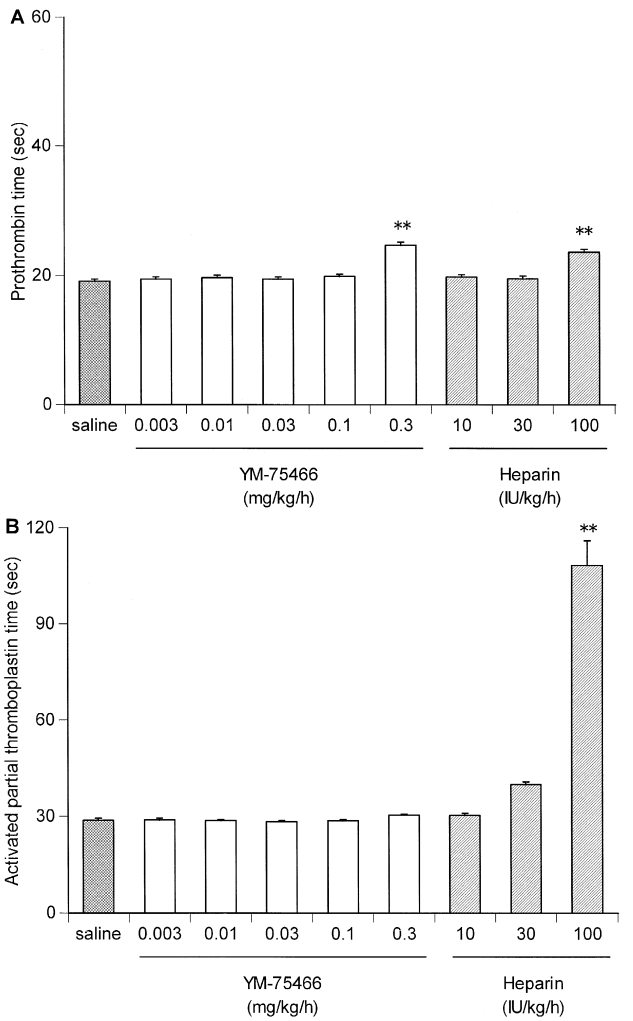

Fig. 3. Effects of YM-75466 (open columns) and heparin (hatched column) on prothrombin time (panel A) and activated partial thromboplastin time (panel B) after intravenous infusion in rats. Data are expressed as means ± S.E.M. ($n = 10$). Statistical analysis was performed by using Dunnett's multiple comparison test. ** $P < 0.01$ compared with the saline group.

75466 and heparin significantly inhibited thrombus formation at doses of 0.03 mg kg^{-1} h^{-1} and 30 IU kg^{-1} h^{-1}, respectively, compared with the saline group (2.52 ± 0.228 mg). ID_{50} values of YM-75466 and heparin, which were estimated from the dose-inhibition curve, were 0.023 mg kg^{-1} h^{-1} and 28 IU kg^{-1} h^{-1}, respectively.

3.3. Effects on coagulation parameters

Both YM-75466 and heparin insignificantly prolonged prothrombin time even at doses which significantly inhibited thrombus formation (Fig. 3A). In contrast, YM-75466 did not prolong activated partial thromboplastin time at all, while heparin prolonged it in a dose-dependent manner and prolonged about 4-fold greater than that of the saline group at the dose of 100 IU kg^{-1} h^{-1} (Fig. 3B).

The basal level of TAT in the control group (only infusion with saline, but no shunt) was 4.02 ± 0.610 μg l^{-1} ($n = 6$) and thrombus formation significantly increased the level of TAT in plasma in the saline group (12.1 ± 2.23 μg l^{-1}, $P = 0.0158$ by Student t-test). Both YM-75466 and heparin decreased the level of TAT in plasma in a dose-dependent manner in proportion to their inhibition of thrombus formation. YM-75466 and heparin significantly decreased the level of TAT in plasma at doses of 0.03 mg kg^{-1} h^{-1} and 30 IU kg^{-1} h^{-1}, respectively (Fig. 4).

Both YM-75466 and heparin exerted anti-FXa activity in a dose-dependent manner. YM-75466 and heparin significantly exerted anti-FXa activity at doses of 0.01 mg kg^{-1} h^{-1} and 30 IU kg^{-1} h^{-1}, respectively (Fig. 5A). In contrast, YM-75466 did not exert anti-thrombin activity at all, while heparin significantly exerted anti-thrombin activity at a dose of 30 IU kg^{-1} h^{-1} (Fig. 5B).

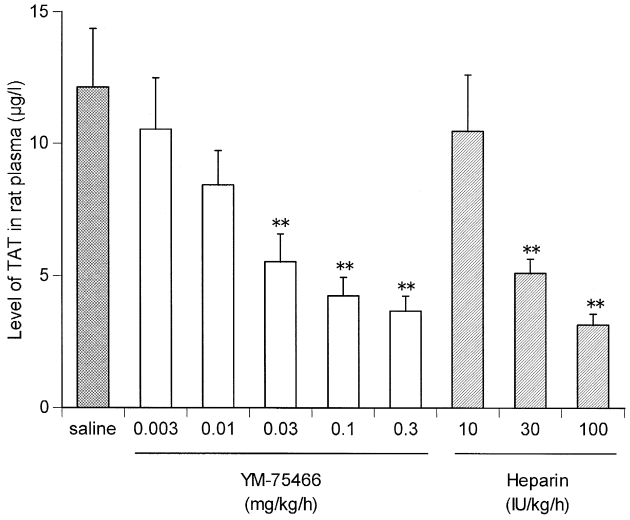

Fig. 4. Effects of YM-75466 (open columns) and heparin (hatched column) on the level of TAT in rat plasma after intravenous infusion. Data are expressed as means ± S.E.M. ($n = 10$). Statistical analysis was performed by using Dunnett's multiple comparison test. $**$ $P < 0.01$ compared with the saline group.

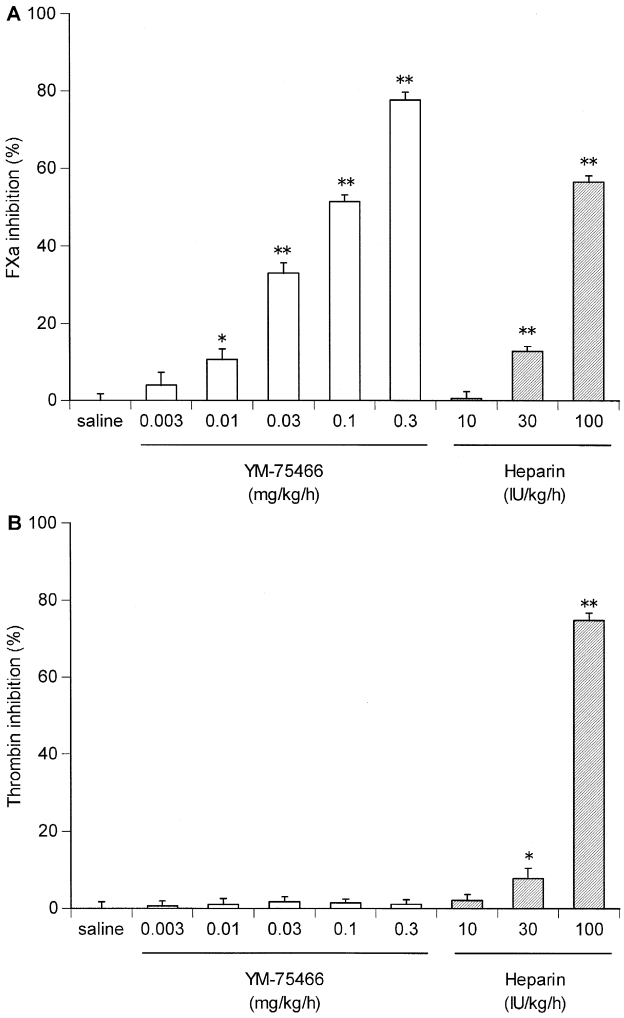

Fig. 5. Anti-FXa (panel A) and anti-thrombin (panel B) effects of YM-75466 (open columns) and heparin (hatched column) after intravenous infusion in rats. Data represent the percent inhibition compared with FXa and thrombin activity in the saline group and are expressed as means ± S.E.M. ($n = 10$). Statistical analysis was performed by using Dunnett's multiple comparison test. $*$ $P < 0.05$, $**$ $P < 0.01$ compared with the saline group.

4. Discussion

In this study, decreases in the levels of TAT in plasma correlated with the antithrombotic effects of YM-75466 and heparin. Heparin exerted both anti-FXa and anti-thrombin activity, while YM-75466 exerted anti-FXa activity, but not anti-thrombin activity.

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., 1998). 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.

Thrombin–antithrombin III complex is formed by the binding of thrombin and its inhibitor, antithrombin III. The level of TAT in plasma is considered a sensitive index of thrombin generation and has been used for clinical diagnosis (Pelzer et al., 1988; Boneu et al., 1991; Estivals et al., 1991; Deguchi et al., 1991). A sandwich-ELISA immunoassay kit (Enzygnost® TAT micro) is now commercially available. Although absolute values of TAT must be considered with care due to the human origin of the calibration standards, this kit has already been shown to be applicable to detect thrombin generation in rat plasma (Ravanat et al., 1995, 1996; Dickneite et al., 1994). In this study, thrombus formation significantly increased the level of TAT in the saline group compared with the basal level, but the administration of YM-75466 and heparin decreased the level of TAT toward the basal level in proportion to their antithrombotic effects. These results suggest that YM-75466 and heparin exert their antithrombotic effects through inhibition of thrombin generation and the level of TAT in plasma is an appropriate parameter for monitoring their antithrombotic effects in the arterio–venous shunt model in rats. The two agents, YM-75466 and heparin, which have different modes of action such as direct inhibition of FXa and indirect and antithrombin III-dependent inhibition of FXa and thrombin, inhibited TAT formation to the same degree in proportion to their antithrombotic effects. This may be because TAT formation in this model is due to thrombin generated when the thrombus is formed on the surface of the copper wire, and therefore, if only thrombus formation is inhibited through any mechanism, the increase in TAT level in plasma can be inhibited.

Prothrombin time and activated partial thromboplastin time have been widely used for monitoring the antithrombotic effects of conventional anticoagulant agents such as heparin or warfarin. Also in this study, the antithrombotic effects of heparin correlated with its prolongation of activated partial thromboplastin time. Since the therapeutic dose of heparin prolongs activated partial thromboplastin time by 1.5–2.0 times, it is reasonable that heparin at a dose of 30 IU kg^{-1} h^{-1}, which prolonged activated partial thromboplastin time by 1.4 times, inhibited thrombus formation significantly. In contrast, YM-75466 hardly prolonged prothrombin time and activated partial thromboplastin time even at a dose which significantly inhibited thrombus formation. Although the mechanism of this has yet to be clarified in detail, YM-75466 may efficiently inhibit thrombus formation through the inhibition of FXa on thrombus and exert significant antithrombotic effects even at the dose which hardly prolongs coagulation time of peripheral blood. In our previous study, it was demonstrated that not only YM-75466 but also argatroban, a thrombin inhibitor, and dalteparin, a low-molecular-weight heparin, exert significant antithrombotic effects at the doses which do not prolong coagulation time, and that the prolongation of activated partial thromboplastin time correlates closely with the prolongation of the bleeding time for all agents (Sato et al., 1997). These results cast doubt on the suitability of coagulation time as parameters for monitoring the antithrombotic effects of new-generation anticoagulant agents and should probably be used to indicate bleeding tendency only.

YM-75466 has been reported to specifically inhibit FXa with a K_i value of 1.3 nM, but not thrombin ($K_i > 100$ μM) in vitro (Taniuchi et al., 1998). However, it has been unclear whether YM-75466 exerts its antithrombotic effect by specific inhibition of FXa in plasma. In this study, YM-75466 exerted anti-FXa activity, but not anti-thrombin activity, in a dose-dependent manner. Although they can not be quantitatively compared with the antithrombotic effects in rats due to the use of human FXa and thrombin, this result suggests that YM-75466 exerts its antithrombotic effect by specific inhibition of FXa. In contrast, heparin exerted both anti-FXa and anti-thrombin activity and it is thought to exert its antithrombotic effects through inhibition of both FXa and thrombin. The difference in thrombin inhibition may result in their different effects on bleeding tendency.

In conclusion, this study suggests that TAT is a suitable parameter for monitoring the antithrombotic effects of YM-75466 in the arterio–venous shunt model in rats and that YM-75466, unlike heparin, exerts its antithrombotic effect through specific inhibition of FXa without any effect on thrombin.

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