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Antithrombotic effects of a synthetic inhibitor of activated factor X, JTV-803, in animals

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Abstract

JTV-803, 4-[(2-amidino-1,2,3,4-tetrahydroisoquinolin-7-yloxy)methyl]-1-(4-pyridinyl)piperidine-4-carboxylic acid monomethanesulfonate trihydrate, at ≥ 0.1 mg/kg/h inhibited the increase in plasma thrombin–antithrombin III complex in response to continuous infusion of thromboplastin in rats. JTV-803 inhibited thrombus formation in an arteriovenous shunt model by intravenous infusion at ≥ 0.3 mg/kg/h and prolonged the occlusion time of photochemically induced arterial thrombus in the middle cerebral artery at > 1.5 mg/kg/0.5 h. Activated partial thromboplastin time was prolonged at 10 mg/kg/h. Intravenous administration of JTV-803 prolonged bleeding time at 30 mg/kg/h, a dose 10–100 times higher than the dose that inhibited thrombus formation. Compared with thrombin inhibitor, JTV-803 had less of an effect on the bleeding time. In the arteriovenous shunt model in cynomolgus monkey, JTV-803 prolonged the occlusion time when administered by continuous infusion at 0.3 mg/kg/h or orally at 10 mg/kg. These results suggest that the human factor Xa inhibitor JTV-803 is an orally active anticoagulant that does not affect bleeding time and is useful for the prevention of thrombus.

Keywords: JTV-803; Factor Xa; Anticoagulant; Bleeding time; Oral administration

1. Introduction

Regulation of thrombin activity is an important factor in the prevention of thrombosis. Heparin, a cofactor in the antithrombin III-dependent inactivation of thrombin and factor Xa, is useful for prophylaxis and the treatment of thrombus diseases such as disseminated intravascular coagulation, reocclusion following thrombolytic therapy and deep vein thrombosis (Coon, 1985; Gold et al., 1986; Ockelford, 1986). However, its action is restricted to the freely circulating forms of these enzymes and it is unable to access clot-bound thrombin or prothrombinase-complexed factor Xa (Miletich et al., 1978; Teitel and Rosenberg, 1983). A direct inhibitor of coagulation factor is thus preferable to an antithrombin III-dependent anticoagulant. In fact, recombinant versions of tick anticoagulant peptide (rTAP), a direct factor Xa inhibitor, have been shown to exert an anticoagulant effect on venous and arterial thrombosis and also to bring about a stronger improvement in time to reperfusion in a dog model as compared to heparin (Lynch et al., 1995; Nicolini et al., 1996; Ragosta et al., 1994; Vlasuk et al., 1991). We therefore focused on developing a nonpeptide and orally active direct inhibitor of human factor Xa.

JTV-803 (Fig. 1) was developed as a highly selective, competitive direct inhibitor of human factor Xa by Japan Tobacco Pharmaceutical Laboratory. Its K_i value for factor Xa, thrombin, trypsin and plasmin was 0.019 ± 0.001, > 100, 13.6 ± 1.8 and 78.2 ± 2.8 μM, respectively. In this paper, we examined the antithrombotic and hemorrhagic effects of JTV-803, argatoroban (direct thrombin inhibitor) and low-molecular-weight heparin (antithrombin III-dependent anticoagulant) in rats and also examined the antithrombotic effect of JTV-803 in cynomolgus monkeys

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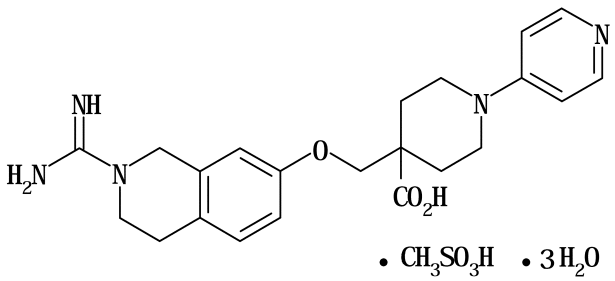

Fig. 1. Chemical structure of JTV-803. 4-[(2-amidino-1,2,3,4-tetrahydroisoquinolin-7-yloxy)methyl]-1-(4-pyridinyl)piperidine-4-carboxylic acid monomethanesulfonate trihydrate.

following its administration by the oral route and by continuous infusion.

2. Materials and methods

2.1. Agent

JTV-803, 4-[(2-amidino-1,2,3,4-tetrahydroisoquinolin-7-yloxy)methyl]-1-(4-pyridinyl) piperidine-4-carboxylic acid monomethanesulfonate trihydrate, was synthesized at Japan Tobacco (Takatsuki, Japan). Other agents were purchased as follows: argatroban (Slonnon®) from Daiichi Pharmaceutical (Tokyo, Japan); low-molecular-weight heparin (Fragmin®) from Kissei Pharmaceutical (Matsumoto, Japan); tissue thromboplastin (Dade®) from Dade International (Miami, USA); and enzyme-linked immunosorbent assay kit (ELISA; Enzygnost ® TAT) from Roche Diagnostic Japan (Tokyo, Japan).

2.2. Animals

Male Sprague–Dawley rats (CRJ, Yokohama, Japan) weighing 250–450 g and male cynomolgus monkeys (Keari, Osaka, Japan) weighing 4.2–4.7 kg were used. All procedures related to the use of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee at the Environmental Biological Life Science Research Center and Japan Tobacco.

2.3. Effect on thromboplastin-induced increase in thrombin–antithrombin III complex

Sprague–Dawley rats were anesthetized with urethane intraperitoneally (1.3 g/kg). After continuous infusion of thromboplastin (1 U/kg/h) into the left femoral vein for 1 h, the concentration of thrombin–antithrombin III complex (TAT) in plasma was determined by using a commercially available ELISA kit (Pelzer et al., 1988). From 1 h before the continuous infusion of thromboplastin, each test compound was infused continuously into the right femoral vein. Plasma was prepared from citrated blood by centrifugation at $2000 \times g$ for 10 min.

2.4. Arteriovenous shunt thrombosis model

A 2-cm length of copper wire, 0.45 mm in diameter, was inserted into a polyethylene tube (PE-120). Two polyethylene tubes (PE-60) were connected to both sides of the PE-120 tubing. This arrangement was used to form a bypass between the left jugular vein and the right carotid artery. The copper wire was removed after 10 min of blood circulation and placed in 1 ml of 0.1 N NaOH–2% Na_2CO_3 solution. After complete dissolution, the thrombus was evaluated in terms of protein weight using a commercial assay kit (BIORAD, Tokyo, Japan).

2.5. Photo-irradiation induced arterial thrombosis model

Sprague–Dawley rats were anesthetized with halothane and maintained at 37°C with a heating pad. A window of 2-mm diameter was opened in the cranial bone at the peripheral end of the exposed artery, through which a flow probe (ADVANCE LASER FLOWMETER, model ALF2100) was inserted. The middle cerebral artery was irradiated at a wavelength of 540 nm, using a xenon lamp. In addition, rose bengal was infused intravenously to induce thrombosis in the middle cerebral artery. The time required to reduce cerebral blood flow by 50% was defined as the time to thrombus formation, and the antithrombotic effect of each test compound was based on this time. From 15 min before administration of rose bengal, each test compound was given by continuous infusion into the femoral vein.

2.6. Effect on activated partial thromboplastin time and bleeding time

Sprague–Dawley rats were anesthetized intraperitoneally with urethane (1.3 g/kg) and each test compound was continuously infused into the femoral vein for 1 h, after which a cut was made on the tail 5 mm from the tip. Filter paper was applied to the cut surface at 15-s intervals. The time until the filter paper no longer absorbed blood was defined as the bleeding time and at this time a blood sample was collected from the abdominal aorta and centrifuged at $2000 \times g$ for 10 min. Plasma thus obtained was analyzed with an automatic blood coagulation analyzer (STA Compact, Roche, Basel, Switzerland) for determination of activated partial thromboplastin time .

2.7. Arteriovenous shunt thrombosis model in anesthetized monkey

Male cynomolgus monkeys were anesthetized intravenously with pentobarbital (10–20 mg/kg). After dissection of both femurs, the artery was detached from one limb, and the vein from the other limb. The artery and the vein were connected to a catheter made of vinyl chloride

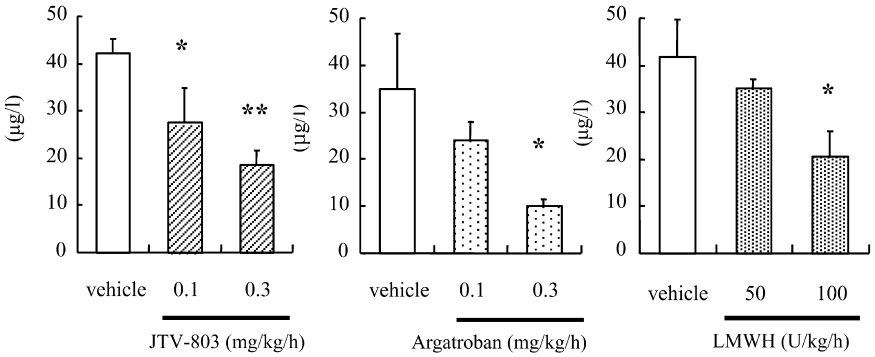

Fig. 2. Inhibitory effect of JTV-803, argatroban, and low-molecular-weight heparin (LMWH) on TAT elevation induced by continuous infusion of thromboplastin in rats. (mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$, Dunnett's test, $n = 4–11$).

(of variable internal diameter over the length to facilitate the arrest of blood flow) to form a shunt, in which an external electromagnetic blood flow probe was placed. After blood perfusion through the shunt, the process of thrombus formation was monitored based on changes in blood flow, and the time to occlusion was evaluated when the blood flow was reduced to zero. The antithrombotic effect of JTV-803 was evaluated as the time to occlusion. From 1 h before blood perfusion through the shunt, JTV-803 was given by continuous infusion. In the case of oral administration, JTV-803 was given 1.5 h before the injection of pentobarbital. To measure the plasma concentrations of JTV-803 by infusion, a blood sample was collected from the vein just before blood perfusion through the shunt. The plasma concentrations of JTV-803 were determined by liquid chromatography.

3. Results

3.1. Effect on thromboplastin-induced increase in TAT

JTV-803 inhibited plasma TAT concentration at 0.1 and 0.3 mg/kg/h, the effect of which was significant at 0.1 and 0.3 mg/kg/h. A similar effect was seen with argatroban at 0.3 mg/kg/h and low-molecular-weight heparin at 100 U/kg/h (Fig. 2).

3.2. Arteriovenous shunt thrombosis model

JTV-803 inhibited thrombus formation at 0.1–1 mg/kg/h, the effect of which was significant at 0.3 and 1 mg/kg/h. A similar antithrombotic effect was seen with argatroban at 0.3 and 1 mg/kg/h, and the effect was significant. The amount of thrombus formed was reduced to about half of that in those receiving low-molecular-weight heparin 300 U/kg/h, although the effect was not statistically significant (Fig. 3).

3.3. Photo-irradiation induced arterial thrombosis model

Both JTV-803 and argatroban prolonged the time to thrombus formation at a dose of 0.5 mg/kg/0.5 h. The prolongation was statistically significant at 1.5 mg/kg/h and above (Fig. 4).

3.4. Effect on activated partial thromboplastin time and bleeding time

JTV-803 did not prolong the activated partial thromboplastin time at doses up to 3 mg/kg/h, but at higher doses, 10 and 30 mg/kg/h, it significantly prolonged the activated partial thromboplastin time. Argatroban and low-molecular-weight heparin also significantly prolonged

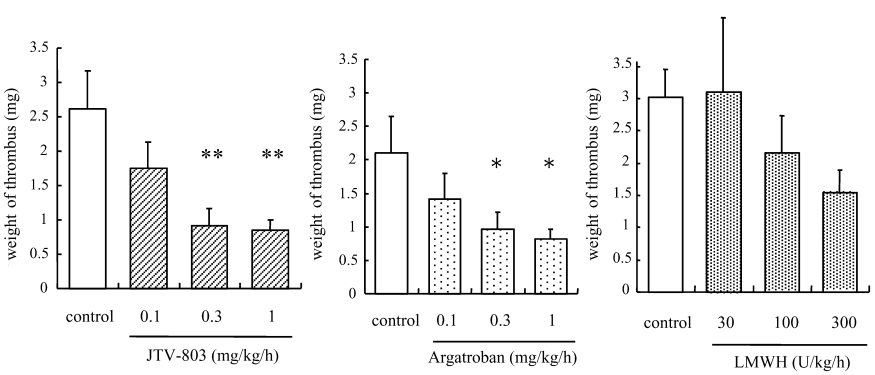

Fig. 3. Anti-thrombotic effect of JTV-803, argatroban, and low-molecular-weight heparin (LMWH) in the arteriovenous shunt thrombosis model in rats. (mean ± S.E.M., $^{*}P < 0.05$, $^{**}P < 0.01$, Dunnett's test, $n = 6–11$).

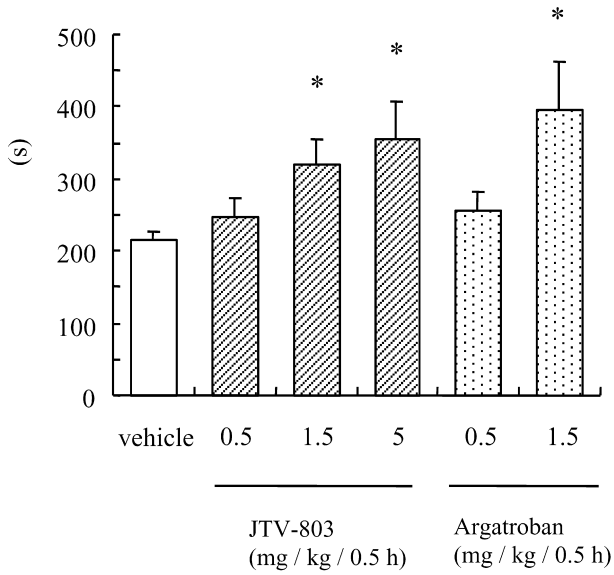

Fig. 4. Effect of JTV-803 and argatroban on middle cerebral arterial thrombosis in rats. (mean ± S.E.M., $^{*}P < 0.05$, Steel's test, $n = 10–14$).

the activated partial thromboplastin time at 1 mg/kg/h and 300 U/kg/h, respectively.

Bleeding time was not significantly changed by JTV-803 at 1–10 mg/kg/h but was significantly prolonged at 30 mg/kg/h. A significant prolongation of bleeding time was observed with argatroban at 3 mg/kg/h and low-molecular-weight heparin at 300 U/kg/h (Table 1).

Table 1
Prolongation of activated partial thromboplastin time (aPTT) and bleeding time by JTV-803, argatroban, or low-molecular-weight heparin (LMWH) given by continuous intravenous infusion

Drug	Dose	aPTT (s)	*n*	Bleeding time (s)	*n*
Vehicle		33.5 ± 2.6	6	187.5 ± 20.6	10
JTV-803	0.3 mg/kg/h	34.6 ± 2.4	6	–	
	1 mg/kg/h	30.2 ± 1.5	6	222.0 ± 21.1	10
	3 mg/kg/h	35.8 ± 1.1	6	219.0 ± 21.1	10
Vehicle		25.2 ± 0.9	11	182.7 ± 16.8	11
JTV-803	10 mg/kg/h	39.3 ± 2.7[a]	10	225.0 ± 15.2	10
	30 mg/kg/h	48.7 ± 2.7[a]	10	282.0 ± 27.0[a]	10
Vehicle		27.9 ± 1.0	6	157.5 ± 12.7	10
Argatroban	0.3 mg/kg/h	30.7 ± 1.3	6	–	
	1 mg/kg/h	40.4 ± 2.5[a]	6	205.5 ± 27.7	10
	3 mg/kg/h	51.7 ± 4.5[a]	6	258.0 ± 22.9[a]	10
Vehicle		29.9 ± 0.6	6	215.0 ± 21.1	10
LMWH	30 U/kg/h	39.2 ± 3.8	6	–	
	100 U/kg/h	35.0 ± 1.9	6	213.0 ± 13.7	10
	300 U/kg/h	119.9 ± 25.1[a]	6	270.0 ± 16.6[b]	9

Each drug was given by continuous intravenous infusion at a volume of 1 ml/kg for 1 h. In animals assigned to the JTV-803 high-dose group, drug was given at a volume of 2 ml/kg. The bleeding time in argatroban-treated animals was measured after administration at a dose of 6 ml/kg.

[a] $P < 0.01$, Dunnett's test.

[b] $P < 0.05$, Dunnett's test.

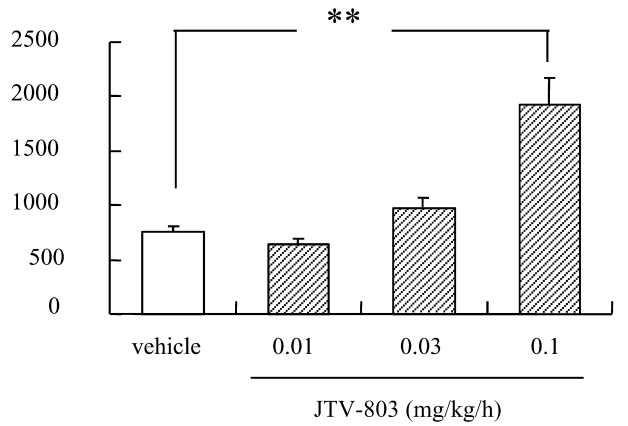

Fig. 5. Effect of JTV-803 on arteriovenous shunt thrombosis induced by continuous infusion in cynomolgus monkey. (mean ± S.E.M., $^{**}P < 0.01$, Dunnett's test, $n = 6$).

3.5. Arteriovenous shunt thrombosis model in anesthetized monkey

The time to blood flow arrest due to thrombosis in the shunt was prolonged at a dose of 0.1 mg/kg/h given by infusion and 10 mg/kg given by oral administration. This

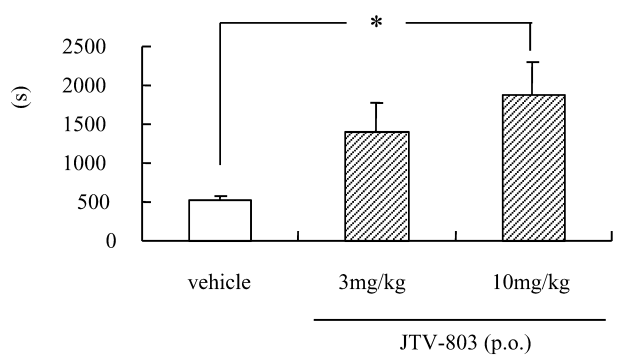

Fig. 6. Effect of oral JTV-803 on arteriovenous shunt thrombosis in cynomolgus monkey. (mean ± S.E.M., $^{*}P < 0.05$, Dunnett's test, $n = 6$).

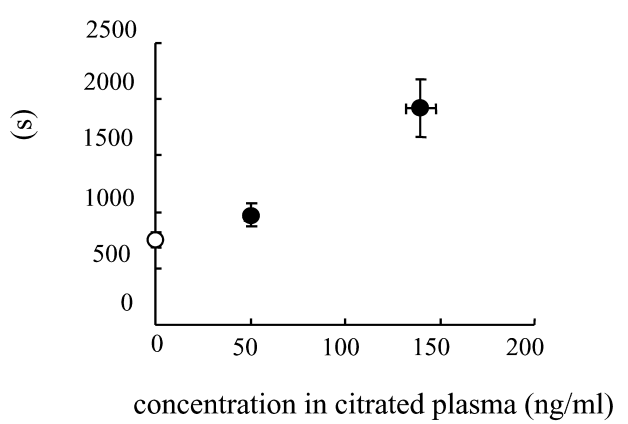

Fig. 7. Correlation between occlusion time of arteriovenous thrombosis and plasma concentration of JTV-803 in cynomolgus monkey. (mean ± S.E.M., $n = 6$).

effect was significantly greater than that seen in animals treated with physiological saline (Figs. 5 and 6). The time to occlusion was correlated with the increase in plasma concentrations of JTV-803 (Fig. 7). One hour after administration of 0.1 mg/kg/h, the concentration of the intact form of JTV-803 in citric acid-treated plasma was 139 ± 8 ng/ml.

4. Discussion

Thrombin plays an important role in thrombosis. It activates platelets, catalyzes the conversion of fibrinogen to fibrin and activates factor V and factor VIII to amplify the production of thrombin (Butenas et al., 1997; Ofosu et al., 1996; Wang et al., 1990). Since one molecule of factor Xa produces 138 molecules of thrombin (Elodi and Varadi, 1979), inhibition of factor Xa is considered to be more efficient than inhibition of thrombin in this pathway. In a thromboplastin infusion rat model, we measured the amount of thrombin–antithrombin III complex (TAT) after the infusion of JTV-803, argatroban and low-molecular-weight heparin. Generated thrombin is quickly neutralized by antithrombin III and becomes TAT in plasma. The measurement of TAT in plasma is considered an appropriate method for measuring thrombin generation. As shown Fig. 2, JTV-803 inhibited the formation of TAT at ≥ 0.1 mg/kg/h. In the arteriovenous shunt thrombosis model, JTV-803 also decreased thrombus formation at ≥ 0.3 mg/kg/h. JTV-803 exhibited almost the same antithrombotic effect as argatroban and low-molecular-weight heparin in both the above models. Thrombi induced by copper coil insertion are considered to be useful to evaluate the efficacy of anticoagulant agents, because the induced thrombi are fibrin-rich (Bush and Shebuski, 1990).

In the photo-irradiation induced arterial thrombosis model, JTV-803 prolonged thrombus formation in the middle cerebral artery at ≥ 1.5 mg/kg/h. Since the thrombus as a result of photo-irradiation mainly consists of aggregated platelets (Horisawa et al., 1999; Inamo et al., 1996; Saniabadi et al., 1995), it is difficult for JTV-803 to exert an antithrombotic effect in this model due to the lack of inhibition of platelet aggregation (agonist: collagen, ADP, arachidonic acid, IC_{50}: 50, ≥ 54, ≥ 60 μM, respectively). The prolongation of the occlusion time may inhibit thrombin-induced platelet aggregation by reducing the amount of thrombin or by inhibiting fibrin formation connected with platelets. However, in platelet-rich thrombosis, 10 times the dose of JTV-803 was needed. Taking this result into account, JTV-803 is considered to be a more efficacious anticoagulant in venous thrombosis than in arterial thrombosis.

As shown in Table 1, JTV-803 prolonged the activated partial thromboplastin time and bleeding time to the same extent as 10 or more times the dosage of argatroban. Compared to the antithrombotic effect at 0.3 mg/kg/h in the arterial-venous thrombosis model, JTV-803 prolonged the bleeding time only at a much higher dose (30 mg/kg/h). This pharmacological profile is a typical feature of factor Xa inhibitors (Fioravanti et al., 1993; Hara et al., 1995b; Tanabe et al., 1999) and is considered a merit in an anticoagulant drug. JTV-803 may thus be an easy and ideal anticoagulant. Clinically, patients may be able to use this antithrombotic drug without worrying about excess bleeding.

The prothrombinase complex, which consists of factor Xa, factor Va and calcium ions on a phospholipid membrane, changes prothrombin to thrombin more efficiently than factor Xa alone (Nesheim et al., 1979). A specific factor Xa inhibitor, recombinant tick anticoagulant peptide (rTAP), is considered to inhibit the coagulation pathway much more strongly at clot-bound sites where prothrombinase complexes exist than in plasma (Krishnaswamy et al., 1994) and to exhibit an effective antithrombotic effect that does not require changes in systemic hemostatic parameters (Fioravanti et al., 1993). The inhibition of prothrombinase complex by JTV-803 may be one of the reasons why the antithrombotic effect and the prolongation of bleeding were produced at different doses; however, this remains to be proven in further experiments.

Although low-molecular-weight heparin is an inhibitor of antithrombin III-dependent factor Xa, there is not much difference in dose between its antithrombotic effect and its prolongation of the bleeding time. This may depend on the different regulation of the prothrombinase complex by antithrombin III-dependent anticoagulant in vivo (Schoen and Lindhout, 1991).

In some studies, it has been shown that a species difference exists in inhibition of factor Xa in vitro (Hara et al., 1995a; Nutt et al., 1991; Tidwell et al., 1980). We therefore examined the antithrombotic effect in the arteriovenous shunt model in cynomolgus monkey. As Figs. 5 and 6 show, JTV-803 prolonged the occlusion time after both oral administration and intravenous infusion. Although statistically 10 mg/kg prolonged the occlusion time, the occlusion time was prolonged more than two times by oral administration of 3 mg/kg ($P = 0.14$). The arteriovenous shunt was prepared under anesthesia 90 min after oral administration of JTV-803. The standard deviation of the occlusion time after oral administration was larger than that after intravenous infusion. This may because anesthesia affects the absorption of JTV-803 from the digestive system. The plasma concentration of JTV-803 at 10 mg/kg in the arteriovenous shunt model was lower than that in conscious animals, after oral administration (data not shown).

We consider that it is necessary to maintain a certain concentration of anticoagulant in plasma in order to exert a constant antithrombotic effect. It is quite important to know the correlation of plasma concentration and antithrombotic effect. In our experiment, the pharmacologically effective plasma concentration of JTV-803 in the

cynomolgus monkey was estimated to be 197 ng/ml from the volume of citric acid per plasma sample and the hematocrit value (assumed to be 40). This concentration of JTV-803 inhibited approximately half of the factor Xa derived from monkey plasma activated with thromboplastin (data not shown).

In conclusion, JTV-803, given intravenously and orally, showed an antithrombotic effect in several models of experimental thrombosis without prolonging the bleeding time in the rat and in nonhuman primates.

Thus, JTV-803 is a promising antithrombotic agent with a reduced bleeding tendency when administered by the oral as well as intravenous route.

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