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Inhibitory effect of JTV-803, a new cyclic guanidine derivative, on factor Xa in vitro and in vivo

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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 showed a competitive inhibitory effect on human factor Xa, with a K_i value of 0.019 μM. This compound was 100 times more selective in inhibiting human factor Xa as compared to its inhibitory activity against thrombin, plasmin, and trypsin. JTV-803 was also examined for its inhibitory effect on activated factor Xa obtained from plasma of various animal species. JTV-803 exerted a potent inhibitory effect on human factor Xa (IC_{50}: 0.081 μM). JTV-803 prolonged activated partial thromboplastin time and prothrombin time in a dose-dependent manner. Oral anticoagulant efficacy of JTV-803 was examined ex vivo for its inhibition of human factor Xa in cynomolgus monkeys. JTV-803 produced more than 20% inhibition of human factor Xa for 8 h. Taken together, the results indicate JTV-803 is a long-acting oral anticoagulant which exerts its effect via specific inhibition of human factor Xa.

Keywords: JTV-803; Oral administration; Coagulation; Factor Xa

1. Introduction

Thrombosis is a major cause of mortality in many countries. Intravascular thrombus formation causes a number of diseases, such as stroke in the brain, myocardial infarction in the heart and pulmonary embolism in the lungs (Giroud et al., 1998; Heye et al., 1992; Kakkar and Adams, 1986; Sakamoto et al., 1994; Stein and Fuster, 1989; Uchiyama et al., 1997). Heparin and warfarin are the most widely used anticoagulants for the prophylaxis and treatment of thrombus-based diseases (Alessandri et al., 1994; Broaddus and Matthay, 1986). However, these anticoagulants have clinical limitations due to dependence on antithrombin III and acting via antagonism against vitamin K, respectively. For instance, heparin is sometimes associated with the generation of an antibody against platelet factor (PF4) that causes thrombocytopenia (Greinacher, 1998; Kaplan and Francis, 1999), while warfarin requires a longer time for exertion of its drug effect and clinical monitoring, and sometimes interacts with food or other drugs (Booth et al., 1997; Wells et al., 1994). Inasmuch as the blood coagulation system is an amplification reaction, the inhibition of the generation of thrombin at an early stage is considered to be more efficient than the direct inhibition of the activity of generated thrombin (Elodi and Varadi, 1979). Since factor Xa is the confluence in intrinsic and extrinsic coagulation pathways, the inhibition of factor Xa is considered to be extremely effective to stop coagulation. We therefore aimed to develop a new type of anticoagulant that directly inhibits factor Xa strongly following oral as well as intravenous administration. JTV-803 (Fig. 1) is a novel synthetic compound designed by a computer and based on the crystal structure of human factor Xa at the Japan Tobacco Inc. Chemical Research Institute. In this paper, we show that JTV-803 is a selective inhibitor of human factor Xa, is more potent in human than in other animal species, and has anticoagulant activity orally as well as intravenously in cynomolugus monkey.

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

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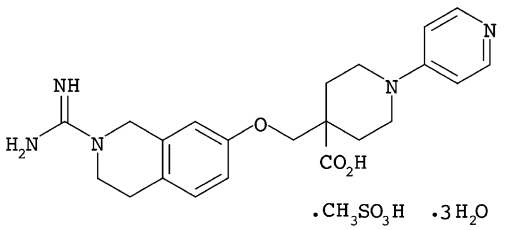

Fig. 1. Chemical structure of JTV-803. 4-[(2-amidino-1,2,3,4-tetrahydroisoquinolin-7-yloxy)methyl]-1-(4-pyridinyl)piperidine-4-carboxylic acid monomethanesulfonate trihydrate.

acid monomethanesulfonate trihydrate (Fig. 1), was synthesized at Japan Tobacco Inc. (Takatsuki, Japan). Other agents were purchased as follows: human factor Xa, from Enzyme Research Lab Inc., (South Bend, USA); human thrombin, trypsin and plasmin, from Sigma (St. Louis, USA); chromogenic substrates, S-2222, S-2238 and S-2403, from Chromogenix (Mölndal, Sweden); thromboplastin, from Dade International Inc. (Miami, USA).

2.2. Animals

Male cynomolgus monkeys (Keari, Osaka, Japan) weighing 4.2–4.7 kg were used. Drug administration and blood withdrawal were performed at Environmental Biological Life Science Research Center Inc. All procedures related to the use of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Environmental Biological Life Science Research Center Inc. and Japan Tobacco Inc.

2.3. Inhibitory effect on human factor Xa

Forty microliters of human factor Xa of 0.16 U/ml and 40 μl of JTV-803 solution adjusted to various concentrations were incubated in 40 μl of 0.1 M Tris (Tris-hydroxymethyl-aminomethane) buffer containing 0.2 M sodium chloride at 37 °C for 10 min. Forty microliters of a synthetic substrate, S-2222, was added and the mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 60% acetic acid. Enzyme activity was estimated based on the level of *para*-nitroaniline, a decomposition product of the synthetic substrate, determined at a wavelength of 405 nm by a spectrometer (Model 3550, BIO-RAD, Hercules, USA). Enzyme activity in the presence of JTV-803 at each concentration was expressed on Lineweaver–Burk plot, from which the mode of inhibitory action was examined. K_i value was determined from Dickson's plot constructed at two concentrations of the substrate.

2.4. Inhibitory effect on thrombin, plasmin and trypsin

Chromogenic substrates S-2238, S-2403 and S-2222 were used for the measurement of inhibition of thrombin, plasmin and trypsin. The K_i values for thrombin, plasmin and trypsin were determined from Dickson's plot constructed at two concentrations of the substrate.

2.5. Species specificity of inhibition on plasma factor Xa

Citrated blood was collected from volunteers and animals (rat, dog, hamster and monkey). Plasma was prepared by centrifugation of blood at $2000 \times g$ for 15 min at 4 °C. One hundred microliters of 1 mM S-2222, 10 μl of plasma diluted twice with distilled water and 10 μl of JTV-803 adjusted to various concentrations were mixed with 80 μl of 0.1 M Tris–0.2 M NaCl buffer (pH 8.4) at 37 °C for 5 min. The mixture was then activated with thromboplastin for 5 min, and the activity of factor Xa in plasma sample was estimated based on the level of *para*-nitroaniline at a wavelength of 405 nm. For the control, vehicle was used instead of JTV-803. Control value was taken as 100% and the drug concentration showing 50% absorbance of control taken as IC_{50}.

2.6. Coagulation assays

Activated partial thromboplastin time and prothrombin time were measured with automatic coagulometer (STA Compact, Roche, Basel, Switzerland). For activated partial

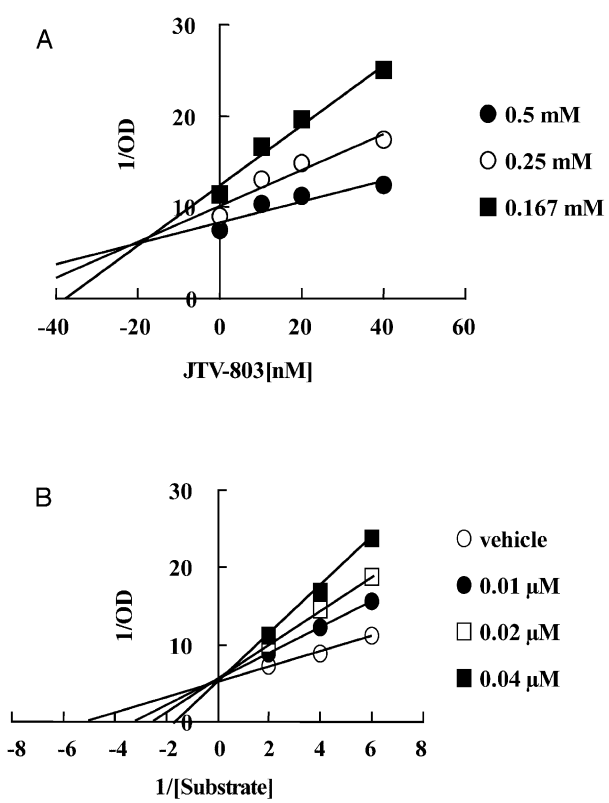

Fig. 2. Mode of inhibition of JTV-803 for human factor Xa. (A) Dickson plot (substrate ■: 0.167 mM, ○: 0.25 mM, ●: 0.5 mM). (B) Lineweaver–Berk plot (○: vehicle; JTV-803 ●: 0.01 μM, □: 0.02 μM, ■: 0.04 μM).

Table 1
Selectivity of JTV-803 for serine protease enzymes

Enzyme	K_i value (μM)
Factor Xa	0.019 ± 0.001
Thrombin	> 100
Plasmin	78.2 ± 2.8
Trypsin	13.6 ± 1.8

The K_i values for each enzyme were determined from Dickson's plot constructed at two concentrations of the substrate. Data represent mean ± S.E.M. ($N = 3$)

Table 2
Concentration of JTV-803 required to inhibit activated plasma factor Xa of various animals. Data represent IC_{50} mean ± S.E.M. ($N = 3$)

Species	IC_{50} (μM)
Human	0.081 ± 0.0080
Monkey	0.25 ± 0.062
Rat	2.9 ± 0.36
Dog	0.22 ± 0.0047
Hamster	0.36 ± 0.013

thromboplastin time, 50 μl of plasma was incubated with 50 μl of commercially available reagent (STA APTT LT, Roche, Basel, Switzerland) for 240 s and to this 50 μl of 25 mM $CaCl_2$ was added. For prothrombin time, 50 μl of plasma was incubated for 240 s at room temperature and 100 μl of thromboplastin with $CaCl_2$ (STA neo plus, Roche, Basel, Switzerland) was added. Anticoagulant activity was evaluated with plasma clotting time doubling concentration.

2.7. Collection of plasma for anti-human factor Xa assay in cynomolgus monkey

JTV-803 dissolved in physiological saline was administrated intravenously to male cynomolgus monkey at a dose of 1 mg/kg. Before administration and at 5, 10, 15, 30, 60 and 120 min following administration, 1500 μl blood samples were collected from the saphenous vein of each monkey into a syringe containing 300 μl of 3.8% citric acid. Plasma of each sample was prepared by centrifugation at 2000 × g for 10 min at 4 °C.

JTV-803 was dissolved in deionized distilled water and administered orally to fasted male cynomolgus monkeys at a dose of 10 mg/kg. Before administration and at 15, 30, 60, 120, 240, 360 and 480 min following oral administration, 1500 μl blood samples were collected and plasma were obtained as described above.

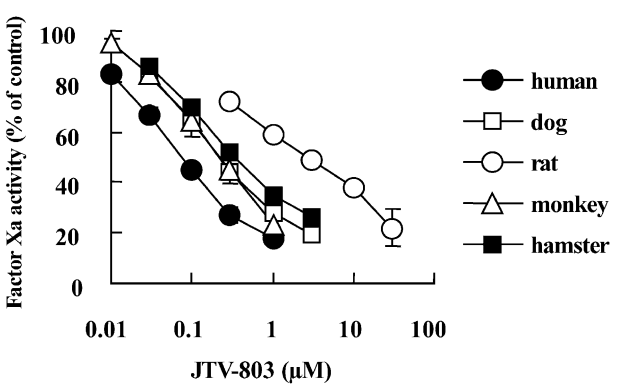

Fig. 3. Dose–response curve of inhibition of JTV-803 on the activated plasma factor Xa of various animals. Data represent mean ± S.E.M. ($N = 3$). The activity of factor Xa (p-NA/min) was 28 ± 2 μM/min in human, 27 ± 1 μM/min in monkey, 24 ± 0 μM/min in rat, 32 ± 1 μM/min in dog and 45 ± 1 μM/min in hamster.

2.8. Ex vivo anti-human factor Xa assay in cynomolgus monkey plasma

Forty microliters of human factor Xa (0.5 U/ml) and 40 μl of a fourfold diluted plasma sample were incubated in 40 μl of 0.1 M Tris–0.2 M NaCl buffer (pH 8.4) at 37 °C for 10 min. Then, 40 μl of a synthetic substrate, S-2222, adjusted to 0.8 mM was added and the mixture was incubated at 37 °C for 3 min. The reaction was stopped by the addition of 60% acetic acid and the absorbance at 405 nm was measured by a spectrometer (Model 3550, BIO-RAD, Hercules, USA). For the control, plasma obtained prior to JTV-803 administration was used for measurement. Human factor Xa inhibitory activity was calculated as percent inhibition against control.

3. Results

3.1. Selective inhibition to human factor Xa

JTV-803 exerted a competitive inhibitory effect on human factor Xa as shown in Fig. 2. Human factor Xa was competitively inhibited by JTV-803 with an average K_i value of 0.019 ± 0.001 μM. Human thrombin, plasmin and trypsin were inhibited by JTV-803 with K_i values of > 100, 78.2 ± 2.8 and 13.6 ± 1.8 μM, respectively (Fig. 2, Table 1).

Table 3
Effects of JTV-803 on prothrombin time (PT) and activated partial thromboplastin time (aPTT) in various animals. Data represent CT_2 mean ± S.E.M. ($N = 3$)

Species	CT_2 (μM)	
	PT	aPTT
Human	1.0 ± 0.1	2.4 ± 0.2
Monkey	1.7 ± 0.3	3.0 ± 0.0
Rat	15.5 ± 1.4	> 33.3
Dog	6.0 ± 0.4	9.2 ± 1.3
Hamster	3.4 ± 1.3	3.0 ± 0.0

3.2. Species specificity of inhibition of plasma factor Xa in various animals

As shown in Fig. 3, JTV-803 produced concentration-dependent inhibition of factor Xa from human and various animal species. In terms of IC_{50} value, the inhibitory activity of JTV-803 was most potent in human ($IC_{50} = 0.081 \pm 0.0080$ μM), slightly less potent in monkey and dog ($IC_{50} = 0.25 \pm 0.062$ μM, 0.25 ± 0.36 μM), and much less potent in rat ($IC_{50} = 2.9 \pm 0.36$ μM) (Fig. 3, Table 2).

3.3. Measurement of plasma coagulation parameters

The concentrations of JTV-803 required to double prothrombin time and activated partial thromboplastin time in human plasma were 1.0 ± 0.1 and 2.4 ± 0.2 μM, respectively. As shown in Table 3, the prolongation effect on prothrombin time and activated partial thromboplastin time of JTV-803 was different in various animal species. The concentrations required to double prothrombin time were 1.7 ± 0.3, 15.5 ± 1.4, 6.0 ± 0.4 and 3.4 ± 1.3 μM in monkey, rat, dog and hamster, respectively, while the concentrations required to double the activated partial thromboplastin time were 3.0 ± 0.0, > 33.3, 9.2 ± 1.3 and 3.0 ± 0.0 μM in monkey, rat, dog and hamster, respectively.

3.4. Ex vivo anti-factor Xa activity

The anti-factor Xa activity of JTV-803 after intravenous and oral administration in cynomolgus monkey was evaluated based on the inhibition of human factor Xa in plasma. After intravenous administration of JTV-803 at a dose of 1 mg/kg, the inhibition was $80 \pm 1\%$, $76 \pm 1\%$, $71 \pm 2\%$, $64 \pm 2\%$, $53 \pm 2\%$ and $42 \pm 4\%$ at 5, 10, 15, 30, 60 and 120 min, respectively.

In contrast, oral administration of JTV-803 at a dose of 10 mg/kg produced inhibition of $1 \pm 1\%$, $9 \pm 3\%$, $33 \pm 6\%$, $41 \pm 4\%$, $33 \pm 3\%$, $23 \pm 3\%$ and $22 \pm 3\%$ at 15, 30, 60, 120, 240, 360 and 480 min, respectively (Fig. 4). Peak inhibitory effect was observed at 2 h after oral administration and over 20% inhibition continued for 8 h.

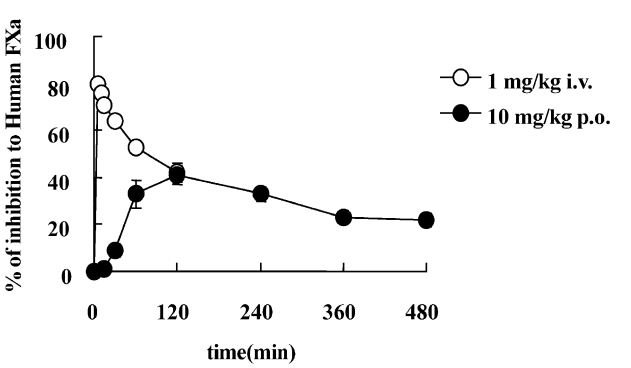

Fig. 4. Percent inhibition of human factor Xa after intravenous and oral administration of JTV-803 in cynomolgus monkey. Data represent mean ± S.E.M. ($N = 6$).

4. Discussion

Factor Xa forms the prothrombinase complex in the presence of factor Va and calcium ions on phospholipid membrane (Harker, 1994). This complex converts prothrombin to thrombin 300,000-fold more efficiently than factor Xa alone (Nesheim et al., 1979). An inhibitor of factor Xa may therefore inhibit the coagulation pathway much more strongly at clot-bound sites where prothrombinase complexes exist than in the plasma where factor Xa is free. This is considered to be the reason why factor Xa inhibitors express stronger antithrombotic effect than the prolongation of bleeding time and plasma parameters such as prothrombin time or activated partial thromboplastin time in plasma (Morishima et al., 1997; Sitko et al., 1992), and is the most important feature distinguishing factor Xa inhibitors from thrombin inhibitors. JTV-803 inhibited factor Xa more than 1000 times stronger than thrombin, which is a typical feature of a selective factor Xa inhibitor. JTV-803 is also considered to have little effect on the fibrinolytic pathway, because the selectivity of JTV-803 for factor Xa was 100 times higher than for tissue plasminogen activator (t-PA), urokinase and protein C. In our preliminary experiment, t-PA was inhibited by JTV-803 with K_i values of 2.7 μM, and urokinase and activated protein C were hardly inhibited by 10 μM of JTV-803 when they were measured by chromogenic substrate method.

Factor Xa consists of a light chain and a heavy chain linked by a single disulfide bond. Recently, the crystal structure of human factor Xa has been determined as a complex with its inhibitor such as FX-2212a, (2*S*)-(3′-amidino-3-biphenylyl)-5-(4-pyridylamino) pentanoic acid, DX-9065a, (+)-(2*S*)-2-[4-[[(3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-[7-amidino-2-naphthyl]propanoic acid hydrochloride pentahydrate (Brandstetter et al., 1996; Kamata et al., 1998; Wei et al., 1998). Although the exact structure of factor Xa of many animal species has not been determined yet, it is important to elucidate species differences in the efficacy of factor Xa inhibitors to speculate on their effect in human. In our experiment, JTV-803 was shown to have its strongest inhibitory effect on human factor Xa ($IC_{50} = 0.081$ μM). This activity was about three times more potent than in monkey and dog, and about 30 times more potent than in rat ($IC_{50} = 0.25$, 0.22, 2.9 μM, respectively). This variation is likely due to minor, species-dependent differences in the structure of factor Xa active sites. In some studies, the prolongation of prothrombin time or activated partial thromboplastin time has been used to compare species difference (Hara et al., 1995; Nutt et al., 1991; Tidwell et al., 1980). However,

more than 10 times the dose of factor Xa inhibitor was needed to affect prothrombin time as compared with the K_i value of the enzyme. As shown in Table 3, the concentrations of JTV-803 required to double prothrombin time were much higher than the results of Table 2. In the present paper, species difference was therefore examined using plasma as the origin of animal factor Xa and substrate to measure the inhibitory effect. This direct technique to detect species differences of factor Xa is considered superior in terms of ease and sensitivity as compared to using coagulation time.

Recently, DX-9065a and YM-60828, [*N*-[4-[(l-acetimidoyl-4-piperidyl)oxy]phenyl]-*N*-[(7-amidino-2-naphthyl) methyl]sulfamoyl]acetic acid dihydrochloride, have been described as orally active inhibitors of factor Xa (Taniuchi et al., 1998; Yokoyama et al., 1995). The C_{max} value of DX-9065a after oral administration at 50 mg/kg in baboons obtained at 10 min was 6.9 μg/ml (Yokoyama et al., 1995), while the profile of YM-60828 after oral administration at 3 mg/kg in squirrel monkey revealed a C_{max} of 0.79 μg/ml at 1 h, and a $T_{1/2}$ of 1.5 h (Taniuchi et al., 1998). We also examined the profile of JTV-803 after oral administration at 10 mg/kg.

The activity of JTV-803 after oral administration in cynomolgus monkey was evaluated based on the inhibition of human factor Xa in plasma. After administration, the inhibition increased to $41 \pm 4\%$ at 120 min then gradually decreased to $22 \pm 3\%$ at 480 min. The correlation of human factor Xa inhibition and plasma concentration of JTV-803 determined by liquid chromatography was quite linear. The plasma concentration of JTV-803 at a dose of 10 mg/kg was 0.07, 0.33, 0.39, 0.25, 0.15 and 0.11 μg/ml at 30, 60, 120, 240, 360 and 480 min, respectively. A C_{max} value was obtained at 120 min and $T_{1/2}$ calculated from the concentration of JTV-803 was estimated at 3.64 h. The concentration of JTV-803 at 8 h (0.11 μg/ml = approximately 0.2 μM) was about the same as the IC_{50} value of the enzyme inhibition used in cynomolgus monkey, which also suppressed about 70% of factor Xa derived from human plasma (Fig. 3). Prothrombin time and activated partial thromboplastin time were also measured at 120 min (prothrombin time: 14.1 ± 1.0 s; activated partial thromboplastin time: 38.6 ± 2.7 s) and they were not statistically different from normal plasma (prothrombin time: 10.9 ± 0.4 s; activated partial thromboplastin time: 41.4 ± 2.6 s). In our previous paper, the antithrombotic effect has been exerted at a dose of 10 mg/kg given by oral administration. The correlation of plasma concentration of JTV-803 and antithrombotic effects has been shown in arteriovenous shunt model in cynomolgus monkey (Hayashi et al., 2001). From this respect, JTV-803 is considered to express long anti-thrombotic effect following oral administration for up to 480 min without the prolongation of prothrombin time and activated partial thromboplastin time. The profile of long-acting anti-thrombotic effect is advantageous because clinically an oral anticoagulant is required to maintain drug concentration for a long period to prevent coagulation at all times.

In conclusion, JTV-803 is a potent anticoagulant agent which specifically inhibits human factor Xa. JTV-803 may show promise as a drug for venous thrombotic diseases by oral as well as intravenous administration.

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