CHARACTERISTICS OF THE HEMOSTATIC ACTION OF KFA-1411, AN INHIBITOR OF COAGULATION FACTOR Xa (FXa), IN HUMANS AND VARIOUS ANIMALS

Kiyoto HARA1, Toshiki HONMA2, Akane MATSUZAWA2, Atsushi MATSUZAWA2, Masahiko UCHIDA2, Takashi KOIZUMI2, Satoshi AKAHANE2 and Masami KOJIMA1

1Pharmacology Research Laboratory, 2Discovery Research Laboratory, Research & Development, Kissei Pharmaceutical Co., Ltd., 4365 Kashiwabara, Hotaka, Minamiazumi, Nagano 399-8304, Japan

(Received September 14, 2002; Accepted January 10, 2003)

ABSTRACT — This study examined a low-molecular-weight factor-Xa inhibitor, KFA-1411 (3-[N-(3-amidinophenyl)]-N-[4-[1-(1-iminoethyl)piperidin-4-yl]phenyl]carbamoylmethyl)aminomethylphenoxyacetic acid monosulfonate dihydrate). KFA-1411 selectively inhibited FXa among the serine proteases in the human blood-coagulation cascade with a Ki value of 1.73 nM, (selectivity ratio, 15000 versus its action on thrombin). The anticoagulant action of KFA-1411 in human plasma almost equaled that of the selective thrombin inhibitor, argatroban. KFA-1411 did not inhibit platelet aggregation at the concentration at which it showed an anticoagulant action. In contrast, argatroban, heparin, and low-molecular-weight heparin (LMWH; dalteparin) inhibited thrombin-induced platelet aggregation at concentrations lower than those needed for their anticoagulant actions. The FXa-inhibiting action of KFA-1411 differed among animal species, the maximum effect being seen in humans, followed by monkeys and rabbits, with rats and mice showing about one-tenth the potency seen in humans. A species variation was also observed among the values obtained for KFA-1411 in respect of anticoagulant activity in plasma (monkeys again being closest to humans).

These results indicate that KFA-1411 may exhibit antithrombotic efficacy without an unwanted platelet-related action in the future treatment of various thrombotic diseases. The experimental model of monkeys is recommended for estimation of the clinical effects and safety of KFA-1411 in humans.

KEY WORDS: Factor-Xa inhibitor, Factor-Xa, Coagulation, Hemostatic action, Platelet, Thrombin

INTRODUCTION

The blood-coagulation cascade is initiated either via an extrinsic pathway following the expression of tissue factors (TF) during disruption of the vascular wall and by the increasing number of cancer cells or via an intrinsic pathway following contact with negatively charged materials such as foreign bodies. FXa, which is located at the intersection of the two pathways, is produced through activation of FX (in the extrinsic pathway by TF and FVIIa, and FIXa and in the intrinsic pathway by FIIa with FVIIa acting as a cofactor). FXa forms a prothrombinase complex with phospholipid, Ca2+, and FV (FVa), and converts prothrombin into thrombin. Since FXa is located upstream of thrombin in the coagulation cascade, it efficiently controls the coagulation system, according to Davie et al. (1991). Heparin produces its anticoagulant and antithrombotic effects by enhancing the inhibitory actions of antithrombin III (ATIII) on serine proteases such as FXa and thrombin. However, heparin is known to have a variety of clinically adverse effects in addition to its anticoagulant action; for example, it also acts on platelets and causes heparin-induced thrombocytopenia (HIT) in some patients (Warkentin et al., 1995; Burgess and Chong, 1997), and its anticoagulant action may be neutralized by platelet factor 4 (PF4) secreted by activated platelets (Lane et al., 1984). Low-molecular-
weight heparin (LMWH), developed as an improved variant of heparin, has been widely used due to the reduced risk of bleeding as compared with heparin (Cade et al., 1984). LMWH is considered to be an excellent anticoagulant, as the ratio of its inhibitory activity against FXa to that against thrombin is greater than the corresponding ratio for standard heparin. This is due to the difference in their mode of binding with ATIII. However, since the basic action of LMWH is also dependent on ATIII, it is not entirely free of the problems associated with heparin (Burgess and Chong, 1997). ATIII, a macromolecular protein with a molecular weight of 59 kD, cannot reach thrombus-bound FXa or thrombin, which help to strengthen and extend thrombi, and is considered to be ineffective against these enzymes in the thrombus (Beimond et al., 1996). For these reasons, a selective and small molecular-size FXa inhibitor that has more a selective action on FXa than LMWH and can exert its inhibitory action even on enzymes within a thrombus might represent an ideal anticoagulant (McKenzie et al., 1996).

We recently developed KFA-1411, a synthetic selective FXa inhibitor (Fig 1). To clarify the characteristics of KFA-1411 as an anticoagulant, we evaluated its in vitro actions on hemostatic mechanisms or their components, such as the serine proteases of the coagulation cascade, blood coagulation, and platelet function. In addition, as species variation has been reported in the effects of other FXa inhibitors (Hara et al., 1995), we looked for species variation in the action of KFA-1411. We also tried to search for an optimal animal model for the estimation of the clinical effects and safety of KFA-1411 in humans.

**MATERIALS AND METHODS**

**Reagents and Animals**

KFA-1411 was synthesized at Kissei Pharmaceutical Co., Ltd. (Kissei) (Fig. 1). It was dissolved just before use in dimethylsulfoxide (DMSO), acetate buffer, or physiologic saline, the concentration depending on the experimental method being employed. Novastan® injection (argatroban) was purchased from Mitsubishi Pharma. (Osaka, Japan). Heparin (Novoheparin®) was purchased from Aventis Pharma Ltd. (Tokyo, Japan). Dalteparin sodium (Fragmin injection®) marketed by Kissei was used as low-molecular-weight heparin. Russell’s Viper Venom-X (RVV-X) manufactured by Enzyme Research Laboratory Inc. (IN, USA) and normal human frozen plasma manufactured by George King Bio-Medical Inc. (KS, USA) were purchased from Cosmobio Inc. (Tokyo, Japan). Purified human enzyme FXa and Activated Protein C (APC) were purchased from Serbio (Gennevilliers, France), ATIII from SIGMA Chemical Co. (MO, USA), FXIIa, trypsin, and kallikrein from Calbiochem (CA, USA), plasmin from Wako (Osaka, Japan), tissue plasminogen activator (t-PA) from Biopool (Umea, Sweden), and FIXa from American Diagnostica Inc. (CT, USA). Recombinant human FvIIa (Novo Seven®) from Novo Nordisk Pharma. Ltd. (Tokyo, Japan) and recombinant human tissue thromboplastin (Hemoliance Recombiplastin®) from DIY-İATORON (Tokyo, Japan), respectively. The synthetic substrates for enzyme-activity assays (S-2222, S-2238, S-2302, S-2251, S-2288, and S-2366), which are manufactured by CHROMOGENIX (Molndal, Sweden), were purchased from Daiichi Chemical Industries (Tokyo, Japan). The synthetic enzyme-substrates, SPECTORZYME® FVIIa and SPECTORZYME® FIXa, were purchased from American Diagnostica Inc. (CT, USA). The Neoplastin Plus® used for the measurement of the prothrombin time (PT) and the PTT reagent® used for the measurement of the activated partial thromboplastin time (APTT) were purchased from Roche Diagnostics (Tokyo, Japan).

Rats (male Wistar rats) and mice (male ICR mice) were purchased from Japan SLC (Hamamatsu, Japan), rabbits (male Japanese whites) from Japan Kearie (Osaka, Japan), and beagle dogs (males aged 0.8-2.0 years) from CLEA Japan Inc. (Tokyo, Japan). When animal blood was needed for the various experiments, it was obtained according to the rules laid down by the Laboratory Animal Committee of Kissei Pharmaceutical Co., Ltd. After 3.13% citrated blood was obtained, the plasma was isolated by centrifugation at 3000 rpm for 10 min at 4°C, then stored by freezing at −80°C until measurement. Cynomolgus monkey frozen plasma collected using 3.13% citrate was purchased from Japan SLC.

![Fig. 1. Structural formula of KFA-1411](image-url)
Measurement of enzyme activities

Enzyme activity in vitro was measured at room temperature by taking the initial velocity of degradation of synthetic substrates by the purified enzyme as the specific absorption (405 nm). This was done using a plate reader (Spectra MAX 250; Molecular Devices, CA, USA). The test drug was deposited with each reaction mixture in a 96-well microtiter plate. The Ki value was calculated by the Dixon-plot method. FXa activity was measured after allowing purified human FXa (0.03 unit/ml) and the synthetic substrate S-2222 (0.1 mM, 0.2 mM) to react in 200 mM NaCl, 100 mM tris (hydroxymethyl) aminomethane (Tris)/HCl buffer, pH8.4, for 5 min. For the other enzymes examined, the corresponding details were as follows. For (i) thrombin: purified human thrombin (0.2 unit/ml); synthetic substrate S-2238 (0.1 mM and 0.2 mM); 50 mM Tris/HCl, 50 mM NaCl buffer, pH8.4, for 5 min; (ii) FXIIa: purified human FXIIa (0.004 unit/ml); synthetic substrate S-2302 (0.1 mM and 2 mM); 50 mM Tris/HCl buffer, pH7.8, for 5 min; (iii) TF-mediated FVIIa (FVIIa/TF): recombinant TF (0.333 µM) and recombinant human FVIIa (0.5 µM); synthetic substrate SPECTROZYME FVIIa (0.5 mM and 1.0 mM); 50 mM Tris/HCl, 100 mM NaCl, 2 mM CaCl2, 0.2% PEC6000 buffer pH7.5, for 5 min; (iv) FIXa: purified human FIXa (2.5 µM); synthetic substrate SPECTROZYME FIXa (0.4 mM and 0.8 mM); 50 mM Tris/HCl 100 mM NaCl, 5mM CaCl2, 33% (v/v) ethylene glycol buffer pH7.4, for 5 min; (v) trypsin: purified human trypsin (0.0004 unit/ml); synthetic substrate S-2222 (1 mM and 2 mM); 50 mM Tris/HCl buffer, pH8.2, for 5 min; (vi) plasmin: purified human plasmin (1.6 µg/ml); synthetic substrate S-2251 (1 mM and 2 mM); 50 mM Tris/HCl, 10 mM NaCl buffer, pH7.4, for 5 min; (vii) kallikrein: purified human kallikrein (0.006 unit/ml); synthetic substrate S-2302 (1 mM and 2 mM); 50 mM Tris/HCl buffer, pH7.8, for 5 min; (viii) tissue plasminogen activator (t-PA): purified human t-PA (0.32 µg/ml); synthetic substrate S-2288 (1 mM and 2 mM); 500 mM Tris HCl buffer, pH9.0, for 5 min; (ix) APC activity: purified human APC (0.2 µg/ml); synthetic substrate S-2366 (1 mM and 2 mM); 50 mM Tris/HCl, 130 mM NaCl, 10 mM CaCl2 buffer, pH8.0 for 5 min.

Measurement of RVV-X-activated FX activity

The factor X present in the plasma of various species was activated using RVV-X, and was allowed to react in a tube containing the test drug. 0.2 mM S-2222, 50 mM CaCl2, 50 mM NaCl, 100 mM Tris/HCl buffer, pH8.4, for 15 min (25 min in the case of Cynomolgus monkey plasma), and the reaction was stopped using 60% acetic acid. The supernatant was isolated from the reaction fluid by centrifugation, and the enzyme activity was assessed by measuring the absorbance of the product at 405 nm. The reference activity was observed when vehicle alone was added to the reaction mixture, and the inhibitory activity (IC50) of the test drug was calculated from the concentration-inhibition curve.

Measurement of clotting time

PT and APTT were measured in human and animal thawed plasmas by routine procedures using an assay cuvette and a coagulometer (ST4; Roche Diagnostics, Tokyo, Japan). Anticoagulant activity was similarly evaluated by measuring the clotting time after placing a solution of the test drug in DMSO (at 75 times the final concentration) in an assay cuvette. The anticoagulant activity of the test drug was expressed as the CT2, which is the concentration necessary to double the clotting time of the control (in which vehicle alone was added). The CT2 was calculated from the curve relating to concentration to clotting time prolongation.

Measurement of platelet aggregation

Adenosine 5'-diphosphate (ADP), collagen, and epinephrine-induced platelet aggregation

With the approval of the Institutional Review Board of Kissei Pharmaceutical Co., Ltd., blood was collected from healthy adult male volunteers, each of whom gave informed consent. Blood with 3.7% sodium citrate was centrifuged at 1000 rpm for 10 min, and the supernatant was collected as platelet-rich plasma (PRP). Its lower layer was further centrifuged at 3000 rpm for 10 min, and the supernatant was collected as platelet-poor plasma (PPP). Platelet aggregation was measured in these PRP and PPP samples by the light-transmission method using an aggregometer (NBS HEMATRASER 801; MC-medical, Tokyo, Japan). Inhibitory activity towards this aggregation was measured over a 10 min period after placing 2 µl of the test drug and 198 µl of PRP in an assay cuvette, preincubating them for 1 min, and adding 20 µl of aggregation inducer (ADP, collagen, or epinephrine). Control aggregation was induced by adding 2 µl of the vehicle for the relevant drug.

Thrombin-induced aggregation

Gel-filtered platelets were obtained from human
PRP (prepared as above) using a Sepharose 2B column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as described by Timmons and Hawiger (1978). The platelets were suspended (10^6 platelets / ml) and their aggregation measured in a way similar to that used for PRP. The platelets were suspended in Tyrode’s buffer and the inhibitory activity of heparin and that of dalteparin measured after the addition of 1.25 unit/ml of purified exogenous human ATIII to the reaction mixture. The antiplatelet activity of each of the test drugs was expressed as the concentration at which it inhibited the maximum aggregation in the control (observed following addition of the appropriate vehicle) by 50% (IC50). The IC50 was calculated from the concentration-inhibition curve.

Enhancement of epinephrine-induced aggregation

Epinephrine-induced platelet aggregation was used to evaluate platelet activation by the test drugs. The concentration of epinephrine that induced about 20% of the maximum aggregation, but no secondary aggregation, was observed in the presence of the vehicle of a given test drug (aggregation threshold concentration). This aggregation threshold concentration was determined in each individual. Then, in a similar way, the test drug and PRP were placed in an assay cuvette, epinephrine was added at the aggregation threshold concentration after 1 min, and the aggregation was measured. The aggregation-enhancing activity of the test drug was expressed as its EC200, which was calculated from the dose-response curve as the concentration of the drug at which the maximum aggregation at the aggregation threshold concentration (observed following addition of vehicle alone) was enhanced to 200% (i.e. doubled).

Statistical analysis

The IC50 values for RVV-X-activated FXa activity and platelet aggregation, the CT2 values for blood coagulation, and the EC200 values for platelet aggregation were calculated from the respective concentration-response curves (each obtained using mean values from 3-4 individuals). The results obtained for platelet-inhibition activity were expressed as the mean ± S.E. and examined using Dunnett’s multiple comparison test (Dunnett, 1955). p values less than 0.05 were considered significant.

RESULTS

Inhibitory activities against human FXa and other serine proteases

Ki values were calculated for the actions of KFA-1411 against various purified human serine proteases (Table 1). The Ki value obtained for KFA-1411 against FXa was 1.73 nM and the inhibition was of the competitive type (data not shown). When we examined the inhibitory activity of KFA-1411 against other serine proteases of the blood coagulation/fibrinolytic system, we found that its Ki value against thrombin was 26000 nM (15000 times the Ki value against FXa). Among the other proteases, the inhibitory actions of KFA-1411 against kallikrein and plasmin were relatively powerful, but the Ki values were 80 or more times that for FXa. At the range of concentrations needed to inhibit Ki value against FXa, KFA-1411 did not influence FVIIa/TF (located in the extrinsic pathway of the blood-coagulation system) or FIXa (which participates in the formation of FXa at a subsequent stage). The catalytic site in the digestive enzyme trypsin is considered to be similar to that found in FXa (Whitlow et al., 1999), but the Ki value obtained for the former was 6170 nM, 3600 times that for FXa. The above results suggest that KFA-1411 has a selective inhibitory action on FXa.

Effects on human blood coagulation

Since we found KFA-1411 to have an inhibitory action on FXa, its effects on blood coagulation were

<table>
<thead>
<tr>
<th>Purified Human Enzyme</th>
<th>KFA-1411 Ki (nM)</th>
<th>Selectivity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xa</td>
<td>1.73</td>
<td>1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>26000</td>
<td>15000</td>
</tr>
<tr>
<td>Factor IXa</td>
<td>109000</td>
<td>63000</td>
</tr>
<tr>
<td>Factor VIIa/TF</td>
<td>229000</td>
<td>132000</td>
</tr>
<tr>
<td>Factor XIIa</td>
<td>109000</td>
<td>63000</td>
</tr>
<tr>
<td>APC</td>
<td>37200</td>
<td>22000</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>161</td>
<td>90</td>
</tr>
<tr>
<td>Plasmin</td>
<td>141</td>
<td>80</td>
</tr>
<tr>
<td>t-PA</td>
<td>13300</td>
<td>7700</td>
</tr>
<tr>
<td>Trypsin</td>
<td>6170</td>
<td>3600</td>
</tr>
</tbody>
</table>

Ki values were each obtained in a chromogenic assay using the purified human enzyme shown. Each value was determined from a Dixon-plot (n = 3).
Characteristics of factor Xa inhibitor; KFA-1411.

examined in vitro (Table 2). KFA-1411 prolonged both APTT and PT dose-dependently, and its CT2 for PT was about one-third of the value obtained for APTT, indicating that PT is sensitive to the anti-coagulant action of KFA-1411. In the case of the thrombin inhibitor argatroban, the CT2 values for PT and APTT were in a similar range to those of KFA-1411. However, in contrast to the situation noted above for KFA-1411 there was no difference between the CT2 values for PT and APTT.

The actions on PT and APTT exerted by heparin and dalteparin were also measured, although direct comparison with KFA-1141 or argatroban was impossible because of the differences in their concentration units. A CT2 value in respect of APTT was obtained for all four drugs in the present experiment. On the other hand, heparin had to be used at high concentration to cause a prolongation of PT, and dalteparin did not cause a marked prolongation of PT at any concentration tested.

Effects on human platelet aggregation

The function of platelets is as important as blood coagulation for hemostasis and thrombus formation, so we examined the action of KFA-1141 on platelet aggregation in vitro using human gel-filtered platelets. Thrombin, which is related most closely to blood coagulation, was used as the aggregation inducer, and the action of KFA-1141 was compared with those of the other anticoagulants. KFA-1411 inhibited platelet aggregation at a high concentration (10 µM), but its IC50 was about 600 times that of argatroban (16 nM) (Table 3 and Fig. 2A). The anti-aggregation activity of heparin and that of dalteparin were measured by adding exogenous ATIII to the platelet preparation, and they dose-dependently inhibited aggregation with IC50 values of 0.027 U/ml (heparin) and 0.083 IU/ml (dalteparin) (Table 3 and Fig. 2B). To further evaluate the actions of KFA-1411 on platelet aggregation and blood coagulation, we divided the CT2 for APTT by the IC50 for thrombin-induced platelet aggregation (Table 3).

Table 2. Anticoagulant effects of KFA-1411 and other anticoagulants in human plasma.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>APTT (CT2)</th>
<th>PT (CT2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KFA-1411 (µM)</td>
<td>0.87</td>
<td>0.28</td>
</tr>
<tr>
<td>Argatroban (µM)</td>
<td>0.51</td>
<td>0.49</td>
</tr>
<tr>
<td>Heparin (U/ml)</td>
<td>0.18</td>
<td>4.64</td>
</tr>
<tr>
<td>Dalteparin (IU/ml)</td>
<td>0.89</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

CT2 indicates the drug concentration needed to double the clotting time established in the vehicle assay, as calculated from the curve relating concentration to clotting-time prolongation (n=3-7). APTT: activated partial thromboplastin time; PT: prothrombin time. The control clotting times (vehicle alone was added) were 33.5 ± 0.9 sec. (APTT) and 15.3 ± 0.2 sec. (PT).

Fig. 2. Effects of KFA-1411 and other anticoagulants on thrombin-induced gel-filtrated human platelet aggregation. Panel A, Antiplatelet activity of KFA-1411 (○, µM) and that of argatroban (■, µM) expressed as inhibition of maximum aggregation induced by thrombin. Panel B, Antiplatelet activity of heparin (○, U/ml) and that of dalteparin (▲, international units/ml) expressed in same way as in panel A. Mean±S.E. of 4 observations. * p<0.05 vs. vehicle.
The ratio was about 12 for KFA-1411, indicating that KFA-1411 exerts an anticoagulant action at a lower concentration than that needed for its antiplatelet action. On the other hand, the ratio was small at 0.03 for argatroban, suggesting that it inhibits platelet aggregation at a lower concentration than that at which it inhibits blood coagulation. The ratio was also less than unity for both heparin and dalteparin, and they can therefore be considered to be have antiplatelet actions within the concentration range at which they exhibit anticoagulant action.

Heparin is known to induce platelet aggregation. In this study, we made use of epinephrine-induced platelet aggregation in human PRP to examine whether or not KFA-1411 has a similar action. The mean concentration of epinephrine needed to induce about 20% of the maximum aggregation was 0.28 μM in this experiment. KFA-1411 had no effect on this platelet aggregation at concentrations below 10 μM, although a tendency towards inhibition was seen at 30 μM and above (Fig. 3A). In contrast, heparin dose-dependently promoted platelet aggregation at 0.1 U/ml and above, with 300% promotion seen at 10 U/ml and above (compared with the aggregation induced by vehicle alone), and the EC200 value was 0.04 U/ml. Although the action of dalteparin was weaker than that of heparin, it tended to promote aggregation, with the EC200 value in its case being 0.53 IU/ml (Fig. 3B).

The concentration of KFA-1411 needed to inhibit platelet aggregation in this experiment was consistent with the results observed in standard epinephrine-induced sub-maximal aggregation of human PRP (78% aggregation, IC50: 25.9 μM). In addition, although KFA-1411 inhibited standard ADP-induced and collagen-induced platelet aggregation in human PRP, a high concentration was needed in each case. The IC50 values were 39.9 μM and 41.0 μM, respectively, higher than the concentrations at which it inhibits blood coag-

<table>
<thead>
<tr>
<th>Table 3. Ratio of anticoagulant activity to antiplatelet activity for KFA-1411 and other anticoagulants.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>KFA-1411 (1)</td>
</tr>
<tr>
<td>Argatroban (1)</td>
</tr>
<tr>
<td>Heparin (2)</td>
</tr>
<tr>
<td>Dalteparin (3)</td>
</tr>
</tbody>
</table>

Antiplatelet* indicates the IC50 for thrombin-induced gel-filtrated human platelet aggregation. Anticoagulation** indicates the CT2 for APTT in human plasma. Ratio*** was calculated by dividing the IC50 for thrombin-induced gel-filtrated human platelet aggregation by the CT2 for APTT in human plasma. The concentration units of each drug for IC50 and CT2 were as follows: (1): KFA-1411, Argatroban (μM), (2): Heparin (U/ml), (3): Dalteparin (IU/ml).

Fig. 3. Effects of KFA-1411 and other anticoagulants on epinephrine-induced human platelet aggregation. Panel A, Effects of KFA-1411 (●, μM) and argatroban (□, μM) on maximum aggregation induced by epinephrine. Panel B, Effects of heparin (○, unit/ml) and dalteparin (▲, international units/ml) expressed as in panel A. Mean ± S.E. of 4 observations. *p<0.05 vs vehicle.
Characteristics of factor Xa inhibitor; KFA-1411.

Species differences in the inhibitory actions of KFA-1411 against RVV-X-activated FX and anticoagulant actions

The species variation in the inhibitory action of KFA-1411 against FXa was examined using plasma from humans and animals. The inhibitory activity was expressed as the concentration producing 50% inhibition of reference enzyme activity (IC50). For the present purposes, we used a method that does not require purification of the enzyme from plasma (i.e. by activating the endogenous FX in plasma with RVV-X, and assaying RVV-X-activated FX activity by the chromogenic method). The RVV-X used to activate FX was confirmed to have little cross-reaction with respect to S-2222, a synthetic substrate for FXa (data not shown). Although RVV-X activates FIX as well as FX (see Table 1), KFA-1411 did not affect the activity of FIXa at the concentrations at which it KFA-1411 inhibited FXa. KFA-1411 dose-dependently inhibited RVV-X-activated FX in all species (Fig. 4).

To confirm the species variation in the inhibitory action of KFA-1411 against FXa, the anticoagulant action of KFA-1411 was measured in plasma samples from various species. The anticoagulant action was evaluated by calculating the CT2 values for PT and APTT in a way similar to that used for the evaluation of anticoagulant actions in humans. Table 4 shows the CT2 values for APTT and PT as well as the IC50 values for KFA-1411 against RVV-X-activated FX in various species (as the absolute value and relative to that obtained in humans). The IC50 and CT2 values were lowest in human plasma, and the values obtained for Cynomolgus monkeys were closest to the human values. In contrast, the values in dogs, rabbits and rodents were different from the human values, although the IC50 value in rabbits was quite close to the monkey IC50 and close to human IC50.

**DISCUSSION**

LMWH has been shown by preclinical studies to carry less risk of hemorrhage than heparin due to its ratio of inhibitory effects (on FXa versus thrombin) being greater than that of heparin (Cade et al., 1984). However, LMWH is still an ATIII-dependent anticoag-

**Table 4.** Effects of KFA-1411 on APTT, PT, and RVV-X-activated FX in human and animal plasma samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>APTT (CT2: µM) [Ratio]</th>
<th>PT (CT2: µM) [Ratio]</th>
<th>RVV-X-activated FX (IC50: µM) [Ratio]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.87 [1.1]</td>
<td>0.28 [1.1]</td>
<td>0.051 [1.1]</td>
</tr>
<tr>
<td>Monkey</td>
<td>1.28 [1.5]</td>
<td>0.36 [1.3]</td>
<td>0.080 [1.6]</td>
</tr>
<tr>
<td>Dog</td>
<td>6.18 [7.1]</td>
<td>1.85 [6.6]</td>
<td>0.38 [7.5]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3.33 [3.8]</td>
<td>0.86 [3.1]</td>
<td>0.11 [2.2]</td>
</tr>
<tr>
<td>Rat</td>
<td>4.18 [4.8]</td>
<td>1.44 [5.1]</td>
<td>0.42 [8.3]</td>
</tr>
</tbody>
</table>

The effects of KFA-1411 on APTT and PT are expressed as the CT2 value, with the ratios being obtained by dividing the animal CT2 by the human CT2 in each case. The inhibitory activity of KFA-1411 towards RVV-X-activated FX is expressed as the IC50. The ratio was calculated by dividing the animal IC50 by the human IC50 in each case. Human, monkey (Cynomolgus), dog (beagle), rabbit (Japanese white), rat (Wistar), mouse (ICR).

The control clotting times (vehicle alone was added) were as follows; APTT: human 33.6 ± 1.2 sec., monkey 22.7 ± 0.6 sec., dog 13.2 ± 0.1 sec., rabbit 21.3 ± 0.2 sec., rat 15.2 ± 0.4 sec. and mouse 21.6 ± 0.1 sec. and PT: human 15.1 ± 0.4 sec., monkey 16.0 ± 0.5 sec., dog 7.6 ± 0.1 sec., rabbit 8.6 ± 0.3 sec., rat 19.0 ± 0.4 sec. and mouse 12.5 ± 0.2 sec.
ulant not entirely free of the problems associated with heparin (Petitou et al., 1999). The advantages possessed by LMWH would be even greater in any anticoagulant with a greater selectivity for FXa if one could be developed. In fact, the newly developed agent KFA-1411 has a high selectivity for FXa with little action on other enzymes involved in blood coagulation. In addition, as its actions on fibrinolytic enzymes are also weak, it should be possible to use it concomitantly with thrombolytic agents for the treatment of thrombosis.

We initially assumed that KFA-1411, an FXa inhibitor, would act equally on the intrinsic and extrinsic pathways of the coagulation cascade, since FXa is located at the intersection of the two pathways. In this study, however, the action of KFA-1411 on PT was about 3 times stronger than that on APTT not only in humans, but also in other animal species, and so it had a stronger action on the coagulation induced via the extrinsic pathway. These results are in agreement with a report concerning another FXa inhibitor, DX9065a (Hara et al., 1994). However, Herbert et al. (1996), who used a reassembled prothrombinase and measured thrombin production as an index in human plasma, reported that DX9065a had a greater effect on the intrinsic system than on the extrinsic system. Argatroban, a thrombin inhibitor, showed similar CT2 values for PT and APTT in this study. Since argatroban acts after the integration of the intrinsic and extrinsic pathways of the coagulation cascade, it is considered to exert similar effects on both APTT and PT. This difference in action may lead to practical differences depending on whether a selective FXa inhibitor or selective thrombin inhibitor is used as an anticoagulant.

In this study, KFA-1411 had little effect on platelets in the range of concentrations at which it inhibited FXa. Platelets are important primarily in hemostasis when the vascular wall is disrupted. From an antithrombotic viewpoint, inhibition of both platelet aggregation and blood coagulation is considered to be advantageous. However, from a hemostatic viewpoint, inhibition of both platelet aggregation and blood coagulation increases the risk of hemorrhage. Moreover, antiplatelet agents with diverse mechanisms of action have already been developed, and there appears to be no merit in a single drug having both of these actions.

Argatroban suppressed thrombin-induced platelet aggregation at a much lower concentration than needed for its anticoagulant effect. This difference in threshold concentration between its antiplatelet action and its anticoagulant action indicates the possibility that the risk of hemorrhage due to a decrease in platelet function might occur before the concentration was reached at which the anticoagulant action would appear. A similar result, with regard to platelet aggregation and anticoagulant action, was observed for both heparin and LMWH. In contrast, KFA-1411 did not inhibit thrombin-induced platelet aggregation, nor indeed the platelet aggregation induced by other inducers, even at higher concentrations than those needed for anticoagulation. These results suggest that KFA-1411 would exert no antiplatelet action at the concentrations reached in the body when it is given as an anticoagulant, so that we may anticipate a low risk of hemorrhage with this agent.

On the other hand, heparin is reported to activate platelets, and the PF4 secreted from activated platelets is known to neutralize the anticoagulant activity of heparin (Burgess and Chong, 1997). Moreover, this phenomenon is causally related to HIT (Warkentin et al., 1995). In the present study, platelet activation by heparin was reproduced in vitro by epinephrine-stimulated platelet aggregation, and platelet activity was enhanced at heparin concentrations of 1 U/ml and above, so this phenomenon may occur even within its range of effective circulating concentrations (0.1-1 U/ml) (Abildgaard, 1989). LMWH is considered to have a smaller potency towards platelets than heparin (Salzman et al., 1980; Holmer et al., 1980), but platelet activation could not be avoided with dalteparin in this study, although it was less notable than with heparin treatment. In fact, platelet activation could not be eliminated in a study using a synthetic polysaccharide that represents the minimum synthetic unit of heparin (Petitou et al., 1999). As KFA-1411 induced no platelet activation, it ought not to cause untoward events in which platelets are involved (such as neutralization of anticoagulant activity and HIT), and which pose problems during routine heparin treatment.

Generally, species variation must be taken into consideration when an assessment of any developing drug is carried out using experimental animal models. In the present study, to evaluate species variation in the effect of KFA-1411 we devised an evaluation system using RVV-X-activated FX, since the affinity of RVV-X for S-2222, which is a synthetic substrate of FXa, is low, and since trypsin, which can also use S-2222 for a substrate, is not usually present in blood. Although RVV-X also activates FIX, KFA-1411 does not inhibit FIX at the concentrations at which it inhibits FXa, and the results obtained in this study are considered to be ascribable to an action on FXa (Jesty, 1986). This
method is convenient for estimation of the actions of a drug on an enzyme in various animal species, but purification of each enzyme is necessary to obtain accurate results. We found the activity of KFA-1411 to be very similar between humans and monkeys.

The species difference in FXa inhibition between rat and human was about 10-fold, a finding similar to that reported for another FXa inhibitor, DX9065a (Hara et al., 1995). The KFA-1411 activity in the dog was also markedly different from that in humans, so that the dog is unsuitable as an experimental model for the evaluation of KFA-1411. Experimental animal models are indispensable for the estimation of the clinical efficacy and safety of any new drug in humans, and KFA-1411 is no exception. On the basis of our species-variability data, confirmation in pre-clinical tests using monkeys will be necessary for the estimation of the clinical potential of KFA-1411 both as an FXa inhibitor and as an anticoagulant in humans. Safety data of KFA-1411, including bleeding risk in monkeys, will also be necessary to predict the clinical safety of this drug.

ACKNOWLEDGMENT

We sincerely thank Dr. Ichirou Kudo, Professor of the Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University, for valuable advice and discussion.

REFERENCES


