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Inhibition of arterial thrombosis by a protease-activated receptor 1 antagonist, FR171113, in the guinea pig

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Abstract

The antiplatelet and antithrombotic effects of FR171113, 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonyl methylene)-1,3-thiazolidin-4-one, a non-peptide protease-activated receptor 1 (PAR1) antagonist, were evaluated in guinea pigs. FR171113 inhibited Ser–Phe–Leu–Leu–Arg–Asn–NH_2 (a synthetic PAR1 agonist peptide)-induced and thrombin-induced aggregation of guinea pig platelets in a concentration-dependent manner in vitro (IC_{50}=1.5 and 0.35 μM, respectively). Subcutaneous administration of FR171113 (0.1–3.2 mg/kg) produced a dose-dependent inhibition of platelet aggregation ex vivo. The ED_{50} value of FR171113 for platelet aggregation was 0.49 mg/kg s.c. However, FR171113 did not have an inhibitory effect on ADP- or collagen-induced platelet aggregation in vitro and ex vivo. One hour after FR171113 treatment at 1.0 mg/kg s.c., significant inhibition of arterial thrombosis without a prolongation of thrombin time or coagulation time was seen in the $FeCl_3$-induced carotid artery thrombosis model in guinea pigs. Furthermore, FR171113 did not prolong bleeding time even at 32 mg/kg s.c., which is a much higher dose than that required in the thrombosis model. These observations indicate that FR171113 has desirable antiplatelet effects both in vitro and in vivo and that its in vivo antithrombotic activity is efficacious without causing a prolongation of bleeding time.

Keywords: FR171113; PAR-1 antagonist; Thrombin receptor; Arterial thrombosis

1. Introduction

Platelet aggregation plays a critical role in the pathophysiology of thrombotic diseases, and as a consequence antiplatelet agents have been used clinically in patients at risk for brain ischemia, unstable angina and acute myocardial infarction (Gregory, 1995). Thrombin is a serine protease which catalyzes the cleavage of fibrinogen and the thrombin receptor and activates the coagulation system and platelet function. Therefore, thrombin is considered to play a central role in hemostasis and in thrombosis. The cloning of a functional thrombin receptor (protease-activated receptor 1, PAR1) (Vu et al., 1991) and elucidation of the proteolytic mechanism for its activation (Coughlin et al., 1992) were subsequently followed by the cloning of PAR2 (Nystedt et al., 1994), PAR3 (Ishihara et al., 1997) and PAR4 (Xu et al., 1998).

We recently discovered an unique non-peptide antagonist compound, FR171113, 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonyl methylene)-1,3-thiazolidin-4-one (Fig. 1) (Kato et al., 1999), which is more potent in causing inhibition of human platelet aggregation induced by Ser–Phe–Leu–Leu–Arg–Asn–NH_2, a PAR1 agonist peptide, in vitro than C186-65, with the known thrombin receptor antagonist (3-mercapto-propionyl-Phe–Cha–Cha–Arg–Asn–Pro–Asn–Asp–Lys–Tyr–OH) (Scarborough, 1994; Scarborough et al., 1992). FR171113 appeared to be a specific inhibitor of PAR1-mediated platelet aggregation in humans. It also appeared to act directly on the thrombin receptor, since it does not have thrombin protease activity (Kato et al., 1999). FR171113

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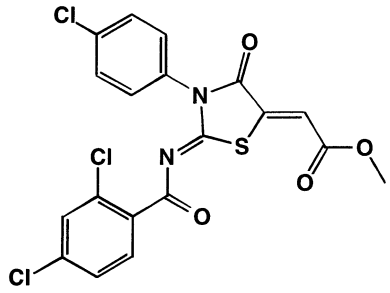

Fig. 1. The chemical structure of FR171113.

may be an antithrombosis agent without side effects such as hemorrhage. However, the antithrombotic effects of FR171113 in vivo had not been reported. It has been shown that C186-65 inhibits the deposition of platelets onto a Dacron vascular graft and collagen-coated grafts in baboon in an in vivo experiment (Lindahi et al., 1993).

In this study, we investigated the antiplatelet and antithrombotic effects of FR171113 in normal guinea pigs and in the $FeCl_3$-induced carotid artery thrombosis model in guinea pigs. In addition, we compared the pharmacological profile of FR171113 with the profiles of anticoagulant, heparin and argatroban by evaluating occlusive thrombus formation, plasma coagulation activity and bleeding time in vivo.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs were purchased from Japan SLC (Hamamatsu, Japan). They were housed in temperature- and humidity-controlled animal quarters with access to food and water ad libitum before the experiment. The protocol of the present study was approved by the Animal Care and Use Committee of Fujisawa Pharmaceutical.

2.2. In vitro platelet aggregation

Human blood was collected from healthy volunteers into plastic vessels containing 3.8% sodium citrate (1:10 volume). Blood from male guinea pigs weighing 500–800 g, which were anesthetized with diethyl ether, was collected from the abdominal aorta into plastic vessels containing 3.8% sodium citrate (1:10 volume). Human or guinea pig platelet-rich plasma was obtained from the supernatant fraction of the blood after centrifugation at 150×*g* for 10 min at room temperature. Human or guinea pigs platelet-poor plasma was obtained by centrifugation of the remaining blood at 1500×*g* for 10 min at room temperature.

To obtain guinea pig washed platelets, prostaglandin I_2 (1 μM) was added to the prepared guinea pig platelet-rich plasma. Plasma was removed by centrifugation at 800×*g* for 10 min at room temperature, and the pellet was resuspended in a equal volume of modified Tyrode's buffer which contained 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 8.9 mM $NaHCO_3$, 10 mM HEPES and 5.5 mM glucose (pH 6.5). The final cell count of the washed platelet suspension was adjusted to 3×10^8 platelets/ml with the above buffer (pH 7.2).

Platelet aggregation was measured according to Born's turbidimetric method (Born and Cross, 1968) with an aggregometer (Hema Tracer 801, MC Medical, Tokyo, Japan). Platelet-rich plasma was used as described previously (Kato et al., 1999). To 194 μl of washed platelets in a cuvette, 2 μl of each drug or vehicle and 4 μl of 50 mM $CaCl_2$ were added, and the mixture was preincubated at 37 °C for 2 min. After the incubation, platelet aggregation was induced by the addition of 4 μM of aggregating agent. In order to quantify the inhibitory effects of each drug, the maximum increase in light transmission was determined from the aggregation curve for 7 min after the addition of agonist. The effect of each drug was expressed as percent inhibition of agonist-induced platelet aggregation compared with vehicle treatment.

2.3. Ex vivo platelet aggregation

After anesthetizing the animals, blood samples anticoagulated with sodium citrate were collected. Platelet-rich plasma and platelet-poor plasma were prepared, and platelet aggregation studies were performed as described for in vitro platelet aggregation. The guinea pigs were subcutaneously treated with the vehicle, FR171113, argatroban and aspirin dissolved in dimethyl sulfoxide or saline 1 h before collecting the blood.

2.4. In vivo thrombosis model

The carotid thrombosis model was established by a modification of the method of Broersma et al. (1991). Male Hartley guinea pigs (650–950 g) were anesthetized with urethane (1.25 g/kg, i.p.). The carotid artery was carefully dissected and a pulse Doppler flow probe (φ: 1.5 mm, Primetech, Tokyo, Japan) was placed around it to record the blood-flow velocity and to monitor the patency of the vessels. The carotid blood flow was recorded on a polygraph (Nihon Koden, Tokyo, Japan). Carotid artery thrombosis was induced by the $FeCl_3$ method as follows: a square (1×1 mm) of Advantec No. 2 filter paper was immersed in 20% $FeCl_3$ solution and placed on the carotid artery. Thrombosis was monitored as the reduction in carotid artery blood flow. The time at which the blood-flow velocity decreased to zero was recorded as the time to thrombotic occlusion of the vessel. When the blood-flow velocity did not occlude within 120 min, time to thrombotic occlusion was assigned a value of 120 min. Each of the drugs or vehicle was administered subcutaneously 1 h before applying the filter paper to the carotid artery.

2.5. Measurement of coagulation times in the thrombosis model

Male guinea pigs (650–950 g) were anesthetized with urethane (1.25 g/kg, i.p.). The carotid thrombosis model was established as described above, and plasma was obtained from the supernatant fraction of the blood after centrifugation at 1500×*g* for 10 min at 4 °C. Clotting time was determined as the time to clot formation at 37 °C with a coagulometer (Amelung Coagulometer, Heinrich Amelung, Lemgo, Germany). To measure the activated partial thromboplastin time, as a measure of the intrinsic coagulation pathway, 100 μl of plasma was preincubated for 2 min at 37 °C after the addition of 100 μl of PLANTERIN Plus Activator which contained rabbit brain phospholipids and Celite. Then, coagulation was initiated with 100 μl of 0.025 M calcium chloride. To measure the prothrombin time, as a measure of the extrinsic coagulation pathway, 100 μl of plasma was preincubated for 2 min at 37 °C and coagulation was initiated with 200 μl of SIMPLASTIN which contained 2.25 mg of thromboplastin. To measure the thrombin time, 100 μl of plasma was added to 100 μl of Owren's Veronal buffer, which contained 28 mM sodium barbital and 125 mM sodium chloride (pH 7.35) and was preincubated for 2 min at 37 °C. The coagulation was initiated with 100 μl of 3 U/ml of human thrombin.

2.6. Bleeding time

Template bleeding time was measured with Simplate® R (Organon Teknika Durham, NC). Male Hartley guinea pigs (650–950 g) were anesthetized by intraperitoneal injection of pentobarbital (25 mg/kg). Each drug or vehicle was given subcutaneously 1 h before incising the ear. The template bleeding device was placed on the dorsal surface of the auricle and triggered. Blood from the incision was blotted with filter paper every 30 s until bleeding had stopped completely, without making contact with the cut surface when blotting. Time elapsed until bleeding stopped was measured as the bleeding time.

2.7. Measurement of intracellular Ca^{2+} mobilization

Normal human umbilical cord vein endothelial cells were obtained from Kurabo (Osaka, Japan). The cells were cultured in HuMedia-EB2 medium (Kurabo) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2. Only four to six passages of the cells were used in the experiments.

The cytosolic Ca^{2+} concentration was measured using the fluorescent Ca^{2+} indicator, Fura 2-AM. For the experiments, cells were grown on fibronectin-coated cover slips (6×10 mm) placed in petri dishes (60 cm^2) to 80% confluency and loaded with 3.3 μM of Fura 2-AM in buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 2 mM HEPES and 0.1% bovine serum albumin adjusted to pH 7.4 for 30 min at 37 °C. After the incubation, cells were washed with two changes of the buffer to remove the excess dye. The mobilization of internal stores of calcium was measured using the calcium-ion analyzer FS-100 (Kowa, Tokyo, Japan). To 297 μl of buffer and a cover slip in a cuvette, 3 μl of FR171113 or vehicle was added and incubated at 37 °C for 2 min. After the incubation, calcium mobilization was induced by the addition of 5 μl of the PAR2 agonist peptide Ser–Leu–Ile–Gly–Lys–Val. The fluorescence was measured at wavelengths of 340 and 380 nm excitation and 510 nm emission. Cytosolic calcium ion concentrations were calculated as described (Grynkiewicz et al., 1985).

2.8. Statistical analysis

The data are presented as the means±S.E.M. or +S.E.M. for the number of experiments indicated. The IC_{50} and ED_{50} values are expressed as the drug concentration required to produce 50% inhibition of agonist-induced platelet aggregation in comparison to vehicle treatment; this was obtained by linear regression. For comparison between two groups and multiple comparison, the data were analyzed using Student's *t*-test and one-way analysis of variance followed by Dunnett's test, respectively.

2.9. Materials

FR171113 (Fig. 1) was synthesized by the Medicinal Chemistry Research Laboratories, Fujisawa (Osaka, Japan) and the purity was >98% by high-pressure liquid chromatography. Argatroban was purchased from Mitsubishi-kasei (Tokyo, Japan). Adenosine 5′ -diphosphate (ADP), prostaglandin I_2 and aspirin were obtained from Sigma (St. Louis, MO). Collagen (equine tendon) (Packham, 1984) was bought from Nycomed (Munich, Germany). Heparin was purchased from Shimizu Pharmaceutical (Tokyo, Japan). Ser–Phe–leu–Leu–Arg–Asn–NH_2 (PAR1 agonist peptide) and Gly–Tyr–Pro–Gly–Gln–Val (PAR4 agonist peptide) were from Kurabo (Tokyo, Japan). Ser–Leu–Ile–Gly–Lys–Val–NH_2 (PAR2 agonist peptide) was purchased from Bachem (Bubendorf, Switzerland). PLANTERIN Plus Activator and SIMPLASTIN were obtained from Organon Teknika. Owren's Veronal buffer was from Toa Medical Electronics (Hyogo, Japan). Fura 2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan).

In in vitro, ex vivo and in vivo experiments, FR171113 and aspirin were dissolved in dimethyl sulfoxide for the in vitro, ex vivo and in vivo experiments and in a 100-fold concentrated stock solution for the in vitro experiment. The subcutaneous injection was given in a volume of 0.2 ml/kg of animal weight. Argatroban was dissolved in saline for the in vitro, ex vivo and in vivo experiments and added to a 100-fold concentrated stock solution for the in vitro experiment. The subcutaneous injection was given in a volume of

2 ml/kg of animal weight. Heparin was dissolved in saline and given in a volume of 2 ml/kg of animal weight.

3. Results

3.1. Effects of FR171113 and antithrombotic drugs on platelet aggregation in vitro

The inhibitory effects of FR171113, argatroban and aspirin on PAR1 agonist peptide-, ADP- and collagen-induced platelet aggregation in guinea pig platelet-rich plasma are shown in Fig. 2. FR171113 (0.1–10 μM) dose-dependently inhibited the aggregation induced by the PAR1 agonist peptide in guinea pigs. The IC_{50} value of FR171113 for PAR1 agonist peptide-induced platelet aggregation was 1.5±0.55 μM. However, FR171113 did not inhibit the ADP- and collagen-induced aggregation of platelet even at 100 μM. Argatroban, a specific protease inhibitor of thrombin (Kikumoto et al., 1984; Hara et al., 1986), did not significantly inhibit the platelet aggregation induced by PAR1 agonist peptide, ADP or collagen even at 100 μM. Aspirin, a cyclooxygenase inhibitor (Smith and Willis, 1971), was only effective in preventing the aggregation induced by collagen, via the arachidonic acid cascade. The IC_{50} value of aspirin for collagen-induced platelet aggregation was 33±7.6 μM.

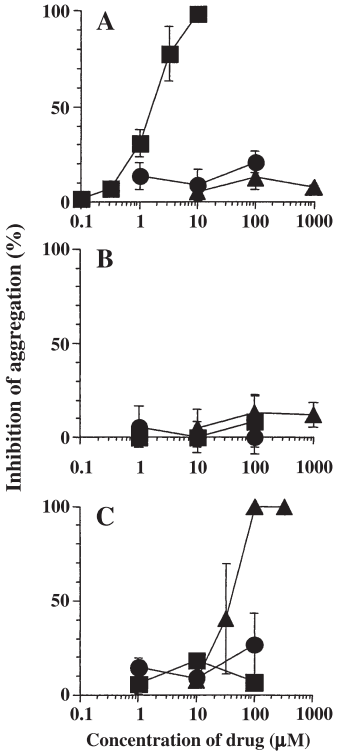

Fig. 2. The inhibitory effects of FR171113 (■), argatroban (●) and aspirin (▲) on (A) PAR1 agonist peptide-induced, (B) ADP-induced and (C) collagen-induced aggregation of guinea pig platelets in vitro. The final concentration of PAR1 agonist peptide, ADP and collagen were 6 and 1 μM and 1 μg/ml, respectively. The effects of each drug are expressed as percent inhibition of platelet aggregation compared with vehicle treatment. Each value represents the mean±S.E.M. for five experiments.

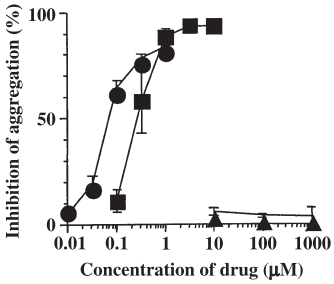

Fig. 3. The inhibitory effects of FR171113 (■), argatroban (●) and aspirin (▲) on thrombin-induced platelet aggregation in washed guinea pig platelets. The final concentration of thrombin was 0.5 U/ml. The effects of each drug are expressed as percent inhibition of platelet aggregation compared with vehicle treatment. Each value represents the mean±S.E.M. for five experiments.

The effect on the aggregation of washed platelets induced by thrombin in guinea pigs is shown in Fig. 3. FR171113 (0.1–10 μM) and argatroban (0.01–1 μM) dose-dependently inhibited the aggregation. Their IC_{50} values for thrombin-induced platelet aggregation were 0.35±0.10 and 0.077±0.0094 μM, respectively. Aspirin did not significantly inhibit the aggregation of washed platelets induced by thrombin, even at 1000 μM.

3.2. Effects of FR171113 and antithrombotic drugs on platelet aggregation ex vivo

Fig. 4 shows the effects of FR171113, argatroban and aspirin on platelet aggregation in guinea pigs ex vivo. Subcutaneous injection of FR171113 (0.1–3.2 mg/kg) inhibited the aggregation induced by PAR1 agonist peptide dose dependently. The ED_{50} value of FR171113 for this aggregation was 0.49 mg/kg. Argatroban at 3.2 mg/kg did not inhibit the platelet aggregation induced by PAR1 agonist peptide. Aspirin also did not inhibit PAR1 agonist peptide-induced aggregation at 100 mg/kg. These three drugs did not inhibit the aggregation of platelets induced by ADP. Only aspirin inhibited collagen-induced platelet aggregation. The percent inhibition by aspirin at 100 mg/kg s.c. of platelet aggregation was 87.8±5.8%.

3.3. Effects of FR171113 and antithrombotic drugs in an in vivo thrombosis model

The effects of FR171113, argatroban and heparin on the $FeCl_3$-induced carotid artery thrombosis model in guinea pigs are shown in Fig. 5. Time to thrombotic occlusion was taken as the time until blood flow stopped during 2 min. The control range for time to thrombotic occlusion after subcutaneous injection of dimethyl sulfoxide and saline was 18.3±1.0 and 14.8±0.9 min, respectively. Pretreatment

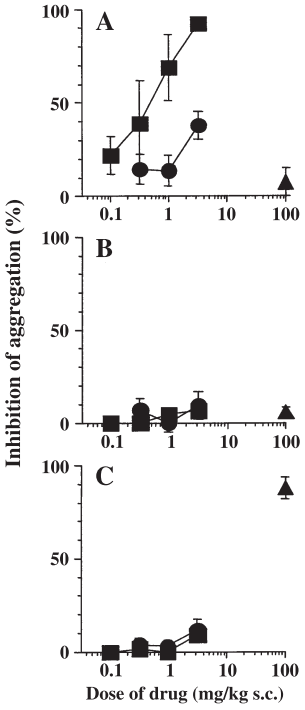

Fig. 4. The inhibitory effects of FR171113 (■), argatroban (●) and aspirin (▲) on PAR1 agonist peptide-induced (A), ADP-induced (B) and collagen-induced (C) platelet aggregation in guinea pig platelet-rich plasma ex vivo. The final concentrations of PAR1 agonist peptide, ADP and collagen were 6 μM, 1 μg/ml and 1 μM, respectively. Each value represents the mean±S.E.M. for five experiments.

with FR171113 prolonged this parameter in a dose-dependent manner. The time to thrombotic occlusion for 0.32, 1.0 and 3.2 mg/kg of FR171113 was 30.7±5.36, 44.7±8.41 (P<0.05) and 92.6±9.79 (P<0.01), respectively. Argatroban also dose-dependently (0.32–3.2 mg/kg) prolonged it. The mean time to thrombotic occlusion for 0.32, 1.0 and 3.2 mg/kg of argatroban was 18.6±1.9, 33.8±4.8 and 67.3±12.4 (P<0.01), respectively. Heparin had a prolonging effect at 100 and 320 U/ml (66.7±17.4 min [P<0.01], 112.3±7.0 min [P<0.01]).

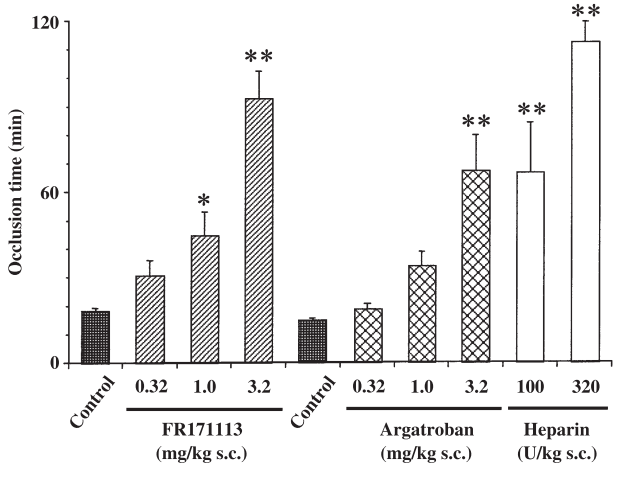

Fig. 5. Effects of FR171113, argatroban and heparin on thrombus formation in guinea pig carotid artery. The time at which the blood-flow velocity decreased to zero was recorded as the time to occlusion of vessels. When the vessels did not occlude within 120 min, the time to occlusion was assigned a value of 120 min. Each value represents the mean±S.E.M. of 10 experiments. *P<0.05 vs. control, **P<0.01 vs. control (Dunnett's test).

Table 1
Effects of FR171113 and argatroban on coagulation time in the $FeCl_3$-induced guinea pig arterial thrombosis model

Drug	Dose (mg/kg s.c.)	Clotting time (s)		
		APTT	PT	TT
Control		21.02±0.57	44.18±2.01	21.04±1.15
FR171113	3.2	22.44±0.20	42.88±2.05	24.76±2.04
Control		20.84±0.32	36.92±2.70	18.50±0.89
argatroban	3.2	30.44±1.21[a]	79.78±2.01[a]	120.00±0.58[a]

Each drug or vehicle was administered 1 h before the stimulation by $FeCl_3$. One hour after the stimulation, blood was collected from the abdominal aorta into plastic vessels containing 3.8% sodium citrate (1:10 volume), and plasma was prepared. Each value represents the mean±S.E.M. for five experiments.

[a] Indicates P<0.01 in comparison with control (Student's t-test).

Table 1 shows the effects of FR171113 and argatroban on coagulation time in the $FeCl_3$-induced arterial thrombosis

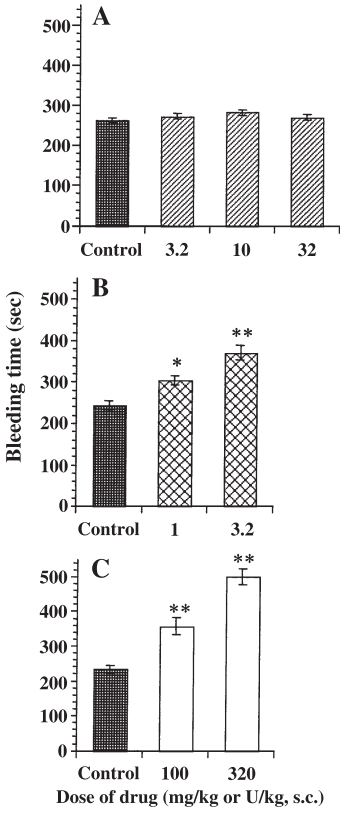

Fig. 6. Effects of FR171113 (mg/kg) (A), argatroban (mg/kg) (B) and heparin (U/kg) (C) on bleeding time in anesthetized guinea pigs. Bleeding time was measured 1 h after dosing by template methods. Each value represents the mean±S.E.M. of 10 experiments. *P<0.05 vs. control, **P<0.01 vs. control (Dunnett's test).

model. FR171113 did not influence the activated partial thromboplastin time, the prothrombin time or the thrombin time at 3.2 mg/kg s.c. at which concentration it significantly showed antithrombotic effects. However, argatroban significantly prolonged the activated partial thromboplastin time, the prothrombin time and the thrombin time in the model at 3.2 mg/kg s.c., at which concentration it showed significant antithrombotic activity but had less of an effect than FR171113.

3.4. Bleeding time

The control range for bleeding time after subcutaneous injection of dimethyl sulfoxide and saline was 4.3±0.1 and 4.2±0.1 min, respectively. Template bleeding time was not prolonged by treatment with FR171113 at 32 mg/kg s.c. (Fig. 6). Heparin and argatroban caused a prolongation of bleeding time that was dose dependent. Significant effects of heparin and argatroban were observed at 100 U/kg and 1 mg/kg s.c., respectively.

3.5. Effect of FR171113 on other protease-activated peptides at human tissues in vitro

Fig. 7 shows the effect of FR171113 (100 μM) on the PAR2-evoked Ca^{2+} response in normal human umbilical cord vein endothelial cells and the effect on PAR4-induced platelet aggregation in human platelet-rich plasma. FR171113 did not significantly inhibit the PAR2-induced cytosolic Ca^{2+} response or PAR4-induced platelet aggregation at 100 μM.

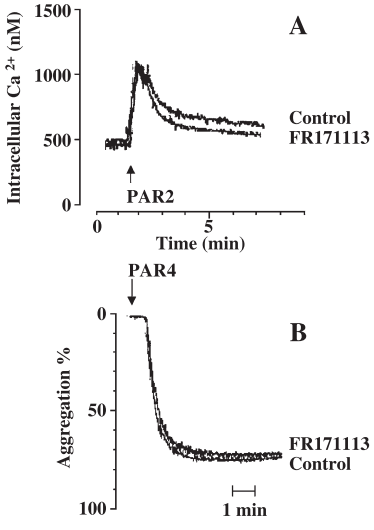

Fig. 7. Characterization of FR171113 on protease-activated receptors. (A) Effect of FR171113 (100 μM) and vehicle on PAR2-evoked Ca^{2+} responses in normal human umbilical cord vein endothelial cells. (B) Effect of FR171113 (100 μM) and vehicle on platelet aggregation induced by PAR4 in human platelet-rich plasma. The final concentrations of PAR2 and PAR4 were 100 μM and 1 mM, respectively.

4. Discussion

In the present study, the antithrombotic effects of a PAR1 antagonist, FR171113, in guinea pigs was demonstrated in vitro and in vivo. The antiplatelet effect in vitro of FR171113 on the aggregation of guinea pig platelets induced by PAR1 agonist peptide and thrombin (IC_{50}: 1.5 and 0.35 μM, respectively) was nearly equal to that on the aggregation of human platelets (IC_{50}: 2.5 and 0.29 μM, respectively) (Kato et al., 1999). FR171113 did not inhibit the aggregation induced by ADP and collagen even at 100 μM. We compared the antiplatelet profile of FR171113 in guinea pigs with the profiles of antithrombosis drugs with other functional mechanisms. Aspirin, a cyclooxygenase inhibitor (Smith and Willis, 1971), was only effective against collagen-induced platelet aggregation acting via the arachidonic acid cascade. Argatroban, a thrombin protease inhibitor (Hara et al., 1986), inhibited the aggregation induced by thrombin but not induced by other agonists in guinea pig platelets. Thus, a PAR1 antagonist, FR171113, which inhibits only PAR1 agonist peptide-induced platelet aggregation, has a different spectrum of antiplatelet effects in vitro from aspirin and argatroban in guinea pigs.

Next, we examined the effect of FR171113 on thrombosis in vivo using a guinea pig model. It was reported that platelets were depleted by more than 50% in a model of arterial thrombosis using $FeCl_3$ (Kurz et al., 1990a). It was reported that the occlusion of $FeCl_3$-damaged arteries is related to thrombin-mediated platelet aggregation and a fibrin-dependent process (Broersma et al., 1991). In this model, a platelet-rich thrombus forms similar to the arterial thrombi causing acute cardiovascular syndromes (Kurz et al., 1990b). Argatroban and heparin dose-dependently prolonged the occlusion time at 0.32–3.2 mg/kg and 100–320 U/kg, respectively. The antithrombotic effect of these drugs entails inhibitory action against the blood coagulation system. The effective dose of argatroban and heparin in this model correlated with the effective dose in terms of bleeding time. Argatroban significantly prolong the activated partial thromboplastin time, the prothrombin time and the thrombin time at 3.2 mg/kg s.c. in $FeCl_3$-induced arterial thrombosis. The preventive effect of argatroban and heparin on thrombosis was similar to the prolonging effect on bleeding time or coagulation time in guinea pigs in vitro.

In contrast, in the carotid artery thrombosis model, FR171113 suppressed occlusive thrombosis dose dependently and caused significant prolongation at 1 mg/kg s.c. The range of the effective dosage of FR171113 in the carotid artery thrombosis model paralleled the effective dosage ex vivo on platelet aggregation. Thus, the prevention of occlusive thrombosis by FR171113 may be closely reflected by the inhibitory effect of FR171113 on platelet aggregation via activation of the PAR1 receptor. However, FR171113 did not prolong activated partial thromboplastin time, prothrombin time or thrombin time in the carotid

artery thrombosis model at 3.2 mg/kg. Moreover, FR171113 did not affect bleeding time, even at 32 mg/kg. Therefore, PAR1 antagonist appears to prevent occlusive thrombosis without prolonging bleeding time in guinea pigs. It seems that the pharmacokinetic profile of the drug is important to its antithrombotic activity in vitro. A bolus subcutaneous administration of argatroban may not provide enough antithrombotic activity. However, the efficacy of argatroban at 3.2 mg/kg s.c. was significant against the $FeCl_3$-induced thrombosis model, coagulation time and bleeding time. FR171113 might better control bleeding time than argatroban in antithrombotic therapy.

PAR1 was cloned by Vu et al. (1991), and so far, four members of this class of receptors have been identified, PAR2 (Nystedt et al., 1994), PAR3 (Ishihara et al., 1997) and PAR4 (Xu et al., 1998). We evaluated the effects of FR171113 on PARs. FR171113 did not inhibit Ser–Leu–Ile–Gly–Lys–Val–NH_2 (PAR2 agonist peptide)-induced Ca^{2+} mobilization in normal human umbilical cord vein endothelial cells, even at 100 μM (only shown at 100 μM of FR171113). FR171113 also did not inhibit Gly–Tyr–Pro–Gly–Gln–Val (PAR4 agonist peptide)-induced platelet aggregation (only shown at 100 μM of FR171113).

In vivo antithrombotic effects of MW-1436, a synthetic PAR1 antagonist peptide, in a baboon arteriovenous shunt model (Lindahi et al., 1993), and the effects of RWJ-58259, another PAR1 antagonist peptide, in a guinea pig arteriovenous shunt model and photochemical model (Andrede-Gordon et al., 2001), were reported as well as the effects of C186-65 described in the Introduction. FR171113, unlike MW-1436, RWJ-58259 and C186-65, is a non-peptide PAR1 antagonist and is considered more stable in plasma and has a more efficient oral absorption than antagonists with peptide structures. This paper described how FR171113 showed antiplatelet and antithrombotic effects in vivo. FR171113 could become a good biological tool for investigating the role of PAR1 in many diseases.

In conclusion, in the $FeCl_3$-induced carotid artery thrombosis model, the non-peptide PAR1 antagonist FR171113 is effective in preventing occlusive arterial thrombosis without prolonging bleeding time or affecting coagulation time. FR171113 can be expected to become a useful agent for investigating antithrombotic actions via PAR1 in vivo.

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References

Andrede-Gordon, P., Derian, C.K., Marianoff, B.E., Zhang, H.-C., Addo, M.F., Cheung, W.-M., Damiano, B.P., D'andrea, M.R., Darrow, A.L., Galavilla, L.D., Eckardt, A.J., Giardino, E.C., Haertlein, B.J., Mccomsey, D.F., 2001. Administration of a potent antagonist of protease-activated receptor-1 (PAR-1) attenuates vascular restenosis following balloon angioplasty in rats. J. Pharmacol. Exp. Ther. 298, 34–42.

Born, G.V.R., Cross, M.J., 1968. The aggregation of blood platelets. J. Physiol. 168, 178–195.

Broersma, R.J., Kutcher, L.W., Heminger, E.F., 1991. The effect of thrombin inhibition in a rat arterial thrombosis model. Thromb. Res. 64, 405–412.

Coughlin, S.R., Vu, T.K.-H., Hung, D.T., Wheaton, V.I., 1992. Characterization of the cloned platelet thrombin receptor: issues and opportunities. J. Clin. Invest. 89, 351–355.

Gregory, W.A., 1995. Antithrombotic agents in cerebral ischemia. Am. J. Cardiol. 75, 34B–38B.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. J. Biol. Chem. 260, 3440–3450.

Hara, H., Tamao, Y., Kikumoto, R., 1986. Effect of argipidine(MD805) on platelet function. Jpn. Pharmacol. Ther. 14, 13–20.

Ishihara, H., Connolly, A.J., Zeng, D., Kahn, M.L., Zheng, Y.W., Timons, C., Tram, T., Coughlin, S.R., 1997. Protease-activated receptor 3 is a second thrombin recetor in humans. Nature 386, 502–506.

Kato, Y., Kita, Y., Nishio, M., Hirasawa, Y., Ito, K., Yamanaka, T., Motoyama, Y., Seki, J., 1999. In vitro antiplatelet profile of FR171113, a novel non-peptide thrombin receptor antagonist. Eur. J. Pharmacol. 384, 197–202.

Kikumoto, R., Tamao, Y., Tezuka, T., Tonomura, S., Hara, H., Ninomiya, K., Hijikata, A., Okamoto, S., 1984. Selective inhibition of thrombin by (2*R*, 4*R*)-4-methyl-1-[*N*2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl) sulfonyl]-L arginyl)]-2-piperidinecarboxylic acid. Biochemistry 23, 85–90.

Kurz, K.D., Jakubowski, J.A., Smith, T., Moore, R.A., Main, B.W., 1990a. Antithrombotic mechanisms involved in prevention of ferric chloride-induced arterial thrombosis in rat. Fed. Am. Soc. Exp. Biol. 4, A1228.

Kurz, K.D., Main, B.W., Sandusky, G.E., 1990b. Rat model of arterial thrombosis induced by ferric chloride. Thromb. Res. 60, 269–280.

Lindahi, A.K., Scarborough, R.M., Naughton, M.A., Harker, L.A., Hanson, S.R., 1993. Antithrombotic effect of a thrombin receptor antagonist peptide in baboons. Thromb. Haemostasis 69, 1196 (Suppl.).

Nystedt, S., Emilsson, K., Wahlestedt, C., Sundelin, J., 1994. Molecular cloning of a potential proteinase activated receptor. Proc. Natl. Acad. Sci. U. S. A. 91, 9208–9212.

Packham, M.A., 1984. Standardization of collagen: consideration of current practices in testing collagen-induced aggregation. Thromb. Haemost. 52, 3, 358–361.

Scarborough, R.M., 1994. Thrombin receptors and their pharmacological modulation. Can. J. Physiol. Pharmacol. 72, 29 (Suppl.).

Scarborough, R.M., Teng, W., Naughton, M.A., Rose, J.W., Alves, V., Arfsten, A., 1992. C186-65, a thrombin receptor antagonist designed from tethered ligand agonist peptides. Circulation 86 (suppl. I), I-151.

Smith, J.B., Willis, A.L., 1971. Aspirin selectively inhibits prostaglandin production in human platelets. Nature 231, 235–242.

Vu, T.K.-H., Humg, D.T., Wheatin, V.I., Coughlin, S.R., 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64, 1057–1068.

Xu, W.F., Andersen, H., Whitmore, T.E., Presnell, S.R., Yee, D.P., Ching, A., Gilbert, T., Davie, E.W., Foster, D.C., 1998. Cloning and characterization of human protease-activated receptor 4. Proc. Natl. Acad. Sci. U. S. A. 95, 6642–6646.