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In vitro antiplatelet profile of FR171113, a novel non-peptide thrombin receptor antagonist

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Abstract

Synthetic peptides (5 to 14 amino acids), identical in sequence to the new amino-terminus of the thrombin receptor generated following cleavage by thrombin, act as thrombin receptor agonist peptides. Whilst thrombin receptor antagonist peptides are known, non-peptide thrombin receptor antagonists have yet to be described. In the present study, we compared the antiplatelet effects of 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonyl methylene)-1,3-thiazolidin-4-one (FR171113), a novel non-peptide thrombin receptor antagonist, with the known thrombin receptor antagonist 3-mercapto-propionyl-Phe–Cha–Cha–Arg–Asn–Pro–Asn–Asp–Lys–Tyr-OH (C186-65), and argatroban, a specific protease inhibitor of thrombin. FR171113 and C186-65 inhibited thrombin-induced platelet aggregation ($IC_{50} = 0.29$ μM and 15 μM, respectively) and Ser–Phe–Leu–Leu–Arg–Asn-NH_2 [a synthetic thrombin receptor agonist peptide (TRAP-6)] induced platelet aggregation (0.15 μM and 20 μM, respectively) in human washed platelets. Argatroban potently inhibited thrombin-induced platelet aggregation ($IC_{50} = 3.5$ nM), but did not inhibit TRAP-6-induced aggregation even at 100 μM. In contrast, these compounds did not show inhibitory effects on ADP- and collagen-induced aggregation in human platelet-rich plasma even at 100 μM. FR171113 caused a parallel shift to the right of the concentration–response curve describing aggregation induced by TRAP-6. The Schild plot of the data had a slope of -0.840 ($r = 0.98$) and the pA_2 was 7.29. In protease activity studies using a chromogenic substrate, argatroban inhibited thrombin protease activity in a dose-dependent manner, whereas FR171113 and C186-65 were inactive, even at 100 μM. Additionally, only argatroban displayed dose-dependent prolongation of thrombin time, activated partial thromboplastin time and prothrombin time. FR171113 and C186-65 showed no effects, even at a concentration of 100 μM. These results suggest that FR171113 has a similar mode of action to C186-65, but with more potent antiplatelet activity. In conclusion, FR171113 is suggested to be the first example of a non-peptide thrombin receptor antagonist.

Keywords: FR171113; Thrombin; Thrombin receptor; Antiplatelet activity

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 that catalyzes the cleavage of fibrinogen and the thrombin receptor and as a result activates the coagulation system and platelet function. As such, thrombin is considered to play a central role in hemostasis and thrombosis. Cloning of a functional thrombin receptor was recently described (Vu et al., 1991) and the proteolytic mechanism for activation was elucidated (Coughlin et al., 1992). The thrombin receptor is a G protein-coupled receptor activated by a unique mechanism involving proteolytic cleavage of

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the receptor by α-thrombin. Cleavage of the receptor between Arg^{41} and Ser^{42} unmasks a new amino-terminal sequence that functions as a ''tethered ligand'' for the receptor. Moreover, peptides based on the revealed amino-terminal sequence, ranging from 5 (SFLLR) to 14 amino acids (SFLLRNPNDKYEPF), have been found to activate the thrombin receptor and mimic the actions of thrombin in platelets (Hui et al., 1992).

It was reported that an undecapeptide analogue of the tethered ligand thrombin receptor [3-mercapto-propionyl-Phe – Cha – Cha – Arg–Asn–Pro–Asn–Asp–Lys–Tyr-OH (C186-65)] acted as a thrombin receptor antagonist, since it specifically suppressed thrombin-mediated platelet aggregation (Scarborough, 1994; Scarborough et al., 1992). Furthermore, in vivo experiments demonstrated that C186-65 inhibited the deposition of platelets onto a Dacron vascular graft and collagen-coated grafts in baboons (Lindahi et al., 1993). Thus, the thrombin receptor mediates the function of platelets and has a significant role in in vivo platelet thrombus formation. Thrombin protease inhibitors such as argatroban have been reported to be useful for the treatment of coronary thrombosis (Fitzgerald and Fitzgerald, 1989). However, antithrombotic therapy with a protease inhibitor is potentially problematical since bleeding caused by simultaneous suppression of the coagulation system and platelet function may be disadvantageous in a clinical setting. Thrombin receptor antagonists should not interfere with the clotting system since thrombin-induced cleavage of fibrinogen to fibrin plays a more dominant role in the process of blood coagulation. Since the target of a thrombin receptor antagonist is distinct from the corresponding target of thrombin inhibitors, a selective antagonist should be devoid of side effects such as spontaneous hemorrhage (Ray et al, 1997).

We thus embarked upon a program to discover the first example of a non-peptide thrombin receptor antagonist. In this study, we evaluated the in vitro antithrombotic effects of a novel thrombin inhibitor, 3-(4-chlorophenyl)-2-(2,4-dichlorobenzoylimino)-5-(methoxycarbonyl methylene)-1,3-thiazolidin-4-one (FR171113) (Fig. 1), which was developed by optimization of a lead molecule found by random screening, in comparison with those of C186-65. In addition, we elucidated the differences in pharmacological profile between FR171113 and argatroban by evaluation of platelet aggregation and plasma coagulation activities.

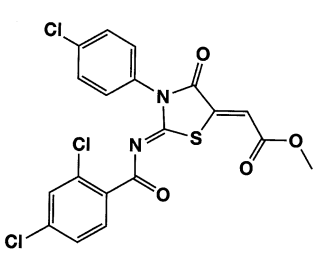

Fig. 1. The chemical structure of FR171113.

2. Materials and methods

2.1. Preparation of platelet-rich plasma and platelet suspension (washed platelets)

Blood from healthy human volunteers was collected into plastic vessels containing 3.8% sodium citrate (1/10 volume). Platelet-rich plasma was obtained from the supernatant fraction of blood after centrifugation at $150 \times g$ for 10 min. Platelet-poor plasma was obtained by centrifugation of the remaining blood at $1500 \times g$ for 10 min. The final cell count in platelet-rich plasma was adjusted to 3×10^8 platelets/ml with the platelet-poor plasma.

Prostaglandin I_2 (1 μM) was added to the prepared platelet-rich plasma. Plasma was removed by centrifugation at $800 \times g$ for 10 min at room temperature and the pellet was re-suspended in an equal volume of a 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, 5.5 mM glucose and 0.3% bovine serum albumin adjusted to pH 6.5. The final cell count of the washed platelet suspension was adjusted to 3×10^8 platelets/ml with the above buffer.

2.2. Measurement of platelet aggregation

Platelet aggregation was measured according to the turbidimetric method of Born and Cross (1968) with an aggregometer (Hema Tracer 801, MC Medical, Tokyo, Japan). In the cuvette, platelet-rich plasma or washed platelets were pre-incubated for 2 min at 37°C after the addition of drug or vehicle. 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 percentage inhibition of agonist-induced platelet aggregation compared with vehicle treatment. Data are presented as the means ± S.E.M. for the indicated number of experiments. The IC_{50} value was obtained by linear regression, and is expressed as the drug concentration required to produce 50% inhibition of agonist-induced platelet aggregation in comparison to vehicle treatment. The slopes of the resulting Schild plots were used to assess competitive antagonism. The pA_2 value was obtained by Schild plot analysis (Tallarida et al., 1979).

2.3. Thrombin protease inhibition activity

Thrombin protease inhibition activity was measured using S-2238, a chromogenic substrate. To 0.1 M Tris–HCl buffer in a cuvette, drug or vehicle and human thrombin (final concentration: 0.01 units/ml) were added and incubated at 37°C for 2 min. S-2238 (final concentration: 50 μM) was added to initiate the enzyme reaction. The rate of

increase in absorbance at 405 nm was measured with a spectrophotometer (UV-2200, Shimadzu, Kyoto, Japan). Inhibition activity was calculated as follows: Inhibition activity = 100 − ((ΔA_{405}/min of the drug/ΔA_{405}/min of vehicle) × 100). Data are presented as the means ± S.E.M. for the indicated number of experiments.

2.4. Materials

FR171113 was synthesized in the Medicinal Chemistry Research Laboratories, Fujisawa (Osaka, Japan). C186-65 and Ser–Phe–Leu–Leu–Arg–Asn-NH_2 (TRAP-6) were purchased from Kurabo (Osaka, Japan). Argatroban was purchased from Mitsubishi-kasei (Tokyo, Japan). Arg–Gly–Asp–Ser (RGDS) was purchased from Peptide Institute (Osaka, Japan). Adenosine 5′-diphosphate (ADP), adrenaline, aspirin, human thrombin and bovine serum albumin were purchased from Sigma (St. Louis, MO). Collagen (equine tendon) (Packham, 1984) was purchased from Hormon Chemie (Munich, Germany). Prostaglandin I_2 was purchased from Cayman Chemical. In all experiments, FR171113, C186-65 and aspirin were dissolved in dimethyl sulfoxide and added as a 100-fold concentrated stock solution. Argatroban and RGDS were soluble in saline and added as a 100-fold concentrated stock solution.

3. Results

3.1. In vitro antiplatelet activities of FR171113, C186-65 and argatroban

The inhibitory effects of FR171113, C186-65 and argatroban on thrombin- and TRAP-6-induced aggregation of human washed platelets are shown in Fig. 2. FR171113 (0.032–1 μM) and C186-65 (3.2–100 μM) dose-dependently inhibited platelet aggregation induced by both thrombin and TRAP-6. Maximum inhibitions were about 100%. The IC_{50} values of FR171113 and C186-65 for thrombin-induced platelet aggregation were 0.29 ± 0.08 μM and 15 ± 3.2 μM, respectively. The IC_{50} values of FR171113 and C186-65 for TRAP-6-induced platelet aggregation were 0.15 ± 0.04 μM and 20 ± 5.9 μM, respectively. Argatroban inhibited thrombin-induced platelet aggregation (IC_{50}: 0.0035 ± 0.0006 μM), but showed only 4 ± 2% inhibition of TRAP-6-induced aggregation at 100 μM.

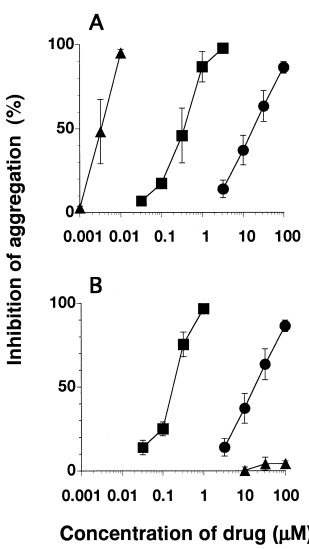

Fig. 2. The inhibitory effects of FR171113 (■), C186-65 (●) and argatroban (▲) on (A) thrombin-induced and (B) TRAP-6-induced platelet aggregation in human washed platelets. The final concentrations of thrombin and TRAP-6 were 0.1 NIH unit/ml and 1 μM, respectively. The effects of each drug are expressed as percentage inhibition of platelet aggregation compared with vehicle treatment. Each value represents the mean ± S.E.M. for five to six experiments.

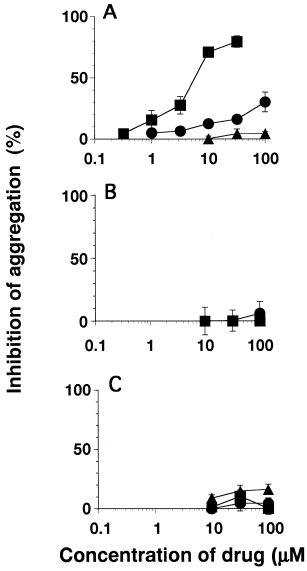

Fig. 3. The inhibitory effects of FR171113 (■), C186-65 (●), and argatroban (▲) on (A) TRAP-6-induced, (B) ADP-induced and (C) collagen-induced platelet aggregation in human platelet-rich plasma. The final concentrations of TRAP-6, ADP and collagen were 2 μM, 2.5 μM and 0.1 μg/ml, respectively. Each value represents the mean ± S.E.M. for five experiments.

The effect on platelet aggregation induced by TRAP-6, ADP and collagen in human platelet-rich plasma is shown in Fig. 3. These three drugs did not significantly inhibit the platelet aggregation induced by ADP or collagen, even at 100 μM. In contrast, FR171113 (0.32–32 μM) dose-dependently inhibited platelet aggregation induced by TRAP-6 in platelet-rich plasma, whilst C186-65 showed a weak inhibitory effect (inhibition: 40 ± 7.5% at 100 μM). Argatroban showed only 16 ± 4.3% inhibition of TRAP-6-induced platelet aggregation at 100 μM.

3.2. Comparison of in vitro antiplatelet activities of FR171113 with those of RGDS and aspirin

A comparison of the inhibitory effects (IC_{50} values) of FR171113 and the antiplatelet agents, RGDS and aspirin, on TRAP-6-, collagen- and ADP-induced platelet aggregation in platelet-rich plasma is shown in Table 1. RGDS

Table 1
The comparison of antiplatelet effect of FR171113, aspirin and RGDS
The IC_{50} values are expressed as the drug concentration required to produce 50% inhibition of agonist-induced platelet aggregation in comparison to vehicle treatment. The final concentrations of TRAP-6, ADP and collagen were 2 μM, 2.5 μM and 0.5 μg/ml, respectively. Each value represents the mean ± S.E.M. for five experiments.

Drugs	IC_{50} (μM)		
	TRAP-6	ADP	Collagen
FR171113	2.5 ± 1.0	> 100	> 100
Aspirin	> 1000	> 1000	58 ± 9
RGDS	72 ± 10	83 ± 16	71 ± 4

dose-dependently (10–100 μM) inhibited platelet aggregation induced by TRAP-6, collagen and ADP (data not shown). In each case, the level of antiplatelet activity was almost the same. Aspirin inhibited collagen-induced platelet aggregation (IC_{50}: 59 ± 10.1 μM), but did not inhibit TRAP-6- and ADP-induced aggregation, even at 1 mM. FR171113 only inhibited TRAP-6-induced platelet aggregation (IC_{50}: 2.5 ± 1.0 μM), as previously described.

3.3. Schild plot analysis

Fig. 4 shows that variable concentrations of FR171113 shifted the dose–response curve for TRAP-6 to the right in a parallel fashion. The pA_2 value was estimated to be 7.29 with a slope of −0.840 ($r = 0.98$).

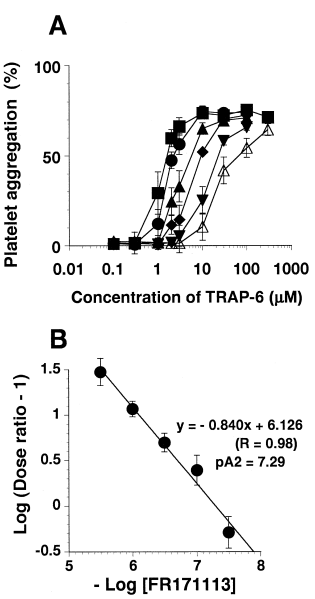

Fig. 4. Analysis of antagonism by FR171113 of TRAP-6-induced aggregation in human washed platelets. (A) Platelets aggregated by variable concentrations of TRAP-6 after the addition of FR171113 (● 0.032, ▲ 0.1, ◇ 0.32, ▼ 1 or △ 3.2 μM) or vehicle (■). Each value represents the mean ± S.E.M. for six experiments. (B) Schild plot analysis of the effect of FR171113 on TRAP-6-induced platelet aggregation. The relationship of platelet aggregation to agonist log-dose was fitted after logic transformation of dependent variables by a weighted least-squares method. The pA_2 value was obtained by Schild plot analysis.

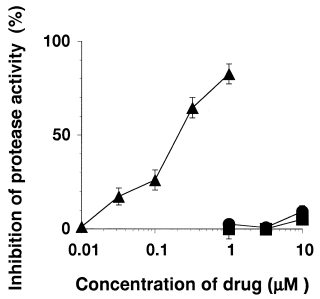

Fig. 5. Inhibitory effects of FR171113 (■), C186-65 (●) and argatroban (▲) on protease activity of thrombin. The final concentrations of S-2238 and thrombin were 50 μM and 5 NIH unit/ml, respectively. In all cases, the effect of drug is expressed as percentage inhibition of the response to thrombin after the addition of vehicle. The data shown are the means ± S.E.M. from three experiments.

3.4. Effects on thrombin-type protease activity

The effects of FR171113, C186-65 and argatroban on thrombin protease activity, assessed using chromogenic substrate S-2238, are shown in Fig. 5. Argatroban (0.01–1 μM) dose-dependently inhibited thrombin protease activity (IC_{50}: 0.29 ± 0.07 μM). In contrast, FR171113 and C186-65 did not influence thrombin protease activity even at 100 μM.

4. Discussion

We have been engaged in a program to find a prototype non-peptide thrombin receptor antagonist, since such a compound has not been reported yet. As a result, we discovered the unique non-peptide antagonist compound, FR171113, which was 130 times and 50 times more potent in causing inhibition of platelet aggregation induced by TRAP-6 and thrombin, respectively, than C186-65. In order to examine whether FR171113 and C186-65 display inhibitory effects on platelet aggregation induced by agents other than TRAP-6 and thrombin, their effect on ADP- and collagen-induced platelet aggregation was tested with human platelet-rich plasma. FR171113 and C186-65 did not inhibit the platelet aggregation induced by ADP and collagen, even at 100 μM. These results suggest strongly that FR171113 has a similar antiplatelet profile to C186-65, but with much more potent activity.

We compared the in vitro antiplatelet profile of FR171113 in human platelet-rich plasma with that of antiplatelet drugs with other functional mechanisms. RGDS, a glycoprotein IIb/IIIa (GP IIb/IIa) antagonist with broad-spectrum antiplatelet activity (Peerschke and Galanakis, 1987), inhibited platelet aggregation induced by TRAP-6, ADP and collagen. Aspirin, a cyclooxygenase

inhibitor (Smith and Willis, 1971), was only effective against collagen-induced platelet aggregation via the arachidonic acid cascade. Thus, FR171113, which did not inhibit ADP- or collagen-induced platelet aggregation, would appear to have a different mechanism of action from RGDS peptide and aspirin. Furthermore, FR171113 did not inhibit platelet aggregation induced by arachidonic acid, the thromboxane A_2 analog U46619, platelet-activating factor (PAF), adrenaline and calcium ionophore A23187 in human platelet-rich plasma, even at 100 μM (data not shown). Therefore, FR171113 appears to be a specific inhibitor of thrombin receptor-mediated platelet aggregation.

Next, we examined the profile of antagonism of FR171113. This compound displayed no agonistic action, since a concentration of 300 μM did not change the shape of the platelets or induce platelet aggregation in human platelet-rich plasma and washed platelets (data not shown). Schild analysis of the profile of antagonism indicated that FR171113 shifted the dose–response curve to the right in a concentration-dependent manner without changing the shape and the maximal aggregation induced by TRAP-6. Thus, the present data indicate that FR171113 is a competitive antagonist of the thrombin receptor.

The thrombin protease inhibitor argatroban inhibited thrombin-induced platelet aggregation, whereas it did not inhibit platelet aggregation induced by the N-terminal peptide of the thrombin receptor. Argatroban inhibited thrombin protease activity in a dose-dependent manner from 0.032 μM in a thrombin enzyme assay using a chromogenic substrate. It has been reported that argatroban inhibits thrombin in a competitive fashion by binding to a hydrophobic pocket close to the enzyme active site of thrombin (Kikumoto et al., 1984; Hara et al., 1986). In contrast, FR171113 and C186-65 did not inhibit thrombin protease activity even at 100 μM. As described earlier, the mode of action of FR171113 is similar to that of C186-65 and is quite distinct from that of argatroban, a known inhibitor of thrombin. Thus, FR171113 appears to act directly on the thrombin receptor.

It is now generally accepted that both platelet aggregation and fibrin formation are causative factors for ischemic diseases such as unstable angina and acute myocardial infarction. Thrombin is one of the most important triggers for platelet aggregation and activation of the coagulation system. Consequently, it is of therapeutic importance to suppress thrombus formation induced by thrombin in patients with ischemic cardiovascular diseases (Harkar et al., 1995; Tapparelli et al., 1995). The thrombin inhibitor argatroban has been demonstrated to show beneficial effects in patients with ischemic ulcer associated with chronic arterial occlusion (Tanabe et al., 1986) and is currently being assessed in clinical trials for prevention of reocclusion after reperfusion therapy in patients with acute myocardial infarction (Tabata et al., 1992). In an in vitro coagulation study, argatroban dose-dependently prolonged the activated partial thromboplastin time, the prothrombin time and the thrombin time. In contrast, FR171113 and C186-65 did not prolong the clotting time of plasma even at a concentration of 100 μM (data not shown). Thrombin protease inhibitors inhibit both thrombin-mediated fibrin formation and platelet aggregation, whereas FR171113 inhibits only thrombin-mediated platelet aggregation and therefore may be devoid of side effects such as hemorrhage. The preventive effects and side effects, such as hemorrhage, of FR171113 in experimental animal models of thrombosis are currently under investigation.

Recently, several new platelet thrombin receptors known as protease-activated receptor 2 (PAR-2), protease-activated receptor 3 (PAR-3) and protease-activated receptor 4 (PAR-4) were cloned (Borm et al., 1996; Ishihara et al., 1997; Xu et al., 1998). In this study, we used a six-amino acid sequence peptide (SFLLRN-NH_2, TRAP-6) as an agonist of the thrombin receptor and demonstrated the effects of compounds on PAR-1. Recently, it was reported that PAR-1 and PAR-4 mediate thrombin signaling of human platelets (Kahn et al., 1999). The effects of FR171113 on PAR-4 are also currently under investigation.

In conclusion, FR171113 displays a mode of action comparable to that of C186-65, but has more potent in vitro antiplatelet activity. FR171113 inhibits thrombin-induced platelet aggregation without affecting coagulation time, in contrast to argatroban. It can be expected that FR171113 will be a useful agent for investigating the role of the thrombin receptor.

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