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Antithrombotic activity of AT-1015, a potent 5-HT_{2A} receptor antagonist, in rat arterial thrombosis model and its effect on bleeding time

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Abstract

The antithrombotic activity of *N*-[2-{4-(5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)piperidino}ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate (AT-1015; a 5-HT_{2A} receptor antagonist) was studied in a photochemically induced arterial thrombosis (PIT) model in the rat femoral artery, and in the tail transection bleeding time test. Ticlopidine (an antiplatelet agent) and sarpogrelate (a selective 5-HT_{2A} receptor antagonist) were studied as reference compounds. Pretreatment with AT-1015 (1 mg/kg, p.o.) significantly prolonged the time required to occlusion of the artery with thrombus, and the effect (3 mg/kg, p.o.) persisted for 24 h with significant inhibition of 5-HT-induced vascular contraction. Ticlopidine and sarpogrelate also significantly prolonged the time to occlusion at 100 mg/kg, p.o. Sarpogrelate (300 mg/kg, p.o.) showed the similar antithrombotic efficacy to AT-1015 (3 mg/kg, p.o.), while the effect disappeared within 6 h. No significant bleeding time prolongation was observed at 10 mg/kg of AT-1015, which is 10 times higher than the antithrombotic effective dose; whereas ticlopidine significantly prolonged bleeding time at the same dose as the antithrombotic effective dose. These results suggested that AT-1015 is a potent and long-acting oral antithrombotic agent in this model, which may be elucidated by its potent and long-acting inhibition of vasoconstriction through 5-HT_{2A} receptor.

Keywords: Arterial thrombosis; Bleeding time; 5-HT (5-hydroxytryptamine, serotonin); Platelet aggregation; Vascular contraction

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is released from activated and aggregated platelets at the site of vascular injury including adenine nucleotide (ADP) and thromboxane A_2. Although 5-HT only weakly induces platelet aggregation, it potentiates aggregation in the presence of other agonists, such as collagen, ADP, epinephrine and thrombin (Holmsen, 1985; De Clerck et al., 1982). 5-HT is also known to be a strong vasoconstrictor at sites of endothelial injury (Van Nueten et al., 1984). Thus, during thrombus formation, platelet-derived 5-HT plays an important role in creating a positive feedback cycle on further platelet aggregation followed by release of vasoconstrictor such as thromboxane A_2. In fact, increased plasma 5-HT levels can be observed during the thrombus formation in various animal models (Ashton et al., 1986; Wester et al., 1992). Moreover, it has been recently reported that elevated plasma 5-HT is associated with coronary artery disease and with cardiac events (Golino et al., 1994; Vikenes et al., 1999). These deleterious effects of 5-HT are mediated by 5-HT_{2A} receptors on platelet and vascular smooth muscle (Roth et al., 1998). These observations provide a rationale to explore a 5-HT_{2A} receptor antagonist for the therapy of cardiovascular disease. In fact, mortality was reduced by ketanserin in patients with various manifestations of arteriosclerotic disease in nonrandomized study (Chalmers and Murray, 1989; Noble and Drake-Holland, 1990; Verstraete, 1996). However, it was shown that this agent has an adverse activity of prolongation of an electrocardiogram QT interval, which is a serious side effect in clinical use (Zehender et al., 1990).

N-[2-{4-(5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)piperidino}ethyl]-1-formyl-4-piperidinecarboxamide monohydrochloride monohydrate, AT-1015, a novel 5-HT_{2A} receptor antagonist, selectively inhibited 5-HT-augmented platelet aggregation and 5-HT-induced vascular contraction with insurmountable antagonism and also ameliorated laurate-induced peripheral vascular lesions in rats (Kihara et al., 2000). Its effect was more potent than those of other 5-HT_{2A} receptor antagonists such as ketanserin and sarpogrelate

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(Kihara et al., 2000). However, the mechanism of AT-1015 in the laurate-induced rat model has not been clarified. As it is reported, that thrombus formation is triggered by endothelial denudation with laurate in the model, the possible mechanism would be elucidated by the potent or long-acting antithrombotic effect due to 5-HT_{2A} receptor antagonism.

In the present study, we evaluated the antithrombotic potency and duration of AT-1015 using arterial thrombosis model induced by endothelial injury in comparison with sarpogrelate. The therapeutic window between antithrombotic effect and bleeding time prolongation was also evaluated compared to a representative antiplatelet agent, ticlopidine.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 250–450 g were obtained from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions. All procedures for the care and use of animals were approved by the Institutional Animal Care and Use Committee of the Pharmaceutical Research Laboratories of Ajinomoto prior to the study being conducted.

2.2. Photochemically induced thrombosis (PIT) model

Transluminal thrombosis was induced by photochemical injury to the endothelium according to the method described previously (Takiguchi et al., 1992). After anesthesia with intraperitoneal (i.p.) injection of urethane (1 g/kg), each animal was placed on a heating pad at 37 °C in the supine position. Then, a 5-mm segment of the right femoral artery distal to the inguinal ligament was dissected out carefully and a pulse Doppler flow probe (DBF-10, Crystal Biotech America, USA) was placed on it to monitor blood flow with a pulse Doppler flow meter (PDV-20, Crystal Biotech America, USA). The left jugular vein was cannulated with polyethylene tube for rose bengal delivery. The femoral arterial blood flow data were continuously recorded on a thermal recorder (WT-645G, Nihon-Koden, Japan). Transillumination with green light (540 nm) was done by using a xenon lamp with a heat-absorbing filter (Hamamatsu Photonics, Japan). The light was directed into an optical fiber positioned 5 mm from the right femoral artery proximal to the flow probe. This part of the artery was left intact. About 10 min after establishing the baseline blood flow, irradiation was started and 8.0 mg/kg of rose bengal (Wako, Japan) was injected intravenously (i.v.). Irradiation was continued until either the artery was occluded or for 10 min, whichever was longer. Formation of an occlusive thrombus was indicated by complete cessation of blood flow for over 1 min. The occlusion time was determined by measuring the time from injection of rose bengal until complete cessation of blood flow up to a maximal observation time of 1800 s. Longer occlusion time was assigned a value of 1800 s. AT-1015 was administered 2, 6, or 24 h before the injection of rose bengal, while ticlopidine was administered 3 h before, and sarpogrelate was administered 1 or 6 h before.

2.3. Ex vivo platelet aggregation

Blood samples were collected into 3.8% trisodium citrate (9:1 v/v) at 2, 6, or 24 h after oral administration of AT-1015 (1–10 mg/kg). Platelet-rich plasma and platelet-poor plasma were prepared by centrifugation at 1300 rpm for 10 min and at 3000 rpm for 15 min, respectively. For the platelet aggregation study, platelets were counted using a Sysmex E-2000 (Toa Medical Electronics, Tokyo, Japan) cell counter and platelet-rich plasma was adjusted to 500,000 cells/μl by dilution with platelet-poor plasma. Platelet aggregation was measured with an aggregometer (NBS Hema Tracer 801, Niko Bioscience, Tokyo, Japan) containing 225 μl of platelet-rich plasma under constant stirring at 1000 rpm and 37 °C. Each test drug (2 μl) was added 3 min before addition of 25 μl of an agonist solution (3 μM 5-HT plus 4–5 μg/ml of collagen).

2.4. Ex vivo 5-HT-induced vascular contraction

Rats were sacrificed by cervical dislocation at 2 or 24 h after oral administration of AT-1015 (1 and 3 mg/kg). The thoracic aorta was quickly removed and placed O_2-saturated (95% O_2–5% CO_2) modified Tyrode solution with the following composition (in mM): NaCl, 158.3; KCl, 4.0; $CaCl_2$, 2.0; NaH_2PO_4, 0.42; $MgCl_2$, 1.05; glucose, 5.0; and $NaHCO_3$. The vessel was cut into rings of 2–3 mm width with intact endothelium. Each ring was mounted in a 30-ml organ bath containing modified Tyrode solution at 37 °C, and was connected to a force transducer (T7-8-240, Orientec, Tokyo, Japan) to record changes in isometric force. The ring was maintained under a tension of 2 g and allowed to equilibrate for 2 h. During the equilibration period, the bath was continuously bubbled with 95% O_2–5% CO_2 and the buffer was changed every 20 min. After the contractile response following exposure to 50 mM KCl became stable, the bath was washed out. The concentration–response curve for 5-HT was estimated as a percentage of the maximal response to 50 mM KCl.

2.5. Bleeding time

The bleeding time was determined using a modified tail cutting method, as described previously (Dejana et al., 1982). Rats were anesthetized by i.p. administration of urethane (1 g/kg) after oral administration of AT-1015 or ticlopidine. Then the tail was transected about 1 mm from the tip using a disposable surgical blade. The tail was placed in 25 ml isotonic saline (pH 7.4, 37 °C) immediately after being cut and the bleeding time was measured from the moment of

transection until bleeding stopped completely. Observation was stopped at 1800 s, if bleeding did not cease, and longer time was classified as 1800 s.

2.6. Drugs and chemicals

AT-1015 was synthesized at Ajinomoto (Kawasaki, Japan). Sarpogrelate was synthesized at Hodogaya Contract Laboratory (Tsukuba, Japan). 5-HT, urethane and ticlopidine were obtained from Sigma (St. Louis, MO, USA). Collagen reagent Horm was obtained from Nycomend Arzneimittel (Munich, Germany). Rose bengal was obtained from Wako (Osaka, Japan).

In the PIT and ex vivo studies, AT-1015 was dissolved in distilled water, while sarpogrelate was suspended in 0.5% gum tragacanth in distilled water. Ticlopidine was dissolved or suspended in distilled water. In the bleeding time study, AT-1015 and ticlopidine were dissolved or suspended in 5% gum arabic in distilled water.

2.7. Statistical analysis

Results are expressed as the mean ± S.E.M. In the ex vivo study, the AT-1015 group was compared with the vehicle group using one-way analysis of variance (ANOVA), followed by Dunnett's (two-tailed) post-hoc test (Stat View Ver 5.0). For the PIT and bleeding time studies, the groups with AT-1015, sarpogrelate or ticlopidine treatment were compared with the vehicle group by a nonparametric Kruskal–Wallis test, followed by Dunnett's test. A probability value less than 0.05 was considered significant.

3. Results

3.1. Antithrombotic effects in the PIT model

The antithrombotic effects of orally administered AT-1015, ticlopidine and sarpogrelate were evaluated in the PIT model (Fig. 1). In the control group, femoral artery was occluded within 10 min after the initiation of endothelial injury by the photochemical irradiation of rose bengal. AT-1015 (1, 3 and 10 mg/kg) significantly prolonged the time to occlusion in a dose-dependent manner (Fig. 1A). The minimum effective dose of AT-1015 was 1 mg/kg in this

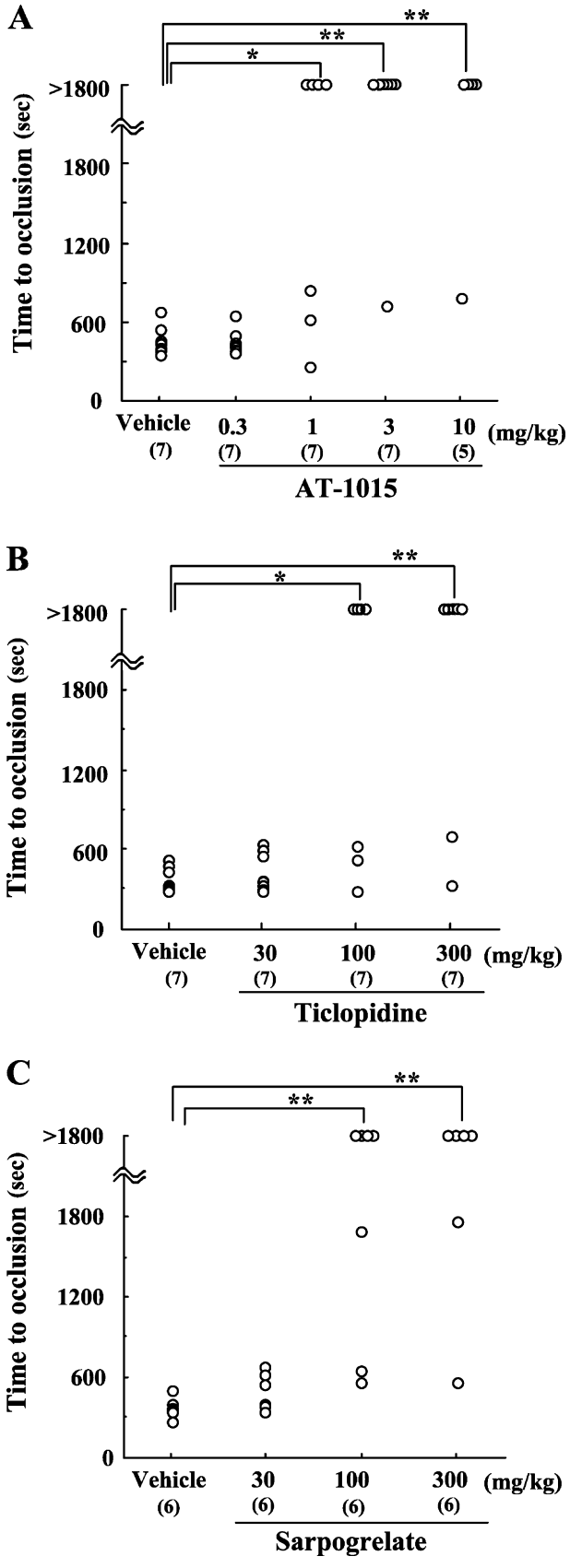

Fig. 1. Effects of AT-1015 (A), ticlopidine (B) and sarpogrelate (C) on photochemically induced thrombosis in the rat femoral artery. Open circles indicate the time to occlusion in each rat. Time to occlusion was measured from the start of rose bengal injection. AT-1015, ticlopidine and sarpogrelate were administered 2, 3 and 1 h before the injection of rose bengal, respectively. Numbers in parentheses indicate number of animals. Time to occlusion in the non-occluded animal was taken as 1800 s. * $P<0.05$ and ** $P<0.01$, as compared with the vehicle by the nonparametric Kruskal–Wallis test, followed by Dunnett's test.

Table 1
Duration of the antithrombotic effect of AT-1015 and sarpogrelate in the rat PIT model

Compound	Dose (mg/kg, p.o.)	Occlusion time (s)	
		6 h	24 h
Vehicle		294 ± 20 (0/7)	435 ± 26 (0/7)
AT-1015	1	983 ± 206 (1/7)*	404 ± 50 (0/7)
	3	1477 ± 209 (5/7)**	1269 ± 252 (4/7)*
	10	1491 ± 200 (5/7)**	1110 ± 245 (3/7)*
Sarpogrelate	300	464 ± 75 (0/5)	NT

NT: not tested.
Data are presented as the mean ± S.E.M.
The number of rats whose occlusion was not observed over 1800 s is shown in parentheses.
* $P < 0.05$ and ** $P < 0.01$, as compared with the vehicle by nonparametric Kruskal–Wallis test, followed by Dunnett's post-hoc test.

model. Ticlopidine (30, 100 and 300 mg/kg) and sarpogrelate (30, 100 and 300 mg/kg) also prolonged the time to vasculature occlusion in a dose-dependent manner and, the minimum effective dose of both agents was 100 mg/kg (Fig. 1B and C).

3.2. Duration of efficacy

The duration of the antithrombotic effect of AT-1015 was compared with sarpogrelate in the PIT model. The effect of AT-1015 was still significant even at 24 h after oral administration at doses of 3 and 10 mg/kg (Table 1); whereas the antithrombotic effect of sarpogrelate already disappeared by 6 h (Table 1).

The time course of the inhibitory effect of orally administered AT-1015 was measured in an ex vivo platelet aggregation induced by 5-HT plus collagen. Significant inhibition of platelet aggregation was observed at 6 h after administration of AT-1015 (1, 3 and 10 mg/kg) (Table 2). The inhibitory effect of AT-1015 at a dose of 10 mg/kg was still significant even after 24 h, while it was weak and not statistically significant at the lower doses.

Table 2
Duration of the inhibitory effect of AT-1015 on ex vivo platelet aggregation induced by 5-HT plus collagen in rat PRP

Compound	Dose (mg/kg, p.o.)	n	Platelet aggregation (%)[a]		
			2 h	6 h	24 h
Vehicle		5	48.8 ± 3.2	32.8 ± 8.4	46.1 ± 3.5
AT-1015	1	5	7.8 ± 5.8**	7.4 ± 5.1*	41.8 ± 6.8
	3	5	− 0.8 ± 0.5**	− 0.8 ± 0.4**	27 ± 9.2
	10	5	− 0.2 ± 0.4**	0.2 ± 0.5**	9 ± 7.7**

Blood was collected at 2, 6, or 24 h after oral administration of AT-1015. Data are presented as the mean ± S.E.M.
[a] Δ%, 5-HT-enhanced platelet aggregation (%), refer to the aggregation response in terms of percentage maximal light transmission through PPP.
* $P < 0.05$ and ** $P < 0.01$, as compared with the vehicle by one-way ANOVA, followed by Dunnett's (two-tailed) post-hoc test.

The inhibitory effect of AT-1015 on 5-HT-induced ex vivo vascular contraction is shown in Fig. 2. 5-HT-induced vascular contractions of rat thoracic aortic rings were significantly and almost completely suppressed at 2 h after the administration of AT-1015 (1 and 3 mg/kg) (Fig. 2A). The significant inhibitory effect of AT-1015 lasted until 24 h at 3 mg/kg (Fig. 2B).

3.3. Effect on bleeding time

The effects of AT-1015 and ticlopidine on the bleeding time are summarized in Table 3. The bleeding time was 227 ± 27 s in the vehicle-treated control group. The bleeding time was dose-dependently prolonged by oral administration of AT-1015 (1–10 mg/kg). However, significant pro-

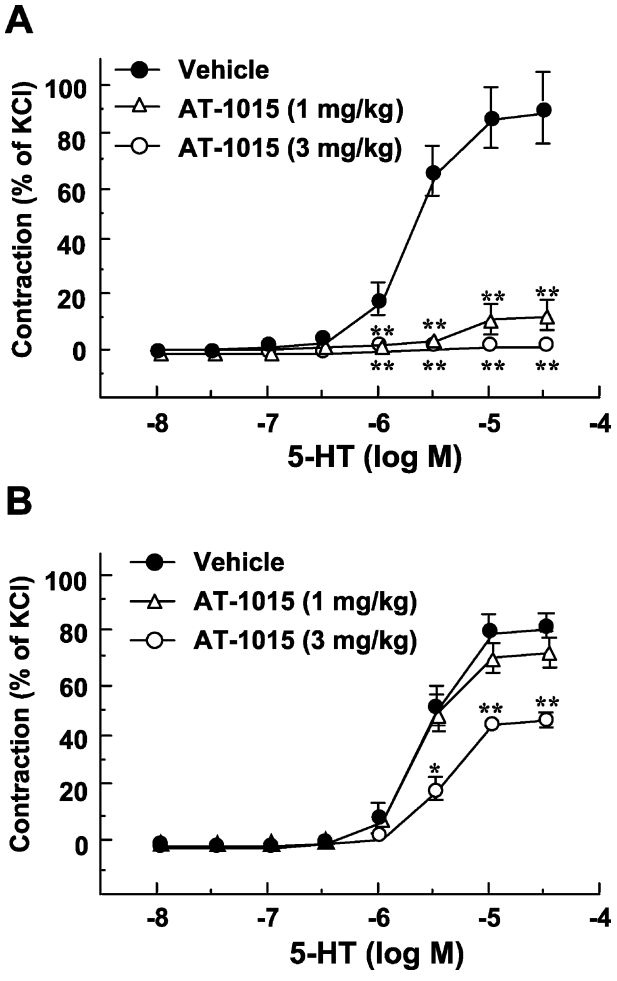

Fig. 2. Duration of the inhibitory effect of AT-1015 on ex vivo 5-HT-induced vascular contraction in rats. Two (A) and 24 h (B) after oral administration of AT-1015 (1 and 3 mg/kg) or vehicle, the thoracic aorta was isolated. The data are expressed as a percentage of the maximal response to 50 mM KCl and are shown as the mean ± S.E.M. ($n = 4–5$). * $P < 0.05$ and ** $P < 0.01$, as compared with the vehicle by one-way factorial ANOVA, followed by Dunnett's (two-tailed) post-hoc test.

Table 3
Effects of AT-1015 and ticlopidine on the tail transection bleeding time in the rat

Compound	Dose (mg/kg, p.o.)	n'/n	Bleeding time (s)
Vehicle		0/10	227 ± 32
AT-1015	1	0/10	203 ± 19
	3	0/10	328 ± 69
	10	0/10	462 ± 83
Ticlopidine	30	0/7	431 ± 96
	100	1/7	699 ± 202*
	300	2/8	986 ± 191**

Data are presented as the mean ± S.E.M.
n': The number of rats whose bleeding did not cease over 1800 s.
* $P < 0.05$ as compared with the vehicle by a nonparametoric Kruskal–Wallis test, followed by Dunnett's post-hoc test.
** $P < 0.01$ as compared with the vehicle by a nonparametoric Kruskal–Wallis test, followed by Dunnett's post-hoc test.

longation was not observed at the highest dose of 10 mg/kg, which was 10-fold higher dose than the minimum effective dose in the PIT model. Ticlopidine prolonged the bleeding time significantly at the same dose level as the effective dose in the PIT model (100 mg/kg).

4. Discussion

In this study, we evaluated the antithrombotic activity (potency and duration) of AT-1015 (a novel 5-HT_{2A} receptor antagonist) after oral administration compared to ticlopidine and sarpogrelate, a selective 5-HT_{2A} receptor antagonist, by using PIT model. In this model, thrombosis is reported to be triggered by endothelial injury caused by photochemically irradiated rose bengal (Saniabadi et al., 1995). The effect of each drug was compared at the time when the blood concentration reached the maximum after oral administration. AT-1015 significantly prolonged the time to occlusion in a dose-dependent manner and the minimum effective dose was 1 mg/kg, while ticlopidine and sarpogrelate needed a much higher dose (100 mg/kg) to achieve the significant prolongation. The efficacy of ticlopidine was almost the same as the previous report (Takiguchi et al., 1992). Therefore, it is suggested that the antithrombotic activity of AT-1015 was the most potent among those antiplatelet agents in the PIT model. An antithrombotic effect of sarpogrelate was observed at 100 mg/kg, which was 100 times higher than that of AT-1015. Although sarpogrelate showed more potent antithrombotic effect in other arterial thrombosis model (Hara et al., 1991), the reason for this discrepancy is not clear. It may be due to methodology differences between the thrombosis models. Their studies were performed using electrically induced thrombosis in the mouse mesenteric artery. This differs from our study in the species, vascular bed and technique of thrombus induction.

The potent antithrombotic effect of AT-1015 may be explained as follows. Thrombus formation in the PIT model is reported to be dependent on both platelet activation at sites of endothelial injury and vasoconstriction induced by constrictors released from aggregating platelets (Takiguchi et al., 1992). Therefore, it is reasonable that AT-1015, an agent having both vasodilating and antiplatelet effects, inhibits thrombus formation more efficiently than the pure antiplatelet agents without vasodilating effect. Indeed, it is reported that aspirin failed to inhibit the thrombus formation in this model (Shimazawa et al., 1997; Takiguchi et al., 1992). The fact that ketanserin, a potent 5-HT_{2A} receptor antagonist, shows a more potent antithrombotic effect than ticlopidine, which only possesses antiplatelet activity and has no vasodilating effect, also supports this hypothesis (Takiguchi et al., 1992). Therefore, it may be argued that agents with a potent inhibitory effect on vascular 5-HT_{2A} receptor-mediated responses inhibit thrombus formation more efficiently.

The duration of the antithrombotic effect of AT-1015 in the PIT model was much longer than sarpogrelate, suggesting the feasibility of once-daily administration of AT-1015. The duration of the antithrombotic effect of AT-1015 correlated well with its vasodilating effect. AT-1015 (3 mg/kg) significantly inhibited 5-HT-induced vascular contraction, while it did not inhibit 5-HT-induced platelet aggregation at 24 h after oral administration. At a dose of 1 mg/kg, the inhibitory effect of AT-1015 on 5-HT-induced vascular contraction as well as platelet aggregation disappeared after 24 h. These results suggest that the long-lasting antithrombotic effect of AT-1015 may be mainly due to its inhibition of 5-HT-induced vasoconstriction. This hypothesis is supported by the fact that vasoconstriction as well as platelet aggregation contributes to the process of thrombus formation in the PIT model (Takiguchi et al., 1992). The long-lasting inhibition of 5-HT-induced vasoconstriction may be elucidated by the slow dissociation of AT-1015 from the 5-HT_{2A} receptor. In the preliminary in vitro study using rabbit femoral artery preparations, the inhibitory activity of AT-1015 lasted for 4 h after changing incubation buffer. In the same experiment, the inhibitory effect of sarpogrelate disappeared more rapidly (data not shown). These data suggest slow dissociation of AT-1015 from arterial 5-HT_{2A} receptors.

AT-1015 showed a trend toward the prolongation of bleeding time in the rat tail transection, but its effect was not statistically significant in the rat tail transection at doses up to 10 mg/kg, which was 10 times higher than the effective dose in the PIT model. In contrast, ticlopidine significantly prolonged the bleeding time at the minimum effective dose in the PIT model. Ticlopidine showed the same results in previous reports (Dejana et al., 1982; Maffrand et al., 1988). The less bleeding tendency of AT-1015 may be due to its selective antagonism on 5-HT-induced platelet activation (Kihara et al., 2000). AT-1015 does not affect other pathways of platelet activation resulting in the small effect on the process haemostasis. On the other hand, ticlopidine prolonged bleeding time due to the inhibition of various pathways of platelet activation induced by ADP, collagen, thrombin, etc. (Ashida and Abiko, 1979).

In summary, the present results indicate that AT-1015 is a potent and long-lasting antithrombotic agent with a low risk of bleeding time prolongation in this model. Its potent and long-lasting antithrombotic effect is based on the inhibition of 5-HT-induced vasoconstriction in addition to the inhibition of 5-HT-mediated platelet aggregation. Accordingly, AT-1015 would be of potential therapeutic value for thrombotic disease.

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