

Antithrombotic Effects of KW-3635, a Thromboxane A₂-Receptor Antagonist, in Guinea Pigs

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ABSTRACT—Antithrombotic effects of KW-3635, a newly synthesized thromboxane (TX) A₂-receptor antagonist, were studied in guinea pigs. In the extracorporeal circulation thrombosis model, the shunt was filled with thrombi, and reduction of platelet count and increase in plasma TXA₂ concentration were observed. KW-3635 (30 and 100 mg/kg, p.o.) inhibited the thrombus formation in the shunt and prevented the decrease in platelet count in the circulating blood without affecting the red blood cell count. BM13,505 (30, 100 mg/kg, p.o.), another TXA₂-receptor antagonist, and ticlopidine (300 mg/kg, p.o.), an antiplatelet drug, also inhibited the thrombus formation, while aspirin (10, 300 mg/kg, p.o.) did not. Peripheral arterial occlusive disease was induced by injection of sodium laurate into the femoral artery in guinea pigs. Daily oral administration of KW-3635 (3–30 mg/kg) significantly prevented the progression of vascular lesions. BM13,505 (3–30 mg/kg, p.o.) and ticlopidine (100 mg/kg, p.o.) also ameliorated the vascular lesions, whereas aspirin (10, 100 mg/kg, p.o.) did not. KW-3635 at concentrations up to 10⁻⁴ M did not affect coagulation parameters in vitro. These results suggest that TXA₂ is involved in the pathogenesis of arterial thrombotic and ischemic disorders. KW-3635 may be useful for the treatment of thrombotic disease and peripheral arterial occlusive diseases.

Keywords: KW-3635, Thromboxane A₂, Thrombosis

Thromboxane A₂ (TXA₂), one of the cyclooxygenase products of arachidonic acid, is a potent inducer of platelet aggregation and vasoconstriction (1–3). The pathophysiological effects of TXA₂ have been implicated in arterial thrombosis, cardiovascular disease (4–6) and circulatory shock (7, 8). There are many reports that TXA₂-synthetase inhibitors and TXA₂-receptor antagonists have antithrombotic effects in various experimental models (9–13). However, the role of TXA₂ in peripheral vascular disease has not fully been clarified. It seems, therefore, interesting to study the effect of a TXA₂ antagonist on the pathogenesis and progression of the thrombotic and ischemic vascular diseases.

In sodium laurate-induced arterial occlusive disease in rats, the injected sodium laurate is supposed to cause endothelial cell damage and lead to precipitation of platelets in the peripheral vascular bed (14). The progression of the disease in this model resembles that reported in the patients with thromboangitis obliterans (TAO) (15, 16). The thrombus occluding the lumen of the femoral artery

was composed of a platelet mass containing fibrin, and red and white blood cells, as was the case with the clinical situation. It has been reported that antiplatelet agents, ticlopidine (14) and cilostazol (17), prevented the progression of the disease.

KW-3635 is a novel specific TXA₂ antagonist with a chemical structure different from those of prostanoid-type TXA₂ antagonists (18, 19). We have previously reported that KW-3635 inhibited platelet aggregation induced by the TXA₂ mimetic U-46619 in various animal species in vitro and that the inhibitory effect of KW-3635 is the most prominent in human platelets (19). In the present study, we examined the effect of KW-3635 on the development of peripheral vascular disease induced by sodium laurate in guinea pigs. Moreover, the effects of KW-3635 on the thrombosis in the arterio-venous shunt and on the blood coagulation parameters were also determined.

MATERIALS AND METHODS

Animals

Male guinea pigs of the Hartley strain (Shizuoka Laboratory Animal, Hamamatsu), weighing 250–350 g, were used. Food and water were given ad libitum prior to the experiment.

Extracorporeal shunt thrombosis

The extracorporeal shunt was produced in the guinea pig with a slight modification of the method of Fujitani and Wakitani (20). The extracorporeal shunt was made of a polyethylene tube (PE-100) and both the ends were connected to polyethylene tubing (PE-50). The extracorporeal shunt filled with a heparin solution (50 U/ml) was inserted between the right carotid artery and the left jugular vein of the guinea pig anesthetized with pentobarbital sodium (35 mg/kg, i.p.). Thereafter, blood was allowed to circulate through the shunt for 1 hr. All drugs except for ticlopidine were orally administered 3 hr before the start of the extracorporeal circulation. Ticlopidine was orally administered 4 hr before the start of the circulation. At the end of the experimental period, the shunt was removed for determining thrombus formation. At the same time, blood was withdrawn from the abdominal aorta for measuring the count of platelets and red blood cells (RBC). The numbers of platelets and RBC were determined with an automatic counter (Sysmex K-2000; Toa Iryo Denshi, Kobe).

Sodium laurate-induced arterial occlusive disease

Sodium laurate-induced arterial occlusive disease was induced in the guinea pig with a slight modification of the method of Ashida et al. (14). 0.1 ml of sodium laurate solution (10 mg/ml) was injected into the right femoral artery of the guinea pig. Drugs were administered 1 hr after the injection of sodium laurate and once a day for 10 days. The degree of gangrene and mummification at the 10th day after the injection was graded from 0 to 4 according to the severity of the lesion as follows: 0: normal appearance; 1: the affected region was limited to the nail parts; 2: to the fingers; 3: to the whole paw and 4: extended to the lower leg.

Measurement of blood coagulation parameters

The guinea pig was anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and 9 ml of blood was collected from abdominal aorta into a plastic tube containing 1 ml of 3.8% sodium citrate. Plasma was obtained by centrifugation at 3,000 rpm for 10 min. Activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were determined with a clinical assay kit (Toa Iryo Denshi).

Measurement of TXB₂ in plasma

To evaluate the change of plasma TXB₂ levels, 2 ml of blood was drawn from the abdominal aorta with a syringe containing 1/10 volume of 77 mM EDTA/2 Na and 10⁻⁴ M of indomethacin 1 hr after the start of the extracorporeal circulation. The blood was centrifuged at 3,000 rpm (KC-70; Kubota, Tokyo) for 10 min at 4°C. The obtained plasma samples were stored at -20°C until the assay for TXB₂ by specific radioimmunoassay according to the method described by Ingerman-Wojenski et al. (21). The working buffer (PBSG) used was as follows: 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.01% NaN₃ and 0.1% gelatin. Antisera for TXB₂ were diluted 30,000 times with PBSG. Then 0.1 ml [³H]-TXB₂ solution (370 kBq), 0.1 ml anti-TXB₂-antiserum, 0.2 ml of PBSG and 0.1 ml of the sample solution were mixed. After incubation for 12 hr at 4°C, 0.7 ml or 1 ml of charcoal/0.05% dextran T70 suspension in PBSG was added to the TXB₂ assay mixture. The mixture was allowed to stand for 15 min and then centrifuged at 1,000 × g for 10 min at 4°C. The radioactivity in the supernatant was counted in a liquid scintillation counter (Model 14530; Packard, Meriden, CT, USA).

Drugs used

KW-3635 (sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylate), BM13,505, another TXA₂ antagonist (22) and ticlopidine, an antiplatelet drug (23), were synthesized in our laboratories. Aspirin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). In the in vivo study, these drugs were suspended in 0.3% sodium carboxymethyl cellulose so as to make the administration volume 0.5 ml suspension per 100 g of animal's body weight and orally administered. In the in vitro study, KW-3635 was dissolved in 10% polyethylene glycol 400.

Statistical analyses

Data except for the incidence of obstruction and coagulation parameters were expressed as means ± S.E. Statistical significance was evaluated by Dunnett's test. Data of the incidence of obstruction in the extracorporeal thrombosis model were analyzed by the χ^2 -test. P values of 0.05 or less were considered to indicate statistically significant differences.

RESULTS

Effects on extracorporeal circulation

The shunt became almost completely filled with thrombus within 60 min following the start of the extracorporeal circulation. The platelet count in the blood decreased significantly, and the RBC count showed a

Table 1. Effects of KW-3635, BM13,505, ticlopidine and aspirin on the incidence of thrombosis and the platelet and RBC counts in the extracorporeal shunt of guinea pigs

Drugs	Dose (mg/kg)	No. occluded /No. tested	Platelet ($\times 10^9$ /ml)	RBC ($\times 10^4$ /mm ³)
Normal			38.5 \pm 0.8	430 \pm 12.5
Control		18/19	28.3 \pm 1.0 [#]	389.5 \pm 8.8
KW-3635	10	7/10	31.5 \pm 1.1	404.0 \pm 13.2
	30	1/11**	33.4 \pm 0.7*	379.3 \pm 6.1
	100	0/5	35.1 \pm 1.0*	380.6 \pm 9.1
BM13,505	3	4/5	30.1 \pm 0.6	385.8 \pm 7.7
	10	5/10	32.6 \pm 2.1	403.2 \pm 4.4
	30	2/6*	33.0 \pm 2.9	406.5 \pm 12.9
	100	0/5**	34.9 \pm 1.7*	409.4 \pm 4.9
Ticlopidine	100	5/5	32.9 \pm 3.0	417.0 \pm 11.2
	300	0/4**	32.1 \pm 2.9	415.5 \pm 9.5
Aspirin	10	4/5	28.1 \pm 1.4	389.3 \pm 3.4
	300	3/5	30.3 \pm 1.0	388.6 \pm 12.3

All values except for the incidence are means \pm S.E.M. #: P < 0.01 vs. normal. * and **: P < 0.05 and P < 0.01 vs. control, respectively.

tendency to decrease (Table 1). Oral administration of KW-3635 at the doses of 30 and 100 mg/kg significantly decreased the incidence of thrombus formation in the shunt. Additionally, KW-3635 (30 and 100 mg/kg, p.o.) significantly inhibited the decrease in platelet count in the circulating blood without any effect on the RBC count. BM13,505 (30 and 100 mg/kg, p.o.) also prevented the thrombus formation and the decrease in platelet count, the anti-thrombotic activity being almost the same as that of KW-3635. Oral administration of ticlopidine (300 mg/kg, p.o.) also reduced the thrombus formation. On the other hand, aspirin failed to protect against the shunt thrombosis even at the high dose of 300 mg/kg. In this model, an increase in plasma TXB₂ was observed in the control group (Table 2). Whereas KW-3635 at a dose of 10 mg/kg did not affect the level of plasma TXB₂, 100 mg/kg of KW-3635 significantly reduced it. Ticlopidine (300 mg/kg, p.o.) tended to reduce plasma TXB₂,

Table 2. Effects on plasma TXB₂ levels in the extracorporeal shunt model of guinea pigs

Drugs	Dose (mg/kg)	Plasma TXB ₂ (ng/ml)	n
Normal		0.21 \pm 0.02	3
Control		0.36 \pm 0.02 [#]	7
KW-3635	10	0.35 \pm 0.02	4
	100	0.28 \pm 0.02*	5
Ticlopidine	300	0.29 \pm 0.01	5
Aspirin	300	0.20 \pm 0.02**	5

Values are means \pm S.E. #: P < 0.01 vs. normal. * and **: P < 0.05 and P < 0.01 vs. control, respectively.

Table 3. Effects of KW-3635, BM13,505, ticlopidine and aspirin on progression of sodium laurate-induced arterial occlusive disease in guinea pigs

Drugs	Dose (mg/kg)	Grade of disease (gangrene or mummification)				
		0	1	2	3	4
Control			2	2	7	17
KW-3635	1	1			1	3
	3	1	1	2	2	2*
	10	2	3	1	3	1**
	30	4	1			**
BM13,505	1	1	1	1		5
	3	2	1		2	*
	10	3	2		3	1**
	30	2	1		2	1**
Ticlopidine	30		1		2	1
	100	6	2	1		1**
Aspirin	10		1		3	1
	100	1			3	

Drugs were orally administered 1 hr and once a day for 10 days after sodium laurate injection. * and **: P < 0.05 and P < 0.01 vs. control, respectively.

although the difference was not statistically significant. Aspirin (300 mg/kg, p.o.) completely inhibited the elevation of plasma TXB₂.

Effects on sodium laurate-induced arterial occlusive disease

KW-3635, BM13,505, ticlopidine and aspirin were evaluated for their effect on sodium laurate-induced arterial occlusive disease in guinea pigs (Table 3). Within 10 days after the injection of sodium laurate, the lesions developed in the whole leg, and the affected parts fell off in most cases in the control group. KW-3635 (1–30 mg/kg, p.o.) prevented the progression of gangrene and mummification in a dose-dependent manner. The protective effect of KW-3635 was statistically significant at doses of 3 mg/kg and higher. BM13,505 also prevented the progression of the disease, and the potency of BM13,505 was almost the same as that of KW-3635. While ticlopidine at a dose of 100 mg/kg (p.o.) prevented the progression of the disease, 30 mg/kg did not. Aspirin at the doses of 10 and 100 mg/kg showed no protective effect on the progression of gangrene and mummification on the 10th day.

Blood coagulation parameters

Figure 1 shows the effects of KW-3635 and heparin on coagulation parameters in vitro. Heparin prolonged PT, APTT and TT in concentration-dependent manners. On the other hand, KW-3635 did not exert any effect on these coagulation parameters.

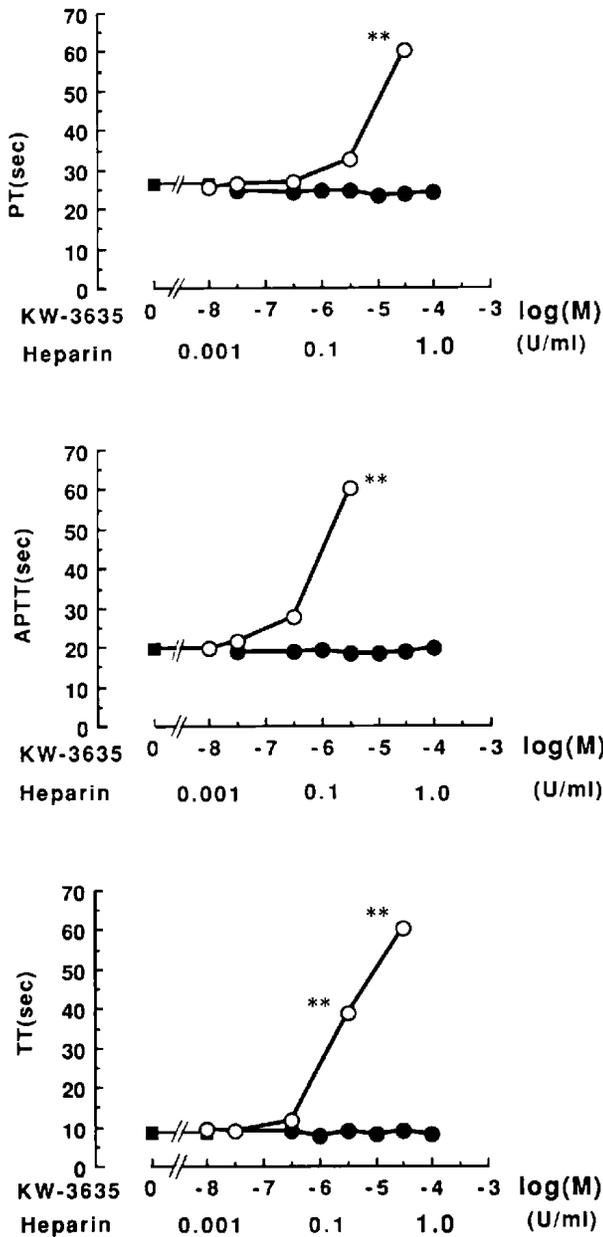


Fig. 1. Effects of KW-3635 and heparin on prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) of plasma from guinea pigs in vitro. ■: Control, ○: Heparin, ●: KW-3635. Each point represents the mean of 3 experiments. **: $P < 0.01$ vs. control.

DISCUSSION

In the extracorporeal shunt model of the guinea pig, an occlusive white thrombus was consistently formed at the connection of polyethylene tubing, where the blood flow is physically disturbed. This disturbance of the blood flow seems to be an important contributing factor for the thrombus formation in the shunt. In similar models of

rats, Ashida et al. (24) demonstrated the deposition of occlusive white thrombi within the polyethylene tubing and the reduction of circulating platelets, both of which were prevented by ticlopidine. Ticlopidine is a well-known antiplatelet agent that inhibits platelet aggregation induced by various stimulators (23). In the present study, reduction of platelet count in the control group was observed, and ticlopidine inhibited the thrombus formation and tended to prevent the reduction of circulating platelets. Therefore, it is likely that platelets are the major component of the thrombus within the polyethylene tubing. In addition, the increased plasma TXB_2 of the guinea pig subjected to the extracorporeal shunt suggested that TXA_2 is related to the thrombus formation in the shunt.

KW-3635 showed significant anti-thrombotic and anti-thrombocytopenic effects in the present arterio-venous shunt model. Similarly, BM13,505 inhibited thrombus formation. These drugs, however, did not affect the RBC count. The antiplatelet drug ticlopidine was also effective, although the activity of this drug was less potent than those of KW-3635 and BM13,505. In addition, coagulation parameters such as TT, PT and APTT were not affected by KW-3635 in vitro. Therefore, the most likely explanation for the inhibitory effects of KW-3635 against thrombus formation in the shunt is the inhibition of platelet aggregation via the antagonism of TXA_2 . In KW-3635-treated animals, the elevation of plasma TXB_2 following the extracorporeal shunt was inhibited. This observation is in accordance with the previous report that BM13,177, a TXA_2 -receptor antagonist, inhibited the increase of TXA_2 production in platelets stimulated by collagen (25). In fact, it is known that TXA_2 per se stimulates the production of TXA_2 in platelets. Therefore, the reduction of plasma TXB_2 by the treatment with KW-3635 was due to the inhibition of the secondary production of TXA_2 by blocking TXA_2 receptors.

Ticlopidine prevented the progression of the arterial occlusive disease induced by sodium laurate in guinea pigs. Ashida et al. (14), using a rat model of arterial occlusive disease produced by sodium laurate, reported that the platelet contributes greatly to the progression of the disease, because thrombocytopenia and the antiplatelet drug ticlopidine were found to prevent the progression of the disease in rats. KW-3635 shows inhibitory effects against U-46619- and collagen-induced platelet aggregation *ex vivo* in guinea pigs (19). These results suggest that the effectiveness of the drug against the disease was primarily due to the inhibition of platelet aggregation, perhaps induced by TXA_2 . In fact, elevation of plasma β -thromboglobulin and serum TXB_2 levels, indicative of platelet activation, were observed in patients with occlusive peripheral arterial disease (26).

Vasodilators are widely used in the treatment of

patients with peripheral circulatory insufficiency (27) and are considered to improve the occlusive ischemic state by increasing blood flow via the collateral circulation. There have been some reports indicating that vasodilating agents such as lipo-PGE₁ (28) and cilostazol (17) were effective against sodium laurate-induced peripheral arterial disease in rats. On the other hand, Reilly and co-workers (29) obtained evidence for the involvement of platelet activation and TXA₂ biosynthesis in patients with peripheral arterial disease. Therefore, it is reasonable to assume that elevated TXA₂ causes the peripheral arterial vasoconstriction and contributes to the peripheral arterial disease. KW-3635 was demonstrated to inhibit U-46619-induced contraction of the vascular preparations in various species (30). Similar results were observed for BM13,505. Therefore, the prevention of TXA₂-induced vasoconstriction may have also contributed to the ameliorating effect of KW-3635 and BM13,505 on the peripheral arterial disease. Thus, as TXA₂ antagonists also have an anti-vasoconstrictive action in addition to antiplatelet action, KW-3635 may be more useful than the antiplatelet agent ticlopidine. In the present study, however, we did not measure plasma TXB₂ levels in the guinea pig following the injection of sodium laurate. Further study is necessary to clarify the mechanism of the protection by KW-3635 against the disease.

In contrast to TXA₂ antagonists and ticlopidine, aspirin was ineffective against the thrombus formation in the extracorporeal shunt, despite the reduction of plasma TXB₂ concentration, and in the sodium laurate-induced peripheral arterial disease. In fact, similar results have been reported by several authors (12, 15, 31–33). The studies of mesenteric artery and venous thrombosis in rats (12, 31) and jugular vein thrombosis in rabbits (32) showed rather a thrombogenic action of the high-dose of aspirin. Moreover, the clinical trial of aspirin resulted in no dramatic beneficial effects (34). The possible explanation for the ineffectiveness of aspirin may be related to its inhibitory effect on prostaglandin I₂ (PGI₂) generation by endothelial cells, because PGI₂ has a potent anti-aggregative (35), vasodilating (36) and cytoprotective (37) actions. In fact, PGI₂ and its analogue had beneficial effects on various experimental thrombosis and occlusive diseases (14, 38, 39). Moreover, PGI₂ has clinically been used to prevent the thrombus formation in the extracorporeal circulation (40, 41) and the arterial diseases such as Burger (42) and Raynaud's disease (43, 44).

In summary, the present study demonstrated that KW-3635, when orally administered, inhibited the thrombosis in the arterio-venous shunt and the peripheral circulation insufficiency in guinea pigs. The mechanism for the inhibitory effect was assumed to be the inhibitory effect on platelet aggregation and/or vasoconstriction induced by TXA₂.

The TXA₂-receptor antagonist may be useful for the treatment of thrombotic and peripheral vascular diseases.

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