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T-686, a novel inhibitor of plasminogen activator inhibitor-1, inhibits thrombosis without impairment of hemostasis in rats

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Abstract

The aim of this study was to evaluate the antithrombotic potential of T-686 ((3*E*,4*E*)-3-benzylidene-4-(3,4,5-trimethoxybenzylidene)-pyrrolidine-2,5-dione), a novel inhibitor of plasminogen activator inhibitor-1 (PAI-1), in rat thrombosis models. T-686 (0.1–100 mg/kg per day, p.o.) dose dependently decreased the weight of venous thrombi induced by a combination of stasis and hypercoagulability. The antithrombotic effect was enhanced by repeated administration of T-686. Warfarin (0.1 mg/kg per day for 3 days) also prevented thrombus formation. The antithrombotic action by warfarin was accompanied by prolongation of coagulation time, while no effect on coagulation time was observed in T-686-treated rats. T-686 lowered the activity of PAI-1 in plasma. In the arterio-venous shunt model, pretreatment with T-686 (10 mg/kg per day) or ticlopidine (100 mg/kg per day) for 8 days inhibited thrombus formation by 33% and 44%, respectively. T-686 had no effect on collagen-induced platelet aggregation ex vivo, while ticlopidine inhibited platelet aggregation. T-686 did not affect bleeding time at 10–100 times the antithrombotic dose, while warfarin dose dependently prolonged bleeding time at and around the antithrombotic dose. These results suggest that T-686 prevents thrombus formation in rats without impairment of hemostasis. © 1997 Elsevier Science B.V.

Keywords: PAI-1 (plasminogen activator inhibitor-1); Fibrinolysis; Hemostasis; Thrombus; (Rat)

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1), a specific inhibitor of both tissue-type plasminogen activator and urokinase-type plasminogen activator, plays an important role in regulation of the fibrinolytic system (Schneider and Loskutoff, 1991). Elevated levels of PAI-1 in plasma have been observed in patients with deep vein thrombosis (Nilsson et al., 1985; Wiman et al., 1985; Juhan-Vague et al., 1987) and unstable angina (Wieczorek et al., 1994). Furthermore, a number of animal studies have shown that PAI-1 is a factor which disturbs fibrinolytic activity in the thrombotic and prethrombotic states (Krishnamurti et al., 1987; Reilly et al., 1991; Carmeliet et al., 1993). Thus, inhibition of PAI-1 activity or reduction of PAI-1 production may shift the balance between thrombogenesis and thrombolysis towards thrombolysis. In fact, it has been reported that an antibody against PAI-1 can enhance clot lysis and decrease thrombus growth in animal models of venous thrombosis (Levi et al., 1992) and arterial thrombosis (Biemond et al., 1995). It has also been shown that the hypolipidemic agents, gemfibrozil and niacin, inhibit PAI-1 expression in vitro and in vivo to stimulate fibrinolysis (Fujii et al., 1993; Avellone et al., 1993; Brown et al., 1995).

We have previously described that a new butadiene derivative, (3*E*,4*E*)-3-benzylidene-4-(3,4,5-trimethoxybenzylidene)-pyrrolidine-2,5-dione (T-686), not only inhibits the expression of PAI-1 mRNA in cultured bovine endothelial cells (Ohtani et al., 1996) but also attenuates the increase in aortic PAI-1 mRNA expression induced by both hypercholesterolemia and mechanical injury in rabbits (Vinogradsky et al., 1997), and can prevent the shutdown of fibrinolysis in an experimental hypofibrinolytic model (Ohtani and Murakami, 1997). However, the antithrombotic potential of T-686 has not yet been defined. The present study was performed to investigate the antithrombotic effects of T-686 on two experimental thrombosis models in rats.

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2. Materials and methods

2.1. Materials

T-686 was synthesized, and ticlopidine was extracted from commercially available tablets, in this laboratory. Warfarin was obtained from Aldrich (Milwaukee, WI, USA), thromboplastin of rabbit brain from Sigma (St. Louis, MO, USA) and Nikkol HCO-60 from Nikko (Tokyo, Japan). All other reagents were of the highest grade available.

2.2. Animal experiments

All protocols conformed to the 'Guide for the Care and Use of Laboratory Animals' published by US National Institutes of Health (NIH publication No. 85-23, revised in 1985), and were approved by the animal studies committee at Tanabe Seiyaku Co. Male Wistar rats (250–380 g) were purchased from Charles River Japan.

2.2.1. Venous thrombosis model

Thrombus formation by a combination of stasis and hypercoagulability was induced as described previously (Vogel et al., 1989). Rats were anesthetized with Nembutal (50 mg/kg, i.p.) and the abdomen was surgically opened to expose the vena cava. All branches of the vena cava from the left renal veins to the bifurcation (2 cm) were ligated. 0.1 ml of diluted thromboplastin (1:10) per rat was injected into the dorsal vein of the penis. Ten seconds after the injection, the vena cava was ligated beneath the left renal vein. The abdominal cavity was provisionally closed. After 15 min of stasis, the cavity was reopened and ligation was performed near the bifurcation. The vena cava was opened longitudinally with a surgical blade, the thrombus formed was removed and its dry weight was measured (Reyers et al., 1980). T-686 and warfarin were suspended in 0.1% Nikkol HCO-60 solution at concentrations of 0.1–100 mg/10 ml and 0.1 mg/10 ml, respectively. Each drug solution was given orally in a volume of 10 ml/kg for the number of days shown. Control groups were treated with 0.1% Nikkol HCO-60 solution only. Two hours after the last administration of each drug, the animals were operated upon as described above and thromboplastin was injected. Blood samples were taken from the jugular vein 3 min after the thromboplastin injection.

2.2.2. Arterio-venous shunt model

The method of Umetsu and Sanai (1978) was employed after slight modification. Rats were anesthetized as described above. A cervical incision was made in the mid-line to expose the left carotid artery and right jugular vein. A 25-cm-long polyethylene tube with a 6-cm-long silk thread fixed in its lumen was filled with saline, then one end of the tube was inserted into the right jugular vein and tied. The proximal side of the left carotid artery was clamped to block the blood flow temporarily, while the free end of the tube was inserted into the artery and tied. The clamp was removed and blood flow through the tube was verified. After 30 min of blood circulation, the silk thread covered with thrombi was removed from the tube and its wet weight was immediately measured. T-686 and ticlopidine were suspended in 0.1% Nikkol HCO-60 solution at concentrations of 10 mg/10 ml and 100 mg/10 ml, respectively. Each drug solution was given orally in a volume of 10 ml/kg per day for 8 consecutive days. Control groups were treated with 0.1% Nikkol HCO-60 solution only. Two and 3 hours after the last administration of T-686 and ticlopidine, respectively, the animals were operated upon as described above and blood was allowed to circulate through the shunt for 30 min.

2.2.3. Collagen-induced platelet aggregation ex vivo

T-686 (10 mg/kg per day) and ticlopidine (100 mg/kg per day) were given orally for 8 consecutive days. Two and 3 hours after the last administration of T-686 and ticlopidine, respectively, blood was taken from the abdominal aorta under Nembutal anesthesia. Nine volumes of blood was collected in a tube containing 1 volume of 2.2% trisodium citrate solution and rapidly centrifuged at $150 \times g$ for 10 min to give platelet-rich plasma. The remaining blood was further centrifuged at $1000 \times g$ for 10 min to give platelet-poor plasma. Platelet aggregation induced by collagen (final concentrations: 5–7 μg/ml) was performed as described previously (Born, 1962). The results were expressed as percent of the difference in absorbance between platelet-rich plasma at maximum aggregation and platelet-rich plasma (Odawara et al., 1994).

2.2.4. Bleeding time

T-686 (10–100 mg/kg per day) and warfarin (0.1 and 0.3 mg/kg per day) were given orally for the number of days indicated. Two hours after the last administration of each drug, an incision 2 mm in depth was made with a surgical blade 3.5 cm from the tip of the tail under Nembutal anesthesia. Blood was blotted in filter paper (No. 2) every 30 s and bleeding time was determined by measuring the time until no blood was seen on the filter paper.

2.3. Assays

Coagulation time in plasma was measured with Thrombotest (Eisai, Tokyo, Japan) in accordance with the manufacturer's directions. Plasma PAI-1 activity was assayed spectrophotometrically (Chmielewska et al., 1983) using Spectrlyse/fibrin (Biopool, Umeå, Sweden). One arbitrary unit (AU) of plasma PAI-1 activity was defined as the amount of activity that inhibited 1 IU of tissue-type plasminogen activator completely over a period of 20 min.

Table 1
Effects of T-686 and warfarin on thrombus weight, coagulation time, and PAI-1 activity in the rat venous thrombosis model

Treatments [a]	(*n*)	Dry weight of thrombus (mg)	Coagulation time with Thrombotest [b] (s)	(AU/ml) [b]
Control	7	4.4 ± 0.5	30.2 ± 0.9	24.3 ± 1.0
Warfarin 0.1 mg/kg per day × 3	6	2.1 ± 0.4 [d]	54.5 ± 9.6 [d]	22.2 ± 1.1
T-686 10 mg/kg per day × 3	7	1.8 ± 0.2 [d]	31.0 ± 0.8	19.9 ± 0.9
T-686 10 mg/kg per day × 8	7	1.2 ± 0.2 [d]	31.0 ± 0.6	18.4 ± 2.1 [c]

[a] T-686 and warfarin were given orally to rats at the doses indicated for the number of days shown and thromboplastin was injected intravenously 2 h after the last administration of each drug.
[b] Blood samples were taken from the jugular vein 3 min after thromboplastin injection. Results were expressed as the means ± S.E.M.
[c] $P < 0.05$ compared with controls.
[d] $P < 0.01$ compared with controls.

2.4. Statistical analysis

The data were expressed as the means ± S.E.M. Statistical analysis was done by two-way analysis of variance with Dunnett's test. A difference with $P < 0.05$ was considered statistically significant.

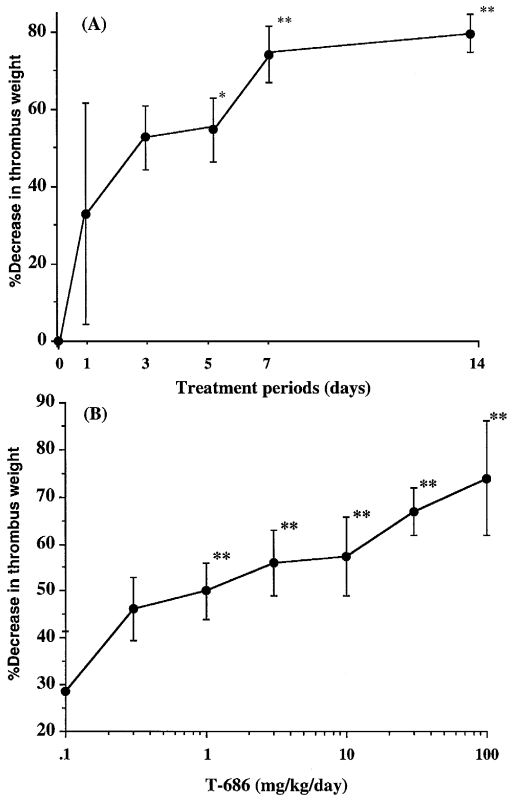

Fig. 1. Antithrombotic effects of T-686 on the venous thrombosis model in rats. (A) T-686 (10 mg/kg per day) was given orally for the number of days indicated. See the text for preparation of the venous thrombosis model. Values are the means ± S.E.M. for 6–7 rats. Separate control experiments were conducted for each treatment period. * $P < 0.05$, ** $P < 0.01$ compared with controls. (B) T-686 was given orally at doses of 0.1–100 mg/kg per day for 8 consecutive days. Values are the means ± S.E.M. for 8–10 rats. ** $P < 0.01$ compared with controls.

3. Results

3.1. Effects of T-686 and warfarin on the venous thrombosis model

T-686 was given orally to rats at a dose of 10 mg/kg per day for the number of days shown. Thrombus weight was decreased by repeated treatment with T-686 (Fig. 1A) and the decrease was statistically significant when the treatment period was longer than 3 days. Treatment with 0.1–100 mg/kg per day of T-686 for 8 days dose dependently inhibited thrombus formation with an estimated ED_{50} value of 1 mg/kg (Fig. 1B). The efficacy of T-686 on the venous thrombosis model was compared with that of warfarin. When warfarin (0.1 mg/kg per day) and T-686 (10 mg/kg per day) were administered for 3 consecutive days, thrombus formation was inhibited by 52% and 59%, respectively, as shown in Table 1. The antithrombotic action of T-686 was markedly enhanced by treatment for 8 days. Warfarin significantly prolonged

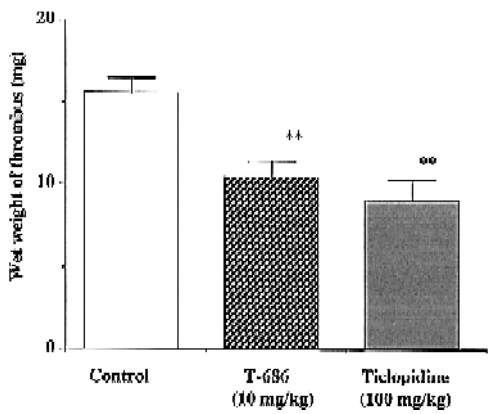

Fig. 2. Antithrombotic effects of T-686 and ticlopidine on the arterio-venous shunt model in rats. T-686 (10 mg/kg per day) and ticlopidine (100 mg/kg per day) were given orally for 8 consecutive days. See the text for preparation of the arterio-venous shunt model. Two and 3 hours after the last administration of T-686 and ticlopidine, respectively, blood was allowed to circulate through the shunt for 30 min. Values are the means ± S.E.M. for 9 rats. ** $P < 0.01$ compared with controls.

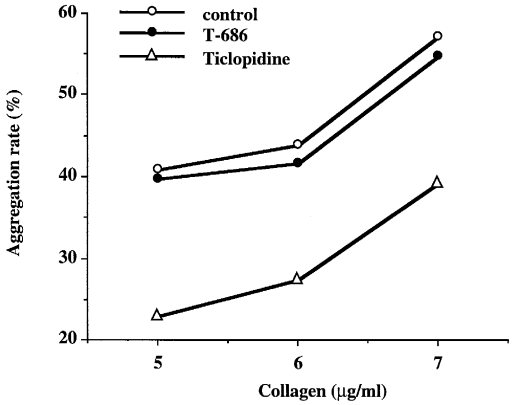

Fig. 3. Effects of T-686 and ticlopidine on collagen (5–7 μg/ml)-induced platelet aggregation in rat platelet-rich plasma ex vivo. T-686 (10 mg/kg per day) and ticlopidine (100 mg/kg per day) were given orally for 8 consecutive days. Values are the means for 3–4 rats.

coagulation time, while no effect was observed in T-686-treated rats. On the other hand, T-686 moderately lowered plasma PAI-1 activity (Table 1).

3.2. Effects of T-686 and ticlopidine on the arterio-venous shunt model

In the arterio-venous shunt model, neither single treatment nor repeated treatment for a period of 4 consecutive days with T-686 at a dose of 10 mg/kg per day affected the thrombus weight (data not shown). However, treatment with T-686 (10 mg/kg per day) or ticlopidine (100 mg/kg per day) for 8 consecutive days inhibited thrombus formation by 33% and 42%, respectively (Fig. 2). In this model, activation of both platelet aggregation and blood coagulation systems is involved in thrombus formation (Vogel et al., 1989). To determine whether T-686 affects platelet aggregation, the ex vivo effect on collagen-induced platelet aggregation was examined. As shown in Fig. 3, ticlopidine inhibited collagen-induced platelet aggregation, while T-686 showed no inhibitory effect. These results suggest that T-686 inhibits thrombus formation without affecting platelet aggregation.

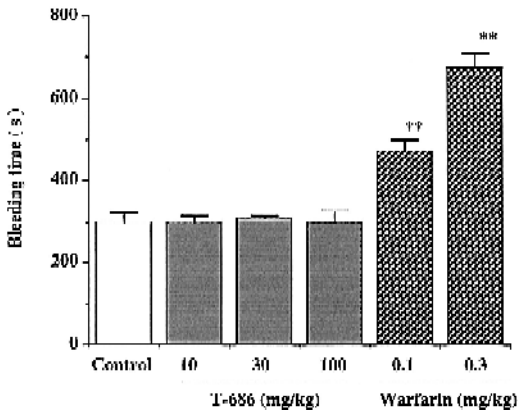

Fig. 4. Effects of T-686 and warfarin on bleeding time in rats. T-686 (10–100 mg/kg per day) and warfarin (0.1 and 0.3 mg/kg per day) were given orally for 8 and 3 consecutive days, respectively. Two hours after the last administration of each drug, an incision 2 mm in depth was made 3.5 cm from the tip of the tail. Values are the means ± S.E.M. for 4 rats. $^{**}P < 0.01$ compared with controls.

3.3. Effects of T-686 and warfarin on bleeding time

Rats were treated with T-686 (10–100 mg/kg per day) and warfarin (0.1 and 0.3 mg/kg per day) for the indicated number of days, and bleeding time was determined 2 h after the last administration of each drug. No effect was observed in T-686-treated rats, while warfarin dose dependently prolonged bleeding time (Fig. 4).

4. Discussion

It has previously been shown that T-686 inhibits the expression of PAI-1 mRNA in vitro (Ohtani et al., 1996) and in vivo (Vinogradsky et al., 1997). Furthermore, T-686 can prevent the shutdown of fibrinolysis induced in rats by endotoxin (Ohtani and Murakami, 1997). In the present study, T-686 showed antithrombotic activity in rat models of venous thrombosis and arterio-venous shunt. To our knowledge, the present study is the first demonstration that an orally active inhibitor of PAI-1 shows antithrombotic action in these two experimental thrombosis models.

In the venous thrombosis model, the combination of coagulation and stasis plays a pivotal role in the development of thrombus formation (Vogel et al., 1989). Warfarin, a vitamin K antagonist, prevented thrombus formation with prolongation of coagulation time. On the other hand, T-686 reduced thrombus weight without affecting coagulation time. The antithrombotic activity was enhanced by repeated administration of T-686. This was accompanied by a reduction of plasma PAI-1 activity (Table 1). Administration of the anti-PAI-1 antibody to rabbits results in increased endogenous thrombolysis and inhibition of thrombus growth in a venous thrombosis model (Biemond et al., 1995). Thus, the antithrombotic mechanism of T-686 may be dissolution of formed thrombi mediated by endogenous fibrinolysis. However, the reduction of plasma PAI-1 activity by T-686 was rather modest compared with the magnitude of the antithrombotic activity. The reason for this discrepancy is not yet clear. In a recent report, an injection of the antibody against PAI-1 to rats given endotoxin inhibited fibrin deposition in lungs by 70% and modestly reduced plasma PAI-1 activity, by 30% (Abrahamsson et al., 1996). Furthermore, our previous investigation had shown that T-686 may act on the vessel wall directly, to reduce the local expression of PAI-1 in rabbits (Vinogradsky et al., 1997). Thus, it is tempting to speculate that the local inhibition of secretion and expression of PAI-1 may not be reflected by the magnitude of the decrease in plasma PAI-1 activity (Padro et al., 1994).

In the arterio-venous shunt model used in the present study, the thrombotic process is initiated by platelet adherence to the silk thread, and the platelet aggregates then being surrounded by clot-like thrombi. Therefore, the silk thread covered with thrombi is considered to be a mixed thrombus (Freiman, 1982). Pretreatment with 10 mg/kg per day of T-686 for 8 days inhibited the growth of thrombi by 33%. However, a longer period of treatment seems to be required to achieve an inhibition of thrombus formation in the arterio-venous shunt model similar to that in the venous thrombosis model. The reason may be that T-686 has no antiplatelet aggregation activity, and that the vessel walls do not exist at the site of thrombus formation. Because the treatment with 10 mg/kg per day of T-686 for 8 days decreased the baseline of plasma PAI-1 activity by 25% (Table 1 and Ohtani et al., 1996), the mechanism of the antithrombotic action is presumably inhibition of PAI-1 production in the endothelial cells proximal to the shunt, shifting the local balance between thrombogenesis and thrombolysis towards thrombolysis. This possibility will be further explored in future studies. Moreover, the efficacy of T-686 in an arterial thrombosis model remains to be examined.

T-686 did not prolong bleeding time in the rat tail at 10–100 times the effective dose in the venous thrombosis model, whereas warfarin, at or near the antithrombotic dose, dose dependently prolonged bleeding time. It has been shown that no impairment of hemostasis is observed in PAI-1 gene-deficient mice (Carmeliet et al., 1993). However, a homozygous PAI-1 deficiency in man results in a relatively mild bleeding tendency (Fay et al., 1992). Whether the absence of anti-hemostatic effect by T-686 could be due to the difference between rodents and man is not clear. T-686 markedly decreases aortic tissue PAI-1 mRNA levels with only a moderate decrease in plasma PAI-1 activity (Vinogradsky et al., 1997). This suggests that the inhibitory effect of T-686 is more potent on 'local PAI-1 (vasculature)' than on 'systemic PAI-1 (in plasma)' activity. Thus, T-686 presumably does not impair the hemostatic system.

In conclusion, the orally active inhibitor of PAI-1 production, T-686, shows antithrombotic activity in two experimental thrombosis models without affecting bleeding time in rats. These results suggest that inhibitors of PAI-1 production might provide a relatively safe and beneficial means to treat disorders involving venous thrombosis.

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