Antithrombotic Effect of TA-993, a Novel 1,5-Benzothiazepine Derivative, in Conscious Rats

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ABSTRACT—Since reported experimental models of thrombosis are not suitable for comparison of several drugs by oral administration, we developed a convenient model for this purpose by applying direct current through an intravascular electrode. In conscious rats, which were implanted with anodal electrodes in the abdominal aorta on the day before the experiment, application of 200 μA of direct current induced the formation of a platelet-rich thrombus around the intravascular electrode. Using this model, we studied the antithrombotic effect of the novel antiplatelet agent TA-993, (-)-cis-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-8-methyl-2-(4-methylphenyl)-1,5-benzothiazepin-4(5H)-one maleate, and compared its effect with other antiplatelet agents. TA-993 at doses of 30 mg/kg, p.o. or more by single administration or at doses of 10 mg/kg or more by repeated administration dose-dependently suppressed the thrombus formation. Aspirin (10 mg/kg, p.o. or more), cilostazol (100 mg/kg, p.o.) and ticlopidine (30 mg/kg, p.o. or more) also suppressed the thrombus formation by single administration. These results suggest that TA-993 has a comparable antithrombotic effect with other antiplatelet agents, and thus it is a possible remedy for thrombotic and embolic diseases.

Keywords: Experimental thrombosis, Antiplatelet agent, Antithrombotic effect, TA-993, 1,5-Benzothiazepine derivative

Although platelets are an important component of blood for hemostasis, they are also known to adhere to the injured vessel wall and to cause an arterial thrombus. Adherence and aggregation of platelets are injury healing processes, but may invite arteriosclerosis and embolism (1). Antiplatelet agents like aspirin and ticlopidine inhibit thrombogenesis experimentally (2, 3) and they are employed for the treatment of patients with occlusive arterial diseases (1).

Among the procedures of experimental thrombosis, direct current application through an intravascular (4) or extravascular (5) electrode is applicable to conscious animals. Since, only relatively big animals like dogs are used for the former procedure (4), this procedure is not suitable for comparison of drug effects. On the other hand, employing the disappearance of the righting reflex as an index of thrombus formation in the latter procedure (5) makes it difficult to differentiate the antithrombotic effect from the protective effect against cerebral ischemia. Therefore, we designed an animal model using rats in which direct current was applied through an implanted intravascular electrode in the conscious state.

TA-993, (-)-cis-3-acetoxy-5-(2-(dimethylamino)ethyl)-2,3-dihydro-8-methyl-2-(4-methylphenyl)-1,5-benzothiazepin-4(5H)-one maleate (Fig. 1), is a novel 1,5-benzothiazepine derivative having a l-cis conformation. Diltiazem, one of the representative calcium antagonists, is a compound very closely related to TA-993 but has a d-cis conformation. Diltiazem is reported to show a weak inhibitory action on platelet aggregation that could be potentiated when used in combination with a thromboxane A2 synthase inhibitor or prostaglandin I₂, like other calcium antagonists (6, 7).

However, the potency of the antiplatelet effect of diltiazem is reported to be too weak to exert any in vivo antithrombotic action (8). Not only has TA-993 been reported to have a more potent antiplatelet effect than diltiazem, but its metabolite MB3 (Fig. 1) also showed a 100 times stronger antiplatelet effect than the parent compound (9). Thus, MB3 is considered to contribute significantly to the antiplatelet effect of TA-993 under in vivo conditions, and unlike diltiazem, TA-993 may show
an antithrombotic effect without any combining drug. For these reasons, we evaluated the antithrombotic effects of various antiplatelet drugs by oral administration in this model and compared them with that of TA-993.

MATERIALS AND METHODS

Animals
Sprague Dawley rats (305 males, 8- to 10-weeks-old) weighing 200-347 g were used. The rats were purchased from Charles River Japan (Tokyo) at the age of 7 weeks and were then kept in our animal room (25 ± 1°C, 12-12 hour light-dark cycle) for at least a week before use. Food and water were freely accessible during this period.

Implantation of electrodes and evaluation of thrombus formation
A schematic representation of the apparatus is shown in Fig. 2. On the day before the experiment, a catheter-type platinum electrode was implanted in the rat by inserting it into the abdominal aorta through the left femoral artery under pentobarbital anesthesia (50 mg/kg, i.p.). After the other end of the catheter was exteriorized at the back of the neck, a plate-type silver-silverchloride electrode was also implanted subcutaneously at the back of the neck as the opposite pole. After suturing the incisions, an electric cell and a variable resistance were placed on the back, each secured by adhesive tape, as a power source and for fine tuning of the current, respectively. Rats were allowed to recover overnight in individual cages. They were fasted but allowed to drink tap water ad libitum during this period.

In the experiment, each electrode was connected to the electric cell and direct current was applied continuously. The current was adjusted to 9 V, 200 μA by the variable resistance. After current application was stopped, rats were anesthetized as described above, and the 500 units/kg of heparin Na solution was injected into the femoral vein of each. Usually these procedures were completed within 5 min. Five minutes later, the rats were exanguinated, and the thrombus was removed by forceps from the longitudinally opened aorta after a laparotomy. The wet weight of the thrombus was measured as an index of thrombus formation.

Procedure
Polarity dependence and the time course of thrombus formation: Polarity dependence of thrombus formation was investigated by studying the time course of thrombus formation for 4 or 6 hr, during which time anodal or cathodal current was applied continuously through the intravascular electrode, respectively. Since the amount of thrombus found on the anode was much larger than that on the cathode as shown in Fig. 3, drug effects were evaluated with an anode throughout the following experiments. In some cases, the formed thrombus was taken for histological study with a scanning electron microscope.

Drug administration: Drugs were administered orally by gavage 1 hr before the current application. Repeated treatment of TA-993 was performed by administering the drug twice daily (9:00 and 16:00) for 4 days before the experiment, and another dosing was also performed on the day of the experiment as described above.

Statistical analyses
All results are expressed as means ± S.E.M. Although variation of thrombus weight within each control group was considered to be reasonable, inter-experimental variation was too large to consider that the control group of each experiment was comparable to those of the other experiments. Thus, each individual experiment had its own control group and statistical analysis was performed.
individually. Results were analyzed by one way analysis of variance and Scheffe’s method, and were considered to be statistically significant when P < 0.05.

**Drugs**

In this report, we used the following compounds: TA-993, ticlopidine and cilostazol (Organic Chemistry Laboratory, Tanabe Seiyaku, Co., Ltd., Osaka) and aspirin (Nacalai Tesque, Tokyo). TA-993 and ticlopidine were dissolved in deionized water at a volume of 10 ml/kg, and aspirin and cilostazol were suspended in 0.5% carboxymethylcellulose solution at the same volume. The control groups received the same volume of the corresponding vehicle solution.

**RESULTS**

**Polarity dependence and the time course of thrombus formation**

Figure 3 shows micrographs of the thrombus formed on the intravascular anodal electrode (Fig. 3A) and cathodal electrode (Fig. 3B), and the time courses of thrombus formation on each electrode are shown in Fig. 4. Both anodal and cathodal current caused time-dependent thrombus formation. When the anodal current was applied intravascularly, wet weight of thrombus reached about 14 mg after 4 hr. In contrast, the cathodal current induced only about 3 mg of thrombus even after the application period of 6 hr.

Scanning electron microscopic observation revealed that the surface of the thrombus formed on the anode was
covered by many aggregated platelets with scattered and/or clustered erythrocytes (Fig. 5A). An abundant amount of fibrous structure, which was considered to be platelets and accumulated fibrin, was present in a shallow part of the cracked section (Fig. 5B). A large number of erythrocytes were found in a deeper part of the cracked section with a sponge-like structure of fibrin that trapped the erythrocytes (Fig. 5C). Exfoliation of endothelial cells and adhered platelets were observed on the adjacent endothelium (Fig. 5D).

Most of the surface of the thrombus formed on the cathode was covered by platelets, and erythrocytes were rarely observed (Fig. 5E). A frost-column-like structure, which should mainly be constructed of fibrin, and platelets were observed in the cracked section (Fig. 5F). Clusters of erythrocytes were also observed in a minor part of that section. The adjacent endothelium showed similar changes to that observed with the anode.

Effects of antiplatelet agents on thrombus formation

Figure 6 shows the effects of antiplatelet agents, aspirin (Fig. 6A), ticlopidine (Fig. 6B) and cilostazol (Fig. 6C), on thrombus formation. Figure 6A also shows the result obtained in a sham group to which direct current was not applied. Only about 0.5 mg of thrombus was obtained in the sham group and about 16 mg of thrombus was obtained in a control group of this experiment. Even though the dose-dependency was not clear, aspirin at doses of 10–100 mg/kg, p.o. significantly suppressed the thrombus formation.

Ticlopidine and cilostazol also significantly suppressed thrombus formation at doses of more than 30 mg/kg, p.o. and 100 mg/kg, p.o., respectively.

Antithrombotic effect of TA-993 by single and repeated administration

Figure 7 shows the suppressive effect of TA-993 on direct current-induced thrombus formation. Single oral administration of TA-993 (Fig. 7A) suppressed thrombus formation dose-dependently, and the effect was statistically significant when doses of more than 30 mg/kg, p.o. were administered.

Consecutive administration of TA-993 (Fig. 7B) showed a stronger effect than that observed by single dosing, and the effect reached a significant level when doses of more than 10 mg/kg, p.o. were employed.

Fig. 3. Micrographs of the thrombus formed on the intravascular anodal electrode (A) and cathodal electrode (B). Bars on the bottom right of the micrographs indicate 10 mm.

Fig. 4. The time courses of thrombus formation in conscious rats by applying direct anodal (---) or cathodal (-----) current of 9 V and 200 μA. Each point and vertical bar indicates the mean ± S.E.M. of 7 to 10 experiments.
Fig. 5. Scanning electron micrographs of the thrombus formed on the intravascular anodal electrode (A–D) and cathodal electrode (E and F). Bars on the bottom right of the micrographs indicate 1 μm.
Fig. 6. Inhibitory effects of aspirin (A), ticlopidine (B) and cilostazol (C) on direct anodal current-induced thrombus formation in conscious rats. Each column and vertical bar indicates the mean±S.E.M. of 5 to 10 experiments. * and ** represent significant differences from the corresponding control group at levels of P<0.05 and P<0.01, respectively.

Fig. 7. Inhibitory effects of single (A) or repeated (B) administration of TA-993 on direct anodal current-induced thrombus formation in conscious rats. Each column and vertical bar indicates the mean±S.E.M. of 6 to 10 experiments. * and ** represent significant differences from the corresponding control group at levels of P<0.05 and P<0.01, respectively.

DISCUSSION

Romson et al. employed scanning electron microscopy to examine the thrombus and adjacent endothelium, which was induced by a direct anodal current of 50 μA applied through an intracoronary arterial electrode for 24 hr in conscious dogs; and they reported that this thrombus was a mixed-type one, containing platelets, erythrocytes and fibrous materials that accompany endothelial injury (4). Since our present scanning electron microscopical findings revealed similar characteristics in the thrombus and the adjacent endothelium of our model to those of Romson's report, our model should share common causal mechanisms with Romson's one despite
the different species and vascular bed.

Certainly, multiple induction of thrombus in the same animal is possible in cases of miniature thrombosis in the hamster cheek pouch (10, 11) or in the mesenteric vascular bed (12, 13) under anesthesia. However, it has been difficult to investigate the dose-dependency of drug treatment and to compare several drugs using conscious animals as mentioned before. Because of the simple procedure and utilization of small animals like rats, the method we used in the present study made it possible to compare the antithrombotic effect of several drugs within a short period by using a reasonable number of animals.

In the present study, we compared the antithrombotic potency of 3 antiplatelet agents by studying their dose-dependency. As a result, their antithrombotic potency was as follows: aspirin > ticlopidine > cilostazol.

Although aspirin, a cyclooxygenase inhibitor, is known to inhibit platelet aggregation by suppressing thromboxane A2 synthesis in platelets, it causes the so-called “aspirin dilemma” by suppressing prostaglandin I2 synthesis in the vascular endothelial cells (14). This phenomenon is also reproducible in experimental thrombosis; i.e., Escudero et al. reported that 1 mg/kg/day, p.o. and 20 mg/kg/day, p.o. aspirin were not effective, but intermediate dose of 5 mg/kg/day, p.o. aspirin showed a significant antithrombotic effect (2, 15). The doses we used might correspond to the peak flat part of the dose-dependency of aspirin.

The inhibitory effect of TA-993 (IC50: 49.3 μmol/l) on platelet aggregation induced by 10 to 20 μg/ml of collagen, which caused an aggregation of about 70% upon addition to platelet-rich plasma, was reported to be as potent as aspirin (IC50: 99.3 μmol/l) and more potent than that of ticlopidine (IC50: 456.7 μmol/l) in rat platelet-rich plasma (9). In addition, MB3, one of the metabolites of TA-993 is reported to show a potent inhibitory effect on platelet aggregation with an IC50 of 0.4 μmol/l (9). Moreover, ex vivo data revealed that the antiplatelet effect of TA-993 (ED50: 3 mg/kg, p.o.) was 4 times more potent than aspirin (ED50: 12 mg/kg, p.o.) and 40 times more potent than ticlopidine (ED50: 119 mg/kg, p.o.) after oral administration, and this discrepancy was explained by the presence of the metabolite MB3 (9). However, the antithrombotic potency of a single dose of TA-993 observed in the present report was one third that of aspirin, as potent as that of ticlopidine and 3 times more potent than that of cilostazol.

Thus, the order of antithrombotic potencies of compounds we studied were different from that of their antiplatelet effects, although their antithrombotic effects should be mainly due to their common effects: i.e., inhibition of platelet aggregation. The reason for this discrepancy could be a contribution of their actions other than antiplatelet effects, but the main reason should be the time period we applied the direct current; i.e., 1 to 5 hr after dosing. Available data about the antiplatelet effect of TA-993 after oral administration is that obtained only 3 hr after dosing and the time course of its inhibitory effect on platelet aggregation has not yet been reported (9). Therefore, we cannot compare the antithrombotic and antiplatelet effects directly, and it should be studied further.

The antithrombotic effect of TA-993 was potentiated by consecutive administration of 5 days. Similar potentiation was also reported about its antiplatelet effect (9). Although pharmacokinetic data of TA-993 in rats with repeated administration is not available, a pharmacokinetic study in humans revealed that the Cmax of TA-993 as a dose of 200 mg, p.o. slightly increased from 612.9 ng/ml by single administration to 727.5 ng/ml by repeated administration of twice daily for 7 days. In addition, the Cmax of MB3, one of the metabolites of TA-993, concomitantly increased from 12.8 ng/ml by single administration to 109.0 ng/ml by repeated administration (K. Ban-no et al., personal communication). Since the inhibitory effect of MB3, a metabolite of TA-993, on platelet aggregation is 100 times more potent than that of its parent compound, as mentioned before, these potentions may be derived from increases in plasma levels of TA-993 and/or MB3 by repeated administration.

In summary, we demonstrated the antithrombotic effect of TA-993, a new 1,5-benzothiazepine derivative, by using a new convenient model of thrombosis in conscious rats. Since TA-993 showed an inhibitory action on thrombus formation by single and repeated oral administration, TA-993 was suggested to be a possible remedy for thrombotic and embolic diseases.

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