Antithrombotic Effect of TRK-100, a Novel, Stable PGI2 Analogue

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Abstract—TRK-100, a stable PGI2 analogue structurally different from carbacyclines, was compared with other antiplatelet drugs for its effect on platelet functions using animal models. TRK-100 (10–300 nM) inhibited rat platelet aggregation induced by ADP (3 μM), collagen (12.5 μg/ml) and A23187 (10 μM), and its potency was about 1/3–1/7 that of PGI2. TRK-100 (0.3–3 mg/kg, p.o.) dose-dependently inhibited rabbit platelet adhesion (ED50: 2.2 mg/kg), and its effect lasted over at least 5 hr. In contrast, aspirin and ticlopidine (both at 300 mg/kg, p.o.) showed only a slight inhibition (4–7%). In the thrombocytopenia induced by collagen injection in rats, TRK-100 (3–300 μg/kg, i.v.; 0.1–3 mg/kg, p.o.) dose-dependently inhibited a decrease in platelet number, and its ED50 was 0.48–0.62 mg/kg orally and 13.7–16.4 μg/kg intravenously, while the inhibition by aspirin and ticlopidine (both at 1000 mg/kg, p.o.) was 40 and 37%, respectively. In the experimental thread thrombosis in rats, TRK-100 (0.03–3 mg/kg, p.o.) dose-dependently inhibited thrombus formation, and its ED50 was 0.46 mg/kg, being 21 and 87 times as potent as aspirin and ticlopidine, respectively. These results reveal that TRK-100 has a potent antiplatelet activity and is orally and intravenously effective for a variety of thrombosis models, suggesting that it may have a therapeutic value as an antithrombotic drug.

Since the finding of PGI2 that is chemically unstable and has both antiplatelet and hypotensive actions, various PGI2 derivatives have been developed in an attempt to dissociate these actions and to make PGI2 chemically stable (1–4). TRK-100 (Sodium dl-4-[(1R, 2R, 3aS, 8bS)-1, 2, 3a, 8b-tetrahydro-2-hydroxy-1-[(3S,4RS)-3-hydroxy-4-methyl-oct-6-yne-(E)-1-enyl]-5-cyclopenta[b]benzofuranyl]butyrate), a novel PGI2 analogue synthesized by Ohno et al. (5), is structurally different from carbacyclines (Fig. 1) and has been found to possess more potent antiplatelet activity than vasodilating activity, to be chemically stable and to be orally effective (6, 7). In this report, we have compared the effect of TRK-100 with some other antiplatelet drugs on rat platelet aggregation in vitro, rabbit platelet adhesion ex vivo and experimental thrombocytopenia and thrombosis in rats in vivo.

Materials and Methods

1. Rat platelet aggregation in vitro: Blood was collected from the abdominal artery of Wistar male rats anesthetized with pentobarbital sodium (NEMBUTAL, Abbott Labo., 50 mg/kg, i.p.) in a ratio of 9 volumes of blood to 1 volume of 3.8% sodium citrate solution. Platelet-rich plasma (PRP) was obtained by centrifugation of the citrated blood at 400×g for 10 min. A part of the
blood was centrifuged at 1500 × g for 10 min, and the supernatant was used as platelet-poor plasma (PPP). The platelet number of PRP was adjusted to be in the range of 90–100 × 10⁴/mm³ with a platelet Counter (PL-100, Toa Medical Electronics). Platelet aggregation was measured with a platelet aggregation meter (Sienco Co., U.S.A.), adjusting the range between 100 and 0 with PRP and PPP, respectively. After pre-incubation of PRP (200 μl) with each drug or its vehicle (20 μl) for 1 min at 37°C while stirring (1000 rpm), 20 μl (in case of A23187, 5 μl) of each aggregating agent was added. Inhibition percent was calculated by comparing the maximal aggregation of the control, which occurred within 3 min in ADP induced aggregation and after 5 min in collagen and A23187 induced aggregation, with that of the drug treated PRP.

2. Rabbit platelet adhesion ex vivo: Male rabbits (2.5–4.0 kg) were fasted overnight and were anesthetized with urethane (1 g/kg, i.p.). A tracheal cannula was inserted after exposure of the trachea, and then the jugular veins were exposed. After oral administration of each drug, blood was drawn from the jugular veins at 0, 0.5, 1, 2, 3 and 4 hr through a glass bead column (Igakushoin Kiki). The platelet number in the blood was counted with a platelet counter (PL-100, Toa). Platelet adhesion was expressed as the percentage of platelets that adhere or stick to the glass beads while the blood is passed through the column.

3. Experimental thrombocytopenia induced by collagen in rats: An extracorporeal polyethylene shunt (PE-100, 15 cm, Clay Adams) for blood sampling was made between the carotid artery and the jugular vein in Sprague-Dawley male rats (350–490 g for p.o., 290–390 g for i.v.) anesthetized by pentobarbital (50 mg/kg, i.p.) as described previously in detail (8). The polyethylene tube was filled with saline. Thirty (TRK-100) and 180 min (ticlopidine and aspirin) after the oral administration of each drug or vehicle, collagen (0.15 mg/kg) was injected into the femoral vein over a 20 sec period. In the case of intravenous administration, collagen was injected 2 min after drug injection except for hydralazine (5 min after). Blood (100–200 μl) was collected 1 min before and 1, 2.5, 5, 10 and 20 min after collagen injection into a sample bottle (SB-45, Toa Medical Electronics). The decrease in platelet numbers 1 min after collagen injection in the animals treated by test drugs was compared with that in the vehicle-treated animals and expressed as percent inhibition. Areas under the curves of change of platelet numbers for the first 20 min after collagen injection in the test drug animals were also expressed as percent inhibition of the vehicle control.

4. Experimental thread thrombosis in rats: A silk thread was inserted into the extracorporeal polyethylene tube (PE-100, 15 cm and PE-200, 6 cm, Clay Adams) filled with a heparin solution (50 IU/ml), as described above (8). The wet weight of the thrombus adhering to the silk thread (#50, 4 cm) in the blood stream for 20 min was measured, and the same procedure was repeated 2 times consecutively. When the silk thread was changed, 0.2 ml of heparin solution (50 IU/ml) was quickly injected into the venous side to remove the blood coagulates on the tubing wall. The total thrombus weight of the three experiments was compared. The first experiment was started 30 min after oral administration of TRK-100 and 3 hr after administration of aspirin or ticlopidine.

5. Drugs: For oral administration, TRK-100 and ticlopidine (Daiich Seiyaku, extracted from tablets) were dissolved in distilled water, and aspirin was suspended in 0.5% carboxymethylcellulose when used. For intravenous administration, TRK-100, pentoxifylline (Hoechst Japan, extracted from tablets) and hydralazine hydrochloride (Tokyo Kasei) were dissolved in saline, and PGI₂ (sodium salt) and PGE₁ (both synthesized in Toray Ind. Inc.) were dissolved in ice-cold water before use. Collagen (0.15 mg/ml, Sigma, from Bovine Achilles Tendon) was suspended in 0.2 M glycine-HCl buffer (pH 2.8) using the same procedure described elsewhere (9). Collagen suspension stored in a refrigerator was sonicated for 3 min intermittently just before its use. ADP (BMG)
and theophylline (Nutri. Biochem. Co.) were dissolved in saline. A23187 (Lilly Research Lab.) was dissolved as a 1% solution in ethanol-dimethylsulfoxide (1:1, v/v). This solution was diluted with ethanol for further use.

6. Statistical analysis: Results are expressed as the means and the standard error of the mean. The significance of the data was evaluated using the paired t-test, non-paired t-test (for two groups) and analysis of variance (for multi-groups). The efficiency ratio was calculated by parallel line analysis.

Results

1. Rat platelet aggregation in vitro: TRK-100 (10–300 nM), similar to PGI2, dose-dependently inhibited rat platelet aggregation induced by ADP (3 μM) or collagen (12.5 μg/ml). As shown in Table 1, the efficacy of TRK-100 was about 1/3–1/7 that of PGI2. Inhibition of A23187-induced aggregation with TRK-100 showed a ceiling effect at higher concentrations, being only about 60% of the inhibition at 300 nM. A similar phenomenon was observed with PGI2.

Then, the combined effect of TRK-100 and theophylline, a phosphodiesterase inhibitor, on ADP-induced aggregation was studied. A combination of TRK-100 with 1 mM theophylline, a concentration that inhibited the aggregation by about 25%, caused a parallel, leftward shift of the concentration-reaction curve of TRK-100, and its inhibitory action was about 14 times more active than TRK-100 alone (Fig. 2).

2. Rabbit platelet adhesion ex vivo: Inhibition of platelet adhesion by drugs was expressed as percent decrease of the adhesion when the adhesion before administration was defined as 100%. In the control group, a slight but significant increase of platelet adhesion was observed 30 min after administration of the vehicle and it was followed by a constant platelet adhesion percent (about 86%) (Fig. 3). TRK-100 produced a significant inhibition of platelet adhesion from 1 hr at 0.3 mg/kg and from 30 min at 1 and 3 mg/kg. After that, platelet adhesion was gradually reduced, but even after 5 hr, significant inhibition was found at 3 doses. In contrast, aspirin and ticlopidine (both at 300 mg/kg) showed a significant inhibition of platelet adhesion from 3 hr after drug administration.

![Fig. 2. Synergistic effect of TRK-100 with theophylline (1 mM) on rat platelet aggregation induced by 3 μM of ADP (n=2–5). ○: TRK-100 and ●: TRK-100+theophylline.](image)

Table 1. Effects of TRK-100 and PGI2 on rat platelet aggregation induced by ADP, collagen and A23187

<table>
<thead>
<tr>
<th>Compound</th>
<th>ADP (3 μM)</th>
<th>Collagen (12.5 μg)</th>
<th>A23187 (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (nM)</td>
<td>Potency ratio</td>
<td>IC50 (nM)</td>
</tr>
<tr>
<td>PGI2</td>
<td>4.2 (3.5–4.9)</td>
<td>1</td>
<td>9.5 (6.9–13.5)</td>
</tr>
<tr>
<td>TRK-100</td>
<td>27.9 (24.5–31.7)</td>
<td>1/7</td>
<td>27.0 (18.9–37.4)</td>
</tr>
</tbody>
</table>

IC50 was determined from 5–6 different experiments. The 95% confidence limits are shown in parentheses.
administration, being less effective than 0.3 mg/kg of TRK-100.

3. Experimental thrombocytopenia induced by collagen in rats: As shown in Fig. 4A, TRK-100 (0.1–3 mg/kg, p.o.) dose-dependently suppressed the decrease of rat blood platelets induced by collagen injection, its ED50 being 0.69 mg/kg calculated from the decrease of platelets at 1 min after collagen injection. Meanwhile, aspirin at a dose of more than 300 mg/kg significantly suppressed the platelet decrease, its effect being far less active than TRK-100. Ticlopidine was shown to be almost inactive. When the inhibitory effect was calculated from AUC₀−₂₀ (Fig. 4B), TRK-100, aspirin and ticlopidine dose-dependently inhibited the platelet decrease, but TRK-100 was about 3700–3800 times more active than aspirin and ticlopidine, its ED₅₀ being 0.48 mg/kg (Table 2). Intravenous TRK-100 dose-dependently inhibited a decrease of platelets in blood similar to PG₁₂ and PGE₁ (Fig. 5). Its ED₅₀ was 13.7 µg/kg as calculated from the decrease of platelets at 1 min after collagen injection and 16.4 µg/kg as calculated from AUC₀−₂₀ (Table 2). TRK-100 was of the same order of activity as PG₁₂ and it was 9–29 times more active than PGE₁. Hydralazine at a dose which
Fig. 4. Effects of TRK-100, ticlopidine and aspirin administered orally on experimental thrombocytopenia induced by collagen-injection in rats (n=6). —: TRK-100, ▲: Ticlopidine and ■: Aspirin. A (upper panel): Data were calculated from the percent decrease of platelet number at 1 min after collagen injection. Collagen alone decreased the platelet number by 62.7±7.7% in the DW treated control (n=11) and by 68.3±9.8% in the CMC treated control (n=9). B (lower panel): Data were calculated from $\text{AUC}_{0-20}$. Control values of $\text{AUC}_{0-20}$ were as follows: DW (p.o.): 566±132%-min (n=11) and CMC (p.o.): 562±124%-min (n=9). *P<0.05, **P<0.01 and ***P<0.001: Significantly different from the control.

Table 2. Comparison of potency of TRK-100 with those of a variety of drugs in experimental thrombocytopenia induced by collagen-injection in rats

<table>
<thead>
<tr>
<th></th>
<th>Oral</th>
<th>Intravenous</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B*</td>
<td></td>
<td>A*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ED50 (mg/kg)</td>
<td>Potency ratio</td>
<td>ED50 (µg/kg)</td>
<td>Potency ratio</td>
</tr>
<tr>
<td>TRK-100</td>
<td>0.48 (0.12–1.40)</td>
<td>1</td>
<td>13.7 (7.4–29.5)</td>
<td>1</td>
</tr>
<tr>
<td>PGI2</td>
<td>11.9 (7.3–21.3)</td>
<td>1.15</td>
<td>16.3 (7.2–46.1)</td>
<td>1.00</td>
</tr>
<tr>
<td>PGE1</td>
<td>400.6 (592.4–69021)</td>
<td>0.034</td>
<td>146.9 (73.3–354.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>1829</td>
<td>0.00026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>1751</td>
<td>0.00027</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: “A” and “B” are as in Figs. 4 and 5. The 95% confidence limits are shown in parentheses.
Fig. 5. Effects of TRK-100 and a variety of drugs administered intravenously on experimental thrombocytopenia induced by collagen-injection in rats (n=6). —○—: TRK-100, —△—: PGI2, —■—: PGE1, —○—: Hydralazine and —▲—: Pentoxifylline. A (upper panel): Data were calculated from percent decrease of platelet number at 1 min after collagen injection. Collagen alone decreased platelet number by 65.5±9.3% in the control (n=10). B (lower panel): Data were calculated from $\text{AUC}_{0-20}$. The control value of $\text{AUC}_{0-20}$ was 582±118%·min (n=10). *P<0.05 and ***P<0.001: Significantly different from the control.

shows antihypertensive action rather aggravated collagen-induced thrombocytopenia, or it further decreased platelet number. Inhibition of platelet decrease with pentoxifylline was small; it only suppressed the decrease significantly at a high dose of 100 mg/kg. The duration of action of TRK-100 was studied by administering 1 and 3 mg/kg of TRK-100 orally (Fig. 6). At a dose of 1 mg/kg, the maximal inhibition was observed 30 min after dosing, and the inhibition lasted for at least 6 hr. At 3 mg/kg, the maximal inhibition was also observed 30 min after dosing, and the inhibition was sustained over 8 hr, recovering after 17 hr.

4. Experimental thread thrombosis in rats: As shown in Fig. 7, TRK-100 administered orally inhibited dose-dependently thrombus formation, its ED50 being 0.46 mg/kg. Aspirin and ticlopidine also inhibited dose-dependently thrombus formation, their ED50 being 9.6 and 39.9 mg/kg, respectively. Therefore, TRK-100 was found to be 21 and 87 times more active than aspirin and ticlopidine, respectively.

Discussion

From the present study, it has been shown that TRK-100, a stable PGI2 analogue structurally different from the carbacyclines, inhibited rat platelet aggregation in vitro induced by a number of aggregating agents, rabbit platelet adhesion ex vivo and experimental thrombocytopenia induced by
Fig. 6. Duration of action of TRK-100 administered orally in experimental thrombocytopenia in rats. ---: 1 mg/kg (n=4-5) and ---: 3 mg/kg (n=5-7). *P<0.05, **P<0.01 and ***P<0.001: Significantly different from the value before drug administration.

Fig. 7. Effects of TRK-100, ticlopidine and aspirin administered orally on experimental thrombosis in rats (n=6). ---: TRK-100, ---: Ticlopidine and ---: Aspirin. *P<0.05, **P<0.01 and ***P<0.001: Significantly different from the control.
collagen and thread thrombosis in rats in vivo.

Inhibition of platelet aggregation with TRK-100 was 1/3–1/7 the inhibition by PGI2; but similar to PGI2, TRK-100 inhibited the aggregations induced by all kinds of agonists such as ADP, collagen and A23187. This indicates that the compound may suppress the process which is common to all three agonists. Inhibition of ADP-induced aggregation with TRK-100 was enhanced by theophylline, suggesting that the antiplatelet effect of TRK-100 is associated with an increase of cyclic AMP in rat platelets similar to human platelets (6).

TRK-100 was found to suppress significantly platelet adhesion in anesthetized rabbits as early as 30 min after dosing, and its action sustained over at least 5 hr. This, coupled with its prolonged action in the collagen-induced thrombocytopenia, demonstrates that TRK-100 given orally may be quickly absorbed from the gastrointestinal tract and has a longer duration of action, in contrast with PGI2 which is unstable in the acidic conditions such as gastric juice (10) and then has little oral activity in vivo. Meanwhile, significant inhibition by aspirin and ticlopidine were observed 3 hr after dosing. This is probably associated with the slow absorption of oral drugs under anesthesia and may be explained, particularly for ticlopidine, by the fact that the antiplatelet activity of its metabolites is greater than that of the drug itself (11).

Experimental thrombocytopenia induced by collagen in rats is a type of thromboembolism (12) and is a good method for assessing antiplatelet activity, because the degree of inhibition can be quantitatively estimated with a small number of animals compared with other methods determining mortality (13). In this model, it is possible to estimate not only the degree of platelet aggregation from the platelet decrease at 1 min after collagen injection but also possible to determine disaggregation from AUC0-20. Inhibition of both parameters with TRK-100 suggests that it has both inhibition of platelet aggregation and disaggregation activities in vivo, in parallel with the results in hamster pouches by Sim et al. (7).

Although the in vitro antiplatelet effect of TRK-100 was about 1/3–1/7 that of PGI2, it had the same level of intravenous activity as PGI2 for collagen-induced thrombocytopenia. This may be due to the greater effectiveness of TRK-100 on collagen-induced aggregation in vitro than ADP and A23187-induced aggregation and its resistance to the decomposition in plasma compared with PGI2 (5). The efficiency of PGE1 in this study was found to be 1/30 and 1/10 that of PGI2 calculated from the platelet decrease induced by collagen at 1 min after dosing and AUC0-20, respectively. Our results are almost in accordance with the report that PGI2 is 30 times more active than PGE1 in vitro (10, 14).

TRK-100 administered orally inhibited platelet adhesion ex vivo, platelet decrease in collagen-induced thrombocytopenia and experimental thread thrombosis. This indicates that TRK-100 is an antithrombotic compound which is orally effective.

Hydralazine as a vasodilator had no effect on platelet decrease induced by collagen and rather tended to stimulate it. This is attributable to an increase of blood viscosity, because blood flow becomes slow as a consequence of a decrease of blood pressure and heart rate under anesthesia (T. Murata et al., unpublished data). Therefore, the action of TRK-100 in this model seems to be due to its antiplatelet effect rather than a vasodilative one. Pentoxyfylline with a weak vasodilating action is likely to exert its antithrombotic action through lowering blood viscosity by improved red cell deformability and suppressing platelet function by inhibition of cAMP phosphodiesterase. Its action, however, was far less than that of TRK-100.

In the present thread thrombosis, formation of thrombus is initiated by adherence and aggregation of platelets on the silk thread inserted into the shunt (8). In this model, TRK-100 suppressed the formation of thrombus similar to aspirin and ticlopidine. Therefore, TRK-100 seems to block thrombus formation by inhibiting platelet adhesion and aggregation. The efficiency ratio between TRK-100 and aspirin or ticlopidine in this model was smaller than that in the thrombocytopenia induced by collagen. The reason for this difference is not clear, but it
may be due to the difference of experimental conditions. In this model, the activity of each drug showed higher potency than in the other experiments, suggesting that the experimental conditions of this model are less drastic than those of intravenous collagen injection. In addition, the anticoagulating effect of added heparin appears to exert a synergistic effect with the antiplatelet effect of each drug.

PGI₂ has clinically been utilized as an alternative to heparin to prevent blood coagulation in the extracorporeal circulation (15, 16) and renal dialysis (17), and it has been shown to be effective for peripheral arterial diseases such as Burger (18) and Raynaud disease (19). PGE₁ which possesses a similar pharmacological profile to PGI₂ has also been used for treating peripheral arterial diseases as an intraarterial drug (20, 21). Oral TRK-100 has been found to be far more potent than aspirin and ticlopidine in a variety of thrombotic models, although PGI₂ orally shows little effect. Therefore, this compound can be replaced by PGI₂ or PGE₁, because it is suitable for oral administration, with a long duration of action and chemical stability. In addition, its antiplatelet action seems to be separated from the blood pressure lowering effect compared with PGI₂ and PGE₁ (6). This compound is suggested to have a potential thrombotic effect in subjects with thrombotic diseases and clinical trials are under way.

References
15. Bunting, S., O’Grady, J., Fabiani, J.N., Terrier,


