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Tissue-type plasminogen activator transgenic rats for evaluating inhibitors of the activated form of thrombin-activatable fibrinolysis inhibitor

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No rodent models are currently available for evaluating inhibitors of the activated form of thrombin-activatable fibrinolysis inhibitor (TAFIa) without exogenous supplementation of tissue-type plasminogen activator (tPA). Characterization of tPA transgenic rats as a tool for the nonclinical evaluation of TAFIa inhibitors is the objective of the current study. tPA transgenic rats were subjected to rat models of tissue-factor-induced thromboembolism, $FeCl_3$-induced deep vein thrombosis (DVT) and arterial thrombosis, and tail bleeding. Potato tuber carboxypeptidase inhibitor (PCI), a selective TAFIa inhibitor, was used as an experimental compound at doses of 0.1, 1, or 10 mg/kg, and its antithrombotic effects and bleeding prolongation effect were compared with nontransgenic rats. Intravenous PCI showed significant and dose-related increase in plasma D-dimer levels in the tissue-factor-induced thromboembolism model. Intravenous PCI also significantly and dose-dependently reduced thrombus weights in the two thrombosis models only in the tPA transgenic rats. These results suggest that sensitive in-vivo evaluation of TAFIa inhibitors can be achieved using tPA transgenic rats without exogenous supplementation of recombinant tPA. On the other hand, no statistically significant prolongation of bleeding times by PCI was observed in either strain, whereas increased bleeding times were observed with 10 mg/kg of intravenous recombinant tPA, suggesting that the low bleeding risk of TAFIa inhibitors is further confirmed in the tPA transgenic rats whose basal tPA levels are elevated. tPA transgenic rats may be beneficial for the pharmacological and toxicological evaluation of TAFIa inhibitors and further confirm that TAFIa is a promising target for various thrombotic disorders. ***Blood Coagul Fibrinolysis*** **29:314–321 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Thromboembolic diseases, such as ischemic stroke, acute coronary syndrome (ACS), and venous thromboembolism (VTE), pose serious health problems worldwide [1–5]. Pharmacological therapies for such disorders include antiplatelets, anticoagulants, and thrombolytics. In these antithrombotic agent classes, balance between effectiveness and potential side effects (bleeding complications) remains a major challenge.

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a circulating basic carboxypeptidase zymogen primarily produced in liver [6]. TAFI is converted into the active form (TAFIa) by proteases, including thrombin, thrombin/thrombomodulin complex, and plasmin. TAFIa removes lysine residues at the carboxy terminal (C-terminal) of fibrin degradation products in fibrin clots. As the C-terminal lysine residues function as cofactors for efficient interaction with plasminogen and tissue-type plasminogen activator (tPA), TAFIa attenuates plasmin generation and fibrin degradation [7]. In clinical perspective, elevated TAFI concentration in plasma has been reported in patients with acute ischemic stroke and VTE, suggesting involvement of TAFIa in clinical outcomes of such thrombotic disorders [8–10]. It has been reported that pharmacological inhibition of TAFIa displays antithrombotic effects in animal thrombosis models, with reduced bleeding risk compared with human recombinant tissue-type plasminogen activator (rt-PA), a marketed thrombolytic drug [11,12]. These distinct profiles of TAFIa inhibitors are explained by their mechanisms of action. TAFIa inhibitors protect the C-terminal lysine residues and increase plasmin generation. Cofactor-mediated plasmin generation avoids neutralization by alpha 2 plasmin inhibitor (α_2-PI) in the circulation and thereby leads to efficient fibrinolysis. On the other hand, intravenous rt-PA converts plasminogen into plasmin in fluid phase as well as on fibrin clots. Plasmin generated in the fluid phase is immediately blocked by α_2-PI and this results in inefficient fibrinolysis. In addition, rt-PA causes excess plasmin generation in the fluid phase to cause fibrinogenolysis, which is one of the possible causes of bleeding in fibrinolytic treatment. Thus, TAFIa inhibitors would be next-generation drugs for thrombotic disorders and drug discovery studies and clinical trials are underway [13–15].

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However, a well known obstacle to the nonclinical evaluation of TAFIa inhibitors is that this class of compounds needs subtherapeutic supplementation of rt-PA in animal thrombosis models, including arteriovenous thrombosis in rats, jugular vein thrombosis in rabbits, and coronary artery thrombosis in dogs [11–13,16–18]. To date, this exogenous rt-PA supplementation has hampered the efficient evaluation and screening of TAFIa-inhibiting compounds in animal thrombosis models. As rats have larger body size than mice, several experimental advantages exist in using rats over mice for investigating TAFIa inhibitors and more elaborate thrombotic disease models are easily created.

We previously generated tPA transgenic rats to circumvent species differences in the responsiveness to rt-PA between rats and humans [19]. The confirmed profile of the rats is as follows: conservation of pathophysiological response of fibrinolysis (transgene is controlled by its endogenous promoter), normal hemostasis, and effective doses of rt-PA in a thromboembolic stroke being closer to those of human patients. Thus, tPA transgenic rats, which overexpress tPA under an endogenous regulation system, can potentially be used for efficient screening development and pharmacological/toxicological evaluations of TAFIa inhibitors without exogenous rt-PA supplementation.

In this study, we investigated easy assessment of TAFIa inhibitor effects with fibrinolytic markers, antithrombotic effects of TAFIa inhibition in venous and arterial thrombosis models without exogenous rt-PA administration, and lower risk of TAFIa inhibitor bleeding compared with rt-PA using tPA transgenic rats with elevated basal tPA levels.

Materials and methods

Animal care

All experimental procedures were performed according to the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. The frequency of animal health monitoring by animal care personnel was twice/day on weekdays and once/day during weekends. Microbial monitoring was conducted using sentinel animals once every 2 months. Proper care was taken or directed by attending veterinarians for abnormal animals (displaying distress in drinking water, feeding, breathing or other abnormal behaviors such as self-injury or abnormal posture). All surgeries were performed under sodium thiopental (100 mg/kg, intraperitoneal; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) or isoflurane anesthesia (3.5% for induction and 1.5% for maintenance, Pfizer Inc., New York City, New York, USA); all efforts were made to minimize suffering. Humane endpoints were applied to disease model experiments when the subjected animals showed above-listed abnormalities. Intravenous sodium thiopental (100 mg/kg) was administered to euthanize animals if these endpoints were applied. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Daiichi Sankyo (Permit Number: A1502377).

Animals

Male tPARecBACTg (tPA transgenic) rats and nontransgenic rats were generated and systematized as described [19]. Male tPA transgenic and nontransgenic rats were maintained at the Institute of Immunology Co., Ltd., and supplied at 6–7 weeks of age. The rats were maintained in cages ($\leq$3 animals per cage) with free access to chlorinated water and food (FR-2; Funabashi Farm Co., Ltd., Tsukuba, Japan). The animal quarters were set at $23 \pm 2\,^{\circ}C$ (allowable range: 18–28 °C), humidity of $55 \pm 10\%$ (allowable range: 30–70%), and 12-h lighting cycle (lights on 7:00–19:00). The acclimation period was more than 3 days. The number of animals used in each experiment were determined by preliminary experiments. In total, 197 of rats were subjected to the following experiments.

Tested compounds

Potato tuber carboxypeptidase inhibitor (PCI; carboxypeptidase inhibitor from potato tuber lyophilized powder, C0279), a selective TAFIa inhibitor [12,20,21], was purchased from Sigma-Aldrich Co., Limited Liability Company. PCI was dissolved in physiological saline (0.9% NaCl, Otsuka Pharmaceutical Factory Inc., Tokyo, Japan) and intravenously administered as a bolus at 0.1, 1, or 10 mg/kg doses. PCI dosage was determined to detect dose dependency regarding previously reported ex-vivo plasma TAFIa inhibiting activity [16]. rt-PA (activacin for injection) was purchased from Kyowa Hakko Kirin Co., Ltd. and dissolved in adjunctive injection solvent and saline for further dilution. The rt-PA was intravenously administered with 1/10 vol. bolus followed by 1-h infusion using an infusion pump (TE-361; Terumo Corporation, Tokyo, Japan) at 1 or 10 mg/kg doses. The saline served as the control solution for PCI and rt-PA. Administration timing is described in each animal experiment section.

Tissue factor-induced venous thromboembolism model

To assess fibrinolytic activity upregulation with PCI administration in tPA transgenic and nontransgenic rats, D-dimer plasma concentrations were determined in a tissue factor (TF)-induced VTE model. Rats were anesthetized with sodium thiopental. TF (Dade Innovin, Siemens AG, Munich, Germany, GTN-200A, 10-ml vial) solution was prepared by adding 5-ml saline, and continuously administered to the rats via the jugular vein over 20 min using an infusion pump (TE-361; Terumo Corporation) at 7.5 ml/kg/h. PCI solutions or the vehicle were intravenously administered as a bolus via the jugular vein 5 min before TF administration. Blood samples (400 μl per time point) were collected from the jugular vein into a

syringe containing 10 vol.% of 3.13% sodium citrate 20 min after TF administration. Citrated plasma was collected on blood sample centrifugation at $900 \times g$ at 4 °C for 10 min. The plasma samples were diluted 20 times with factor diluent (LSI Medience Corporation, Osaka, Japan), and D-dimer concentration, an indicator of fibrin (not fibrinogen) degradation, was measured using LPIA-ACE D-D dimer II (LSI Medience Corporation) on ACL TOP 500 CTS. Five, nine, or 10 rats/group were subjected to the experiment (total 57 rats).

$FeCl_3$-induced deep vein thrombosis model

To assess antithrombotic effect of PCI in tPA transgenic and nontransgenic rats, thrombus weights were determined in a $FeCl_3$-induced deep vein thrombosis (DVT) model, known to have fibrin-rich thrombus formation [22]. Rats were anesthetized with sodium thiopental, and partial stenosis was applied to the vena cava at the renal veins by ligation with a blunt 20-ga needle that was subsequently removed. A filter paper (no. 2, Advantec Toyo Kaisha, Ltd. Tokyo, Japan, cut into 2×5 mm per piece) soaked with 3.5 μl of 10% $FeCl_3$ (Nacalai Tesque, Inc., Kyoto, Japan) was applied to the external surface of the vena cava for 5 min. The PCI solutions or the vehicle were intravenously administered as a bolus via the jugular vein 5 min before thrombus induction. The thrombus was excised 90 min after thrombus induction, and wet weights were measured. Four or five rats/group were subjected to the experiment (total 39 rats).

$FeCl_3$-induced arterial thrombosis model

To elaborate on the antithrombotic effect of PCI in another model in tPA transgenic and nontransgenic rats, thrombus weights were determined in a $FeCl_3$-induced arterial thrombosis model, known as platelet predominant (antiplatelet sensitive) [23,24]. Rats were anesthetized with isoflurane. Arterial thrombosis was induced in the common carotid artery (CCA) by sandwiching the CCA between two filter papers (no. 2, cut into 1×10 mm per piece) soaked with 3.5 μl of 10% $FeCl_3$ for 10 min. The PCI solutions or the vehicle were intravenously administered as a bolus via the jugular vein 5 min before thrombus induction. The rats were then allowed to recover from anesthesia. The thrombus was excised 360 min after thrombus induction under isoflurane anesthesia and wet weights were measured. Five rats/group were subjected to the experiment (total 40 rats).

Tail bleeding model

Rats were anesthetized with sodium thiopental (100 mg/kg, intraperitoneal) and put on heating pads at approximately 37 °C to maintain body temperature. rt-PA (1 or 10 mg/kg) or its vehicle (saline) was intravenously administered as a bolus (1/10 vol.) followed by infusion (9/10 vol.) via the jugular vein using the infusion pump for an hour. The PCI solutions were administered as a bolus followed by an infusion similar to that for rt-PA. A 1-mm incision was made with a blade (FAS-10; Feather Safety Razor Co., Ltd., Osaka, Japan) on the artery of the ventral part of the tail at 4 cm from the tip 30 min after commencement of compound administration, and blood was blotted every 30 s with filter papers (no. 2, Advantec Toyo Kaisha, Ltd.) for 30 min. Bleeding time was defined as the multiplication of detectable blood stain number on the opposite side of the filter paper that touched the blood by 30 s. Five or six rats/group were subjected to the experiment (total 61 rats).

Statistical analysis

Calculations were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA). Data are expressed as the mean ± SEM. Steel test was carried out to compare the test compound-treated groups with the vehicle-treated group using the SAS System Release 9.2. (SAS Institute Inc., Cary, North Carolina, USA). Dose dependency of the tested compounds was evaluated by Spearman's rank correlation coefficient hypothesis testing using SAS System Release 9.2. P values of less than 0.05 were considered statistically significant.

Results

Effect of potato tuber carboxypeptidase inhibitor on plasma D-dimer concentrations in the tissue factor-induced venous thromboembolism model

Plasma D-dimer concentrations are presented in Fig. 1. In nontransgenic rats, plasma D-dimer concentrations were not statistically significantly elevated 20 min after TF administration in PCI-treated groups compared with the vehicle-treated group. In tPA transgenic rats, dose-dependent ($P < 0.0001$) and statistically significant increase in D-dimer concentrations was observed in the 1 or 10 mg/kg of intravenous PCI-treated groups compared with the vehicle-treated group ($P = 0.0040$ and 0.0069, respectively).

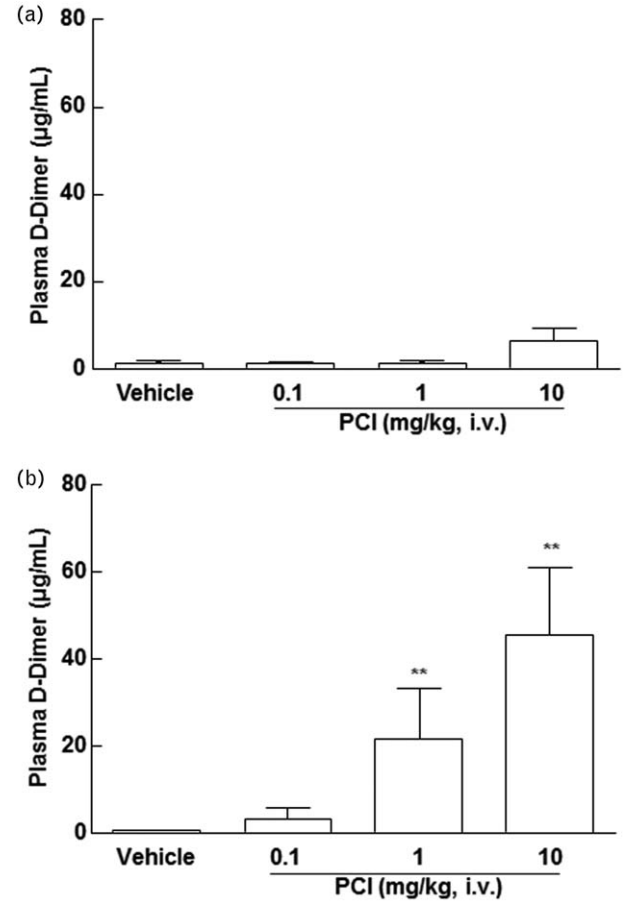
Effect of potato tuber carboxypeptidase inhibitor on thrombus weights in the $FeCl_3$-induced deep vein thrombosis model

Figure 2 shows antithrombotic effect of PCI in the $FeCl_3$-induced DVT model. In nontransgenic rats, a statistically significant reduction in thrombus weight was not observed with PCI treatment. However, PCI (1 and 10 mg/kg, i.v.) exhibited a statistically significant reduction in wet thrombus weights in tPA transgenic rats compared with the vehicle ($P = 0.0247$ and 0.0247, respectively). The effect of PCI was dose dependent ($P < 0.0001$).

Effect of potato tuber carboxypeptidase inhibitor on thrombus weights in the $FeCl_3$-induced arterial thrombosis model

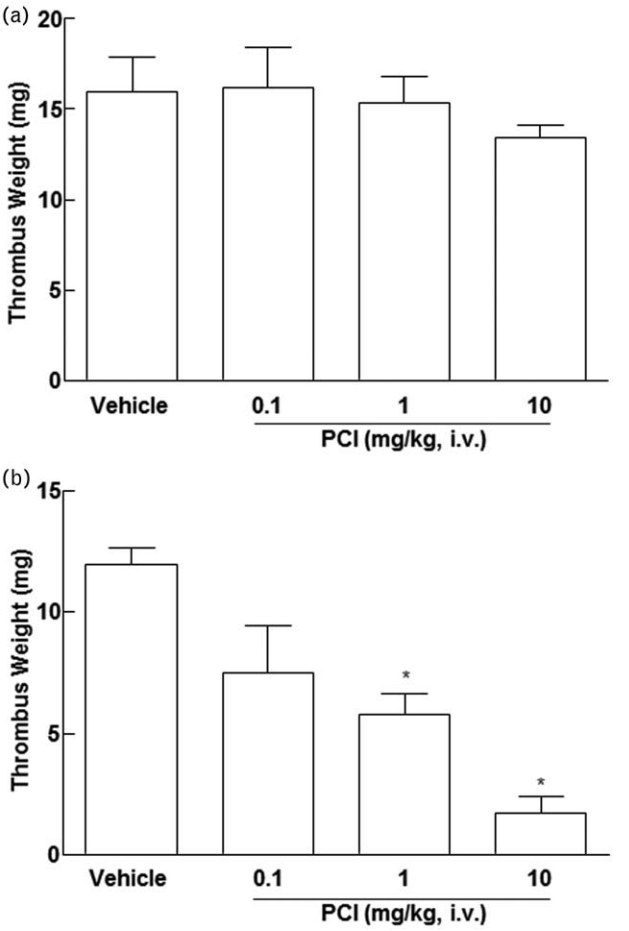
Figure 3 shows the antithrombotic effect of PCI in the $FeCl_3$-induced arterial thrombosis model. In the

Fig. 1



Comparison of the effect of potato tuber carboxypeptidase inhibitor on plasma D-dimer levels between tissue-type plasminogen activator transgenic and nontransgenic rats in a tissue factor-induced venous thromboembolism model. Recombinant tissue factor was intravenously administered (i.v.) to the nontransgenic rats (a) and the tissue-type plasminogen activator transgenic rats (b) using an infusion pump at a rate of 7.5 ml/kg/h for 20 min. The potato tuber carboxypeptidase inhibitor solutions or the vehicle were intravenously administered as a bolus 5 min before tissue factor administration. Blood was collected from the jugular vein 20 min after tissue factor administration. Plasma D-dimer levels were determined as a biomarker of fibrinolysis. Each value represents the mean ± SEM ($n = 5–10$). $^{**}P < 0.01$ compared with vehicle (steel test).

Fig. 2



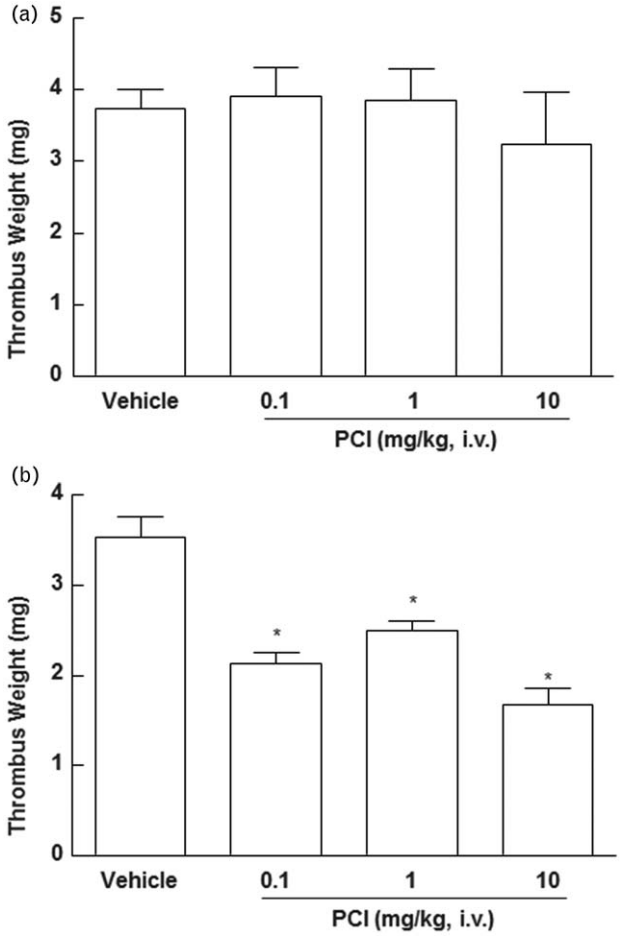
Comparison of the antithrombotic effect of potato tuber carboxypeptidase inhibitor between the tissue-type plasminogen activator transgenic and the nontransgenic rats in a $FeCl_3$-induced deep vein thrombosis model. Venous thrombosis was induced in the inferior vena cava of the nontransgenic rats (a) and the tissue-type plasminogen activator transgenic rats (b) by partial stenosis and topical application of 10% ferric chloride for 5 min. The potato tuber carboxypeptidase inhibitor solutions or the vehicle were intravenously administered (i.v.) as a bolus via jugular vein 5 min before thrombus induction. Wet thrombus weights were measured 90 min after thrombus induction. Each value represents the mean ± SEM ($n = 4, 5$). $P < 0.05$ was regarded as statistically significant. $^{*}P < 0.05$ compared with vehicle (steel test).

nontransgenic rats, there was no statistically significant reduction in thrombus weight with the PCI treatment compared with the vehicle (3.73 ± 0.27 mg in the vehicle, 3.91 ± 0.39 mg in the 0.1 mg/kg of PCI, 3.85 ± 0.45 mg in the 1 mg/kg of PCI, and 3.23 ± 0.74 mg in the 10 mg/kg of PCI, respectively). In the tPA transgenic rats, thrombus weight (mg) of each group was 3.54 ± 0.23 (vehicle), 2.14 ± 0.12 (PCI 0.1 mg/kg), 2.50 ± 0.11 (PCI 1 mg/kg), and 1.68 ± 0.19 (PCI 10 mg/kg). PCI (0.1, 1, and 10 mg/kg, i.v.) exhibited a statistically significant reduction of the wet thrombus weight in tPA transgenic rats compared with the vehicle ($P = 0.0247$, 0.0247, and 0.0247, respectively). The effect of PCI was dose dependent ($P = 0.0002$).

Tail bleeding model

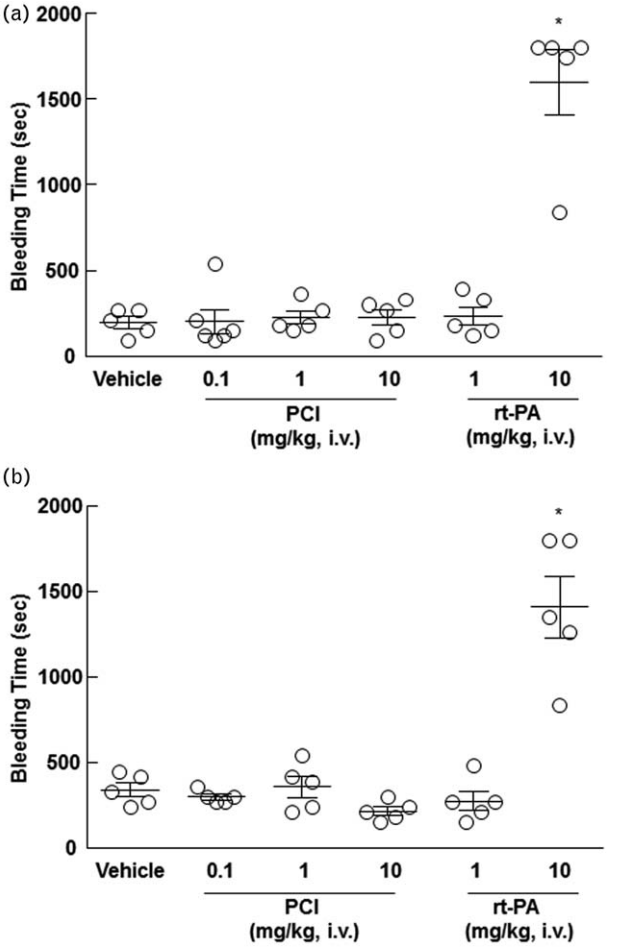
Bleeding profiles of the two agents (PCI and rt-PA) are presented in Fig. 4. The bleeding times (in seconds) of each group in nontransgenic rats were 198 ± 35 (vehicle), 205 ± 69 (PCI 0.1 mg/kg), 228 ± 39 (PCI 1 mg/kg), 228 ± 46 (PCI 10 mg/kg), 234 ± 53 (rt-PA 1 mg/kg), and 1596 ± 189 (rt-PA 10 mg/kg). In tPA transgenic rats with elevated basal tPA concentrations, bleeding times (in seconds) of each group were 342 ± 41 (vehicle), 300 ± 16 (PCI 0.1 mg/kg), 360 ± 61 (PCI 1 mg/kg), 216 ± 26 (PCI 10 mg/kg), 276 ± 56 (rt-PA 1 mg/kg), and 1410 ± 181 (rt-PA 10 mg/kg). Compared with the

Fig. 3



Comparison of the antithrombotic effect of potato tuber carboxypeptidase inhibitor between the tissue-type plasminogen activator transgenic and the nontransgenic rats in a $FeCl_3$-induced arterial thrombosis model. Arterial thrombosis was induced in the common carotid artery) the nontransgenic rats (a) and the tissue-type plasminogen activator transgenic rats (b) by sandwiching the common carotid artery between two filter papers soaked with 3.5 μl of 10% $FeCl_3$ for 10 min. The potato tuber carboxypeptidase inhibitor solutions or the vehicle were intravenously administered (i.v.) as a bolus via jugular vein 5 min before thrombus induction. Wet thrombus weights were measured 360 min after thrombus induction. Each value represents the mean ± SEM ($n = 5$). $P < 0.05$ was regarded as statistically significant. $^*P < 0.05$ compared with vehicle (steel test).

Fig. 4



Comparison of the effects of potato tuber carboxypeptidase inhibitor and recombinant tissue-type plasminogen activator on tail bleeding time between tissue-type plasminogen activator transgenic and nontransgenic rats. Potato tuber carboxypeptidase inhibitor, recombinant tissue-type plasminogen activator, or their vehicle (saline) was intravenously administered (i.v.) via the jugular vein of the nontransgenic rats (a) and the tissue-type plasminogen activator transgenic rats (b). The rat tail was cut by a razor 30 min after the beginning of administration and the bleeding time was monitored for 30 min. Each value represents the mean ± SEM ($n = 5, 6$). $^*P < 0.05$ compared with vehicle (steel test).

vehicle-treated group, PCI (0.1, 1, and 10 mg/kg) and 1 mg/kg of rt-PA did not show a statistically significant prolongation of bleeding time in either strain, whereas 10 mg/kg of rt-PA significantly increased bleeding time in both strains ($P = 0.0153$ in nontransgenic rats, $P = 0.0168$ in tPA transgenic rats, respectively).

Discussion

The tPA transgenic rats were originally generated to address species differences in the responsiveness to rt-PA between wild-type rats and humans [19]. In this study, we investigated the usefulness of tPA transgenic rats for evaluating activated TAFI inhibitors.

A thrombin-activatable fibrinolysis inhibitor potato tuber carboxypeptidase inhibitor increases plasma D-dimer concentrations in the tissue factor-induced thromboembolism model in tissue-type plasminogen activator transgenic rats

Intravenous TF infusion is a common VTE or hypercoagulation model in rats [11,12]. Here, the pharmacodynamics of fibrinolytic enhancers is evaluated using the plasma D-dimer concentration, a kind of fibrin (not

fibrinogen) degradation product [12,14]. Here, PCI showed a dose-dependent increase in plasma D-dimer concentration in the TF-induced VTE model in tPA transgenic rats only, when blood sampling was performed 20 min after the TF administration started. However, D-dimer concentration in vehicle-treated groups was equivalent in the two strains, suggesting that basal differences and TF stimulation-dependent plasma tPA induction are not critical in this model or the evaluation point. These results suggest that sensitive in-vivo evaluation of TAFIa inhibitors may be achieved using tPA transgenic rats and the TF model within a short time period.

Reports have revealed that TAFIa inhibitors exert profibrinolytic effects in TF-induced thromboembolism model without exogenous rt-PA supplementation [11,12,14]. Indispensability of this model can be attributed to local tPA availability in the lung, a microthrombi-accumulating organ under the TF challenge. We previously reported that plasma D-dimer concentrations were elevated in delayed time points (90 and 120 min) [19]. Considering this fibrinolytic profile, selecting a time point at which plasma D-dimer levels are kept basal is important for evaluating TAFIa inhibitors. TAFIa inhibitors may be efficiently screened by plasma D-dimer levels in the TF model using tPA transgenic rats.

Potato tuber carboxypeptidase inhibitor decreases thrombus weight in the $FeCl_3$-induced deep vein thrombosis model without exogenous recombinant tissue-type plasminogen activator in tissue-type plasminogen activator transgenic rats

DVT is a major type of VTE and is at risk of pulmonary embolism [3]. $FeCl_2$ or $FeCl_3$ is frequently employed as a thrombus inducer, and a TAFIa inhibitor is effective in the presence of exogenous rt-PA in DVT models [16]. Here, PCI showed a dose-related reduction of thrombus weight in tPA transgenic but not in nontransgenic rats. These results confirm that TAFIa inhibitors need the subthreshold addition of tPA to exert their antithrombotic effects on deep vein thrombus in rats. Thrombus weights of the tPA transgenic and nontransgenic rats treated with vehicle were comparable, suggesting that tPA overexpression extent in transgenic rats may be suitable for evaluating the antithrombotic effect of TAFIa inhibitors in the DVT rat model. These results also indicate that TAFIa inhibitors may be effective in VTE.

The rat DVT model is known as a fibrin-rich thrombus formation and anticoagulants are effective [22,25]. However, these agents do not directly affect existing thrombus resolution regarding their action mechanism. However, TAFIa inhibitors are thrombolytic enhancers with the potential to resolve existing blood clots; thus, not only preventive but also therapeutic usages should be investigated in VTE using tPA transgenic rats.

Potato tuber carboxypeptidase inhibitor decreases thrombus weight in the $FeCl_3$-induced arterial thrombosis model in tissue-type plasminogen activator transgenic rats

A $FeCl_3$-induced arterial thrombosis model was employed to investigate antithrombotic activity of TAFIa inhibitors in another thrombosis model. Generally, arterial thrombosis models are sensitive to antiplatelet agents [23,24], but the effectiveness of TAFIa inhibitors on arterial thrombosis awaits clarification [11,12,16].

In this study, PCI showed a dose-related thrombus weight reduction in tPA transgenic but not in nontransgenic rats. These findings suggest that tPA transgenic rats are beneficial for pharmacologically evaluating TAFIa inhibitors in the arterial thrombosis model by subthreshold tPA overexpression.

In DVT models, the evaluation point was 90 min after thrombus induction, so continuous intravenous rt-PA supplementation may be feasible with wild-type rats. However, in our arterial thrombosis model, the evaluation point was 360 min after thrombus induction and rats recovered from the inhaled anesthesia after the thrombus induction procedure. In such subacute models or even chronic disease models, continuous rt-PA infusion is not feasible. Therefore, the tPA transgenic rats may be convenient for evaluating TAFIa inhibitors not only in subacute arterial thrombosis models but also in chronic thrombotic models or other disease models in which TAFIa is involved, including pulmonary fibrosis and pulmonary hypertension [26,27].

Thrombus weights in vehicle-treated tPA transgenic and nontransgenic rats were comparable in the arterial thrombosis model as well as in the DVT model, demonstrating that the tPA overexpression extent in the transgenic rats is unexpectedly at an optimal level for evaluating antithrombotic effect of TAFIa inhibitors in various thrombosis models, as previous studies have reported a need for determining the subthreshold rt-PA dose for each disease model [16]. Regarding target validation, TAFIa inhibitors may also be effective in arterial thromboembolic diseases such as ACS and stroke.

Potato tuber carboxypeptidase inhibitor did not show bleeding prolongation in the tail bleeding model in tissue-type plasminogen activator transgenic rats

As basal tPA concentrations are elevated in tPA transgenic rats [19], efficacious PCI doses in the three tested thrombosis models may increase bleeding risk in tPA transgenic rats. To test this hypothesis, tPA transgenic and nontransgenic rats were intravenously treated with PCI (0.1, 1, or 10 mg/kg) or rt-PA (1 or 10 mg/kg) and effects on bleeding time were compared. PCI did not display statistically significant bleeding time prolongation in tPA transgenic or nontransgenic rats at any dose, whereas the higher rt-PA dose showed statistically

significant prolongation of bleeding time in both strains. These results suggest that the low bleeding risk of TAFIa inhibitors is further confirmed using tPA transgenic rats with upregulated endogenous tPA levels and fibrinolytic enhancer (rt-PA)-sensitive bleeding occurs. The findings of these experiments using PCI and tPA transgenic rats, TAFIa inhibitors may prove to be safer thrombolytic enhancers.

A report demonstrated that TAFIa-dependent fibrinolysis is dictated by available tPA concentration [28], and tPA or rt-PA availability differs depending on disease and/or treatment. Therefore, to assess the safety profile of TAFIa inhibitors, appropriate animals or strains in which TAFIa inhibitors are effective in the disease models of interest should be selected. tPA transgenic rats are thus ideal for the following factors: basal tPA expression is upregulated, pathological tPA induction is preserved, and the effectiveness of a TAFIa inhibitor on DVT and arterial thrombosis is confirmed. Further TAFIa inhibitor analyses regarding bleeding risk in different disease models (e.g., stroke) and/or in combination treatment with rt-PA using tPA transgenic rats are necessary to determine the target indication(s) of TAFIa inhibitors and their safety.

Experimental limitations

In this study, we did not confirm TAFIa inhibition extent in each experimental model. Bird *et al.* reported that intravenous bolus injection of PCI (0.3, 1, 3, and 10 mg/kg) in rats provided a dose-dependent plasma TAFIa activity inhibition. Inhibitory activity change at the highest dose was 80% at 5 min and 60% at 60 min after administration [16]. In this study, evaluation time points were 20 min (TF-VTE), 90 min ($FeCl_3$-DVT), 360 min ($FeCl_3$-arterial thrombosis), and 30–60 min (tail bleeding), respectively, so the antithrombotic effect of the TAFIa inhibitors may be underestimated because of insufficient TAFIa inhibition. Due to poor PCI solubility, doses more than 10 mg/kg (e.g., 30 or 100 mg/kg), which may achieve full inhibition, could not be examined. Therefore, pharmacokinetic and pharmacodynamic analyses using more potent TAFIa inhibitors with greater solubility may provide further insight into the therapeutic potential of TAFIa inhibition in various thrombosis models.

In conclusion, we confirm that TAFIa inhibitors can be easily evaluated with fibrinolytic markers in the TF-induced VTE model, TAFIa inhibition exerts antithrombotic effects in venous and arterial thrombosis models without exogenous rt-PA administration, and TAFIa inhibition has lower bleeding risk compared with rt-PA using tPA transgenic rats. tPA transgenic rats seem beneficial for the pharmacological and toxicological TAFIa inhibitor evaluation, with the promise of safer thrombolytics in the future.

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Compliance with ethical standards: all the authors are employees of Daiichi Sankyo Co., Ltd.

Ethical approval: all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflicts of interest

There are no conflicts of interest.

References

1 Fernandez MM, Hogue S, Preblick R, Kwong WJ. Review of the cost of venous thromboembolism. *Clinicoecon Outcomes Res* 2015; **7**: 451–462.
2 Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, *et al.* Heart disease and stroke statistics – 2013 update: a report from the American Heart Association. *Circulation* 2013; **127**:e6–e245.
3 Guyatt GH, Akl EA, Crowther M, Schünemann HJ, Gutterman DD, Lewis SZ. Introduction to the ninth edition: antithrombotic therapy and prevention of thrombosis, 9th ed: American college of chest physicians evidence-based clinical practice guidelines. *Chest* 2012; **141**:S48–S52.
4 GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015; **385**:117–171.
5 Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, *et al.* Heart disease and stroke statistics – 2015 update: a report from the American Heart Association. *Circulation* 2015; **131**:e29–e322.
6 Declerck PJ. Thrombin activatable fibrinolysis inhibitor. *Hamostaseologie* 2011; **31**:165–166; 168–173.
7 Foley JH, Kim PY, Mutch NJ, Gils A. Insights into thrombin activatable fibrinolysis inhibitor function and regulation. *J Thromb Haemost* 2013; **11**:306–315.
8 Leebeek FW, Goor MP, Guimaraes AH, Brouwers GJ, Maat MP, Dippel DW, *et al.* High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke. *J Thromb Haemost* 2005; **3**:2211–2218.
9 Montaner J, Ribó M, Monasterio J, Molina CA, Alvarez-Sabín J. Thrombin-activable fibrinolysis inhibitor levels in the acute phase of ischemic stroke. *Stroke* 2003; **34**:1038–1040.
10 Eichinger S, Schönauer V, Weltermann A, Minar E, Bialonczyk C, Hirschl M, *et al.* Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism. *Blood* 2004; **103**:3773–3776.
11 Wang YX, da Cunha V, Vincelette J, Zhao L, Nagashima M, Kawai K, *et al.* A novel inhibitor of activated thrombin activatable fibrinolysis inhibitor (TAFIa) – Part II: Enhancement of both exogenous and endogenous fibrinolysis in animal models of thrombosis. *Thromb Haemost* 2007; **97**:54–61.
12 Suzuki K, Muto Y, Fushihara K, Kanemoto K, Iida H, Sato E, *et al.* Enhancement of fibrinolysis by EF6265 [(S)-7-amino-2-[[[(R)-2-methyl-1-(3-phenylpropanoylamino)propyl]hydroxyphosphinoyl] methyl]heptanoic acid], a specific inhibitor of plasma carboxypeptidase B. *J Pharmacol Exp Ther* 2004; **309**:607–615.
13 Islam I, Bryant J, May K, Mohan R, Yuan S, Kent L, *et al.* 3-Mercaptopropionic acids as efficacious inhibitors of activated thrombin activatable fibrinolysis inhibitor (TAFIa). *Bioorg Med Chem Lett* 2007; **17**:1349–1354.
14 Sasaki T, Yoshimoto N, Sugimoto K, Takada K, Murayama N, Yamazaki H, *et al.* Intravenous and oral administrations of DD2 [7-Amino-2-(sulfanylmethyl)heptanoic acid] produce thrombolysis through inhibition of plasma TAFIa in rats with tissue factor-induced microthrombosis. *Thromb Res* 2012; **130**:e222–e228.
15 Zhou J, Kochan J, Yin O, Warren V, Zamora C, Atiee G, *et al.* A first-in-human study of DS-1040, an inhibitor of the activated form of thrombin-activatable fibrinolysis inhibitor, in healthy subjects. *J Thromb Haemost* 2017; **15**:961–971.

16 Bird E, Tamura J, Bostwick JS, Steinbacher TE, Stewart A, Liu Y, *et al.* Is exogenous tissue plasminogen activator necessary for antithrombotic efficacy of an inhibitor of thrombin activatable fibrinolysis inhibitor (TAFI) in rats? *Thromb Res* 2007; **120**:549–558.
17 Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagila R, *et al.* An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tPA-induced thrombolysis in a rabbit jugular vein thrombolysis model. *Thromb Res* 2000; **98**:333–342.
18 Björkman JA, Abrahamsson TI, Nerme VK, Mattsson CJ. Inhibition of carboxypeptidase U (TAFIa) activity improves rt-PA induced thrombolysis in a dog model of coronary artery thrombosis. *Thromb Res* 2005; **116**:519–524.
19 Ito Y, Noguchi K, Morishima Y, Yamaguchi K. Generation and characterization of tissue-type plasminogen activator transgenic rats. *J Thromb Thrombolysis* 2018; **45**:77.
20 Ryan CA, Hass GM, Kuhn RW. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J Biol Chem* 1974; **249**: 5495–5499.
21 Bouma BN, Meijers JC. Thrombin-activatable fibrinolysis inhibitor (TAFI, plasma procarboxypeptidase B, procarboxypeptidase R, procarboxypeptidase U). *J Thromb Haemost* 2003; **1**:1566–1574.
22 Hara T, Bhayana B, Thompson B, Kessinger CW, Khatri A, McCarthy JR, *et al.* Molecular imaging of fibrin deposition in deep vein thrombosis using fibrin-targeted near-infrared fluorescence. *JACC Cardiovasc Imaging* 2012; **5**:607–615.
23 Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005; **115**:3355–3362.
24 Mizuno M, Tomizawa A, Ohno K, Jakubowski JA, Sugidachi A. A novel model of intravital platelet imaging using CD41-ZsGreen1 transgenic rats. *PLoS One* 2016; **11**:e0154661.
25 Carlsson S, Elg M. The effects of ximelagatran and warfarin on the prophylaxis of a caval vein thrombosis and bleeding in the anaesthetized rat. *Blood Coagul Fibrinolysis* 2005; **16**:245–249.
26 Fujimoto H, Gabazza EC, Taguchi O, Nishii Y, Nakahara H, Bruno NE, *et al.* Thrombin-activatable fibrinolysis inhibitor deficiency attenuates bleomycin-induced lung fibrosis. *Am J Pathol* 2006; **168**:1086–1096.
27 Qin L, D'Alessandro-Gabazza CN, Aoki S, Gil-Bernabe P, Yano Y, Takagi T, *et al.* Pulmonary hypertension is ameliorated in mice deficient in thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost* 2010; **8**:808–816.
28 Leurs J, Nerme V, Sim Y, Hendriks D. Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism. *J Thromb Haemost* 2004; **2**:416–423.