Effect of tiplaxtinin (PAI-039), an orally bioavailable PAI-1 antagonist, in a rat model of thrombosis

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Summary. Objective: To assess the antithrombotic and profibrinolytic effects of tiplaxtinin (PAI-039), an orally bioavailable antagonist of PAI-1, in rat models of thrombosis. Methods and results: Carotid artery and vena cava vascular injury was produced by application of FeCl3 and blood flow was monitored using ultrasonic technology. To assess efficacy in a thrombosis prevention paradigm, PAI-039 was administered orally 90 min before injury (1–30 mg kg−1). To assess efficacy in a thrombosis treatment paradigm, vascular injury and stable thrombus formation were followed 4 h later by recovery and PAI-039 administration. PAI-039 prevented carotid artery occlusion in 20, 68 and 60% of animals pretreated with 0.3, 1.0 and 3.0 mg kg−1, respectively. Time to occlusive thrombosis was increased from 18.2 ± 4.6 min in controls to 32.5 ± 8.7 (P = ns), 46.1 ± 7.0 (P < 0.05), and 41.6 ± 11.3 min (P < 0.05) in the respective PAI-039 treatment groups. In the vena cava protocol, PAI-039 pretreatment significantly reduced thrombus weight at PAI-039 doses of 3, 10 and 30 mg kg−1. When PAI-039 was dosed in a treatment paradigm 4 h after stable arterial and venous thrombosis, a significant reduction in thrombus weight was observed 24 h later at PAI-039 doses of 3, 10 and 30 mg kg−1. PAI-039 (10, 30 and 100 mg kg−1) had no effect on platelet aggregation in response to ADP or collagen and was not associated with increased bleeding or prolonged prothrombin time. In animals bearing no vascular injury, PAI-039 had no effect on circulating, low-levels of PAI-1 activity. In contrast, circulating PAI-1 activity increased 5-fold following the induction of vascular injury, which was completely neutralized by PAI-039. Conclusions: PAI-039 exerts antithrombotic efficacy in rat models of arterial and venous vascular injury without affecting platelet aggregation.

Keywords: fibrinolysis, PAI-1, thrombosis, vascular injury.

Introduction

Physiological inhibition of tissue-type (tPA) and urokinase-type (uPA) plasminogen activators by plasminogen activator inhibitor-1 (PAI-1) regulates the proteolytic processes of fibrinolysis and thrombolysis. At the site of vascular injury, substantial amounts of tPA are released from endothelial storage pools to counteract intravascular coagulation and dissolve nascent clots [1,2]. This local increase in fibrinolytic activity is subsequently neutralized by the local release of PAI-1, presumably from degranulating platelets accumulating in the growing thrombus [3]. Altering the balance between PAI-1 and tPA via pharmacological neutralization of PAI-1 activity represents a promising therapeutic strategy to augment fibrinolysis and promote clot dissolution. The therapeutic rationale for targeting PAI-1 is further supported by clinical observations showing that PAI-1 antigen and activity are elevated in acute disorders such as deep vein thrombosis, myocardial infarction and unstable angina [4,5].

Inhibitory monoclonal antibodies against PAI-1 have exhibited thrombolytic efficacy rat thrombosis models [6,7]. Mice lacking the PAI-1 gene also have heightened fibrinolytic capacity and exhibit endogenous protection against thrombosis [8,9]. The small molecule PAI-1 antagonist, tiplaxtinin (PAI-039), has demonstrated efficacy in a rodent model of acute arterial thrombosis [10] and potent prothrombolytic effects in a dog model of coronary artery electrolytic injury [11]. In the present study, we further characterize the efficacy of PAI-039 in rat models of arterial and venous injury, focusing on both prevention of thrombosis and treatment of existing intravascular thrombi. Additionally, the effect of PAI-039 on neutralization of plasma PAI-1 has been investigated.
Methods

In vitro assays

Animal studies were conducted in accordance with the guidelines of the Wyeth Collegeville Animal Care and Use Committee and conform to the standards in The Guide for Care and Use of Laboratory Animals (NIH no.86–23). For all in vivo studies, PAI-039 was formulated in a solution of 2.0% Tween 80/0.5% methylcellullose (Fluka BioChemika, Neu-Ulm, Switzerland). The suspension was sonicated for 1 min, followed by stirring for 1 h at room temperature.

Rat arterial thrombosis: prevention model

Male Sprague-Dawley rats (250–350 g) were dosed orally with either vehicle or PAI-039 (0.3–3 mg kg⁻¹) on the morning of the experiment, then anesthetized with sodium-pentobarbital (50 mg kg⁻¹, i.p.). A midline incision was made along the neck, and the right and left carotid arteries and jugular veins were exposed. The left jugular vein was catheterized for tPA delivery and blood sampling. The right carotid artery was cannulated with PE-60 tubing filled with 3.8% sodium citrate solution, and interfaced to a saline-filled pressure transducer (Gould Instrument Systems, Inc., Valley View, OH, USA) for monitoring of heart rate and blood pressure. The left carotid artery was used for vascular injury induction and thrombosis. An ultrasonic flow probe (Transonic Systems, Ithaca, NY, USA) was placed on the left carotid artery, and baseline blood flow was recorded. A 3-mm section of PE-60 tubing was sectioned longitudinally, and a piece of filter paper saturated with 25% FeCl₃ was inserted into the tubing. Injury was initiated 90 min after oral administration of vehicle or PAI-039 by placing the tubing onto the surface of the carotid artery proximal to the flow probe for 5 min. After FeCl₃ injury, flow was recorded for an additional 40 min to determine time to occlusion, defined as zero flow. If occlusive thrombosis did not develop during this period, a value of 40 min was used as the time to thrombosis for statistical comparisons between control and PAI-039-treated animals. Recombinant human tPA (Activase®; Genentech, S. San Francisco, CA, USA) was then infused at 30 μg kg⁻¹ min⁻¹ into the jugular vein for a total of 30 min. This dosage had previously been shown to be ineffective at restoring carotid blood flow (data not shown). Flow was monitored in the damaged vessel during tPA infusion and for an additional 20 min afterwards. At the end of the experiment, the arterial thrombus was excised and weighed.

Rat arterial thrombosis: treatment model

Rats were anesthetized and subjected to FeCl₃-induced vascular injury similar to the methods above but in the absence of PAI-039 or vehicle. The carotid artery and jugular vein were not catheterized for blood pressure assessment and tPA administration. Immediately after confirmation of occlusive carotid artery thrombosis, the flow probe was removed and the surgical incision was closed using surgical staples. Rats were permitted to recover from general anesthesia. Approximately 4 h after induction of vascular injury, conscious rats were dosed orally with vehicle or PAI-039 (1–30 mg kg⁻¹). Twenty-four hours later, animals were re-anesthetized with sodium-pentobarbital (50 mg kg⁻¹, i.p.) and the thrombus removed and weighed.

Rat venous thrombosis: prevention model

Rats were dosed orally with vehicle or PAI-039 (0.3–3 mg kg⁻¹) as described for the arterial thrombosis prevention experiments. In order to compare the efficacy of PAI-039 with other approved anticoagulants, low molecular weight heparin (enoxaparin) was administered subcutaneously at a dose of 3 mg kg⁻¹. Approximately 1 h after dosing either orally or subcutaneously, rats were anesthetized with sodium-pentobarbital (50 mg kg⁻¹, i.p.). The abdomen was subsequently opened by a midline incision, and the inferior vena cava exposed. A stenosis was applied to the vena cava by tying a 4.0 silk suture (Ethicon, Somerville, NJ, USA) around both the vessel and a blunt 20-gauge needle between the renal veins. The needle was removed, and 3.5 μL of a 10% FeCl₃ solution was applied to a 3-mm diameter disk of filter paper that was then placed on the external surface of the vena cava for 5 min. The FeCl₃ application was initiated 90 min after dosing. The exposed viscera were covered with saline soaked gauze for 1 h, and then the thrombus was removed and immediately weighed. Tissue plasminogen activator was not administered in the venous thrombosis experiments.

Rat venous thrombosis: treatment model

Rats were anesthetized and subjected to FeCl₃-induced vascular injury to the inferior vena cava similar to the methods above. Immediately after vascular injury, the midline incision was stapled closed and the animals were permitted to recover from general anesthesia. Approximately 4 h after induction of vascular injury, conscious rats were dosed orally with vehicle or PAI-039 (1–30 mg kg⁻¹). Twenty-four hours later, animals were again anesthetized with sodium-pentobarbital (50 mg kg⁻¹, i.p.) and the thrombus removed and weighed.

PAI-1 activity assay and total PAI-1 antigen

Plasma samples from the rat vena cava thrombosis prevention model were used for these assays. Whole blood samples were drawn in 3.7% sodium citrate (1:10 citrate to blood (vol/vol)) and plasma was isolated by centrifugation at 2000 × g for 20 min. Plasma samples were drawn at baseline (pre-injury) and 1 h after injury, which represents 90 min and 150 min post-dosing PAI-039, respectively. PAI-1 activity was assayed using a commercially available ELISA kit from Molecular Innovations (Southfield, MI, USA). This 96-well assay utilizes uPA coated micro-titer plates that bind functionally active PAI-1. Latent or complexed PAI-1 do not bind to the plate and
are not detected. An anti-rat PAI-1 primary antibody is used to detect PAI-1. Samples were prepared and assayed according to the manufacturer's instructions. To measure total PAI-1 in the rat plasma samples, the Immulon rat PAI-1 ELISA assay was used (American Diagnostica Inc., Stamford, CT, USA). This assay is designed to detect latent, active and inactive PAI-1 as well as tPA/PAI-1 complexes. This sandwich assay uses two monoclonal antibodies specific for rat PAI-1 and a detection antibody raised against rat PAI-1 coupled with horseradish peroxidase. Samples were prepared and assayed according to the manufacturer's instructions.

**Bleeding time and platelet aggregation**

To determine baseline platelet activity, 1 mL of blood was drawn into 3.7% sodium citrate (1:10 citrate to blood (vol/vol)) from Sprague–Dawley rats (weighing 250–350 g) with surgically implanted jugular vein catheters. Animals were then dosed orally with PAI-039 (10–100 mg kg$^{-1}$), vehicle or enoxaparin (30 mg kg$^{-1}$, s.c.) followed by induction of general anesthesia with sodium pentobarbital (50 mg kg$^{-1}$, i.p.) 90 min later. Two hours after dosing a second blood sample was taken to determine the effect of PAI-039 on platelet activity. Platelet-rich plasma (PRP) was prepared by collecting the supernatant from the whole blood centrifuged at 140 × g for 10 min. Platelet poor plasma (PPP) was prepared from the same blood sample by centrifugation at 2000 × g for 10 min. Ex vivo platelet aggregation was determined at 37 °C with a four-channel platelet aggregometer (Bio-Data-PAP-4; Bio Data, Hatboro, PA, USA) by recording the increase in light transmission through a stirred suspension of PRP. Aggregation was induced with adenosine diphosphate (ADP, 20 μM) and collagen (2.5 μg mL$^{-1}$). Values are expressed as percentage of aggregation, representing the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

For assessment of bleeding time in the anesthetized rats, the tail was transected 0.5 cm from the tip using a disposable surgical blade 2 h after dosing. The tail was placed vertically in 25-mL isotonic saline at room temperature immediately after being cut, and the bleeding time measured from the moment of transection until bleeding has stopped completely. A blood sample was taken at conclusion of the bleeding time assessment for determination of prothrombin time (PT) using a Stago coagulation instrument (ST4; Diagnostica Stago, Parsippany, NJ, USA).

**Pharmacokinetics**

Rats were dosed with PAI-039 either i.v. (1 mg kg$^{-1}$) or p.o. (10 mg kg$^{-1}$), and blood was sampled at defined time points after dosing. Quantitation of PAI-039 in plasma was determined using an LC/MS/MS method employing negative electrospray ionization. The lower limit of quantitation of the assay was 0.5 ng mL$^{-1}$ using plasma aliquots of 200 μL or less. Pharmacokinetic parameters were calculated by non-compartmental model analysis using WinNonlin Professional Software, version 3.2 (Pharsight Corporation, Cary, NC, USA).

**Statistical analysis**

Carotid artery blood flow over time in PAI-039-treated rats was compared with vehicle-treated controls using a repeated measures one-way ANOVA. The effect of PAI-039 on carotid artery time to occlusion and thrombus weight was compared with vehicle-treated controls using a one-way ANOVA followed by Dunnett’s post-hoc test. The frequency of occlusion in the acute carotid artery experiments was compared using Fisher’s exact test. Reduction in thrombus weight by PAI-039 in the vena cava clot lysis experiments was compared with vehicle-treated controls using a one-way ANOVA followed by Dunnett’s post-hoc test. Changes in PAI-1 antigen and active PAI-1 within individual treatment groups were compared using paired t-tests. Differences between treatment groups were compared using Student’s t-test. The effects of PAI-039, enoxaparin and vehicle on bleeding time and PT clotting time were compared using a one-way ANOVA followed by Dunnett’s post-hoc test. $P < 0.05$ was considered statistically significant for all analysis.

**Results**

**Rat arterial thrombosis: prevention model**

Oral administration of PAI-039 (0.3, 1.0 and 3 mg kg$^{-1}$) was not associated with any change in heart rate or blood pressure (data not shown). Occlusion was prevented in 20%, 68% and 60% of animals treated with 0.3, 1 and 3 mg kg$^{-1}$ PAI-039, respectively. Time to occlusive thrombosis was increased from 18.2 ± 4.6 min in controls to 32.5 ± 8.7 min ($P > 0.05$), 46.1 ± 7.0 min ($P < 0.05$) and 41.6 ± 11.3 min ($P < 0.05$) after PAI-039 doses of 0.3, 1 and 3 mg kg$^{-1}$, respectively (see Fig. 1). The frequency of occlusion in the initial 40-min period post-injury was recorded and compared with vehicle control animals. Significant reduction in occlusive thrombosis was observed at 1 mg kg$^{-1}$ (3/9 occluded, $P < 0.01$) and 3 mg kg$^{-1}$ PAI-039 (2/5 occluded, $P < 0.05$) compared with control (9/9 occluded). At the 40-min time point after injury, a sub-protective tPA infusion was initiated to determine the effect of PAI-039 thrombolysis. In the occluded control and 0.3 mg kg$^{-1}$ PAI-039 animals no restoration of blood flow was observed over the 50-min recording period. In the 1 and 3 mg kg$^{-1}$ groups, vessel patency was maintained throughout the recording period. PAI-039 produced a significant decrease in thrombus weight at doses of 1 and 3 mg kg$^{-1}$ compared with vehicle-treated controls (Fig. 2, panel A). A small reduction in efficacy was observed as the dose was increased from 1 to 3 mg kg$^{-1}$ PAI-039. This rapid achievement of maximal effect may be attributed to the non-linearity in exposure with PAI-039. The apparent decrease in efficacy with the highest dose is likely to be a result of animal-to-animal variability and the small number of animals per group.
The animals were anesthetized and the thrombi removed and weighed. PAI-039 significantly reduced carotid artery thrombus size at the 24-h time point after doses of 3, 10 and 30 mg kg\(^{-1}\) (Fig. 3, panel A). A similar result was observed in the vena cava, where a significant reduction in thrombus weight was measured in animals treated with 3, 10 and 30 mg kg\(^{-1}\) PAI-039 (Fig. 3, panel B).

**Effect of PAI-039 on PAI-1 antigen and active PAI-1**

Total PAI-1 antigen was measured prior to injury and 1 h after injury in animals that were dosed orally with 3 mg kg\(^{-1}\) PAI-039. As shown in Fig. 4A, plasma PAI-1 antigen increased significantly and to the same extent 1 h after vascular injury in both vehicle control \((0.46 ± 0.23 \text{ to } 2.78 ± 1.06 \text{ ng mL}^{-1})\) and PAI-039-treated animals \((0.81 ± 0.27 \text{ to } 3.77 ± 0.76 \text{ ng mL}^{-1})\). A second assay to determine active PAI-1 was performed using the same plasma samples because PAI-1 antigen represents both active and inactive PAI-1. While PAI-1 activity was increased significantly in the vehicle control animals following vascular injury \((0.034 ± 0.008 \text{ to } 0.124 ± 0.020 \text{ ng mL}^{-1})\), it was dramatically and significantly reduced following injury \((0.01 ± 0.008 \text{ ng mL}^{-1})\) in the PAI-039-treated group. These data suggest that the pool of PAI-1 released due to vascular injury was susceptible to inhibition by PAI-039 and may represent the summation of the increased, albeit neutralized, PAI-1 released upon vascular injury. In addition, the reduction in active PAI-1 was associated with functional benefit as determined by vascular flow and thrombus weight.

**Pharmacokinetics**

The plasma concentrations of PAI-039 were measured in a separate group of rats administered PAI-039 at 0.3, 1, 3 and 10 mg kg\(^{-1}\) by oral gavage. PAI-039 concentration was determined 1.5 and 3 h post-dosing (Fig. 5). Plasma concentrations of PAI-039 ranged from near the limit of detection for the assay \((0.5 \text{ ng mL}^{-1}, 11 \text{ nm})\) at the 0.3 mg kg\(^{-1}\) dose to \(3000 \text{ ng mL}^{-1} (6.8 \mu\text{g L}^{-1})\) at the 10 mg kg\(^{-1}\) dose. We thus achieved a wide range of PAI-039 concentrations in our pharmacology studies that flanked the published IC\(_{50}\) of PAI-039, 2.7 \(\mu\text{g L}^{-1}\) [10].

The pharmacokinetics of PAI-039 were further evaluated in male SD rats administered a 1 mg kg\(^{-1}\) i.v. dose or a 10 mg kg\(^{-1}\) p.o. dose of PAI-039. The compound exhibited an apparent half-life of 2.95 ± 1.15 h with a CI \([0.42 ± 0.08 (\text{L h}^{-1})\text{kg}]\) and \(V_d \text{ (0.62 ± 0.05 L kg}^{-1})\). The low \(V_d\) indicates low tissue uptake. PAI-039 was well absorbed following oral administration. Exposure, based upon mean AUC\(_{0-\infty}\) values, was dose proportional. The relative bioavailability of PAI-039 following oral gavage administration (10 mg kg\(^{-1}\)), based upon a 1 mg kg\(^{-1}\) i.v. dose, assuming linear pharmacokinetics, is \(≈93\%\). The high bioavailability is related to the low clearance of the compound \([0.42 ± 0.08 (\text{L h}^{-1})\text{kg}]\) and may be related to saturation of
metabolism or another clearance pathway following administration of a 10 mg kg\(^{-1}\) oral dose.

**Discussion**

The fibrinolytic system is controlled by a critical balance between plasminogen activators and plasminogen activator inhibitors. tPA is synthesized and stored in vascular endothelial cells and released upon stimulation to increase local fibrinolysis and provide thromboprotective capacity. The actions of tPA are inhibited predominantly through local release of PAI-1 from activated platelets in the growing thrombus [12,13]. In the present study, we assessed the antithrombotic and profibrinolytic effects of the PAI-1 antagonist, PAI-039, in rat models of arterial and venous vascular injury. When administered orally to prevent or treat thrombosis, PAI-039 effectively reduced thrombus weight, prevented or delayed time to occlusive thrombosis and promoted sustained vessel patency in the presence of in vivo vascular injury in both veins and arteries. Efficacy was not associated with increased bleeding time or changes in platelet aggregation.

We have shown that PAI-039 has no measurable effect on the pre-injury circulating PAI-1 antigen levels or activity. The lack of effect of PAI-039 on pre-injury systemic PAI-1 activity might be explained by the inability of PAI-039 to inhibit PAI-1 when bound to vitronectin [14]. These data have been further supported by recent investigations on the mechanism of inactivation of PAI-1 by PAI-039 [15]. Using site-directed mutagenesis, enzymology and BIACore binding assays it was determined that PAI-039 bound near the vitronectin binding site on PAI-1. Thus, because vitronectin circulates at high concentrations and has been shown to bind active circulating PAI-1 [16], this pool of PAI-1 is likely to be resistant to inhibition by PAI-039. Conversely, un-complexed active PAI-1 is rapidly and specifically inhibited by PAI-039 [15]. Our data suggest that the pool of PAI-1 released during vascular injury is not vitronectin bound (at least initially), and is therefore susceptible to inhibition by PAI-039 based on the significantly reduced PAI-1 activity levels in this treatment group. Presumably, the source of PAI-1 originates from activated platelets during vascular injury. However, even \(\alpha\)-granule stored PAI-1 in platelets is associated with vitronectin [17,18], rendering it resistant to inhibition by PAI-039.

![Fig. 2. Effect of PAI-039 on arterial and venous thrombus weight and hemostasis after FeCl\(_3\)-induced vascular injury. Panel A shows thrombus weight 90 min after vascular injury to the carotid artery. Panel B shows thrombus weight 60 min after vascular injury in the abdominal vena cava. Bleeding time and prothrombin time are shown in panels C and D, respectively. Values are shown as mean ± SEM for \(n = 5–9\) rats. Asterisk indicates a significant difference from control after treatment with PAI-039 or enoxaparin (one-way ANOVA followed by Dunnet’s post-hoc test, \(P < 0.05\)).](image-url)
PAI-039 effectively reduced thrombus weight after FeCl₃-induced vascular injury in the carotid artery and abdominal vena cava. This model of vascular injury initiates circumferential damage to the vessel and produces significant endothelial damage via production of reactive oxygen species [19]. Similar antithrombotic and profibrinolytic observations were made after FeCl₃-induced injury of the carotid artery in PAI-1 knockout mice that exhibit accelerated clot lysis times, prolonged time to arterial occlusion and increased thrombolysis after tPA administration [8,20]. The first studies to demonstrate that PAI-1 inhibition was a favorable therapeutic strategy utilized monoclonal antibodies [6,21]. Regardless of the mechanism of antibody-mediated PAI-1 inhibition, monoclonal antibodies against PAI-1 enhanced endogenous thrombolysis in rat, rabbit and canine models of thrombosis. The first small molecule antagonists of PAI-1 lacked oral bioavailability but were effective after i.v. infusion in rat thrombosis models [22]. PAI-039 is the first orally active small molecule PAI-1 antagonist to be tested in rat models of thrombosis. Furthermore, this report describes the first evaluation of

**Fig. 3.** Effect of PAI-039 or vehicle on thrombus weight 24 h after FeCl₃-induced vascular injury to the rat carotid artery (A) or vena cava (B). PAI-039 or vehicle was administered orally after vascular injury and surgical recovery in a treatment paradigm. Values are shown as mean ± SEM for n = 4–8 rats. Asterisk indicates a significant reduction in thrombus weight compared with vehicle-treated controls (one-way ANOVA followed by Dunnet’s post-hoc test, P < 0.05).

**Fig. 4.** Effect of PAI-039 (3 mg kg⁻¹ p.o.) or vehicle on total PAI-1 antigen and PAI-1 activity before and after vena cava vascular injury. Pre-injury plasma samples were drawn from implanted jugular vein catheters and post-injury samples were taken from vena cava venipuncture at the time of euthanasia. Values are shown as mean ± SEM for vehicle (n = 5) and PAI-039 (n = 6) treated animals. *Significant difference in total PAI-1 or PAI-1 activity before and after vascular injury (paired t-test, P < 0.05). Significant difference in PAI-1 activity between vehicle and PAI-039 treated animals after vascular injury (Student’s t-test, P < 0.05).

**Fig. 5.** Plasma concentrations of PAI-039 at 1.5 and 3 h after oral gavage of 0.3, 1.0, 3.0 and 10 mg kg⁻¹. PAI-039 was formulated in 2% Tween and 0.5% methylcellulose. Plasma concentrations were assayed using a LC/MS/MS method employing negative electrospray ionization (limit of detection 0.5 ng mL⁻¹).
PAI-1 inhibitors in a model with existing intravascular thrombi where a drug is tested to assess its possible utility for treatment of thrombosis.

An orally active PAI-1 antagonist holds great therapeutic promise for a variety of clinical indications. Elevated PAI-1 and reduced fibrinolysis are linked to acute conditions such as peripheral arterial disease, deep vein thrombosis and acute myocardial infarction. PAI-1 is also elevated in more chronic diseases such as diabetes and obesity, and PAI-1 antagonism may positively impact the cardiovascular pathologies of these diseases.

**Disclosure of Conflict of Interests**

The authors state that they have no conflict of interest.

**References**