Title page

Enhancement of fibrinolysis by EF6265, a specific inhibitor of plasma carboxypeptidase B

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Running title page

a) Running title: EF6265, a specific inhibitor of plasma CPB

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c)

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d) Abbreviations: CPB, carboxypeptidase B; CPA, carboxypeptidase A; CPN carboxypeptidase N; CPI, potato derived carboxypeptidase inhibitor; MGPA, DL-mercaptmethyl-3-guanidinoethylthiopropanoic acid; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin; C-terminal, carboxy-terminal

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Abstract

Plasma procarboxypeptidase B, also known as thrombin-activatable fibrinolysis inhibitor (TAFI), is converted by thrombin into the active enzyme, carboxypeptidase B (CPB) / activated TAFI (TAFIa). Plasma CPB down-regulates fibrinolysis by removing carboxy-terminal lysines, the ligands for plasminogen and tissue-type plasminogen activator (tPA), from partially degraded fibrin. To target thrombosis in a new way, we have identified and optimized a phosphinic acid-containing inhibitor of CPB, EF6265, and determined both the pharmacological profile and pathophysiological role of CPB in rat thrombolysis. EF6265 specifically inhibited plasma CPB activity with an IC_{50} (50% inhibitory concentration) of 8.3 nmol/L and enhanced tPA-mediated clot lysis in a concentration-dependent manner. EF6265 decreased detectable thrombi (%GFD, control: 98+/-1.1, EF6265: 0.1mg/kg, 27+/-9.1) that had been generated by tissue factor in a rat microthrombosis model with concomitant increases in plasma D-dimer concentration (control: <0.5 μg/mL, EF6265: 0.1mg/kg, 15+/-3.5 μg/mL). EF6265 reduced plasma α2-antiplasmin activity to a lesser extent than tPA. In an arteriovenous shunt model, EF6265 (1 mg/kg) enhanced exogenous tPA-mediated thrombolysis under the same conditions that neither EF6265 nor tPA (600 kIU/kg) alone reduced thrombi. EF6265 (1, 30 mg/kg) did not affect the bleeding time in rats. Moreover, it did not prolong the bleeding time evoked by tPA (600 kIU/kg). These results confirm that circulating proCPB functions as a fibrinolysis inhibitor's zymogen and validates the use of CPB inhibitors as both an enhancer of physiological fibrinolysis in microcirculation and as a novel adjunctive agent to tPA

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for thromboembolic diseases while maintaining a small effect on primary hemostasis.

Thrombosis-related diseases including myocardial infarction, cerebral infarction and disseminated intravascular coagulation are life-threatening and the search for treatments remains challenging. Although thrombolytics including tissue- or urokinase-type plasminogen activator (tPA or uPA) and anticoagulants including heparin have been developed over the last few decades, the risk of hemorrhaging with these antithrombotic agents restricts their clinical use. The dose of tPA must be high enough to overcome the inhibitory effects of plasminogen activator inhibitor-1 (PAI-1) in the plasma, and results in the generation of plasmin in circulating blood (Rijken and Sakharov, 2001). Consequently, these large quantities of generated plasmin can induce thrombolysis and result in hemorrhaging as a side effect (Bloom et al., 1988). Many efforts have therefore been made to identify and develop pharmacologically distinct antithrombotic agents while maintaining a low risk of hemorrhaging.

Plasma procarboxypeptidase B (proCPB; EC 3.4.17.20), also known as TAFI (thrombin-activatable fibrinolysis inhibitor) or procarboxypeptidase U, is produced in the liver and circulates in the blood as a zymogen (Bajzar et al. 1995; Bouma and Meijers, 2003). ProCPB is proteolytically transformed into the active enzyme, carboxypeptidase B (CPB), by thrombin produced during blood coagulation[6]. Thrombin-mediated activation of plasma proCPB is greatly enhanced in the presence of the cofactor protein, thrombomodulin (TM), which is distributed on the plasma membrane of vascular endothelial cells and in circulating plasma in several degraded forms (Ishii, 1994). It has therefore been recognized that activation of proCPB is mainly via thrombin-TM complexes on the surface of endothelial cells or in the

plasma (Bouma and Meijers, 2003).

Generated plasma CPB down-regulates fibrinolysis by removing carboxy-terminal (C-terminal) lysines from fibrin fibers during blood coagulation (Bajzar et al. 1995; Sakharov et al., 1997). These C-terminal lysine residues, initially exposed through the degradation of fibrin by trace amounts of plasmin in the plasma, bind to the lysine-binding sites of plasminogen and tPA (Bajzar et al. 1995; Fleury and Angles-Cano, 1991). Thus, the binding of tPA and plasminogen is facilitated by the C-terminal lysine residues on the surface of fibrin clots and tPA efficiently activates plasminogen bound to the clot surface without any interference of PAI-1 in plasma (Bajzar, 2000) . Plasmin, generated on the fibrin clots, can also escape from the plasmin inhibitor, plasma α2-antiplasmin, resulting in efficient fibrinolysis. The C-terminal lysine residues are therefore very important in physiological fibrinolysis. Additionally, the proCPB level in human plasma correlates with the time for clot lysis in healthy individuals (Mosnier et al., 1998) and with an increased risk of deep vein thrombosis (van Tilburg et al., 2000), coronary artery disease (Schroeder et al., 2002; Zorio et al., 2003) and ischemic stroke (Montaner et al., 2003). Based on a novel pharmacological mechanism, the specific inhibitors of the plasma CPB are therefore expected to enhance fibrinolysis by generating more plasmin on the surface of fibrin clots while maintaining a low risk of hemorrhaging.

Recently, *in vivo* studies have suggested the involvement of plasma CPB in thrombolysis (Bouma and

Meijers, 2003): the induction of plasma CPB activity correlated with the suppression of fibrinolysis in canine coronary thrombosis (Redlitz et al., 1995); CPI (a potato-derived carboxypeptidase inhibitor of carboxypeptidase A, CPA, and CPB), incorporated into a clot created in an isolated segment of the jugular vein, potentiated endogenous thrombolysis (Minnema et al., 1998); administration of CPI or DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGPA, an inhibitor of CPB and carboxypeptidase N, CPN) reduced tissue factor-induced microthrombosis in rats (Muto at al., 2003). Additionally, exogenous tPA-induced thrombolysis was potentiated by combined administration of CPI in jugular vein (Nagashima et al., 2000) and abdominal aorta (Klement et al., 1999) thrombosis models in rabbits. A problem in these studies is that CPI and MGPA are neither specific nor potent enough (Nagashima et al., 2000; Muto at al., 2003) for plasma CPB, and as a result physiological analysis of these inhibitors is not conclusive. In spite of these studies, proCPB (TAFI)-deficient mice unexpectedly showed no overt phenotype (Nagashima et al., 2002). The pathophysiological importance of proCPB in thrombosis/thrombolysis is yet to be elucidated (Bouma and Meijers, 2003). Hence, we searched for a specific CPB inhibitor to determine the importance and therapeutic potential of plasma CPB in thrombotic conditions.

Methods

Reagents and biochemical assays

Human plasma proCPB/TAFI was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Activation of proCPB/TAFI (0.36 μg/mL in 50 mmol/mL Tris-HCl, pH 7.4) to CPB/TAFIa was triggered by addition of 20 μL of thrombomodulin (American Diagnostica, Greenwich, CT) solution (300 ng/mL in 50mmol/mL Tris-HCl, pH 7.4 containing 0.1% Lubrol, 0.1% bovine serum albumin and 0.15 mol/L NaCl) and 20 μL of thrombin solution (3 units/mL in 50mmol/mL Tris-HCl, pH 7.4 containing 0.1% bovine serum albumin and 0.15 mol/L NaCl). After incubation for 30 minutes at 25°C, the generated carboxypeptidase activity was determined. The reaction mixture (10 μL) was incubated with 1.25 mmol/mL of Hippuryl-arginine (Sigma) in 0.1 mol/L Tris-HCl buffer, pH 7.6 for 60 minutes at 25°C (the final volume was 80 μL). The reaction was stopped by adding 100 μL of 0.2 mol/L Pipes-NaOH buffer, pH 7.6containing 12.5% Tween20. Color was developed by adding 100 μL of 1% cyanuric acid in 2-mercaptoethanol, followed by measurement of absorbance at 405 nm (Hosaka et al., 1998). Carboxypeptidase N (CPN) was partially purified from human plasma (Bajzar et al., 1995; Skidgel et al., 1988). Enzyme activity of CPN was measured in the rate of hydrosis of Hippuryl-Lysine (Sigma) in 0.1 mol/L Tris-HCl buffer, pH 7.6 (Hosaka et al., 1998). CPB- and CPN-like activities in rat plasma were evaluated according to a previously reported method (Schatteman et al., 1999). Bovine pancreas carboxypeptidase A (CPA) (Tanaka et al., 1984) was from Boehringer Mannheim GmbH (Mannheim,

Germany), rabbit lung angiotensin converting enzyme (ACE) (Cushman and Cheung, 1971) was from Wako (Tokyo, Japan). Human plasma plasmin (Kato et al., 1980), human plasma α-thrombin (Kawabata et al., 1985) and bovine pancreas trypsin (Kawabata et al., 1988) were from Sigma (St. Louis, MO, USA). Human Factor Xa (Morita et al., 1977) was from Haematologic Technologies (Essex Junction, VT, USA). Human Factor XIa (Kawabata et al., 1988) was purchased from Enzyme Research Laboratories. TPA (alteplase) (Kyowa Hakko, Tokyo, Japan), tissue factor (Innovine) (Dade International, Miami, FL, USA), MGPA (Calbiochem-Novabiochem, La Jolla, CA, USA) and were used.

Synthesis of EF 6 2 6 5

EF6265 was synthesized in our laboratory by consideration of the previously reported methods (Baylis et al., 1984; Miller et al., 1998; Vassiliou et al., 1999). Detailed procedures and spectroscopic data will be reported on another occasion.

Animals

Male Wistar rats weighing 200 – 300 g (Charles River Japan, Yokohama, Japan) were used. The experiments were approved by the institutional review board of the Pharmaceutical Research Laboratories of our company and were performed in accordance with the guidelines for animal experiments of the National Institute of Health.

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***In vitro* clot lysis assay in human and rat plasma**

Blood from healthy donors was collected into one-tenth volume of 3.8% sodium citrate. Platelet poor plasma was obtained by centrifugation and stored at -80°C. Blood from rats was collected from the abdominal aorta of three rats anesthetized by pentobarbital (50 mg/kg, intraperitoneal injection) into a syringe partially filled (1:10 total volume) with 3.8% sodium citrate. Rat plasma was quickly obtained by centrifugation and stored at 4°C. Experimental condition of clot lysis assay in human and rat plasma should be differently set because of the species difference in the reaction of clot formation/degradation (Schatteman et al., 1999). The clot lysis assays using human plasma (Hosaka et al., 1998) and rat plasma (Muto et al., 2003) were performed in the presence of tPA at 30 IU/ml and 60 IU/ml, respectively. A fibrin clot was formed by the addition of $CaCl_2$ (final concentration 10 mmol/L in human plasma and 20 mmol/L in rat plasma) to reach the final volume of the solution 250 μL (human and rat plasma dilutions were 3.75 and 5 times, respectively) in a microtiter plate well, and turbidity was monitored at 600 nm at 37°C for 180 minutes using a microplate reader (THERMO max; Molecular Devices Corp., Sunnyvale, CA, USA). Effect of EF6265 on the clot lysis assay was evaluated by adding EF6265 at final concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/L into the assay mixture. In the human plasma assay, the time for clot lysis (clot lysis time) was determined by measuring the time required for absorbance to reach the value halfway between baseline and plateau (Hosaka et al., 1998). Clot lysis time in rat plasma assay could not be measured since the turbidity did not return to the baseline level in the turbidity-time profiles in the clot lysis assay using rat plasma under the condition described above without EF6265. So the total clot

remaining in the assay was evaluated by integrating the area under the curve (AUC) above the baseline from the turbuduty-time profile observed over 180 minutes, and expressed in "total clot" (Muto et al., 2003).

***Ex vivo* clot lysis assay in rats**

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). EF6265 (at doses of 0.1 or 1 mg/kg) or saline (control) was intravenously administered (n=5 for each group). Blood samples were collected from the jugular vein into a syringe partially filled (1:10 total volume) with 3.8% sodium citrate. The samples were obtained at 0 (just before EF6265 administration), 5, 15, 30 and 60 minutes after the intravenous administration of EF6265 or saline. Plasma was quickly obtained by centrifugation and stored at 4°C until clot lysis assay which was performed as described above.

Plasma concentration of EF6265 after intravenous injection

The blood concentration of EF6265 was measured after intravenous administration to male Wister rats (n=5). EF6265 was separated by a Symmetry Shield RP18 column (Waters, Milford, MA, USA) using 0.1% formic acid-acetonitrile (75:25, v/v) as a mobile phase and quantified using a TSQ7000 tandem mass spectrometry system (Finnigan, San Jose, CA, USA). Pharmacokinetic parameters were calculated using a non-compartment model by processing with WinNonlin professional version 3.1 (Pharsight Corporation, Mountain View, CA). The elimination constant (kel) was calculated from the terminal three points. The

area under the plasma concentration curve (AUC) and the area under the moment curve (AUMC) were calculated using the linear trapezoidal rule. The area under the curve from hour 0 to infinity (AUC0-inf), the area under the moment curve from hour 0 to infinity (AUMC0-inf), the total clearance (CLtot) and the volume of distribution at steady-state (Vdss) were calculated as shown below.

AUC0-inf = AUC0-t + Ct / kel

AUMC0-inf = AUMC0-t +t* Ct / kel+Ct/kel^2

CLtot = Dose / AUC0-inf

Vdss= CLtot*AUMC0-inf/AUC0-inf,

where t is the time of the last blood sample and Ct is the corresponding plasma concentration.

Microthrombosis model induced by tissue factor

Male Wister rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Microthrombi were induced by the continuous infusion of 0.44 µg/kg tissue factor (volume 5 mL/kg) via the femoral vein of rats over a period of 20 minutes (Takahashi et al., 1997). EF6265 (at doses of 0.01, 0.1 and 1 mg/kg) or tPA (at doses of 12, 60 and 120 kIU/kg) was intravenously administered (bolus injection) five minutes after the infusion of tissue factor ended (25 minutes), and the rats were sacrificed at 45 minutes after the beginning of the tissue-factor infusion (n=4-7). The kidneys were excised and fixed in 10% neutral buffered formalin. Sections of the kidney were histologically examined after phosphotungstic acid-hematoxylin staining for fibrin thrombi. The percentage of glomerular fibrin deposition (%GFD), a

marker for thrombi, was determined as follows: 100 glomeruli were examined, and the number of glomeruli with clear fibrin deposits was expressed as a percentage (Muto et al., 2003). The plasma D-dimer concentration was determined in the latex agglutination test (Aoshima et al., 1998) using the assay kit of LPIA ACE DD dimmer (Dia-Iatron, Tokyo, Japan). The D-dimer concentration which was determined to be less than the detection limit (0.5 μg/mL) was assumed to be 0.5 μg/mL for the purpose of statistical analysis. Plasma α2-antiplasmin activity was determined using the Enzyme test of APL2 kit (Matsuda et al., 1984) (Daiichi Chemical, Tokyo, Japan).

Thrombus formation/degradation was also evaluated from the radioactivity levels of the blood and organs (Hasegawa et al., 1996) following the injection of ^{125}I-fibrinogen (Amersham Bioscience, Tokyo Japan) at a dose of approximately 37 kBq/kg five minutes before the infusion of tissue factor started. Radioactivity was measured by a gamma counter (Aloka, Tokyo, Japan). The microthrombi index (MI) was defined as a ratio of the radioactivity in the organ to the radioactivity in the reference blood that was obtained at 0 time (just before the start of tissue factor infusion) and determined in each rat (Hasegawa et al., 1996).

Arteriovenous shunt thrombosis model

An arteriovenous shunt model in rats (n=10 for each group) was prepared as reported previously (Hara et al., 1995) with slight modifications. The polyethylene catheter that held a copper wire inside was placed to connect the right carotid artery and the left jugular vein of each rat under pentobarbital anesthesia (50

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mg/kg i.p.). The copper wire was removed 20 minutes after the blood began to circulate through the catheter, and the thrombus weight was measured (Hara et al., 1995). EF6265 at a dose of 1 mg/kg or saline was intravenously administered (bolus injection) five minutes before the circulation of blood. The dose of EF6265 was determined as the dose that showed the maximum enhancement of fibrinolysis in the *ex vivo* clot lysis assay. TPA at a dose of 600 kIU/kg or saline was administered (bolus injection) 10 minutes after circulation of the blood in the shunt via the artery just in front of the shunt region. The dose of tPA (600 kIU/kg) was determined because tPA at that dose didn't show any reduction of the weight of thrombi in preliminary experiments to evaluate the dose-response effects of tPA.

Bleeding time

The tails of rats anesthetized with pentobarbital (50 mg/kg, i.p.) were incised with disposable surgical blades (Hara et al., 1995). Bleeding of the incised tails was checked by blotting the blood with a filter paper every 30 seconds for 30 minutes (n=5). We measured the control value of bleeding time of each rat before the administration of the drug (pre value). Thereafter, we measured the bleeding time one minute after the starting of the bolus administration of the test sample (post value). The ratio of the post value to the pre value was calculated as a fold-increase in bleeding time. The test compounds were dissolved in saline and intravenously administered into the femoral vein. The bleeding time longer than 30 minutes was assumed to be 30 minutes for the purpose of statistical analysis. In case of evaluation for the effects of combined administration of tPA and EF6265, EF6265 was administered immediately after the tPA

administration and a total injection volume for one rat was identical to 2 mL/kg in all groups.

Results

Inhibitory effects of EF6265 on human plasma CPB and its selectivity

We have selected EF6265 as an optimized compound (Fig. 1). EF6265 inhibited more selectively plasma CPB activity at nanomolar concentrations than existing carboxypeptidase inhibitors, CPI and MGPA (Table 1). The IC_{50} (50% inhibitory concentration) values of EF6265 for CPB and CPN were calculated to be 8.26 nmol/L and 5930 nmol/L, respectively (Table 1). The inhibitory effects of MGPA on plasma CPN (IC_{50} was 73.3 nmol/L) and of CPI on CPA (IC_{50} was 535 nmol/L) were much stronger than that of EF6265 (IC_{50} were 5930 and 8240 nmol/L, respectively) (Table 1). The IC_{50} values of EF6265 for plasmin, thrombin, factor Xa, factor XIa, ACE and trypsin were over 500 μmol/L. Similar to human CPB, EF6265 inhibited rat plasma CPB and was more potent than CPI and MGPA (Table 1). We concluded that EF6265 is a potent and highly selective inhibitor of plasma CPB.

Effect of EF6265 in clot lysis in human and rat plasma

Clot lysis assays were performed to examine the effect of EF6265 on clot formation and subsequent clot lysis. A decline in turbidity after clot formation reflects clot lysis, or the fibrinolytic process. EF6265 enhanced tPA-mediated clot lysis in human (Figs. 2A and B) and rat plasma (Figs. 2C and D) in a concentration-dependent manner (Figs. 2B and D). EF6265 maximally enhanced clot lysis at a concentration of about 1 μmol/L in human and rat plasma..

Enhanced fibrinolysis with the systemic administration of EF6265 into rats was confirmed by an *ex vivo* assay. tPA-mediated clot lysis was enhanced by EF6265 at a dose of 0.1 mg/kg at five minutes (Fig. 3A) and 30 minutes respectively (Fig. 3B) after intravenous administration, but this enhancement in plasma was reduced in a time-dependent manner (Fig. 3C). In contrast, this enhancement in plasma was maintained until 60 minutes after intravenous administration of EF6265 at a dose of 1 mg/kg (Fig.3). These results indicate that systemically administered EF6265 enhanced fibrinolysis in a dose- and time-dependent manner.

Pharmacokinetic profiles of EF6265 after intravenous administration

The plasma concentration of EF6265 decreased with an elimination half-life of 0.96+/-0.10 and 0.97+/-0.06 hour at 0.1 and 1 mg/kg, respectively (Fig. 4). The total body clearance (CLtot) was 390 mL/hr/kg at 0.1 mg/kg and 316 mL/hr/kg at 1 mg/kg. The volume of distribution at steady-state (Vdss) was 316+/-32 mL/kg at 0.1 mg/kg and 223+/-25 mL/kg at 1 mg/kg. The fibrinolysis enhancement by EF6265 in *ex vivo* clot lysis in rat plasma was explained by the plasma concentration of EF6265. The plasma concentrations at five minutes post-dose of 0.1mg/kg and 1 mg/kg were 391 ng/mL (0.92 μmol/L) and 6120 ng/mL (14.4 μmol/L), respectively, and those at 60 minutes post-dose of 0.1 and 1 mg/kg were 54.3 ng/mL (0.13 μmol/L) and 589 ng/mL (1.38 μmol/L), respectively. Considering that the greatest enhancement of fibrinolysis occurred with a concentration of 1 μmol/L in the *in vitro* clot lysis assay (Figs.

2C and D), it is likely that at a dose of 0.1 mg/kg the EF6265-mediated enhancement was attenuated in a time-dependent manner. In agreement, the greatest enhancement of clot lysis was identified 60 minutes after the intravenous administration of 1 mg/kg EF6265. Thus, the enhanced *ex vivo* clot lysis can be explained by the plasma concentrations of EF6265.

Activity of EF6265 in a microthrombosis model induced by a tissue factor

The antithrombotic effect of EF6265 was assessed in a thrombosis model induced by tissue factor (Fig. 5), This model is characterized by microthrombi occluding both the capillaries and small vessels of organs. Histological evaluation showed that the infusion of tissue factor formed distinct thrombi in the rat kidney, and the %GFD in the tissue-factor infused control group was 98 ± 1.1%, whereas in saline infused control group %GFD was 0 ± 0.0% (Fig. 5A). At doses ranging from 0.01 to 1 mg/kg, EF6265 attenuated the increase in %GFD in a dose-dependent manner (Fig. 5A) in conjunction with a simultaneous dose-dependent increase in plasma D-dimer levels (Fig. 5B), a marker of fibrin degradation, when the compound was intravenously administered at 25 minutes (five minutes after the end of tissue factor infusion). A similar change in these parameters was observed in rats injected with tPA at doses of 60 kIU/kg or higher (Figs. 5A and B). These results indicate that EF6265, as well as tPA, ameliorated microthrombosis through the enhancement of fibrinolysis. The effects of EF6265 and tPA in this model were also confirmed by experiments using ^{125}I-labeled fibrinogen. The level of radioactivity was markedly increased in the kidney (Fig. 5C hatched bar) and lung (Fig. 5D hatched bar). Accordingly, the levels of

radioactivity in the blood (Fig. 5E open circle) decreased to about 35% at 25 minutes and were retained for a further 20 minutes (Fig. 5E). These results indicate that the labeled fibrinogen in circulating plasma was transformed into fibrin and deposited into the vessels of various tissues such as the kidney and lung. The administration of EF6265 or tPA, at 25 minutes, significantly inhibited this increase in radioactivity in these organs in a dose-dependent manner (Figs. 5 C and D). The injection of EF6265 slowly re-elevated plasma radioactivity levels compared with tPA (Fig. 5E). Additionally, treatment with EF6265 in the thrombosis model did not markedly reduce the plasma levels of free α2-antiplasmin, while 60 and 120 IU/kg tPA reduced the free levels to 70% and 40%, respectively, at 35 minutes (Fig. 5F). These results suggest that EF6265 reduced thrombi in this model and enhanced fibrinolysis, while EF6265 produced much less plasmin in circulating plasma than tPA.

Activity of EF6265 in an arteriovenous shunt thrombosis model

While the intraarterial administration of 600 kIU/kg tPA, 10 minutes after the recirculation of the blood, didn't reduce the weight of the thrombus, administration of tPA combined with pretreatment of 1 mg/kg EF6265 significantly reduced thrombus weight to less than 50% of that in the saline control (Fig. 6). This indicates that pretreatment with EF6265 enhanced exogenous tPA-mediated thrombolysis of arterial thrombi formed in the larger vessels.

Effects of EF6265 on a bleeding time

The effect of EF6265 on primary hemostasis was evaluated by determining the bleeding time in rats. TPA produced a dose-dependent increase in the fold-increase in bleeding time, and 600 kIU/kg tPA increased it about six-fold (Fig. 7A). EF6265 did not prolong the bleeding time at doses of 1 mg/kg and 30 mg/kg (Fig. 7A). The group which received the combined treatment of tPA and EF6265, at the same doses used in the arteriovenous shunt model (Fig. 6), did not have a significantly prolonged fold-increase in bleeding time compared with the group administered with tPA alone (Fig. 7B). These results show that there is a small effect of EF6265 on primary hemostasis in both condition of the single use and combined use with tPA.

Discussion

We have developed a novel agent that target plasma CPB. EF6265, a phosphinic acid-containing molecule (Fig. 1), was shown to be a potent and specific inhibitor of human and rat plasma CPB (Table 1).

EF6265 enhanced *in vitro* tPA-mediated clot lysis. Clot lysis assays of human and rat plasma showed that this enhanced lysis occurred at concentrations greater than 10^{-8} mol/L EF6265 and that the greatest effects occurred at 10^{-6} mol/L. These concentrations were almost the same for the inhibition of plasma CPB (Figs. 2B and D). The *ex vivo* clot lysis assay showed that the intravenous administration of EF6265 into rats enhanced tPA-dependent fibrinolysis in a dose- and time-dependent manner (Fig. 3). The pharmacokinetic study after intravenous administration of EF6265 (Fig. 4) revealed that the enhanced fibrinolysis (Fig. 3C) could be explained by the blood concentrations of EF6265. Hence, we concluded that to use EF6265 in further pharmacological studies, it was rational to determine both the antithrombotic potency of the CPB inhibitor and the role of plasma CPB in thrombus formation and/or degradation.

EF6265 alone showed clear antithrombotic effects in the tissue factor-induced microthrombosis model (Figs. 5A-E). The injection of EF6265 as well as tPA attenuated the increases in %GFD (Fig. 5A) and deposition of radio-labeled fibrinogen (fibrin) in the tissues of rats with microthrombosis (Figs. 5C and D). This inhibition was accompanied by increased levels of D-dimer (Fig. 5B) and plasma radioactivity (Fig. 5E), indicating degradation of fibrin. However, the increases in plasma radioactivity after the

administration of EF6265 were slower than after the administration of tPA. These rates of increase in radioactivity could reflect the rate of fibrinolysis. This result suggests that the slow rate of fibrinolysis by EF6265, compared to tPA which is a direct plasminogen activator (Rijken and Sakharov, 2001; Bouma and Meijers, 2003), could be explained by the enhancement of endogenous fibrinolysis (Speiser et al., 1988), since EF6265 protects the C-terminal lysine residues, which are the binding sites for endogenous tPA and plasminogen, from being cleaved by plasma CPB. Thrombomodulin plays a pivotal role in thrombin-mediated activation of plasma proCPB and thrombin-mediated activation of protein C as a natural anticoagulant (Esmon, 1989). Protein C activation is stimulated by low concentrations of thrombin and higher concentrations of thrombomodulin, whereas proCPB activation is stimulated by high concentrations of thrombin and low concentrations of thrombomodulin (5 nmol/L) and reduced at higher thrombomodulin concentrations (10 nmol/L) (Bouma and Meijers, 2003). Thus, thrombomodulin seems to play a dual role; it dampens the generation of thrombin by enhancing the activation of protein C, and it down-regulates fibrinolysis via the activation of proCPB. Although activation of proCPB and protein C can occur simultaneously, the concentration of thrombomodulin was found to be the factor determining the overall effect. In this context, vessel size might be an important factor, as the effective concentration thrombomodulin increases as blood moves from the aorta to the capillaries (Bouma and Meijers, 2003; Esmon, 1989). Therefore, the effective antithrombotic action of EF6265 in the microthrombosis model might be characterized by the direct enhancement of fibrinolysis through the inhibition of plasma CPB, and the anticoagulant effects of activated protein C generated by high concentrations of endothelial

thrombomodulin in the microvessels and capillaries.

EF6265 enhanced fibrinolysis without significantly reducing plasma α2-antiplasmin levels in this model, even though effective doses of tPA did reduce it (Fig. 5F). These results indicate that CPB inhibitors enhance fibrinolysis on the surface of clots (Bouma and Meijers, 2003), while effective doses of tPA directly activates plasminogen in circulating blood (Weitz et al., 1993). These results might be related to the low level of bleeding seen with EF6265 *in vivo* (Fig. 7). Correspondingly, Nagashima *et al*. reported that a deficiency in TAFI did not lead to increased bleeding (Nagashima et al., 2002). These results strongly suggest that the effects of CPB inhibitors on primary hemostasis are small.

Using the arteriovenous shunt model, we next evaluated the antithrombotic effects of EF6265, as well as combined with tPA, on the larger thrombi formed on a wire in tube (not in blood vessels). EF6265 enhanced lysis induced by exogenously administrated tPA, while EF6265 alone did not reduce the thrombus in the arteriovenous thrombosis model (Fig. 6). This effect of the CPB inhibitor with exogenous tPA has been previously reported in venous (Nagashima et al., 2000) and arterial (Klement et al., 1999) thrombosis rabbit models. With the exogenous administration of tPA, plasmin generated in the plasma is important for activation of proCPB as well as the degradation of fibrin (Bouma and Meijers, 2003). This occurs because plasmin can activate plasma proCPB with a low Michaelis constant (Km 55 nmol/L) without thrombomodulin and exposes the C-terminal lysine residues on fibrin by partial degradation of

fibrin clots (Bouma and Meijers, 2003). Therefore, potentiation of EF6265 on tPA-mediated thrombolysis can be explained: plasmin produced by administration of tPA partially degrades fibrin clots and exposes the new C-terminal lysine residues on the clot; the plasmin also activates proCPB, although active CPB is inhibited by EF6265 and the newly exposed C-terminal lysine is protected from CPB; therefore, the binding of both tPA and circulating plasminogen to the newly exposed C-terminal lysine is recruited to the tPA-dependent efficient activation of plasminogen (Bajzar, 2000). In contrast, the lack of antithrombotic effects with a single administration of EF6265 might occur because endogenous tPA was insufficient to dissolve larger thrombi even when plasma CPB was inhibited. When the doses of both compounds were set to levels, such that when combined, both compounds showed antithrombotic effects in the arteriovenous model (Fig. 6), EF6265 did not further lengthen the bleeding time prolonged by tPA (Fig. 7B). Thus, EF6265 may also be expected to be an enhancer of tPA treatment for various thrombosis including myocardial and cerebral infarctions while maintaining a low risk of hemorrhaging.

As described above, EF6265 showed different antithrombotic effects on the two models that were performed in this study. The difference could be explained by the size of thrombi and involvement of the blood vessels around the thrombi. EF6265 was clearly effective on the microthrombosis model in the treatment setting. In order to further clarify the potential utility of EF6265 in single use for microthrombotic diseases, a model of organ injury induced by microthrombi would be particular interest (such as endotoxemia and thrombotic microangiopathy). On the other hand, pre-treatment of EF6265

enhanced the fibrinolysis induced by exogenous tPA in the shunt model. To clarify the potential utility of EF6265 for clinical use in arterial thrombotic diseases with or without tPA, a model of clot formation/degradation on arterial side would be of particular interest (such as $FeCl_3$ injury on an carotid model), since it is in such condition that endogenous tPA is delivered. These further studies including are needed to reveal possibilities and limitations of plasma CPB inhibitors in clinical usage.

In conclusion, our results show that plasma CPB regulates pathophysiological fibrinolysis and that targeting plasma CPB is an attractive strategy to uncover novel antithrombotic agents. Using EF6265, a selective inhibitor of plasma CPB, we show the crucial role of plasma CPB in thrombotic conditions especially in small vessels and capillaries. CPB inhibitors can be novel antithrombotic agents and a fibrinolytic enhancer while maintaining a small effect on primary hemostasis, especially for thrombolysis in the microcirculation and in conjunction with drugs such as tPA for the treatment of various thromboembolic diseases.

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Legends for figures

Fig. 1. Structure of EF6265.

Fig. 2. Enhancement of clot lysis *in vitro*.

EF6265 enhanced tPA-mediated clot lysis in human (A and B) and rat plasma (C and D). A, EF6265 enhanced clot lysis in diluted human plasma. Control: open circles. EF6265: closed diamonds, 1 nmol/L; closed squares, 10 nmol/L; closed circles, 100 nmol/L; closed triangles, 1 μmol/L; crosses, 10 μmol/L. tPA-free control: open diamonds. Each point represents the mean of three experiments. B, EF6265 reduced the time for clot lysis in a concentration-dependent manner. Each point represents the mean ± SE of three experiments. *P < 0.01; ANOVA followed by Dunnett's test (n=3). C, EF6265 enhanced clot lysis in diluted rat plasma. Symbols are the same as shown in A. Each point represents the mean of three experiments. D, EF6265 enhanced clot lysis in a dose-dependent manner. The total amount of clot remaining in the assay was determined by integrating the area under the curve (AUC) above the baseline from the turbidity-time profile observed over 180 minutes and is expressed as "total clot". Each point represents the mean ± SE of three experiments. *P < 0.01; ANOVA followed by Dunnett's test (n=3).

Fig. 3. The effects of EF6265 on *ex vivo* tPA-mediated clot lysis.

Rats were intravenously administered saline (control, open circles) or EF6265 at doses of 0.1 mg/kg (closed

triangles) and 1 mg/kg (closed squares). Clot lysis in plasma sampled at five minutes (A) and 30 minutes (B) after intravenous administration of EF6265. C, Time course of the total clot in *ex vivo* clot lysis assay. *P < 0.05; **P < 0.01 versus vehicle-treated value at each point; ANOVA followed by Dunnett's test (n=5).

Fig. 4. Plasma concentration of EF6265 after intravenous administration.

Rat plasma concentrations of EF6265 were determined after a single intravenous bolus of 0.1 mg/kg (closed triangles) and 1 mg/kg (closed squares) EF6265. Each value represents mean ± SD (n=5).

Fig. 5. EF6265 ameliorated tissue factor-induced microthrombosis in rats.

Microthrombosis in a range of tissues was induced by the continuous infusion of tissue factor for 20 minutes. Two methods were adopted to determine the antithrombotic effects, histology (A and B) and quantification of ^{125}I-labeled fibrinogen (C, D and E). A, EF6265 and tPA reduced %GFD in a dose-dependent manner. Each value represents the mean ± SE. **P < 0.01 versus vehicle-treated control; ANOVA followed by Dunnett's test (n = 4-7). B, Increases in D-dimer concentration in plasma sampled at 45 minutes. Each value represents the mean ± SE. *P < 0.05; **P < 0.01 versus vehicle-treated control; Kruskal-Wallis's H test followed by Steel's test (n = 4-7). The antithrombotic effects were determined using ^{125}I-labeled fibrinogen. EF6265 reduced radioactivity in both the kidney (C) and lung (D), and coincidentally increased radioactivity of the blood (E). For each rat, the microthrombi index (MI) was

defined as the ratio of radioactivity in the organ to that of the reference blood obtained at 0 time (just before the start of tissue factor infusion as described below). Radioactivity of the blood was also expressed as a ratio to the reference blood. Control: open circles. EF6265: closed diamonds, 0.01 mg/kg; closed triangles, 0.1 mg/kg; closed squares, 1 mg/kg. TPA: open diamonds, 12 kIU/kg; open triangles, 60 kIU/kg; open squares, 120 kIU/kg. Each value represents the mean ± SE. $^{\#\#}P < 0.01$ versus non-treated group (Non, without the infusion of tissue factor), $*P < 0.05$; $**P < 0.01$ versus vehicle-treated control; ANOVA followed by Dunnett's test (n = 4-7). F, Effects of EF6265 and tPA on plasma α2-antiplasmin concentrations. Symbols were same as E. Each value represents the mean ± SE. $*P < 0.05$; $**P < 0.01$ versus vehicle-treated control; ANOVA followed by Dunnett's test (n = 4-7).

Fig. 6. Effects of EF6265 on arteriovenous shunt thrombosis model.

EF6265 (1 mg/kg) was intravenously injected five minutes before the start of blood circulation, and 600 kIU/kg tPA was administered via the arteries 10 minutes after the start of the blood circulation. After 20 minutes of circulation, the wet weight of thrombus stuck to the copper wire was measured to evaluate antithrombotic effects. Each value represents the mean ± SE. $*P < 0.05$ versus vehicle treated control; ANOVA followed by Dunnett's test (n = 10).

Fig. 7. Effects of EF6265 on a bleeding time.

A, Effects of EF6265 at doses of 1 mg/kg and 30 mg/kg. Effects of tPA was also evaluated at doses of 10

kIU/kg and 600 kIU/kg. B, Effects of EF6265 combined with tPA at a dose of 600 kIU/kg. This dose was used in the arteriovenous shunt model. Each value represents the mean ± SE. *P < 0.05 versus vehicle-treated control; Kruskal-Wallis's H test followed by Steel's test (n=5).

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Table 1. Inhibitory effects of EF6265 on human plasma CPB and other enzymes. Values of IC_{50} were evaluated from the fitting of a sigmoidal curve from data of more than five concentrations of EF6265.

	IC_{50} (nmol/L)		
Target Enzyme	EF6265	CPI	MGPA
CPB (human plasma)	8.26	134	8380
CPN (human plasma)	5930	>500000	73.3
CPA (bovine pancreas)	8240	535	459000
CPB (rat plasma)	3.89	79.4	27800
CPN (rat plasma)	12800	>500000	887

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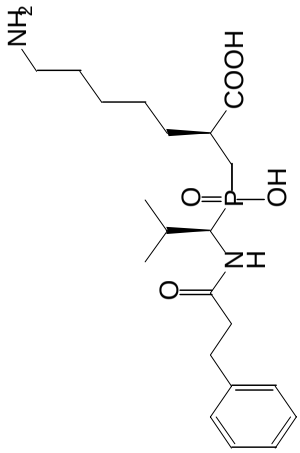

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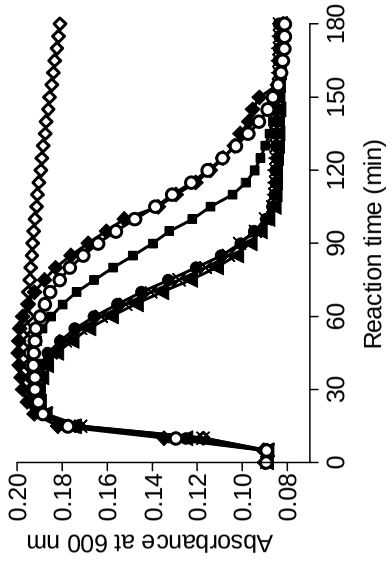

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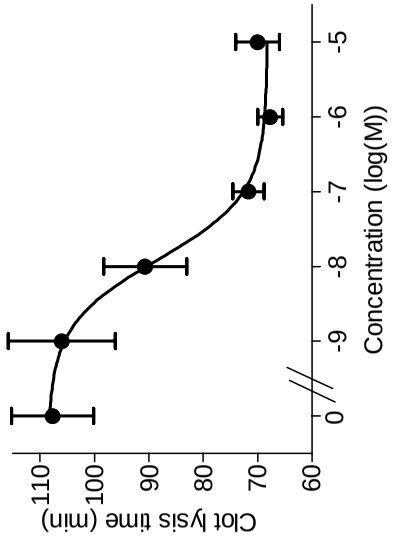

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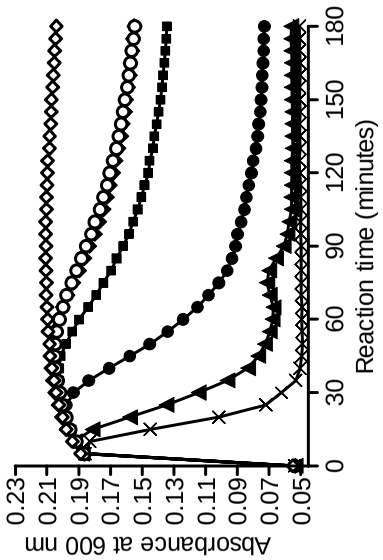

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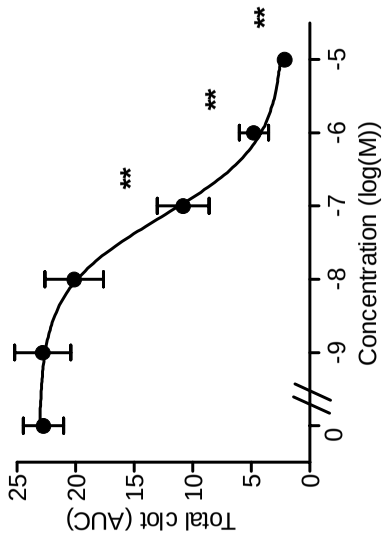

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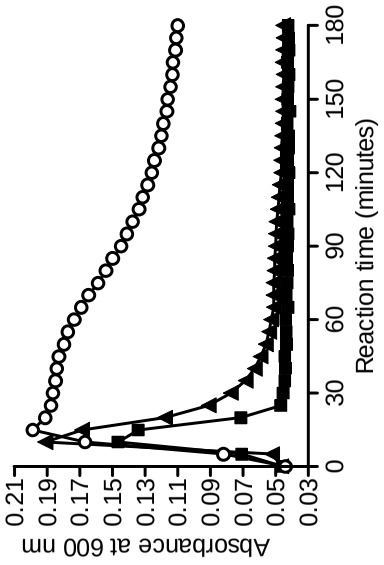

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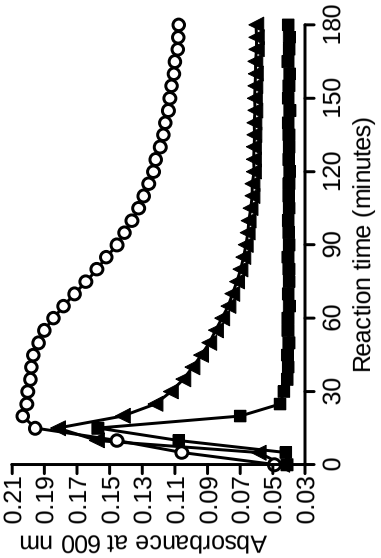

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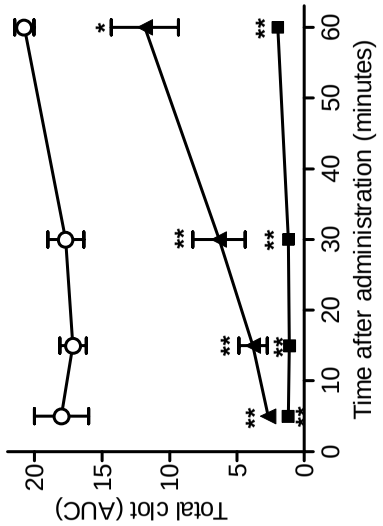

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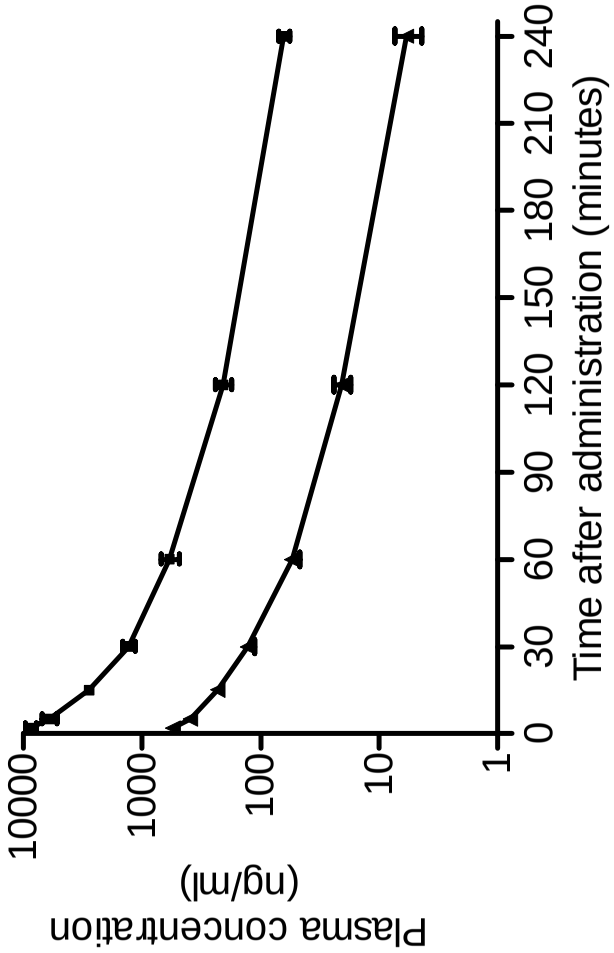

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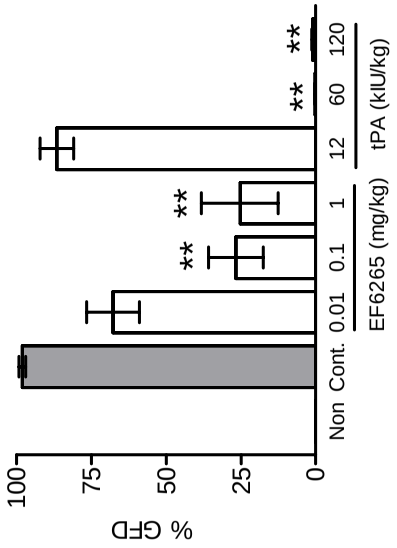

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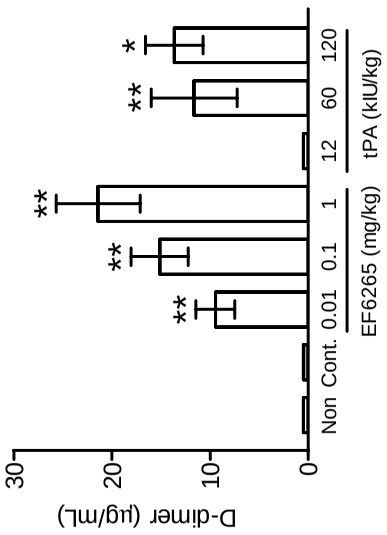

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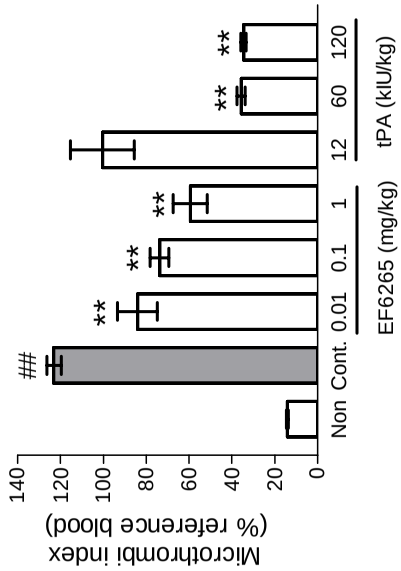

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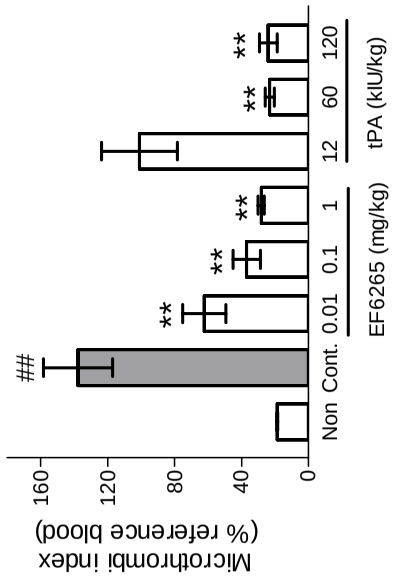

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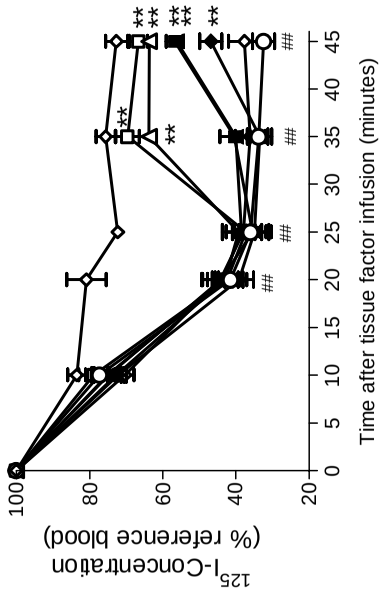

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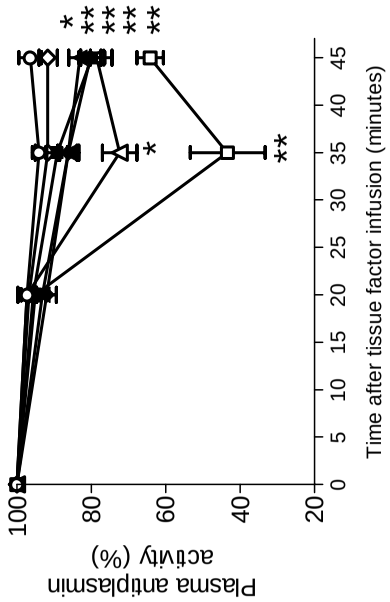

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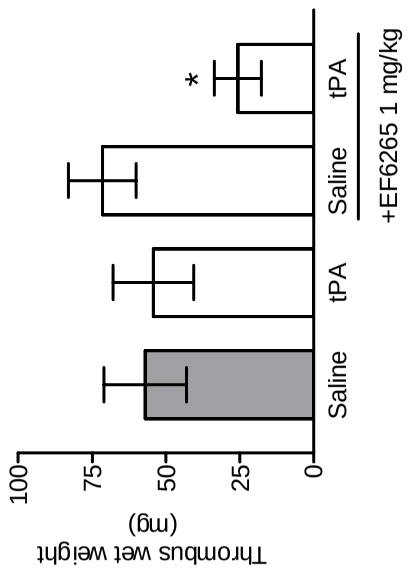

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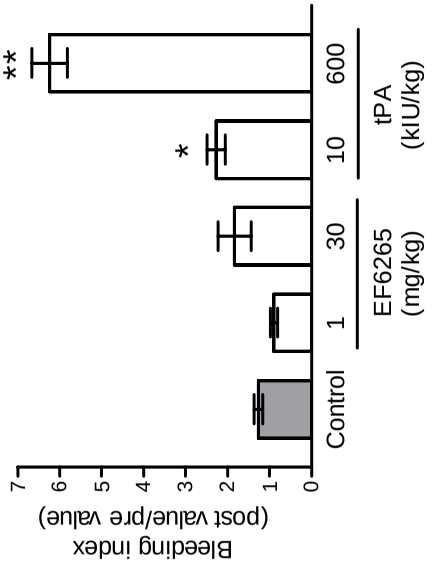

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