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

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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. 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 [(S)-7-amino-2-[[[(R)-2-methyl-1-(3-phenylpropanoylamino)propyl]hydroxyphosphinoyl]methyl]heptanoic acid] and determined both the pharmacological profile and pathophysiological role of CPB in rat thrombolysis. EF6265 specifically inhibited plasma CPB activity with an IC50 (50% inhibitory concentration) of 8.3 nM and enhanced tPA-mediated clot lysis in a concentration-dependent manner. EF6265 decreased detectable thrombi (percentage of glomerular fibrin deposition; control, 98 ± 1.1; EF6265, 0.1 mg/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, 0.1 mg/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 and 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 procarboxypeptidase B 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 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-type plasminogen activator (tPA) or urokinase-type plasminogen activator 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 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 thrombin-activatable fibrinolysis inhibitor (TAFI) or procarboxypeptidase U, is produced in the liver and

ABBREVIATIONS: tPA, tissue-type plasminogen activator; proCPB, procarboxypeptidase B; TAFI, thrombin-activatable fibrinolysis inhibitor; CPB, carboxypeptidase B; CPI, potato-derived carboxypeptidase inhibitor; CPA, carboxypeptidase A; MGPA, DL-mercaptmethyl-3-guanidinoethylthiopropanoic acid; CPN carboxypeptidase N; ACE, angiotensin-converting enzyme; AUC, area under the plasma concentration-time curve; GFD, glomerular fibrin deposition; EF6265, (S)-7-amino-2-[[[(R)-2-methyl-1-(3-phenylpropanoylamino)propyl]hydroxyphosphinoyl]methyl]heptanoic acid.
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. Thrombin-mediated activation of plasma proCPB is greatly enhanced in the presence of the cofactor protein thrombomodulin, 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-thrombomodulin 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 (Fleury and Angles-Cano, 1991; Bajzar et al., 1995). 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 plasminogen activator inhibitor-1 in plasma (Bajzar, 2000). Plasmin, generated on the fibrin clot, 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] inhibited 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); and 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 et 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 et 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.

Materials and Methods

Reagents and Biochemical Assays. Human plasma proCPB/TAFI was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Activation of proCPB/TAFI (0.36 μg/ml in 50 mmol/ml Tris-HCl, pH 7.4) to CPB/activated TAFI was triggered by addition of 20 μl of thrombomodulin (American Diagnostica, Greenwich, CT) solution (300 ng/ml in 50 mmol/ml Tris-HCl, pH 7.4, containing 0.1% Lubrol, 0.1% bovine serum albumin, and 0.15 M NaCl) and 20 μl of thrombin solution (3 units/ml in 50 mmol/ml Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin and 0.15 M NaCl). After incubation for 30 min at 25°C, the generated carboxypeptidase activity was determined. The reaction mixture (10 μl) was incubated with 1.25 mmol/ml hippuryl-arginine (Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris-HCl buffer, pH 7.6, for 60 min at 25°C (the final volume was 80 μl). The reaction was stopped by adding 100 μl of 0.2 M PIPES-NaOH buffer, pH 7.6, containing 12.5% Tween 20. 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). CPN was partially purified from human plasma (Skidgel et al., 1988; Bajzar et al., 1995). Enzyme activity of CPN was measured in the rate of hydrosis of hippuryl-lysine (Sigma-Aldrich) in 0.1 M 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 pancreatic CPA (Tanaka et al., 1984) was from Roche Diagnostics (Mannheim, Germany), rabbit lung angiotensin-converting enzyme (ACE) (Cushman and Cheung, 1971) was from Wako Pure Chemicals (To-kyo, 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-Aldrich. Human factor Xa (Morita et al., 1977) was from Hematologic Technologies (Essex Junction, VT). Human factor XII (Kawabata et al., 1988) was purchased from Enzyme Research Laboratories Inc. tPA (alteplase) (Kyowa Hakko, Tokyo, Japan), tissue factor (Innovine) (Dade International, Miami, FL), and MGPA (Calbiochem-Novabiochem, La Jolla, CA) were used.

Synthesis of EF6265. EF6265 was synthesized in our laboratory with 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 to 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 Institutes of Health.

In Vitro Clot Lysis Assay in Human and Rat Plasma. Blood from healthy donors was collected into 1/10 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 CaCl₂ (final concentration 10 mM in human plasma and 20 mM 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 min using a microplate
reader (THERMO max; Molecular Devices Corp., Sunnyvale, CA). Effect of EF6265 on the clot lysis assay was evaluated by adding EF6265 at final concentrations of 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M 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 because 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 plasma concentration-time curve ( AUC ) above the baseline from the turbidity-time profile observed over 180 min, 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 3.85% sodium citrate. The samples were obtained at 0 (just before EF6265 administration), 5, 15, 30, and 60 min 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 Wistar rats ( n = 5). EF6265 was separated by a Symmetry Shield RP18 column (Waters, Milford, MA) using a mobile phase and quantified using a TSQ7000 tandem mass spectrometry system (Thermo Finnigan, San Jose, CA). Pharmacokinetic parameters were calculated using a noncompartment model by processing with WinNonlin professional version 3.1 (Pharsight, Mountain View, CA). The elimination constant ( k e l ) was calculated from the terminal three points. The 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⁻∞ ), the area under the moment curve from hour 0 to infinity (AUMC⁰⁻∞), the total clearance ( C L tot ), and the volume of distribution at steady state ( V d ss ) were calculated as shown below.

\[
AUC_{0-\infty} = AUC_{0-t} + C_c/k_e
\]

\[
AUMC_{0-\infty} = AUMC_{0-t} + t \cdot C_c/k_e + C_c/k_e^2
\]

\[
C L_{tot} = Dose/AUC_{0-\infty}
\]

\[
V d_{ss} = C L_{tot} \cdot AUMC_{0-\infty}/AUC_{0-\infty}
\]

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

Microthrombosis Model Induced by Tissue Factor. Male Wistar 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 min (Takahashi et al., 1997). EF6265 (at doses of 0.01, 0.1, and 1 mg/kg) or IFA (at doses of 12, 60, and 120 kIU/kg) was intravenously administered (bolus injection) 5 min after the infusion of tissue factor ended (25 min), and the rats were sacrificed at 45 min 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 test (Aoshima et al., 1998) using the assay kit of LPIA ACE DD

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

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\text{NH}_2 &
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Fig. 1. Structure of EF6265.
plasma CPB activity at nanomolar concentrations than existing carboxypeptidase inhibitors, CPI and MGPA (Table 1). The IC\textsubscript{50} (50% inhibitory concentration) values of EF6265 for CPB and CPN were calculated to be 8.26 and 5930 nM, respectively (Table 1). The inhibitory effects of MGPA on plasma CPN (IC\textsubscript{50} of 73.3 nM) and of CPI on CPA (IC\textsubscript{50} of 535 nM) were much stronger than that of EF6265 (IC\textsubscript{50} of 5930 and 8240 nM, respectively) (Table 1). The IC\textsubscript{50} values of EF6265 for plasmin, thrombin, factor Xa, factor Xla, ACE, and trypsin were over 500 \mu M. 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 (Fig. 2, A and B) and rat plasma (Fig. 2, C and D) in a concentration-dependent manner (Fig. 2, B and D). EF6265 maximally enhanced clot lysis at a concentration of about 1 \mu M 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 5 (Fig. 3A) and 30 min (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 min 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 h at 0.1 and 1 mg/kg, respectively (Fig. 4). The CL\textsubscript{tot} was 390 ml/h/kg at 0.1 mg/kg and 316 ml/h/kg at 1 mg/kg. The Vd\textsubscript{ss} 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 5 min postdose of 0.1 and 1 mg/kg were 391 ng/ml (0.92 \mu M) and 6120 ng/ml (14.4 \mu M), respectively, and those at 60 min postdose of 0.1 and 1 mg/kg were 54.3 ng/ml (0.13 \mu M) and 589 ng/ml (1.38 \mu M), respectively. Considering that the greatest enhancement of fibrinolysis occurred with a concentration of 1 \mu M in the in vitro clot lysis assay (Fig. 2, C 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 min 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

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**TABLE 1**

<table>
<thead>
<tr>
<th>Target Enzyme</th>
<th>IC\textsubscript{50} (nM)</th>
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<tbody>
<tr>
<td></td>
<td>EF6265</td>
</tr>
<tr>
<td>CPB (human plasma)</td>
<td>8.26</td>
</tr>
<tr>
<td>CPN (human plasma)</td>
<td>5930</td>
</tr>
<tr>
<td>CPA (bovine pancreas)</td>
<td>8240</td>
</tr>
<tr>
<td>CPR (rat plasma)</td>
<td>3.89</td>
</tr>
<tr>
<td>CPN (rat plasma)</td>
<td>12,800</td>
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</tbody>
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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 min (5 min 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 (Fig. 5, A 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 125I-labeled fibrinogen. The level of radioactivity was markedly increased in the kidney (Fig. 5C, hatched column) and lung (Fig. 5D, hatched column). Accordingly, the levels of radioactivity in the blood (Fig. 5E, open circle) decreased to about 35% at 25 min and were retained for a further 20 min (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 min, significantly inhibited this increase in radioactivity in these organs in a dose-dependent manner (Fig. 5, C and D). The injection of EF6265 slowly reelevated 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, whereas 60 and 120 IU/kg tPA reduced the free levels to 70 and 40%, respectively, at 35 min (Fig. 5F). These results suggest that EF6265 reduced thrombi in this model and enhanced fibrinolysis, whereas EF6265 produced much less plasmin in circulating plasma than tPA.

Activity of EF6265 in an Arteriovenous Shunt Thrombosis Model. Although the intraarterial administration of 600 kIU/kg tPA, 10 min after the recirculation of the blood, did not 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 6-fold (Fig. 7A). EF6265 did not prolong the bleeding time at doses of 1 and 30 mg/kg (Fig. 7A). The group that 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 $10^{-8}$ M EF6265 and that the greatest effects occurred at $10^{-6}$ M. These concentrations were almost the same for the inhibition of plasma CPB (Fig. 2, B 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 (Fig. 5, A–E). The injection of EF6265 as well as tPA attenuated the increases in %GFD (Fig. 5A) and deposition of radiolabeled fibrinogen (fibrin) in the tissues of rats with microthrombosis (Fig. 5, C 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 with 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), because 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 nM) and reduced at higher thrombomodulin concentrations (10 nM) (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 a2-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), whereas effective doses of tPA directly activate 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. (2002) reported that a deficiency in TAFI did not lead to increased bleeding. 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 exog-
enously administrated tPA, whereas 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 ($k_m$ of 55 nM) 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 thromboses, 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. To further clarify the potential utility of EF6265 in single use for microthrombotic effects on the two models that were performed in this study, farctions while maintaining a low risk of hemorrhaging.

References


Ishii H (1994) The detection and measurement of thrombinomul, in Thrombin, Thrombinomul and the Control of Haemostasis (Giddings JC ed) pp 121–141, R. Indus, Austin, TX.


Schroeder V, Catterjette T, Mehta H, Windecker S, Pham T, Devantay N, Meier B,


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