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Intravenous and oral administrations of DD2 [7-Amino-2-(sulfanylmethyl)heptanoic acid] produce thrombolysis through inhibition of plasma TAFIa in rats with tissue factor-induced microthrombosis

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ABSTRACT

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that is activated by thrombin in plasma. In fibrinolytic processes, carboxy-terminal lysine (Lys) residues in partially degraded fibrin are important sites for plasminogen binding and activation, and an active form of TAFI (TAFIa) inhibits fibrinolysis by eliminating these residues proteolytically. We synthesized DD2 [7-Amino-2-(sulfanylmethyl)heptanoic acid], a Lys analogue containing sulfur, as an inhibitor of TAFIa and investigated its pharmacological profile and pathophysiological role in thrombolysis via *in vitro* and *in vivo* studies. DD2 specifically inhibited plasma TAFIa activity with an apparent IC_{50} (50% inhibitory concentration) value of 3.4×10^{-8} M under the present experimental condition and enhanced tissue plasminogen activator-mediated clot lysis in a concentration-dependent manner. In order to study tissue factor (TF)-induced microthrombosis in an animal model, rats were given intravenous injection (2.5 mg/kg and higher) or oral administration (10 mg/kg and higher) of DD2. This attenuated TF-induced glomerular fibrin deposition and increased the plasma levels of fibrin degradation products and D-dimer in a dose-dependent manner. A DD2 dose approximately 4X higher than the dose used in intravenous injections was required to achieve an equivalent thrombolytic effect to that seen following oral administration. Moreover, the oral absorption efficiency of DD2 into the vasculature was 29.8%. These results indicate that both intravenous and oral administration of DD2 enhanced endogenous fibrinolysis and reduced thrombi in a TF-induced microthrombosis model.

Introduction

Myocardial infarction and cerebral infarction are thrombotic diseases with high associated morbidity and mortality rates in developed countries. Since the thrombi consist of fibrin clots and aggregated platelets, tissue plasminogen activator (tPA) has been injected intravenously to dissolve fibrin clots in recent medical treatments of thrombosis. Fibrinolysis is initiated by partial degradation of fibrin fibers by plasmin, which is produced by tPA-mediated plasminogen activation [1]. Since plasmin hydrolyzes the peptide bond between lysine (Lys)-X (X indicates any amino acid), carboxy (C)-terminal Lys residues are created on partially degraded fibrin and provide high-affinity binding sites for the kringle domains of plasminogen and tPA [2]. The tPA bound on C-terminal Lys residues amplifies plasmin generation through activation of plasminogen on the surface of fibrin clots, and this ultimately leads to efficient lysis of fibrin clots. However, large quantities of plasmin generated by tPA injection result in hemorrhaging as a side effect [3]. Many efforts have therefore been made to develop pharmacologically distinct fibrinolytic agents while maintaining a low risk of hemorrhage.

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as plasma procarboxypeptidase B (proCPB), is synthesized by the liver and released into circulating plasma as a 56 kDa glycosylated zymogen [4,5]. TAFI is cleaved at arginine (Arg) 92 by trypsin-like enzymes such as thrombin, plasmin or the thrombin/thrombomodulin (TM) complex,

Abbreviations: tPA, tissue plasminogen activator; Lys, lysine; C, carboxy; TAFI, thrombin-activatable fibrinolysis inhibitor; proCPB, procarboxypeptidase B; Arg, arginine; TM, thrombomodulin; CPB, carboxypeptidase B; EF6265, (*S*)-7-amino-2-[[[(*R*)-2-methyl-1-(3-phenylpropanoylamino)propyl] hydroxyphosphinoyl] methyl]heptanoic acid; TF, tissue factor; CPI, potato derived carboxypeptidase inhibitor; CPA, carboxypeptidase A; MGPA, DL- mercaptomethyl −3-guanidnoethylthiopropanoic acid; CPN, carboxypeptidase N; DD2, 7-Amino-2-(sulfanylmethyl)heptanoic acid; IC_{50}, 50% inhibitory concentration; AUC, area under the concentration-time curve; Cmax, maximal concentration; Tmax, time to reach maximal concentration; FDP, fibrin degradation products; PTAH, phosphotungstic acid hematoxylin; GFD, glomerular fibrin deposition; DIC, disseminated intravascular coagulation; LC-MS, liquid chromatography-mass spectrometry.

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generating activated TAFI (TAFIa) [4]. TAFIa exhibits carboxypeptidase B (CPB)-like activity, cleaving basic amino acids such as Arg or Lys from the C-termini of proteins or peptides. The C-terminal Lys residues that appear in partially degraded fibrin and provide high-affinity binding sites for plasminogen and t-PA are TAFIa substrates. Cleavage of C-terminal Lys residues by TAFIa attenuates fibrinolysis by inhibiting t-PA - induced plasmin formation [6].

Epidemiological studies have revealed that elevated TAFI levels in circulating plasma are correlated with an elevated risk for venous thrombosis [7–10]. Montaner et al. [11] reported that high TAFI levels were found in the acute phase of ischemic stroke. On the other hand, it has been reported that high TAFI levels were not associated with an increased risk of venous and arterial thromboembolism in thrombophilic families [12] and that high TAFI levels are associated with a low risk of hard coronary events [13]. The discrepancies between the conclusions drawn from these different studies have been explained as follows [14]. First, different methods were used to determine the TAFIa activity and/or TAFI antigen levels, including different calibrators and reference samples [15,16]. Second, various isoforms of TAFI display different relativities in some commercially available ELISAs, leading to an underestimation of the isoform (TAFI-I^{325}) that exhibits a more pronounced antifibrinolytic effect [17].

We demonstrated previously that an intravenous injection of EF6265 [(*S*)-7-amino-2-[[[(*R*)-2-methyl-1-(3-phenylpropanoylamino) propyl]hydroxyphosphinoyl] methyl]heptanoic acid], a novel TAFIa inhibitor, decreased thrombi that had been generated by injection of tissue factor (TF) in a rat microthrombosis model, while maintaining a low risk of hemorrhage [18]. Injection of CPI (a potato-derived carboxypeptidase inhibitor of carboxypeptidase A (CPA) and CPB) or MGPA (DL-mercaptomethyl-3- guanidnoethylthiopropanoic acid), an inhibitor of CPB and carboxypeptidase N (CPN)), also reduced TF-induced microthrombosis in rats [19]. Furthermore, CPI that was incorporated into a clot created in an isolated segment of the jugular vein potentiated endogenous thrombolysis [20]. Mishara et al. [21] reported that intravenous injection of anti-TAFI monoclonal antibodies, which mainly impaired plasmin-mediated TAFI activation, could also reduce clot lysis time significantly. These results suggest that TAFIa inhibitors may act as effective thrombolytic agents while maintaining a low risk of hemorrhage. So far, several inhibitors of TAFIa or CPB activity have been identified [22]: 2-guanidinoethyl mercaptosuccinic acid [23,24], SQ24798 [25], leech carboxypeptidase inhibitor [26], tick carboxypeptidase inhibitor [27], cysteine derivatives [28], and heavy-chain antibodies (termed nanobodies) [29]. Recently, one aminopyridine mercaptane coded *AZD 9684*, 2-(6-aminopyridin-3-ylmethyl)-3-mercaptobutyric acid progressed into Phase II clinical trials as the novel TAFIa inhibitor for thrombosis and pulmonary embolism but was discontinued in 2007 [30,31]. Thus, no orally available TAFIa inhibitors have yet been reported as thrombolytic agents that could be effective and convenient to clinical use.

Here we report that DD2 [7-Amino-2-(sulfanylmethyl)heptanoic acid], a Lys analogue, inhibited enzymatic activity of TAFIa and enhanced tPA-induced clot lysis activity in *in vitro* experiments, and that intravenous and oral administrations of DD2 increased thrombolytic activity in a rat microthrombosis model.

Materials and methods

Materials

TM was purchased from American Diagnostica, (Greenwich, CT, USA). Human plasma thrombin, CPI, hippuryl-arginine (Hip-Arg), and hippuryl-lysine (Hip-Lys) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CPN from human plasma was purchased from Elastin Products (St. Louis, MO, USA). tPA (alteplase) (Kyowa Hakko Kirin, Tokyo, Japan) was used. EF6265 was donated from the Pharmaceutical Research Center, Meiji Seika Kaisha Ltd. (Yokohama, Japan).

Synthesis of DD2

DD2 was synthesized in the laboratory of Drug Design and Medicinal Chemistry of Showa Pharmaceutical University (Tokyo, Japan). The structure of DD2 is shown in Fig. 1. DD2 was purified by the medium pressure liquid chromatography (ODS-SM, 26×300 mm, 50 μm, 50% methanol/milliQ + 0.2% trifluoroacetate, 12 mL/min). Purity was determined by the high performance liquid chromatography [CAPCELL PAK UG120, 4.6×150 mm, 70% milliQ/methanol + 0.2% trifluoroacetate, flow rate 1.0 mL/min] and was >95% for DD2 tested. The structure of DD2 was confirmed by the spectral data: ^{1}H nuclear magnetic resonance (300 MHz, D_2O) δ 1.39 (4 H, m, CH_2×2), 1.62 (4 H, m, CH_2×2), 2.65 (1 H, m, *CH*), 2.73 (2 H, m, CH_2SH), 2.98 (2 H, t, J = 7.6 Hz, CH_2NH_2). Low resolution mass spectrometry (electrospray positive ionization): 192.1 (MH^+). Further detailed procedures and spectroscopic data will be reported separately.

Inhibitory effects of DD2 on human plasma TAFIa and other enzymes

TAFI was purified from normal human plasma as described previously [33]. Healthy human plasma was passed over a Lysine-Sepharose 4B column and plasminogen-depleted plasma was obtained. The plasminogen-depleted plasma was brought to 80 mM $BaCl_2$ and centrifuged. The obtained supernatant, barium-adsorbed plasma (BAP), was precipitated between 35% and 70% $(NH_4)SO_4$ saturation. TAFI in the preparation was purified by chromatography on Q-Sepharose Fast Flow, Heparin-Sepharose CL-6B, Sephacryl S-300, and plasminogen –Sepharose column, and stored at −80 °C. These chromatographic steps achieved 3000-fold purification with a 14% recovery from TAFI activity from the BAP and the specific activity of purified TAFI was 28 U/mg protein. The purified TAFI migrated as a single band with a molecular weight 58 kDa on sodium dodecyl sulfate (SDS)-PAGE (12% polyacrylamide) under reducing condition. To activate TAFI, the purified TAFI (4.3 μg/mL TAFI, 20 μL) was mixed with 20 μL of 300 ng/mL TM in buffer A (50 mM Tris–HCl buffer, pH 7.4, supplemented with 0.1% BSA and 0.15 M sodium chloride) and incubated at 25 °C for 3 min. Next, 20 μL of 3 U/mL thrombin solution in buffer A was added and mixed, and TAFI in the mixture was activated by incubation for 30 min at 25 °C. The prepared TAFIa solution (25 μL) was transferred into another tube and incubated with 25 μL of 3.2 mM Hip-Arg in buffer B (0.1 M Tris–HCl buffer, pH 7.6) at 25 °C for 30 min (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 [32]. One unit of TAFIa enzymatic activity is defined as production of 1 μmol of hippuric acid per min.

To determine inhibition of TAFIa activity by DD2 and EF6265, TAFIa solutions were prepared at 20 mU/mL concentration and 25 μL aliquots

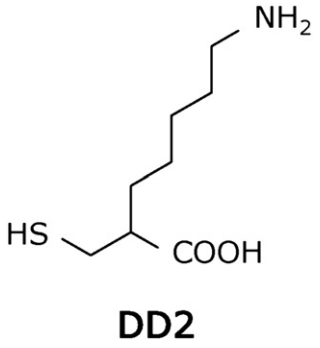

Fig. 1. Structure of DD2.