Factor XIIa as a Novel Target for Thrombosis: Target Engagement Requirement and Efficacy in a Rabbit Model of Microembolic Signals

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ABSTRACT

Coagulation Factor XII (FXII) plays a critical role in thrombosis. What is unclear is the level of enzyme occupancy of FXIIa that is needed for efficacy and the impact of FXIIa inhibition on cerebral embolism. A selective activated FXII (FXIIa) inhibitor, recombinant human albumin-tagged mutant Infestin-4 (rHA-Mut-inf), was generated to address these questions. rHA-Mut-inf displayed potency comparable to the original wild-type HA-Infestin-4 (human FXIIa inhibition constant = 0.07 and 0.12 nM, respectively), with markedly improved selectivity against Factor Xa (FXa) and plasmin. rHA-Mut-inf binds FXIIa, but not FXII zymogen, and competitively inhibits FXIIa protease activity. Its mode of action is hence akin to typical small-molecule inhibitors. Plasma shift and aPTT studies with rHA-Mut-inf demonstrated that calculated enzyme occupancy for FXIIa in achieving a putative aPTT doubling target in human, nonhuman primate, and rabbit is more than 99.0%. The effects of rHA-Mut-inf in carotid arterial thrombosis and microembolic signal (MES) in middle cerebral artery were assessed simultaneously in rabbits. Dose-dependent inhibition was observed for both arterial thrombosis and MES. The ED50 of thrombus formation was 0.17 mg/kg i.v. rHA-Mut-inf for the integrated blood flow and 0.16 mg/kg for thrombus weight; the ED50 for MES was 0.06 mg/kg. Ex vivo aPTT tracked with efficacy. In summary, our findings demonstrated that very high enzyme occupancy will be required for FXIIa active site inhibitors, highlighting the high potency and exquisite selectivity necessary for achieving efficacy in humans. Our MES studies suggest that targeting FXIIa may offer a promising strategy for stroke prevention associated with thromboembolic events.

Introduction

Factor XII (FXII) is the initiator of the intrinsic coagulation cascade and can be activated via contact with charged surfaces to form FXIIa, which then activates FXI and plasma kallikrein. Plasma kallikrein can activate FXII in a reciprocal manner to further amplify contact activation (Renné and Gailani, 2007; Renné et al., 2012; Björkqvist et al., 2014). Although human genetics and epidemiologic studies to date do not yield a clear relationship between FXII deficiency and the risk of thrombosis, FXII deficiency in humans is not associated with bleeding diathesis, despite marked activated partial thromboplastin time (aPTT) prolongation (Lämmle et al., 1991; Renné and Gailani, 2007). FXII knockout mice were protected from arterial thrombosis, ischemic stroke, and deep vein thrombosis, while maintaining normal hemostasis (Renné et al., 2005; Kleinschnitz et al., 2006; Müller et al., 2009; von Brühl et al., 2012), suggesting that FXII is critically involved in pathologic thrombus formation but is dispensable for hemostasis. Emerging studies also suggest that FXII is a key mediator in host defense and additional immune-inflammatory responses, via its downstream kallikrein/kinin system (Schmaier, 2016) and interactions with endothelial and immune cells (Göbel et al., 2016; Long et al., 2016). FXII is thus a promising target for treating not only thrombotic events but also a range of other disorders (Martini et al., 2014; Göbel et al., 2016; Hopp et al., 2016; Nickel et al., 2016; Zamolodchikov et al., 2016) without conferring a bleeding risk.

In recent years, inhibitors for FXII(a) have been generated and described in various preclinical models in vitro or in vivo. These agents include monoclonal antibodies (Larsson et al., 2014; Matafonov et al., 2014), natural peptide or protein inhibitors (Hagedorn et al., 2010; Yau et al., 2012), small-molecule inhibitors (Kleinschnitz et al., 2006; Baeriswyl et al., 2015), RNA aptamer (Woodruff et al., 2013), small interfering
RNA (Cai et al., 2015), and antisense oligonucleotide (Revensko et al., 2011). Although results with these various tool molecules in aggregate strengthen the notion that targeting FXII may provide a benefit in a range of disease settings, some of the small-molecule or peptide inhibitors used in generating in vivo data have insufficient selectivity (Hansson et al., 2014; Korneeva et al., 2014; Xu et al., 2014), hampering the precise understanding on target-based pharmacology. Despite the difficulty in achieving the required target selectivity and potency using a small-molecule inhibitor, this modality potentially maintains favorable attributes such as oral bioavailability and shorter pharmacokinetics that are unavailable in certain other therapeutic classes. Because small-molecule drugs need to balance among potency, selectivity, and pharmacological properties (e.g., permeability and bioavailability), understanding the levels of target engagement (e.g., enzyme occupancy, residence time) needed for efficacy is an important component in establishing chemical tractability of the target. The monoclonal antibody modality, although likely carrying a superior selectivity profile, is mechanistically distinct from small-molecule inhibitors and is limited in applicable patient settings due to its parenteral route of administration.

It has been observed that FXIIs can be localized to thrombi and can directly interact with fibrin, thereby potentially modulating clot structure and thrombus stability (Konings et al., 2011; Kuipers et al., 2014). Although FXII inhibition or loss of function markedly reduces thrombus formation in various models and species, thrombi in FeCl3-injured mesenteric vessels of FXII knockout mice appeared unstable and prone to embolization in intravital microscopy studies (Renné et al., 2005), and FXIIa inhibitor treatment has resulted in increased shedding of emboli in an ultrasound-induced model of atheroatherosclerosis in ApoE−/− mice (Kuipers et al., 2014). As FXII has been proposed to be a target for stroke and other thromboembolic disorders (e.g., device-mediated thrombosis in extracorporeal membrane oxygenation and cardiopulmonary bypass) (Dobrovolskaia and McNeil, 2015; Krupka et al., 2016), it is important to further understand the role of FXII in thrombus embolization.

In this report, we set out to examine the potency, selectivity, and mode of action of a previously reported FXIIa inhibitor, Inf4mut15 (Campos et al., 2012). We then used this molecule to address the following two questions: 1) levels of target engagement (calculated enzyme occupancy) needed for FXIIa in generating efficacy; and 2) effects of FXIIa inhibition in a novel model of cerebral microembolic signals (MESs) induced by FeCl3 injury of the carotid artery in rabbits (Zhou et al., 2012) cDNA sequences, respectively. Recombinant expression of Inf4mut15 (Campos et al., 2012) and Inf4mut15 (P2-P4’ sequence TRPFA) (Campos et al., 2012) CDNA sequences, respectively. Recombinant expression methods were as reported earlier (Xu et al., 2014). Briefly, the recombinant pUC57 plasmids containing HA-linker ((Gly-Gly-Ser)3)-WT- or Mut-inf were transiently transfected into HEK293 cells. Cell culture supernatant was harvested for purification on day 6 post-transfection. The recombinant protein was captured from the cell culture supernatant by Blue Sepharose 6 Fast Flow resin and eluted in 50 mM Tris with 3 M NaCl, pH 8.5. The purified protein was buffer exchanged into Dulbecco’s modified PBS (pH 7.0) and analyzed by SDS-PAGE and Western blot with HA antibody (Abcam, Cambridge, MA) for confirmation.

**In Vitro Protease Inhibition Assays.** Human FXIIa, plasma kallikrein, thrombin, activated protein C, and plasmin were purchased from Enzyme Research Laboratories (South Bend, IN). Human FXIa, FXIIa, and Xa were purchased from Sekisui Diagnostics (Lexington, MA). Human FXIIa was purchased from Haematologics Technologies (Essex Junction, VT). Recombinant human tissue plasminogen activator (TPA) was purchased from Hyphen Biomed, S.A.S. (Neuville-sur-Oise, France). Cyanomolgus macaque and rabbit FXIIa were prepared by Evotec, Inc (Princeton, NJ). The 7-amido-4-thioluminomethyl containing fluorescence substrate CH3SO2-cyclohexyl-Gly-Gly-Arg-AFC was custom synthesized by CPC Scientific (Sunnyvale, CA), whereas substrates N-CBZ-Gly-Pro-Arg-AFC and n-acetyl-Gly-Pro-Arg-AFC were purchased from Sigma-Aldrich (St. Louis, MO).

All enzymatic reactions were carried out in 50 mM HEPES, 150 mM NaCl, 5 mM CaCl2, and 0.1% polyethylene-glycol (8000 nominal molecular weight), at pH 7.4 and 25°C. The change in fluorescence during reaction progression was monitored using a Tecan m200 (TECAN, Männedorf, Switzerland) plate reader in kinetic mode set at 405 nm excitation and 500 nm emission with 9- and 20-nm bandwidths, respectively. For all enzymes tested, except for FXIIa, the slope of the fluorescence versus time plots was determined by linear regression and plotted versus rHA-Mut-inf concentration to determine IC50, which was subsequently converted to inhibition constant (K1) using the Cheng-Prusoff equation. For rHA-Mut-inf and rHA-WT-inf inhibition of human FXIIa, K1 and binding kinetic parameters (k_on and k_off) were determined using global progress curve analysis for one-step slow and tight binding (Zhang and Windsor, 2013). Additional characterization of human, cynomolgus macaque, and rabbit FXIIA activity was performed after a 2-hour preincubation of the enzyme with either rHA-WT-inf or rHA-Mut-inf to enable the enzyme-rHA-inf complex to reach equilibrium and was analyzed using global progress curve analysis for tight binding.

**Plasma Shift Studies.** For plasma shift studies, standard FXIIa enzymatic assays were performed as described above except that human, cynomolgus macaque, or rabbit FXIIA was preincubated with rHA-Mut-Inf in either the absence or presence of human, cynomolgus macaque, or rabbit 30% heat-inactivated plasma for 2 hours prior to initiation of the reaction by the addition of substrate. It has been determined that the 30% heat-inactivated plasma retains protein binding activity but is no longer able to be activated for the endogenous coagulation machinery (data not shown). Fluorescence versus time plots were analyzed from global progress curve analysis for one-step fast and tight binding (Zhang and Windsor, 2013).

To determine the percentage unbound concentration of rHA-Mut-inf in 100% plasma, % Free, the following equation was used: 

\[
\% \text{Free} = \frac{100}{1 + \frac{\% \text{PlasmaTarget}}{\% \text{PlasmaAssay}}} \times \frac{k_{1}^{\text{PlasmaAssay}}}{k_{1}^{\text{PlasmaTarget}}} \times \frac{1}{K_{1}}
\]

Where % PlasmaTarget is the target percentage plasma (100% in these studies), % PlasmaAssay is the percentage plasma used in the assay (30% in these studies), and K_{1}^{\text{PlasmaAssay}} is the plasma-shifted K_{1}.

**Surface Plasmon Resonance.** For surface plasmon resonance (SPR) experiments, rHA-Mut-inf was immobilized to a standard CM3 sensor chip in a BioCore T200 SPR instrument (GE Healthcare, Piscataway, NJ) using the Amine Coupling Kit and the supplied pH 5.0 sodium acetate buffer to a density of ~2000 response units. The kinetics of binding of FXIIa and FXIII zymogen were monitored using the single-cycle kinetic method. Running buffer was 10 mM HEPES sodium salt, 150 mM NaCl, 5 mM CaCl2, and 0.005%/P-20 at pH 7.4. Solutions of FXIIa and FXII at 0.1, 1, 10, 30, and 100 nM were

**Materials and Methods**

**Expression and Purification of Human Albumin-Tagged Mutant Infestin-4 and Recombinant Human Albumin-Tagged Wild-Type Infestin-4.** Purified recombinant human albumin (HA)–tagged wild-type Infestin-4 (rHA-WT-inf) and recombinant HA-tagged mutant Infestin-4 (rHA-Mut-inf) were custom generated by GeneScript (Piscataway, NJ) based on the previously reported Infestin-4 (Campos et al., 2004b) and Inf4mut15 (P2-P4’ sequence TRPFA) (Campos et al., 2012) CDNA sequences, respectively. Recombinant expression methods were as reported earlier (Xu et al., 2014). Briefly, the recombinant pUC57 plasmids containing HA-linker ((Gly-Gly-Ser)3)-WT- or Mut-inf were transiently transfected into HEK293 cells. Cell culture supernatant was harvested for purification on day 6 post-transfection. The recombinant protein was captured from the cell culture supernatant by Blue Sepharose 6 Fast Flow resin and eluted in 50 mM Tris with 3 M NaCl, pH 8.5. The purified protein was buffer exchanged into Dulbecco’s modified PBS (pH 7.0) and analyzed by SDS-PAGE and Western blot with HA antibody (Abcam, Cambridge, MA) for confirmation.
sequentially injected at a flow rate of 25 μl/min for 240 seconds each. Dissociation was monitored for 3600 seconds after the last injection. Data were analyzed using the 1:1 interaction model and the heterologous ligand model in the BiACore analysis software (GE Healthcare Life Sciences). Both fits yielded similar results for the dominant binding event.

**aPTT, Prothrombin Time, Thrombin Generation Assay, and Thromboelastography Assay.** In vitro and ex vivo aPTT (triggered by aPTT-XL, Pacific Hemostasis, Middletown, VA) and prothrombin time (PT) (triggered by TriniCLOT PT Excel; Teag, Bray, Ireland) were measured as described before (Xu et al., 2014; Cai et al., 2015). Thrombin generation assay (TGA) triggered by 0.83 μM ellagic acid (r2 Diagnostics, South Bend, IN) was performed using standard methods as reported previously (Xu et al., 2014; Cai et al., 2015). Briefly, rHA-Mut-inf at different concentrations was spiked into 60 μl of citrated plasma (human, cynomolgus macaque, or rabbit) and incubated at 37°C for 1 hour; a 15-μl trigger was then added to the plasma and incubated for an additional 10 minutes at 37°C. Fifteen microliters of FluCa was subsequently injected into the samples to initiate thrombin generation. Thrombinoscope software (Thromboscop, Maastricht, The Netherlands) was used to calculate peak thrombin potential as well as additional parameters [lag time, endogenous thrombin potential, time to peak, and slope (peak divided by the difference between lag and time to peak)].

Thromboelastography (TEG) analysis was carried out on the thromboelastograph coagulation analyzer model 5000 (Haemonetics, Braintree, MA) as described in detail previously (Zhou et al., 2015). Briefly, rHA-WT-inf or rHA-Mut-inf at various concentrations was spiked into freshly collected citrated human whole blood and incubated at room temperature for 10 minutes. Three hundred forty microliters of FluCa was subsequently injected into the sample to initiate thrombin generation. Thrombinoscope software (Thromboscop, Maastricht, The Netherlands) was used to calculate peak thrombin potential as well as additional parameters [lag time, endogenous thrombin potential, time to peak, and slope (peak divided by the difference between lag and time to peak)].

**Rabbit MES Studies.** Male New Zealand White rabbits (weight, 2.4–5.0 kg; age range, 10–13 weeks of age; Charles River Canada; Saint-Constant, QC, Canada) were used for the current studies. All of the procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Animal Care and Use Committee of Merck & Co., Inc. (Kenilworth, NJ).

The rabbit model of cerebral MCAO was induced by FeCl3 injury of the carotid artery as described in detail previously (Zhou et al., 2016a,b). Briefly, animals were anesthetized with a cocktail (50 mg/kg i.m. ketamine HCl; Pfizer Inc., New York, NY; and 5 mg/kg xylazine; LLOYD Inc., Shenandoah, IA) as a nonrecovery procedure. The left common carotid artery was surgically exposed. A Doppler flow probe (Model 15.1 or 2.0 PRB; Transonic Systems, Ithaca, NY) was connected to a flowmeter (Model T403; Transonic Systems) to measure the average carotid blood flow over 60 minutes. Integrated carotid blood flow over 60 minutes was quantitated using area under the curve (AUC), calculated by the trapezoidal rule, and expressed as a percent of control blood flow as described previously (Wong et al., 2008; Zhou et al., 2016b). At end of study (i.e., 60 minutes after FeCl3 injury), the wet thrombus weight was measured using a balance with a detection limit of 0.001 mg (Mettler Toledo Excellence Plus XP Series Analytical Balances; Mettler-Toledo, LLC, Columbus, OH).

The clinical SONAR™ TCD System (Neurolet Natus Neurology Inc., Middleton, WI) was used for continuously monitoring the blood flow velocity and MES in the ipsilateral MCA for 60 minutes upon FeCl3 injury. A customized pulse-wave 2-MHz probe (optical density, 11.3 mm; 90 mm long; focused at 12–25 mm; MTB Medizintechnik Basler AG, Regensdorf, Switzerland) was fixed by a flexible-arm magnetic-base holder (McMASTER-CARR, Princeton, NJ) at the posterior end of the zygomatic bone of the rabbits, at an angle of ∼80° against the bony surface. The MCA was insonated at a depth between 19 and 22 mm as described in detail previously (Zhou et al., 2016a,b). MES (defined as high-intensity transient signals) was saved and further validated based on the criteria defined by the International Consensus Committee as described previously (Ringelstein et al., 1998; Zhou et al., 2016a,b).

Vehicle (10 mM phosphate buffer, pH 7.0) or various doses of rHA-Mut-inf (0.1–1.0 mg/kg) were administrated at 2 ml/kg, as a bolus, through the ear vein 20 minutes prior to the FeCl3 injury. Doses for rHA-Mut-inf were selected based on its comparable potency to rHA-WT-inf in vitro and in our pilot in vivo studies (not shown), and the efficacy of rHA-WT-inf in our previous studies in rabbits (Xu et al., 2014).

**Ex Vivo Measurement of rHA-Mut-inf Plasma Concentrations and Clotting Time Assays.** Plasma levels of rHA-Mut-inf were measured by a standard liquid chromatography-mass spectrometry method as reported previously (Xu et al., 2014) based on the peptide sequence of Mut-inf (Inf4mut15) (Campos et al., 2012). Ex vivo plasma aPTT and PT were determined by standard methods using aPTT-XL (Pacific Hemostasis, Waltham, MA) and TriniCLOT PT Excel (Teag) on a KC4 Delta coagulation analyzer (Teag).

**Statistical Analysis.** Data are presented as the mean ± S.E. and analyzed using one-way analysis of variance followed by Bonferroni post hoc test in GraphPad Prism (version 7; GraphPad, La Jolla, CA) for comparison among groups with different treatment concentrations or doses. ED50 was determined by a nonlinear four-parameter dose-response curve fit using GraphPad Prism under the dose ranges described in each figure legend. Results were considered significant at $P < 0.05$.

**Results**

rHA-Mut-inf Is a Highly Potent and Selective Inhibitor of FXIIa. Despite its picomolar potency for FXIIa inhibition, the original rHA-WT-inf carries off-target activity on several other coagulation and fibrinolytic factors, including inhibitory activity on Factor Xa (FXa) and plasmin (Campos et al., 2004b; Xu et al., 2014; Kolyadko et al., 2015). To fully understand the selectivity profile of rHA-Mut-inf, we examined its impacts on the enzymatic activities of a comprehensive panel of human and rabbit serine proteases that include coagulation factors, fibrinolytic factors, and trypsin. We chose to determine the apparent $K_I$ values for all of the enzymes tested (Table 1; Supplemental Fig. 1) to accurately assess the occupancy of rHA-Mut-inf for these enzymes in various studies. To facilitate a direct comparison of the activity of these enzymes, we used similar fluorescently labeled tripeptide substrates for Michaelis-Menten constant ($K_M$) determinations. For the human enzymes, no less than 4000-fold selectivity was observed for any off-target human enzyme.
tested and no less than 3200-fold selectivity was observed for rabbit enzyme. Using the same method, the K_I value for rHA-WT-inf on FXIIa was determined to be 0.12 nM (Supplemental Fig. 2). rHA-Mut-inf is thus comparable to rHA-WT-inf for on-target (FXIIa-inhibitory) activity and is much more selective than rHA-WT-inf against other serine proteases.

**Mode of Action of rHA-Mut-inf.** Figure 1A shows the inhibitory effects of rHA-Mut-inf on the human FXIIa-mediated cleavage of a fluororescent substrate. As has been previously (Xu et al., 2014) described for rHA-WT-inf, this inhibition is slow to reach equilibrium and occurs at concentrations of inhibitor that are less than the concentration of enzyme used for this assay, necessitating taking these factors into account in the analysis of the data to determine the K_I. Also, since under the conditions used in this experiment the time to reach equilibrium was slow relative to the data-recording timescale, the binding kinetics of the rHA-Mut-inf-FXIIa interaction has been elucidated directly from this inhibition study. This slowly evolving inhibition is driven by the long half-life of dissociation of the rHA-Mut-inf-FXIIa complex (half-life = 4.1 hours, k_off = 4.8 × 10^{-5} s^{-1}), although the association rate (k_on = 6.6 × 10^{8} M^{-1} s^{-1}) is within normal ranges for this type of interaction.

Previous studies (Xu et al., 2014; Kolyadko et al., 2015) demonstrated that the inhibition of rHA-WT-inf was not directed against thezymogen FXII, but solely against FXIIa. To confirm the activated enzyme specificity and binding kinetics for rHA-Mut-inf we used SPR to kinetically monitor the interactions of FXIIa and FXII zymogen with immobilized rHA-Mut-inf (Fig. 1B). Results indicated that rHA-Mut-inf is directed against FXIIa, since no significant binding was observed for zymogen FXII. In addition, kinetic data from SPR (equilibrium dissociation constant [K_D] = 250 pM, k_on = 1.0 × 10^{6} M^{-1} s^{-1}, k_off = 3.0 × 10^{-5} s^{-1}) agree well with the binding kinetics determined directly from the inhibition assays. Furthermore, the mode of FXIIa inhibition by rHA-Mut-inf was characterized using standard enzymatic methods and was shown to be peptide substrate competitive (Fig. 1C). These findings are in accordance with the earlier characterization of the rHA-WT-inf mode of inhibition (Xu et al., 2014) and strongly support the idea that the inhibitory effects of these molecules are mediated through their direct interactions with the active site of FXIIa.

**Effects of rHA-Mut-inf in aPTT, PT, TGA, and TEG.** Functional activity of rHA-Mut-inf was examined in vitro in human, cynomolgus macaque, and rabbit plasma in aPTT and PT assays, as well as in ellagic acid–triggered TGA. rHA-Mut-inf prolonged aPTT in a dose-dependent manner in all three species (Fig. 2A), with the potency in rabbit far exceeding that in human and cynomolgus macaque. Concentrations needed for doubling aPTT are 19, 8.5, and 0.26 μM, respectively, in human, cynomolgus macaque, and rabbit plasma. In the PT assay (Fig. 2B), no change in PT was observed at concentrations <5 μM and a very modest (~10%) PT prolongation was observed in human and cynomolgus macaque plasma at 30 μM. rHA-Mut-inf reduced ellagic acid–triggered thrombin generation in TGA in a dose-dependent manner (Fig. 2C). IC_50 of peak thrombin is 5.5, 0.69, and 0.05 μM, respectively, in human, cynomolgus macaque, and rabbit plasma.

Effects of rHA-Mut-inf on blood coagulation and fibrinolysis were also assessed in the whole-blood TEG assay (Fig. 3), in a direct comparison with rHA-WT-inf, as described in Materials and Methods. A threshold concentration (1.5 nM) of tPA was present in the assay system to generate a fibrinolysis phase without affecting the clotting phase appreciably. Representative TEG traces of the human blood samples treated with 0 (vehicle), 1, 2, and 4 μM rHA-Mut-inf or rHA-WT-inf are shown in Fig. 3A. Both agents exerted a dose-dependent
prolongation on R (Fig. 3B, left panel), in a similar manner, with rHA-Mut-inf being slightly more potent than rHA-WT-inf at the highest concentration tested (4 μM). In contrast, although rHA-WT-inf produced marked and dose-dependent inhibition of tPA-induced fibrinolysis (Ly60), rHA-Mut-inf did not modulate fibrinolysis (Fig. 3B, right panel).

Effects of rHA-Mut-inf in Plasma Shift Studies. An essential component of the calculation of target engagement of a therapeutic molecule is its unbound fraction in the compartment of its target. Since coagulation occurs within the plasma, and since rHA-Mut-inf is a large molecule that is intractable for deriving free fraction in plasma via the conventional equilibrium dialysis method, we assessed the impacts of human, cynomolgus macaque, or rabbit heat-inactivated plasma on the observed inhibition of the respective FXIIa protein by rHA-Mut-inf (Fig. 4; Table 2) to indirectly determine the free fraction in plasma. To minimize the impact of slow binding by rHA-Mut-inf on the determination of $K_I$, each species of FXIIa was preincubated in rHA-Mut-inf for 2 hours, enabling FXIIa × rHA-Mut-inf interactions to reach equilibrium prior to reaction initiation with substrate (Supplemental Fig. 3). For the plasma of each species, a shift toward lower potency was observed, with the largest shift occurring for human plasma, followed by cynomolgus macaque, and the...
smallest shift occurring for rabbit plasma. These shifts were used to estimate the amount of rHA-Mut-inf not bound to plasma proteins and thus available for binding to the target FXIIa (Table 2). From these values, the percentage of FXIIa bound to rHA-Mut-inf at steady state was calculated using the concentration of rHA-Mut-inf that prolonged aPTT by 2-fold. Target engagement of 99.2% is needed for doubling aPTT in rabbit plasma, although target engagement of 99.9% is needed for doubling aPTT in cynomolgus macaque and human plasma.

**Effects of rHA-Mut-inf in Rabbit MES Studies.** Dose-dependent inhibition of rHA-Mut-inf in both thrombus formation in the carotid artery and MES incidents in the MCA was observed (Fig. 5). The integrated blood flow (AUC, illustrated as the percentage of blood flow relative to baseline) was increased from 55.0 ± 5.9% in the vehicle group (n = 8) to 92.3 ± 1.1% for the 1 mg/kg rHA-Mut-inf i.v. group (n = 6, P < 0.01), with an ED50 of 0.174 mg/kg rHA-Mut-inf (Fig. 5, A and B). Clot weight was reduced from 5.86 ± 0.39 mg (vehicle) to 0.16 ± 0.10 mg (1 mg/kg rHA-Mut-inf, P < 0.001), with an ED50 of 0.162 mg/kg rHA-Mut-inf (Fig. 5C).

Figure 5D illustrates a representative MES recording in the MCA in rabbits treated with 0.1 mg/kg rHA-Mut-inf. The mean MES frequency was reduced from 4.35 ± 1.13 (vehicle) to 0.0 ± 0.0 (1 mg/kg rHA-Mut-inf, P < 0.01), with an ED50 of 0.057 mg/kg rHA-Mut-inf (Fig. 5E). The incidence of MES detected in animals was also decreased from 100% (vehicle) to 0% (1 mg/kg rHA-Mut-inf) (Fig. 5F).

**Pharmacokinetic and Pharmacodynamic Analysis of rHA-Mut-inf in the Rabbit MES Model.** Figure 6A illustrates the dose-related increase in plasma rHA-Mut-inf exposure, with 78.3 ± 3.6, 192.2 ± 10.4, and 478.5 ± 47.0 nM observed at 20 minutes, and 76.3 ± 2.9, 182.1 ± 14.0, and 397.8 ± 37.9 nM observed at 80 minutes, for 0.1, 0.3, and 1 mg/kg i.v. bolus dose, respectively. Thus, drug exposures during the course of arterial thrombosis and MES monitoring were relatively stable.

Ex vivo aPTT confirmed the pharmacodynamic response for rHA-Mut-inf (Fig. 6B). Significant increase in aPTT was observed for 0.3 and 1 mg/kg rHA-Mut-inf dosing groups. The fold aPTT increases at 0.3 and 1 mg/kg (~1.3-fold and ~2.5-fold, respectively) with their specific drug exposures are highly consistent with the in vitro spike-in study (Fig. 2A). A very modest numerical increase in PT post-treatment was observed in all treatment groups, including vehicle, with the 0.3 and 1 mg/kg groups reaching statistical significance at 80 minutes (Fig. 6C).

**Discussion**

FXII is an emerging promising target for a number of diseases. rHA-WT-inf has been used extensively for interrogating the role of FXII in thrombosis, hemostasis, and additional pathologic settings. However, studies from us (Xu et al., 2014) and others (Kolyadko et al., 2015) demonstrated
that rHA-WT-inf carries significant off-target activity on FXa and plasmin that may skew its effect on thrombosis and hemostasis in vivo at high concentrations. We were thus interested in exploring alternative FXIIa inhibitors that may carry an improved selectivity profile. Our in vitro enzyme assays (Table 1) demonstrated that the HA fusion with Inf4mut15, dubbed as rHA-Mut-inf in this report, has an on-target effect on FXIIa that is comparable with that on

#### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_i$</th>
<th>$K_{\text{app, 30% plasma}}$</th>
<th>% Free in 100% Plasma</th>
<th>rHA-Mut-inf at 2× aPTT</th>
<th>% XIIa&lt;sub&gt;Bound&lt;/sub&gt; at 2× aPTT</th>
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<td>Human</td>
<td>0.08</td>
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<td>3.21%</td>
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<td>99.93%</td>
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<td>Rabbit</td>
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<td>0.82</td>
<td>9.97%</td>
<td>260</td>
<td>99.15%</td>
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$^aK_{\text{app, 30\% plasma}}$ indicates the value of $K_i$ observed in the presence of 30% heat-inactivated plasma.

$^b$Estimated using the equation described in the Materials and Methods.

$^c$Mut-inf at 2× aPTT is the concentration of rHA-Mut-inf associated with a 2-fold increase in aPTT time.

$^d$% XIIa<sub>Bound</sub> at 2× aPTT is the percentage of XIIa in complex with rHA-Mut-inf at concentration yielding 2× aPTT.

Fig. 5. Dose-dependent effect of rHA-Mut-inf on FeCl<sub>3</sub>-induced arterial thrombosis and cerebral MES. rHA-Mut-inf dose-dependently inhibited 30% FeCl<sub>3</sub>-induced carotid arterial thrombosis (vehicle, $n = 8$; 0.1 mg/kg i.v., $n = 8$; 0.3 mg/kg i.v., $n = 9$; and 1.0 mg/kg i.v., $n = 6$) as illustrated using carotid blood flow within 60 minutes upon FeCl<sub>3</sub> injury (A), AUC of the integrated blood flow (B), and reduction in clot weight (C). MES was monitored simultaneously in the ipsilateral MCA to the FeCl<sub>3</sub> injury. (D) Representative image for MES detection by TCD in a vehicle-treated animal is illustrated with a snapshot of TCD recording in a period of 4 seconds. The MES was indicated in an arrow determined by Sonora software and confirmed manually. Mean frequency (E) and incidence (F) of MES in MCA was also dose-dependently reduced. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus vehicle.
WT-inf, with higher selectivity against FXa and plasmin, which is consistent with the findings of another report of in vitro characterization of the Infestin-4 mutants without HA fusion (Kolyadko et al., 2015). In the whole-blood TEG assay, at 1 and 2 μM, rHA-Mut-inf and WT-inf prolonged clotting time (R) to the same extent, whereas at 4 μM rHA-Mut-inf was more potent; rHA-Mut-inf did not modulate tPA-induced fibrinolysis, whereas rHA-WT-inf dose-dependently suppressed fibrinolysis, likely due to its off-target activity on plasmin. It is possible that at the highest dose (4 μM) tested, the strong inhibition of plasmin by rHA-WT-inf has dampened its anticoagulant activity, resulting in a lesser prolongation of R compared with rHA-Mut-inf at 4 μM. Although the current tPA-induced fibrinolysis TEG may not mimic all components in vivo by the way in which FXIIa modulates fibrinolysis, the system has been previously benchmarked with a number of thrombin inhibitors yielding results that are consistent with expectations (Xu et al., 2013), and the contrast we observed between rHA-Mut- and WT-inf in fibrinolysis is consistent with their in vitro activity in plasmin in a purified system. Overall, these in vitro results demonstrate that rHA-Mut-inf indeed is a much more selective molecule than rHA-WT-inf, which is consistent with findings from another group using different methods (Kolyadko et al., 2015). As the potency of rHA-Mut-inf exceeds that of 3F7 (a monoclonal antibody inhibitor of FXIIa, $K_I = 6.2$ nM) (Larsson et al., 2014) and FXII618 (a peptide inhibitor of FXIIa, $K_I = 22$ nM) (Baeriswyl et al., 2015), rHA-Mut-inf is an attractive tool molecule for future research on the role of FXII in vivo in various pathologic settings.

With regard to the in vitro aPTT studies, rHA-Mut-inf was much less potent in human and nonhuman primate plasma compared with rabbit plasma (Fig. 2A). This is consistent with what was observed with 3F7, and with the hypothesis that FXII-independent activation of FXI may play a more prominent role in primates than in lower species (Gailani et al., 2015). For the in vitro PT studies, appreciable increases in PT were not observed except at very high concentrations (20-30 μM), and these PT increases could possibly be due to the residual off-target activity of rHA-Mut-inf in FXa, FIIa, and/or FVIIa (Table 1). It is therefore important to monitor the actual exposures of rHA-Mut-inf to appropriately interpret the results of in vivo studies.

With regard to the mode of inhibition, our previous analysis (Xu et al., 2014) as well as X-ray crystallography (Campos et al., 2004a, 2012) and docking studies (Kolyadko et al., 2015) with the active proteases suggest that the Infestin family of peptides acts as competitive, reversible inhibitors of the active site of the coagulation factors and additional serine proteases. Our kinetics studies with rHA-Mut-inf using the small tripeptide substrate (Fig. 1) confirmed this notion. Our SPR-based binding studies (Fig. 1B) further demonstrated that rHA-Mut-inf specifically binds FXIIa but not FXII zymogen. It is therefore unlikely that rHA-Mut-inf blocks the activation of FXII zymogen to form FXIIa.

As rHA-Mut-inf is a highly selective inhibitor of FXIIa and its mode of inhibition is akin to typical small-molecule inhibitors of serine proteases, we were interested in using it in assessing the level of target engagement or enzyme occupancy needed for FXIIa for the active site inhibitor mechanism. From a drug discovery standpoint, this is a very relevant question, as higher levels of requisite on-target enzyme occupancy will dictate a more stringent selectivity profile and often a higher hurdle for the absorption, distribution, metabolism, and excretion properties of the molecule, especially for an intended oral agent. Using a novel plasma shift assay (Fig. 4), we first determined the percentage of plasma protein binding (likely mediated by HA) for rHA-Mut-inf across species. We then selected a putative target of aPTT doubling in calculating enzyme occupancy. Although the levels of aPTT prolongation needed for antithrombotic efficacy via targeting FXII in humans are unknown, previous findings with a FXII monoclonal antibody (15H8, targeting heavy chain
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of FXII) in baboon (Matafonov et al., 2014) suggest that 2× aPTT prolongation is associated with appreciable antithrombotic efficacy in nonhuman primate and is a reasonable aPTT target, recognizing that aPTT target for translatable efficacy for different modes of inhibition could be different. The calculated enzyme occupancies for FXIIa active site inhibitors for doubling aPTT in human, cynomolgus macaque, and rabbit plasma were 99.9%, 99.9%, and 99.2%, respectively (Table 2). This is in contrast with the previous findings that 75% of the antisense oligonucleotide–mediated loss of FXII zymogen (Revenko et al., 2011) or 82% of the small interfering RNA–mediated loss of FXII zymogen (Cai et al., 2015) was sufficient in delivering robust antithrombotic efficacy in various models in rodents, with the caveat that there could be a species difference in target-based pharmacology. The very high level of calculated enzyme occupancy needed for FXIIa active site inhibitors is perhaps unsurprising considering that FXIIa is the most upstream factor in the intrinsic cascade and any “leakage” with a tiny amount of FXIIa may be subsequently cascaded and amplified. One precedent of a very high level of target engagement for an active site inhibitor of a coagulation factor is apixaban, a FXa inhibitor, requiring >99% calculated enzyme occupancy to deliver clinically relevant efficacy (Ankrom et al., 2016). A third potential reason could be that thrombin-mediated activation of FXII will bypass FXII, thus requiring more complete inhibition of FXIIa and immediate shut-down of thrombin generation.

As FXII deficiency confers no bleeding risk, targeting FXII for the treatment of ischemic stroke could be a promising strategy. MES has been considered as an independent predictor of stroke or transient ischemic attack (Gao et al., 2004; Markus et al., 2005), and MES originating from the carotid artery is considered to be one of the primary sources of stroke in patients (Bonati et al., 2010; Yavin et al., 2011). Our findings in the rabbit MES study thus provided preclinical evidence in supporting the therapeutic potential of FXIIa inhibition in the treatment and prevention of ischemic stroke and additional thromboembolic disorders. Our findings also strengthened the findings of previous reports (Kleinschnitz et al., 2006; Krupka et al., 2016) on the beneficial effects of targeting FXII in cerebral ischemia and reperfusion from different models and species. On the other hand, more than 2.0× aPTT prolongation was needed to fully restore blood flow in this rabbit MES model (Fig. 5A; Fig. 6B), more than 99.0% target engagement will be needed, based on our estimate from in vitro analysis (Table 2). It is to be noted that although our scheme of dosing at 20 minutes prior to vascular injury may ensure adequate and consistent coverage of rHA-Mut-inf during the study, based on the findings of a prior pharmacokinetic study with rHA-WT-inf (Hagedorn et al., 2010), the implications of FXIIa inhibition for stroke prophylaxis may need to be further addressed in studies with tool molecules that may allow more flexible dosing paradigms. Another limitation of our study is that neurologic deficits and ischemic brain injury could not be assessed under the current experimental setting (i.e., a nonrecovery procedure and with FeCl3 injury of the carotid artery for 60 minutes to end of study).

In summary, rHA-Mut-inf is a highly potent and selective active site inhibitor of FXIIa. Rabbit MES studies with rHA-Mut-inf suggest that FXIIa inhibition may effectively reduce both thrombus formation and thrombus embolization. However, significant challenges in chemical tractability may exist in developing an oral small-molecule drug-targeting active site of FXIIa. Alternative strategies and alternative modes of inhibition in targeting FXII thus should be considered.

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Authorship Contributions

Participated in research design: Barbieri, Wang, Seiffert, Gutstein, and Chen

Conducted experiments: Barbieri, Wang, Wu, Zhou, Ogawa, O’Neill, Chu, and Castriotia

Contributed new reagents or analytic tools: Barbieri, Wang, and Chen

Performed data analysis: Barbieri, Wang, Wu, Zhou, Ogawa, O’Neill, Chu, Castriotia, and Chen

Wrote or contributed to the writing of the manuscript: Barbieri, Wang, Seiffert, Gutstein, and Chen

References


Korneeva VA, Trubetskov MM, Korshunova AV, Lushcheikina SV, Kolyadko VN, Sergienko OV, Lunin VG, Pantelikos MA, and Asaulakhanan FI (2014) Interactions...
Factor XIIa as a novel target for thrombosis: target engagement requirement and efficacy in a rabbit model of microembolic signals

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Supplemental Figure 1. *rHA-Mut-inf inhibition of selected human and rabbit serine proteases.* Concentration dependent effects of rHA-Mut-inf on selected human (A) or rabbit (B) serine proteases. The enzymes tested are indicated in the legend. Solid lines represent the nonlinear least squares analysis of the data using a four parameter dose-response analysis.
Supplemental Figure 2. *rHA-WT-inf inhibits human FXIIa with pM potency*. Time course of inhibition of FXIIa proteolysis of fluorescent substrate by rHA-WT-inf. Concentrations in nM of rHA-WT-inf are indicated. Solid lines represent the nonlinear least squares analysis of the data as described in Materials and Methods.
Supplemental Figure 3. rHA-Mut-inf inhibition of human, cynomolgus, and rabbit FXIIa

FXIIa Inhibition of human (A), cynomolgus (B), or rabbit (C) FXIIa protease activity by rHA-Mut-inf following a 2 hr pre-incubation of the enzymes with the inhibitor.
Concentrations of rHA-Mut-inf are indicated in the legend. Solid lines represent the nonlinear least squares analysis of the data as described in Materials and Methods.