Inhibition of Factor XIa Reduces the Frequency of Cerebral Microembolic Signals Derived from Carotid Arterial Thrombosis in Rabbits


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Running Title Page:

a) Running Title: FXIa inhibitor reduces cerebral microembolic signals

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aPTT: activated partial thromboplastin time; ASO: antisense oligonucleotide; FXa: Factor Xa; FXIa: Factor XIa; MCA: middle cerebral artery; MES: microembolic signals; NOACs: novel oral anticoagulants; PT: prothrombin time; SPAF: stroke prevention in atrial fibrillation; TCD: transcranial Doppler; TIA: transient ischemic attack; VKA: vitamin K antagonists; VTE: venous thromboembolism

d) A recommended section assignment: Cardiovascular
Abstract

Factor XI (FXI) is an integral component of the intrinsic pathway of the coagulation cascade and plays a critical role in thrombus formation. Since its role in the pathogenesis of cerebral microembolic signals (MES) is unclear, this study utilized a potent and selective small molecule inhibitor of FXIa, Compound 1, to assess the effect of FXI blockade in our recently established preclinical model of cerebral MES induced by FeCl₃ injury of the carotid artery in male New Zealand White rabbits. Ascending doses of Compound 1 were evaluated simultaneously for both carotid arterial thrombosis by a Doppler flowmeter and MES in the middle cerebral artery by a transcranial Doppler. Plasma drug exposure and pharmacodynamic responses to Compound 1 treatment were also assessed. The effective dose for 50% inhibition (ED₅₀) of thrombus formation was 0.003 mg/kg/h Compound 1, i.v. for the integrated blood flow, 0.004 mg/kg/h for reduction in thrombus weight, and 0.106 mg/kg/h for prevention of MES. The highest dose, 3 mg/kg/h Compound 1, achieved complete inhibition in both thrombus formation and MES. In addition, we assessed potential bleeding liability of Compound 1 (5 mg/kg/h, i.v., >1,250-fold ED₅₀ levels in arterial thrombosis) in rabbit using a cuticle bleeding model, and observed about 2-fold (non-significant) prolongation in bleeding time. In summary, our study demonstrates that Compound 1 produced a robust and dose-dependent inhibition of both arterial thrombosis and MES, suggesting that FXIa blockade may represent a novel therapeutic strategy for the reduction in MES in patients at risk for ischemic stroke.
Introduction

Factor XI (FXI) is an integral component in the coagulation cascade. Upon contact activation of the intrinsic pathway of the coagulation cascade, the zymogen form of FXI is activated by FXIIa to form the enzymatically active form FXIa. FXIa in turn cleaves and activates its physiological substrate, FIX, which ultimately leads to thrombin generation and clot formation (Gailani et al., 2015). FXI is a dimer composed of identical 80 KDa subunits, and each subunit starting from the N-terminus consists of four apple domains (A1-A4) and a catalytic domain.

Multiple lines of evidence have demonstrated a crucial role for FXI in thrombus formation, with a relatively small contribution to hemostasis (Duga and Salomon, 2013). Human genetics and epidemiological studies showed that severe FXI deficiency (hemophilia C) confers a reduced risk of ischemic stroke and deep vein thrombosis, whereas increased levels of FXI are associated with a higher risk for venous thromboembolism (VTE) and ischemic stroke (Duga and Salomon, 2013; Gailani et al., 2015). Likewise, preclinical models of FXI deficiency demonstrated profound protection from both arterial and venous thrombosis (Wang et al., 2005; Wang et al., 2006), as well as ischemic brain injury (Kleinschnitz et al., 2006). Furthermore, a recent phase 2 clinical study revealed superior antithrombotic efficacy for the prevention of VTE in patients undergoing total knee arthroplasty treated with FXI antisense oligonucleotide (ASO) compared with enoxaparin (an indirect inhibitor of FXa) (Buller et al., 2015).

Cerebral embolism is one of the critical causes for ischemic stroke, and microembolic signal (MES) is characterized as an independent predictor of stroke or transient ischemic attack (TIA) (Gao et al., 2004; Markus et al., 2005). In spite of genetic evidence from both clinical and preclinical models that suggested a potential association between FXI deficiency and protection
from ischemic stroke (Kleinschnitz et al., 2006; Salomon et al., 2008), no evidence has shown whether FXI/FXIIa blockade could provide therapeutic benefit from ischemic brain injury, or have salutary effects on translatable predictors of ischemic brain injury such as MES. In an effort to explore therapeutic potential for FXI/FXIIa mechanism in ischemic brain injury, we took advantage of our recently established preclinical model of cerebral MES and demonstrated the therapeutic benefits of both clopidogrel and aspirin (Zhou et al., 2016a). These two antiplatelet agents have been shown for their strong correlation between reduction of MES and stroke prevention in patients (Markus et al., 2005; Wong et al., 2010). Since there was no evidence available for the effects of anticoagulants on MES, we used apixaban, a small molecule inhibitor of FXa, as a tool compound and demonstrated its protection from MES and thrombotic events in this rabbit cerebral MES model (Zhou et al., 2016b).

In the current study, we investigated the effects of a potent and selective small molecule inhibitor of FXIIa, Compound 1 in the same model of cerebral MES. Compound 1 was synthesized following the procedures described in detail previously (International Publication Number WO 2011/100401 A1). Along with the assessment of effects of ascending doses of Compound 1 on arterial thrombosis and cerebral MES, we evaluated the correlation between plasma drug exposure and pharmacodynamic parameters in response to treatment. Meanwhile, we evaluated the potential bleeding liability of Compound 1 using a rabbit model of cuticle transection.
Material and Methods

Animals

All animal studies were carried out in male New Zealand White rabbits weighing 2.4–3.0 kg and approximately 10-13 weeks of age (obtained from Charles River Canada). All 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 Institution’s Animal Care and Use Committee of Merck Research Laboratories.

Rabbit Model of Cerebral Microembolic Signals Derived from FeCl₃-induced Carotid Arterial Thrombosis

The rabbit model of cerebral MES was induced by FeCl₃ injury of the carotid artery as described in detail previously (Zhou et al., 2016a) (Zhou et al., 2016b). Briefly, animals were anesthetized with a cocktail (50 mg/kg ketamine HCl, Pfizer Inc., and 5 mg/kg xylazine, LLOYD Inc., IM) as a non-recovery procedure. The left common carotid artery (CCA) was surgically exposed. A Doppler flow probe (Model 1.5 or 2.0 PRB, Transonic Systems, Ithaca, NY, USA) connected to a flow meter (Model T403, Transonic Systems) was used to measure the artery blood flow with continuous data acquisition by a PowerLab 16/35 and LabChart Pro system (AD Instruments, Colorado Springs, CO, USA). Thrombosis was induced by applying two pieces of filter papers (7.4 mm in diameter, 0.5 mm thick each, above and beneath the vessel; Life Technologies, Grand Island, NY, USA) pre-saturated with 30% FeCl₃ (Anhydrous 98%, Cat# 169430050, ACROS Organics/Thermo Fisher Scientific, Waltham, MA, USA) to the adventitial surface of the vessel. A piece of para-film was placed underneath the vessel to protect the surrounding tissue from injury. The filter papers were removed after 5 min followed by washout of residue FeCl₃ with
warm saline. The blood flow was monitored for 60 min from the time of FeCl₃ application (as time zero). Integrated carotid blood flow over 60 min was measured by area under the curve (AUC), calculated by the trapezoidal rule, and expressed as percent of control blood flow as described previously (Wong et al., 2008b). At end of study (i.e., 60 min 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, USA).

For MES detection, we used the clinical SONARA™ TCD System (Nicolet Natus Neurology Inc., Middleton, WI, USA) and continuously monitored the blood flow velocity and MES in the ipsilateral MCA for 60 min upon FeCl₃ injury. A pulse wave 2 MHz probe (OD=11.3 mm, 90 mm long, focused at 12 to 25 mm, customized by MTB Medizentechnik Basler AG, Switzerland) was fixed by a flexible-arm magnetic-base holder (McMaster-Carr, Princeton, NJ, USA) at the posterior end of zygomatic bone of the rabbits, at an angle of ~80 degree against the buccal surface. The MCA was insonated at a depth between 19 to 22 mm as described in detail previously (Zhou et al., 2016a). MES (defined as High Intensity Transient Signals, HITS) was recorded and confirmed based on the criteria defined by the International Consensus Committee (Ringelstein et al., 1998; Symposium, 1995) as described previously (Zhou et al., 2016a) (Zhou et al., 2016b).

**Rabbit Cuticle Bleeding Model**

The rabbit cuticle BT model was carried out as described previously (Wong et al., 2008a). Briefly, rabbits were anesthetized, and the apex of the cuticle was cut with a razor blade. The wound site was super-fused with 37 °C lactated Ringer’s solution to allow blood flow freely.
Bleeding time was recorded when bleeding ceased, with a maximal bleeding time set for 30 min. Three nail cuticles per rabbit were measured, and an average of the bleeding time from the three cuticles was considered as one data point.

**Drug Administration**

The FXIa inhibitor Compound 1 (structure shown in Fig. 1), with a molecular weight of 596.6 Daltons, was synthesized following the procedures described in detail previously (International Publication Number WO 2011/100401 A1) at Merck Research Laboratories (Kenilworth, NJ, USA). Table 1 summarizes the potency and selectivity parameters (details on Methods described in the following section) of the compound.

For the efficacy study (i.e., the 30% FeCl₃-induced carotid arterial thrombosis and cerebral MES study described above), various doses (0-3 mg/kg/h) of Compound 1 or vehicle (35% hydroxypropyl β-cyclodextrin in 10 mM phosphate buffer, pH 7.0) were continuously infused at 2 mL/kg through the marginal ear vein. The i.v. dosing started 60 min prior to vessel injury and ended upon study completed. A dose of 5 mg/kg/h Compound 1 was infused for the cuticle bleeding study; otherwise the dosing regimen was the same as that which was used for the efficacy studies. This dosing regimen for Compound 1 was predicted to accomplish sufficient exposures in our study based on a previous internal standard pharmacokinetics study in rat (not shown), and validated in selected time points in the current rabbit MES and cuticle bleeding models under current dosing regimen. Apixaban (a FXa inhibitor, synthesized at Merck; (Zhou et al., 2016b)) at 3 mg/kg/h, i.v., was used as a control for the cuticle bleeding study.

**In Vitro Protease Inhibition Assays**
Human FXIa was purchased from Sekisui Diagnostics (Lexington, MA, USA). Rabbit FIIa and FXa were purchased from Enzyme Research Laboratories. Rabbit plasma kallikrein, FIXa, and FVIIa were prepared internally (Merck Research Laboratories, Kenilworth, NJ, USA). Rabbit FXIa was purified internally after activating rabbit FXI with human FXIIa, both purchased from Enzyme Research Laboratories. Rabbit FXIIa was prepared by Evotec, Inc. (Princeton, NJ, USA). The 7-amido-4-thifluoromethylcoumarin (AFC) containing fluorescence substrate, CH3SO2-cyclohexyl-Gly-Gly-Arg-AFC (cyclohexyl-G-G-R-AFC) was custom synthesized by CPC Scientific (Sunnyvale, CA, USA), while substrates N-CBZ-Gly-Pro-Arg-AFC (Z-G-P-R-AFC), and n-acetyl-Gly-Pro-Arg-AFC (Acetyl-K-P-R-AFC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All enzymatic reactions were carried out in 50 mM HEPES, 150 mM NaCl, 5 mM CaCl_2, and 0.1% PEG (8000 NMW), at pH 7.4 and 25°C using Corning 3575 384-well assay plates. Fluorescence signal was measured after 60 minutes elapsed reaction time using an Envision 2101 plate reader set at 405 nm excitation and 510 nm emission. Calculated percent inhibition values were plotted versus inhibitor concentration to determine IC_{50}. IC_{50} values were subsequently converted to K_I using the Cheng-Prusoff equation (Zhang and Windsor, 2013).

**Plasma Drug Exposure and Ex Vivo Clotting Time Assays**

Blood samples were collected into sodium citrate (at 3.2% final concentration) vacutainers (Becton Dickinson, Franklin Lakes, NJ) from either the central ear artery or the carotid artery at terminal bleed. Blood samples were centrifuged at 2000 x g for 15 min at 4 °C for plasma preparation and evaluated for PK and ex vivo clotting time assays.
For Compound 1 PK analysis, the plasma samples, plasma standards and quality control stock (10 μL plasma) were assessed after protein precipitation in an acetonitrile crash solution (300 μL) containing labetalol (200 nM), diclofenac (200 nM), imipramine (200 nM) and alprazolam (100 nM). Following automated sample preparation, the supernatant was then analyzed by liquid chromatography (LC)-mass spectrometry (MS)/MS for Compound 1 using a Thermo Scientific LX-2 system (Thermo Fisher Scientific, Waltham, MA) for LC and Applied Biosystems / MSD Sciex API 5500 Q-Trap (Applied Biosystems, Forster City, CA) for MS analysis. Specifically, water, 0.1% formic acid was used as mobile phase A solution, and acetonitrile, 0.1% formic acid as mobile phase B solution. An autosampler wash 1, containing 2% ammonium hydroxide in 5 mM aqueous ammonium acetate / acetone/ isopropanol / acetonitrile / methanol (1/1/1/1/1, v/v/v/v/v), and autosampler wash 2, containing methanol / water / formic acid (10/89/1, v/v/v), were used. The MonChrom C18, 100x2.0 mm, 3 μm column was used at 50 ºC. A gradient solution containing 95-5% of mobile phase A solution in combination with 5-95% of mobile phase B solution was used to elute the column. Plasma drug exposure for apixaban was analyzed as described previously (Zhou et al., 2016b).

*Ex vivo* plasma activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined by standard methods using aPTT-XL (Pacific Haemostasis, Waltham, MA, USA) and TriniCLOT PT Excel (Tcoag, Bray, Ireland), respectively, on a KC4 Delta coagulation analyzer (Tcoag, Bray, Ireland).

**Data Analysis and Statistics**

Data are presented as mean ± standard error (SE), and analyzed using one-way ANOVA followed by Bonferroni post hoc test in GraphPad Prism (Version 7, Graphpad, La Jolla, CA) for
comparison among groups at different doses. Paired t test was applied for comparisons between paired groups. EC₅₀, defined as doses for half-maximal effect, was determined by a non-linear four-parameter dose-response curve fit using GraphPad Prism under the dose ranges described in each figure legend. A Chi-square and Fisher’s test was applied to determine the association between drug exposure at each treatment dose and efficacy or pharmacodynamic read-out. Results were considered significant when p < 0.05.
Results

Effect of Compound 1 on cerebral MES induced by carotid arterial thrombosis in rabbits

Compound 1 dose-dependently inhibited thrombus formation in the carotid artery and MES incidence in the MCA induced by 30% FeCl₃ injury of the carotid artery in rabbits (Fig. 2). The integrated blood flow (AUC, illustrated as % of blood flow relative to baseline) was increased from 57.2 ± 7.7% in vehicle group (n=9), to 90.6 ± 3.6% for the 3 mg/kg/h Compound 1 group (n=6, p<0.01), with an ED₅₀ of 0.003 mg/kg/h Compound 1, i.v. (Fig. 2A and B). Clot weight was reduced from 8.9 ± 1.1 mg (vehicle) to 0.0 ± 0.0 mg (3 mg/kg/h Compound 1, p<0.001), with an ED₅₀ of 0.004 mg/kg/h Compound 1, i.v. (Fig. 2C).

The simultaneous monitoring of cerebral MES demonstrated the dose-dependent inhibition of Compound 1 on MES. Fig. 2D illustrates a representative MES recording in the MCA in rabbits treated with 0.0003 mg/kg/h Compound 1. The mean MES frequency was reduced from 4.0 ± 0.4 (vehicle) to 0.0 ± 0.0 (3 mg/kg/h Compound 1, i.v., p<0.001), with an ED₅₀ of 0.106 mg/kg/h Compound 1, i.v. (Fig. 2E). The incidence of MES detected in animals was also decreased from 100% (vehicle) to 0% (3 mg/kg/h Compound 1, i.v., p<0.001) (Fig. 2F).

Plasma drug exposure and pharmacodynamic analysis of Compound 1 in the rabbit MES model

We measured the plasma drug exposure of Compound 1 under the current dosing regimen to assess the correlation between plasma drug levels and in vivo efficacy, and ex vivo pharmacodynamic readouts (PT and aPTT). As illustrated in Fig. 3A, Compound 1 achieved a steady state plasma exposure during the period (i.e., between 1 and 2 hours of i.v. dosing) of arterial thrombosis and MES monitoring. It also confirmed the dose-related increase in drug
exposure, with 0.07 ± 0.01, 0.66 ± 0.26, 2.16 ± 0.49 and 9.94 ± 1.00 µM at 1 hour, and 0.04 ± 
0.01, 0.59 ± 0.19, 1.60 ± 0.30 and 7.54 ± 0.50 µM at 2 hours post treatment with 0.003, 0.03, 0.3 
and 3 mg/kg/h, i.v., doses of Compound 1, respectively.

*Ex vivo* PT and aPTT analysis confirmed pharmacodynamic responses for Compound 1 (Fig. 3B 
and C). Compound 1 significantly elevated aPTT, with 86.6 ± 9.8% (p<0.001) and 220.3 ± 10.3% 
(p<0.001) increase at 1 hour, and 78.7 ± 15.0% (p<0.001) and 290.4 ± 10.9% (p<0.001) increase 
at 2 hours post treatment with 0.3 and 3 mg/kg/h, i.v., respectively, vs. vehicle (Fig. 3B). In 
contrast, Compound 1 had no effects on PT (Fig. 3C).

Fig. 4 illustrates strong correlations between Compound 1 plasma exposure and inhibition of 
carotid arterial thrombosis (the integrated blood flow, r² = 0.94; clot weight, r² = 0.80), cerebral 
MES (r² = 0.74), and pharmacodynamic biomarker aPTT (r² = 0.99). As expected for the FXIa 
mechanism, weak correlation was observed between Compound 1 plasma exposure and PT 
inhibition (r² = 0.39).

**Effects of Compound 1 on cuticle bleeding time in rabbits**

Fig. 5A illustrates the effects of 5 mg/kg/h Compound 1, i.v., on potential bleeding liability in a 
rabbit cuticle bleeding model. Under this particular dose (>1,250x antithrombotic ED₅₀ as shown 
in Fig. 2), a modest and non-significant increase in bleeding time (102% increase over vehicle 
treatment) was observed for Compound 1. In contrast, 3 mg/kg/h apixaban, i.v. (a positive 
control; ~ 75x antithrombotic ED₅₀ as shown in the same rabbit model (Zhou et al., 2016b)) 
significantly prolonged the bleeding time (291% increase over the vehicle, p<0.001, n=8). 
Plasma drug analysis confirmed the expected drug exposure for both Compound 1 and apixaban 
upon the rabbit nail cuticle transection (i.e., 60 min after i.v. infusion) (Fig. 5B). Likewise, ex
vivo analysis of pharmacodynamic biomarkers, PT and aPTT, confirmed the desired drug exposure for both Compound 1 and apixaban in this bleeding study (Fig 5C and D).
Discussion

Both FXI deficiency in humans (Duga and Salomon, 2013; Gailani et al., 2015) and in mice (Kleinschnitz et al., 2006) reduced stroke incidence and/or protected the brain from ischemic injury. However, there is no evidence that is currently available to date to show a therapeutic benefit for FXI/FXIIa inhibition in clinical indicators of risk of ischemic brain injury (Gailani et al., 2015), even though FXI inhibition has been clinically validated as an anti-thrombotic approach for the prevention of venous thromboembolism (VTE) in patients undergoing total knee arthroplasty when treated with FXI antisense oligonucleotide (ASO) (Buller et al., 2015).

Anticoagulation with vitamin K antagonists (VKA) has been the gold standard for stroke prevention in atrial fibrillation (SPAF) for decades. However, management of VKA therapy is complicated due to the narrow therapeutic index, slow onset and offset of action, and numerous dietary and drug interactions. In recent years, a new class of non-vitamin K antagonist oral anticoagulants (NOACs; including direct thrombin receptor inhibitor dabigatran, and FXa inhibitors such as rivaroxaban and apixaban) were approved for SPAF based on their similar or superior benefit-risk profiles compared to warfarin (Granger et al., 2011; Lin et al., 2015; Morais and De Caterina, 2016). Nevertheless, NOACs are still associated with a significant bleeding risk. Therefore, there is a need for novel anticoagulants that confer non-inferior efficacy with significantly reduced bleeding liability for the treatment of SPAF. FXI blockade mechanisms may meet those criteria and thus may have promising therapeutic potential for SPAF.

In this work, we describe the use of Compound 1, a small molecule inhibitor of FXIIa, in a rabbit model of cerebral MES. Compound 1 achieved full efficacy in both arterial thrombosis and cerebral MES at 3 mg/kg/h, i.v. (Fig 2), which was similar to that of 0.5 mg/kg/h apixaban, i.v., in this rabbit model (Zhou et al., 2016b). The EDs0 for Compound 1 on inhibition of thrombus
formation was 0.003 mg/kg/h, i.v., for integrated blood flow and 0.004 mg/kg/h for thrombus weight, and inhibition of cerebral MES was 0.106 mg/kg/h (Fig 2). The ED$_{50}$ for apixaban was 0.04 and 0.13 mg/kg/h for the integrated blood flow and thrombus weight, respectively, and 0.03 mg/kg/h for MES as reported previously (Zhou et al., 2016b). In our previous study, the ED$_{50}$ for aspirin was defined as 3.1, 4.2 and 12.7 mg/kg for integrated blood flow and thrombus weight and MES, respectively, and the ED$_{50}$ for clopidogrel was 0.30, 0.28 and 0.25 mg/kg for integrated blood flow, thrombus weight and MES, respectively, using the same rabbit model of MES (Zhou et al., 2016a). Almost complete inhibition of MES could be achieved with 25 mg/kg aspirin or 3 mg/kg clopidogrel treatment, respectively (Zhou et al., 2016a). Since MES has been considered as an independent predictor of stroke or transient ischemic attack (TIA) (Gao et al., 2004; Markus et al., 2005), and MES in the setting of carotid origin is considered as one of the primary sources in patients (Bonati et al., 2010; Yavin et al., 2011), the data we present here provide preclinical evidence to support therapeutic potential of FXI/FXIa blockade in SPAF.

We would like to point out certain caveats or limitations regarding the current preclinical model and results. First, the antithrombotic efficacy of FXIa blockade for arterial thrombosis appears to be ~26x more potent than MES. This difference may reflect in part the sub-optimal condition for arterial thrombosis induced by 30% FeCl$_3$ (Zhou et al., 2016a). As demonstrated previously in rodents, the antithrombotic efficacy may dependent on the concentrations of FeCl$_3$ applied to induce the arterial thrombosis (Wang et al., 2005). Thus, it might be more effective for arterial thrombosis than for MES. In addition, the current model is not able to detect MES less than 100 μM in diameter due to the limitation of the clinical TCD instrument, which might impact the determination of ED$_{50}$ for Compound 1 on MES. Second, while the relatively low frequency of MES in this preclinical model mimics what has been observed in patients, the low MES number
poses difficulty in accurately defining the dose-response curve of Compound 1 for the ED$_{50}$.

Third, the current study covers only the prevention paradigm (i.e., Compound 1 was given one
hour prior to induction of vascular injury with FeCl$_3$) but not the treatment paradigm (i.e., given
the compound post injury). The treatment model remains to be explored for MES to learn if there
might be impact on clot stability and thus efficacy, and if FXIa blockade might be of value as a
treatment option in clinic. Fourth, both neurological deficits and ischemic brain injury could not
be assessed under the current experimental setting since it is a non-recovery procedure and the
carotid artery injury/MES generation occurred only for 60 min. Lastly, while plasma kallikrein
inhibition or deficiency only confers modest thromboprotection (Bird et al., 2012; Chen et al.,
2015), one could not exclude the possibility that some off-target activity on plasma kallikrein
from Compound 1 may have contributed to efficacy (based on ~6-fold selectivity of FXIa over
plasma kallikrein; Table 1). Thus, we evaluated the ex vivo plasma kallikrein activity for our
dose-dependent study on MES (Supplemental Fig. 1). The data may allow us to exclude the
potential contribution by cross-activity with plasma kallikrein in our current study.

Bleeding liability of current anticoagulant therapies is a major concern. Head-to-head
comparison between Compound 1 (5 mg/kg/h, i.v., at >1,250x antithrombotic ED$_{50}$ plasma drug
level) and apixaban (3 mg/kg/h, i.v., at ~ 75x antithrombotic ED$_{50}$ plasma drug level) was
conducted and showed much less bleeding liability for Compound 1 than for apixaban in the
rabbit cuticle bleeding model (Fig. 5). A modest and non-significant increase in bleeding time
(102% increase over vehicle) was noted for 5 mg/kg/h Compound 1. In addition, our current data
appear to be more sensitive in prolongation of bleeding time in response to 3 mg/kg/h apixaban
treatment, i.e., 993 ± 141 seconds (Fig. 5) vs. that of previously reported at ~ 300 seconds for 2.1
mg/kg/h apixaban (Wong et al., 2008b). The reason for this difference is unknown. While a
similar bleeding model was used in these two studies, some variables still exist, such as different operators, laboratories, and animal sources. It also should be pointed out that the doses used for these two bleeding studies are not the same, and a full dose-dependent response might be needed. Overall, cross-validation of the model might be helpful to understand the differences. Nevertheless, from plasma drug exposure perspective, our data appear in agreement with clinical observation for apixaban since 3 mg/kg/h apixaban in rabbit achieved ~ 75x antithrombotic ED$_{50}$ plasma exposure vs. ~ 69x peak plasma levels in clinic (Frost et al., 2014).

In summary, our study demonstrated a dose-dependent inhibition of Compound 1 on both arterial thrombosis and MES. Pharmacokinetic and pharmacodynamic analyses demonstrated an excellent correlation between Compound 1 plasma drug exposure and efficacy, as well as aPTT. Our data showed that adequate doses of Compound 1 may achieve maximal reduction of MES, but with significantly less bleeding liability compared to apixaban as defined by the cuticle bleeding model. Thus, FXIa blockade may provide a promising new therapeutic approach for the treatment of thrombotic diseases such as SPAF.
Acknowledgments

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Authorship Contributions

Participated in research design: Wang, Edmondson, Ali, Andre, Seiffert, Erion, Gutstein, Chen

Conducted experiments: Wang, Kurowski, Wu, Castriota, Zhou, and Chu

Performed data analysis: Wang, Kurowski, Castriota, and Wu

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


Figure Legends

Figure 1. Structure of a small molecule FXIa inhibitor Compound 1.

Figure 2. Dose-dependent effect of Compound 1 on FeCl₃-induced arterial thrombosis and cerebral MES in rabbits. Compound 1 dose-dependently inhibited 30% FeCl₃-induced carotid arterial thrombosis (vehicle, n=9; 0.0003, i.v., n=9; 0.003 mg/kg/h, i.v., n=8; 0.03 mg/kg/h, i.v., n=8; 0.3 mg/kg/h, i.v., n=7; and 3.0 mg/kg/h, i.v., n=6) as illustrated by carotid blood flow within 60 min upon FeCl₃ injury (A), AUC of the integrated blood flow (B), and reduction in clot weight (C). MES was monitored simultaneously in the ipsilateral MCA by TCD for 60 min after FeCl₃ injury. A representative image for MES detection in 0.0003 mg/kg/h Compound 1, i.v., treated animal is illustrated (D). The MES is indicated as determined by the Sonora software (arrow) and was subsequently confirmed manually as described in detail in Methods. Compound 1 dose-dependently inhibited mean frequency (E) and incidence of MES (F) in the MCA. *p<0.05, **p<0.01, and ***p<0.001 vs. vehicle.

Figure 3. Plasma drug exposure and pharmacodynamic responses for Compound 1 on FeCl₃-induced arterial thrombosis and cerebral MES. Plasma samples were prepared in animals of each experimental group as illustrated in Fig. 2 prior to, or 1 and 2 hours after Compound 1 i.v. dosing. Plasma drug exposures (A), and ex vivo aPTT (B) and PT responses (C) are illustrated. *p<0.05, and ***p<0.001 vs. vehicle.

Figure 4. Correlation between Compound 1 plasma drug exposure and efficacy (arterial thrombosis and MES) or pharmacodynamic responses (aPTT and PT). Data on Compound 1 drug exposure are plotted with antithrombotic efficacy (in both arterial thrombosis and MES; Fig. 2) and ex vivo aPTT/PT (Fig. 3). Panels A and B show the correlation between drug exposure
and efficacy on integrated blood flow and clot weight, respectively. Panel C depicts the correlation between plasma drug levels with MES. Panel D illustrates the correlation between plasma drug levels and aPTT/PT. Data were analyzed using the GraphPad Prism 7 software, and the $R^2$ data are indicated.

**Figure 5.** Rabbit cuticle bleeding time study and plasma drug and pharmacodynamic measurements. Vehicle, Compound 1 (5 mg/kg/h, i.v.), or apixaban (3 mg/kg/h, i.v.) was infused for 60 min in anesthetized rabbits (n=9 each group) and subjected to nail cuticle transection as described in Methods. The wound site was super-fused with 37 °C lactated Ringer’s solution to allow blood flow freely, and a maximal bleeding time of 30 min was recorded. Three nail cuticles per rabbit were measured for an average of the bleeding time per data point. The cuticle bleeding data are illustrated in panel A. Plasma drug exposures are shown in panel B, and ex vivo aPTT and PT from the same groups of animals are illustrated in panels C and D. Percentages of increase for Compound 1 and apixaban in bleeding time, aPTT and PT over vehicle are indicated. **p<0.01, ***p<0.001, vs. vehicle.
Table 1. Compound 1 Selectivity Profiles

<table>
<thead>
<tr>
<th>Assays</th>
<th>Ki (nM)/Fold Selectivity</th>
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<tr>
<td>Human FXIa</td>
<td>0.3 nM</td>
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<tr>
<td>Rabbit FXIa</td>
<td>0.9 nM</td>
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<td>Rabbit FIIa</td>
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<td>Rabbit IXa</td>
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<td>Rabbit Xa</td>
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<td>Rabbit FXIIa</td>
<td>&gt;16,000 x</td>
</tr>
<tr>
<td>Rabbit Plasma Kallikrein</td>
<td>6 x</td>
</tr>
</tbody>
</table>
**JPET #238600 Fig 5**

**A**

Bleeding time (sec)

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>Compound 1</th>
<th>Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Increase</td>
<td>0</td>
<td>102</td>
<td>291</td>
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</tbody>
</table>

**B**

Plasma drug levels (µM)

**C**

aPTT (sec)

<table>
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<tr>
<th></th>
<th>Veh</th>
<th>Compound 1</th>
<th>Apixaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Increase</td>
<td>0</td>
<td>317</td>
<td>218</td>
</tr>
</tbody>
</table>

**D**

PT (sec)

<table>
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<th>Veh</th>
<th>Compound 1</th>
<th>Apixaban</th>
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</thead>
<tbody>
<tr>
<td>% Increase</td>
<td>0</td>
<td>3</td>
<td>111</td>
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</tbody>
</table>
Supplemental Data

Inhibition of Factor XIa Reduces the Frequency of Cerebral Microembolic Signals Derived from Carotid Arterial Thrombosis in Rabbits


Cardiometabolic Disease Biology (XW, SK, WW, GAC, XZ, PA, DS, ME, DEG, ZC), Discovery Pharmaceutical Sciences (LC), In Vitro Pharmacology (KE, DC), and Discovery Chemistry, Merck Research Laboratories, Kenilworth, NJ 07033, USA (SE); Lead Optimization Chemistry, Merck Research Laboratories, Rahway, NJ 07065, USA (AA)
Supplemental Methods:

Ex Vivo Plasma Kallikrein Activity Assay: Rabbits were dosed with vehicle or Compound 1, i.v., and subjected to the microembolic signal (MES) model as described in the Methods. The plasma samples were collected prior to dose (time 0 as the baseline), or 60 min (started for FeCl₃ injury of the carotid artery) and 120 min (at end of the study) post dosing. A volume of 2 μL of 100 μM ellagic acid (Product #21-405, R2 Diagnostics, Inc., South Bend, IN, USA) was added to 18 μL of plasma and incubated for 15 min at 37 °C. Plasma was the diluted to 6% in assay buffer (5 mM Tris pH 7.8, 50 mM NaCl, 0.1% BSA) containing 100 μM Z-FR-AMC substrate (Product #ES009, R&D Systems, Inc., Minneapolis, MN, USA). Fluorescence (Excitation 360 nm/ Excitation 465 nm) was read kinetically for 30 min at 37 °C in a Tecan Infinite M200 PRO plate reader (Tecan US, Inc., Morrisville, NC, USA).

Supplemental Figure 1 Legend. Ex vivo plasma kallikrein activity assay for dose-dependent study for Compound 1 on FeCl₃-induced arterial thrombosis and cerebral MES in rabbits. The same experiment groups for vehicle and Compound 1 (vehicle, n=9; 0.0003, i.v., n=9; 0.003 mg/kg/h, i.v., n=8; 0.03 mg/kg/h, i.v., n=8; 0.3 mg/kg/h, i.v., n=7; and 3.0 mg/kg/h, i.v., n=6) were used for the ex vivo assay as described in Fig. 1 and Supplemental Methods. The data are illustrated using mean ± SE. *p<0.05, and **p<0.01, vs. 0 min time point of the same group.
Supplemental Figure 1.

![Graph showing plasma kallikrein activity over time and compound dosage]