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A New Mechanism-Driven Bypassing Strategy for Direct Factor Xa Inhibitors Reveals An Unexpected Property of Target Specific Anticoagulants

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Abstract

Hemostasis is a crucial component of vascular homeostasis that prevents blood loss while also maintaining vascular patency. Hemostasis is achieved, in part, through a cascade of serine proteases that are sequentially activated, culminating in formation of the effector protease, thrombin. Normally, this process is tightly regulated, but loss of regulation can lead to bleeding or excessive clot formation (thrombosis). In the event of thrombosis, anticoagulation is the mainstay of care. Numerous pharmacokinetic problems with the oral anticoagulant warfarin prompted the development of new oral agents that directly inhibit the serine proteases of coagulation. In particular, several active site inhibitors of coagulation factor Xa (FXa) have recently been approved and are at least as effective as warfarin for the prevention of thrombosis. However, they, like warfarin, increase the risk of bleeding, and there are no approved countermeasures to treat or prevent bleeding with these direct FXa inhibitors. We evaluated whether a variant of FXa (FXaI[16]L) could reverse the effects of the direct FXa inhibitor rivaroxaban. FXaI[16]L has poor active site function and a long plasma half-life but has high catalytic activity at the site of vascular injury, making it an effective pro-hemostatic agent. FXaI[16]L potently reversed the effects of rivaroxaban in in vitro studies and animal models of coagulation. Kinetic studies revealed that both FXaI[16]L and wt-FXa are highly inhibited by rivaroxaban at therapeutic concentrations when bound to the cofactor FVa. Despite this high level of inhibition with rivaroxaban, both FXaI[16]L and wt-FXa support thrombin generation. To explain this discrepancy, we measured the kinetics of FXa inhibition by antithrombin III (ATIII), a key regulator of FXa activity in plasma. Rivaroxaban impaired ATIII-dependent FXa inhibition by creating a pool of reversibly-inhibited FXa, and kinetic simulations indicated that, under these conditions, a steady-state of free, uninhibited FXa is established. Thus, there is a paradoxical increase in the level of free FXa which explains how FXaI[16]L can generate thrombin in the presence of rivaroxaban. These results reveal a previously unreported, unintended consequence of direct FXa inhibitors that may have important implications. Further, FXaI[16]L may be able to fill the unmet clinical need for a rapid, hemostatic reversal agent for these new anticoagulants.

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A NEW MECHANISM-DRIVEN BYPASSING STRATEGY FOR DIRECT FACTOR Xa INHIBITORS REVEALS AN UNEXPECTED PROPERTY OF TARGET SPECIFIC ANTICOAGULANTS

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ABSTRACT

A NEW MECHANISM-DRIVEN BYPASSING STRATEGY FOR DIRECT FACTOR Xa INHIBITORS REVEALS AN UNEXPECTED PROPERTY OF TARGET SPECIFIC ANTICOAGULANTS

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Hemostasis is a crucial component of vascular homeostasis that prevents blood loss while also maintaining vascular patency. Hemostasis is achieved, in part, through a cascade of serine proteases that are sequentially activated, culminating in formation of the effector protease, thrombin. Normally, this process is tightly regulated, but loss of regulation can lead to bleeding or excessive clot formation (thrombosis). In the event of thrombosis, anticoagulation is the mainstay of care. Numerous pharmacokinetic problems with the oral anticoagulant warfarin prompted the development of new oral agents that directly inhibit the serine proteases of coagulation. In particular, several active site inhibitors of coagulation factor Xa (FXa) have recently been approved and are at least as effective as warfarin for the prevention of thrombosis. However, they, like warfarin, increase the risk of bleeding, and there are no approved countermeasures to treat or prevent bleeding with these direct FXa inhibitors. We evaluated whether a variant of FXa ($FXa^{I[16]L}$) could reverse the effects of the direct FXa inhibitor rivaroxaban. $FXa^{I[16]L}$ has poor active site function and a long plasma half-life but has high catalytic activity at the site of vascular injury, making it an effective pro-hemostatic

agent. $FXa^{I[16]L}$ potently reversed the effects of rivaroxaban in *in vitro* studies and animal models of coagulation. Kinetic studies revealed that both $FXa^{I[16]L}$ and wt-FXa are highly inhibited by rivaroxaban at therapeutic concentrations when bound to the cofactor FVa. Despite this high level of inhibition with rivaroxaban, both $FXa^{I[16]L}$ and wt-FXa support thrombin generation. To explain this discrepancy, we measured the kinetics of FXa inhibition by antithrombin III (ATIII), a key regulator of FXa activity in plasma. Rivaroxaban impaired ATIII-dependent FXa inhibition by creating a pool of reversibly-inhibited FXa, and kinetic simulations indicated that, under these conditions, a steady-state of free, uninhibited FXa is established. Thus, there is a paradoxical increase in the level of free FXa which explains how $FXa^{I[16]L}$ can generate thrombin in the presence of rivaroxaban. These results reveal a previously unreported, unintended consequence of direct FXa inhibitors that may have important implications. Further, $FXa^{I[16]L}$ may be able to fill the unmet clinical need for a rapid, hemostatic reversal agent for these new anticoagulants.

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Introduction: Comprehensive literature review

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Overview of Hemostasis

Hemostasis is the cellular and biochemical process that prevents blood loss following vascular injury. It is a critical component of cardiovascular homeostasis that stops bleeding while maintaining vascular patency. Hemostasis is achieved through the combined function of cellular components, including activated platelets and endothelial cells, and a cascade of homologous serine proteases and their cofactors. Dysfunction of any of these aspects of hemostasis can lead to human disease, either from excessive bleeding or from thrombosis, pathological clot formation that compromises the flow of blood. On the other hand, selective pharmacologic modulation of coagulation can be used to treat bleeding or thrombotic disorders [1].

The Coagulation Cascade

Coagulation is limited to the site of vascular injury in part because the serine proteases of coagulation and their cofactors are synthesized as inactive precursors known as zymogens or procofactors, respectively. The coagulation cascade, therefore, is the process by which zymogens and procofactors are sequentially and selectively proteolyzed to generate active proteases or cofactors, culminating in highly localized thrombin activation. Thrombin, the primary effector protease of coagulation, has both procoagulant and anticoagulant functions. It cleaves soluble fibrinogen into fibrin, which polymerizes into an insoluble mesh that constitutes the major protein component of a clot. Thrombin also initiates a positive feedback loop which amplifies the upstream coagulation cascade to generate more thrombin. Thrombin can potentiate the platelet response to injury through cleavage of protease-activated receptors (PARs) on the

platelet membrane, which enhances platelet activation. Finally, in addition to thrombin's pro-hemostatic roles, thrombin bound to the transmembrane protein thrombomodulin is also able to modulate coagulation by activating the anticoagulant protease, protein C (PC) [2].

Thrombin is activated from prothrombin, its zymogen precursor, by the prothrombinase enzyme complex made up of the serine protease factor Xa (FXa) and its cofactor Va (FVa), assembled on an anionic phospholipid membrane. FXa is, in turn, activated from the zymogen, factor X (FX) by either the extrinsic or intrinsic tenase (Xase) complexes (**Fig. 1**). The extrinsic Xase complex is generated following endothelial injury by exposure of the integral membrane protein, tissue factor (TF), to the blood. TF binds the protease, factor VIIa (FVIIa) and the complex cleaves FX to FXa. Thus, the extrinsic Xase effectively allows for communication between damaged endothelium and the coagulation cascade. The intrinsic Xase, on the other hand, is critical for signal amplification and positive feedback of coagulation. This complex of the protease FIXa and its cofactor FVIIIa assembled on anionic membranes allows for high levels of FXa to be generated, thereby enhancing the rate of thrombin generation. FIXa can be activated by either TF/FVIIa or factor XIa (FXIa). Thrombin exerts positive feedback on the entire pathway by catalyzing cofactor activation (FVa and FVIIIa), as well as FXI activation [2].

Serine Protease Biochemistry

Serine proteases are a large family of proteases that utilize an active site serine residue as the nucleophile for peptide bond hydrolysis. Two prototypical and extensively studied eukaryotic serine proteases are trypsin and chymotrypsin. The catalytic cycle of serine

proteases consists of two nucleophilic acyl substitution reactions to achieve proteolysis (**Fig. 2**). In the first reaction, the catalytic serine is the nucleophile and the amino group of the scissile peptide bond is the leaving group, thus liberating the carboxy-terminal fragment of the target protein. The amino-terminal fragment remains covalently linked to the protease via an ester bond with the serine hydroxyl. In the second substitution reaction, this fragment is released from the enzyme using free water as the nucleophile. Both substitution steps are catalyzed by a stereotyped active site motif known as the "catalytic triad," which consists of the serine nucleophile ($Ser^{[195]}$ in the chymotrypsin numbering system, described below), a histidine residue ($His^{[57]}$) which acts as a base to enhance the nucleophilicity of the serine, and an aspartate residue ($Asp^{[102]}$) which hydrogen bonds with the histidine and enhances its basicity. In addition to the catalytic triad, a pair of backbone N-H bonds ($Gly^{[193]}$ and $Ser^{[195]}$] form an oxyanion hole to stabilize the tetrahedral oxyanionic intermediates of the substitution reactions [3].

Enzyme Complexes in Coagulation

Despite the high degree of homology between coagulation serine proteases and other serine proteases like chymotrypsin and trypsin, coagulation serine proteases are unique in that they typically function in complex with a large protein cofactor on an acidic membrane surface. This is due in large part to the need to localize coagulation to the injured vascular surface. Assembly of these protease-cofactor complexes on membrane surfaces requires divalent calcium ions. In the absence of cofactor, membranes, and divalent calcium ions, FVIIa, FIXa, and FXa are extremely poor catalysts of macromolecular substrate proteolysis. When complexed with the appropriate cofactor, catalysis by these proteases is accelerated by several orders of magnitude [4]. For this

reason, cofactor activation is an extremely important determinant of coagulation cascade activity, and cofactor activity is tightly regulated to prevent thrombosis. For example, FVIIIa is a heterotrimer that dissociates rapidly such that its half-life following activation is 1-2 minutes [5, 6]. FVa is a much more stable molecule, but is inactivated following hydrolysis by activated protein C (aPC), an anticoagulant protease. Underscoring the critical nature of this process, mutations in FV/FVa that prevent aPC inactivation (i.e. FV Leiden) lead to thrombosis [7, 8].

Most coagulation serine proteases and their zymogens engage membranes via a specialized domain containing between 9 and 12 γ-carboxyglutamic acid (Gla) residues. Upon divalent calcium binding, this "Gla-domain" undergoes a conformational change that allows it to bind to anionic phospholipids with high affinity [4]. The cofactors Va and VIIIa do not have Gla-domains, but bind membranes via their homologous C1 and C2 domains [9-13].

Post-translational γ-carboxylation of glutamic acid residues within the Gla-domains of clotting factors is catalyzed by the vitamin-K-dependent enzyme γ-glutamyl carboxylase. In the process, vitamin K is oxidized from a hydroquinone to a quinone epoxide, which is subsequently recycled back to functional vitamin K by the vitamin K epoxide reductase (VKOR). Factors VII, IX, X, PC, protein S, protein Z, and prothrombin all possess Gla-domains, and are therefore known as the vitamin K-dependent clotting factors [14].

Unlike most enzymes, coagulation serine proteases engage their macromolecular substrates primarily via regions distinct from the active site [15-18]. Thus, the specificity of coagulation serine protease complexes for substrate binding is determined primarily by affinity for exosites. Obviously, the specific sequences surrounding the scissile bond

are critical for catalysis since they must dock in the active site, but this is a rapid, intramolecular process compared to intermolecular binding of the substrate to an exosite [18]. Exosite-mediated substrate binding has important implications with respect to the inhibition kinetics of protease inhibitors. Small active site probes display competitive inhibition with respect to small peptide substrates, but are noncompetitive with respect to macromolecular substrates [17]. Agents that bind exosites, on the other hand, are competitive with respect to macromolecular substrate cleavage [19].

Factor X and Xa

FXa is the penultimate protease in clotting cascade that binds to FVa on a membrane surface to generate the "prothrombinase complex" that activates prothrombin to thrombin. In humans, its zymogen precursor, FX, circulates in plasma at a concentration of 10 μg/mL (~170 nM) [20, 21]. It is synthesized in the liver as a single polypeptide chain that is proteolytically processed into a 59 kDa heterodimer of two disulfide-linked subunits: a 17 kDa 139 residue “light chain” and a 42 kDa, 306 residue “heavy chain.” The light chain, which has no homology with chymotrypsin-like serine proteases, contains the Gla-domain and two epidermal growth factor homology domains. The heavy chain contains the serine protease domain as well as a 52-amino acid “activation peptide” at its amino terminus (**Fig. 3**). Removal of the activation peptide following limited proteolysis between $Arg^{[15]}$ and $Ile^{[16]}$ releases the activation peptide to yield the active protease [22].

Chymotrypsin Numbering System

Because of the substantial homology between chymotrypsin-like serine proteases, a standard nomenclature has been developed based on the residue numbering of chymotrypsin that allows for more meaningful comparison of residues between different proteins [23]. For FX, the amino-terminal light chain is non-homologous with chymotrypsin and is thus numbered sequentially from 1-139. Light chain residue numbers are denoted by an L preceding the residue number (for example, Arg^{L139}). Homology with chymotrypsin exists in the heavy chain, and thus the heavy chain will be numbered in brackets according to the chymotrypsin numbering system (for example, $Ser^{[195]}$).

Structural Features of FXa

The protease domain of FXa shares the same overall fold with other chymotrypsin-like serine proteases. The heavy chain is typically depicted in the "standard orientation," as shown in (**Fig. 3**). In this orientation, the light chain, if shown, would be behind the heavy chain, into the page. The standard orientation places the active site and catalytic triad in the middle, in front of the page. An α-helix, often referred to as the "162-helix," is thought to form at least part of the interface with FVa. Two loop regions, [183]-[189] and [221]-[225], form the sodium binding site in the protease [24].

Regulation of FXa Activity

The activity of FXa in plasma is tightly controlled by several inhibitors, including antithrombin III (ATIII) [25], tissue factor pathway inhibitor (TFPI) [26], and α_2-

macroglobulin (α_2M) [27]. Together, these inhibitors result in a very short (1-2 minute) activity half-life of FXa [28, 29]. These plasma inhibitors are critical to the function of the entire coagulation cascade because they effectively create a high barrier to the propagation of coagulation. This is likely why the intrinsic pathway is so important for hemostasis, since it enables rapid FXa generation to overcome its rapid inhibition. In fact, it was recently demonstrated that pharmacologically disrupting these natural antagonists of FXa mitigates the effects of an intrinsic pathway defect [30].

Perhaps the most prominent coagulation protease inhibitor in plasma is ATIII. A member of the serpin superfamily of protease inhibitors, ATIII is present in plasma at a concentration of 0.12 mg/mL (2.3 μM) and irreversibly inhibits not only FXa, but also thrombin and FIXa. Like other serpins, ATIII contains a reactive center loop (RCL) region which is responsible for binding to the active site of the protease. Once bound, the protease can cleave the RCL like any other protein substrate, rendering ATIII inactive. However, cleavage of the RCL results in a rapid, marked conformational change in ATIII. If this conformational change occurs prior to hydrolysis of the ester intermediate, the protease will become irreversibly denatured [25].

TFPI is a potent inhibitor of not only FXa, but also of FVIIa and TF. Importantly, TFPI inhibition of the extrinsic Xase complex is quite weak in the absence of FXa. TFPI contains three Kunitz-type inhibitory domains (K1, K2, and K3). To inhibit FXa, the K2 domain first binds to free FXa in a reversible, active site-dependent fashion. Binding of FXa enhances the affinity of the K1 domain of TFPI for FVIIa/TF, allowing formation of a tight, irreversible quaternary complex [26].

α_2M is a large homotetrameric glycoprotein that inhibits several plasma proteases, including FXa [27], and is present in plasma at a concentration of 1.2 mg/mL (~1.7 μM) [31]. Its 180 kDa subunits form disulfide-linked homodimers which then further dimerize noncovalently to generate the 720 kDa homotetramer [27]. α_2M inhibits proteases via a cage-like mechanism [32]. The middle of each subunit contains the "bait region," which is susceptible to limited proteolysis by the target protease. Cleavage of the bait region results in a conformational change that traps the protease within the tetramer. For many protease targets, the conformational change also exposes a buried cysteine-glutamate thioester bond that then exchanges with a surface lysine on the protease surface to covalently trap the protease in the cage [27]. In this manner, α_2M sterically prevents the protease from accessing its macromolecular substrates [32]. However, unlike ATIII and TFPI, α_2M does not substantially alter active site function of the protease, as evidenced by the fact that protease-α_2M complexes retain a high degree of catalytic activity towards small peptidyl substrates that can pass through small pores in the complex to access the trapped enzyme [27].

Although ATIII, TFPI, and α_2M are all able to rapidly inhibit FXa, their relative contributions to FXa inhibition in plasma are not equal. Experiments using ^{125}I-labeled FXa and an SDS-polyacrylamide gel electrophoresis method revealed that ATIII is the predominant inhibitor of FXa *in vitro* in human or mouse plasma, with α_2M accounting for only 11% of total inhibition [33]. However, α_2M was found to be the major inhibitor of FXa *in vivo*, with ATIII playing an important but minor (38%) role in regulation of FXa activity. The role of TFPI in the regulation of FXa, on the other hand, it likely indirect because it is reversible when only engaged with FXa, and because its plasma concentration is quite low (2-2.5 nM) [34, 35]. Instead, since TFPI binding to FXa

dramatically enhances its ability to irreversibly inhibit FVIIa/TF, FXa is effectively a cofactor that allows TFPI to inactivate the extrinsic Xase [36].

The common theme of the irreversible regulators of FXa is their dependence on FXa active site maturity and availability. They do not inhibit the FX zymogen to an appreciable extent [37]. Furthermore, ATIII and α_2M require not only conformational availability of the active site, but also catalytic function. Thus, anything that impairs active site binding or catalytic activity should impact the function of these protease inhibitors.

The Zymogen-to-Protease Transition

The negative regulators of FXa described above are opposed by the generation of FXa via the extrinsic or intrinsic pathways. Activation of FXa results in key changes in the enzyme that ultimately allow it to activate prothrombin. Prior to activation, FX binds very weakly, if at all, to FVa [38-40]. Furthermore, FX has extremely poor active site accessibility and function, as evidenced by its virtual inability to be labeled using active site probes such as peptide chloromethyl ketones [41]. In contrast, FXa binds its cofactor on membranes with low nanomolar affinity [42] and prothrombin with high nanomolar/low micromolar affinity [4, 43]. Even in the absence of membranes and FVa, FXa can rapidly hydrolyze small peptide substrates and is highly susceptible to inhibitors that occupy the active site [4, 44].

The marked difference in the biochemical properties of zymogen FX and the FXa protease can be explained in large part by an activation mechanism common to all trypsin and chymotrypsin-like serine proteases. Limited proteolysis of FX by the

intrinsic or extrinsic Xase complexes results in removal of the activation peptide from the amino-terminus of the heavy chain [22, 45]. While it is plausible that removal of the activation peptide may directly contribute to revealing protease characteristics, its primary importance is thought to be through the exposure of a new amino terminus of the heavy chain, which inserts into a hydrophobic pocket and forms a salt bridge between $Ile^{[16]}$ (the newly revealed amino-terminal residue) and $Asp^{[194]}$ [24, 46-48]. This leads to a series of conformational changes that yield the mature protease. Remarkably, despite the dramatic functional difference between zymogen and protease, the conformational changes between the two forms of the protein are quite modest [45]. In fact, crystal structures of bovine chymotrypsinogen [49] and chymotrypsin [50, 51] indicate that the orientation of catalytic triad residues is virtually unchanged between zymogen and protease. Instead, the zymogen-to-protease transition is limited to a few regions of the protein collectively termed the "activation domain." The activation domain consists primarily of the $Ser^{[189]}$-$Asp^{[194]}$, along with the two sodium binding loops, the autolysis loop, and the activation loop ($Ile^{[16]}$-$Gly^{[19]}$) [47, 49-53]. To form the conformationally and catalytically mature active site, the $Ser^{[189]}$-$Asp^{[194]}$ loop rotates to form the mature substrate binding cleft and also repositions $Gly^{[193]}$ such that its backbone N-H is properly oriented for hydrogen bonding to and stabilizing the oxyanion transition state (the oxyanion hole).

Importantly, the zymogen-to-protease transition is actually a dynamic equilibrium between a "zymogen-like" conformation and a "protease-like" conformation [47]. The relative abundance of these conformations is determined by the degree to which each is stabilized. For example, for most serine proteases, insertion of the new amino terminus that is exposed following activation peptide removal stabilizes the protease-like

conformation such that the equilibrium lies far towards the protease-like state. In the uncleaved zymogen, the activation peptide prevents this insertion, and thus the zymogen-like state predominates [45, 47]. However, several studies have demonstrated the tunability of the zymogen-to-protease transition. For example, uncleaved trypsinogen can be driven to adopt a protease-like conformation with short peptide mimics of the new amino terminus or high concentrations of strong active site ligands [46, 53-55]. Conversely, mutations in the activation domain of cleaved proteases (ex. mutations in the sodium binding loop of FXa) result in increased zymogenicity [56-58].

Residues [16]-[19] (H_2N-IVGG-) of the new amino-terminal activation loop are highly conserved in FX/Xa, as is the $Asp^{[194]}$ residue that forms an internal salt bridge pair with $Ile^{[16]}$. Mutation of $Asp^{[194]}$ in FXa results in a catalytically inactive protein (Camire laboratory, unpublished data), further highlighting the importance of amino-terminal insertion in stabilization of the protease conformation. Through mutagenesis of the $Ile^{[16]}$ and $Val^{[17]}$ residues, our group has demonstrated that disruption of amino-terminal insertion can shift the equilibrium between zymogen and protease towards the zymogen-like conformation (**Fig. 4**) [44]. These zymogen-like FXa variants, as expected, have poorly formed active sites and, accordingly, poor amidolytic activity when assessed using oligopeptidyl substrates. In addition, these variants are resistant to active site-dependent plasma protease inhibitors ATIII and TFPI. This results in a much longer activity half-life of these variants compared to wild-type (wt)-FXa, with the degree of prolongation proportional to the zymogenicity of the variant. However, since FVa only binds the protease conformation with high affinity, saturating concentrations of the cofactor thermodynamically rescue active protease conformation and function. Thus, these variants have low catalytic activity and long half-lives when free in plasma, but, when

assembled in the prothrombinase complex, they have near-normal ability to generate thrombin [44, 59].

Because of the unique properties of these amino-terminal zymogen-like FXa variants, they have therapeutic potential as procoagulants. Their longer half-lives make them more suitable pharmacologic agents than WT FXa, and their zymogenicity while free in plasma renders relatively inert. Using several animal models of the bleeding disorder hemophilia, we have previously shown that zymogen-like FXa variants, and in particular, $FXa^{I[16]L}$, are highly effective at restoring normal hemostasis [60, 61].

Pharmacologic Anticoagulation

Anticoagulation is the mainstay of care for the prevention and treatment of thromboembolic diseases including atrial fibrillation, pulmonary embolism (PE) or deep venous thrombosis (DVT), and patients with prosthetic heart valves [62, 63]. Anticoagulants can be grouped into two categories based on route of administration: parenteral and oral. In the hospital setting, parenteral anticoagulation is common and practical for short-to-medium term anticoagulation. However, many patients require chronic outpatient anticoagulation, for which oral anticoagulation is preferred [62].

Parenteral Anticoagulants

Several agents are available for parenteral anticoagulation. The most widely used is heparin and its derivatives. Heparin is a naturally occurring glycosaminoglycan that exists as a heterogeneous mixture of polymers of different length. Unfractionated heparin (UFH) binds to ATIII and accelerates its inhibition of FXa, FIXa, and thrombin

by approximately 2,000 to 4,000-fold [25]. This acceleration is mediated in large part by a specific pentasaccharide sequence within the heparin polymer [64]. In addition, the mechanism of UFH acceleration of thrombin inhibition, but not that of FXa or FIXa, requires the polysaccharide to bridge ATIII and thrombin (~2,000-4,000-fold) on the rate of thrombin inactivation by the serpin [25]. Fractionating heparin such that polymers with a mean molecular weight of around 5 kDa remain results in low molecular weight heparin (LMWH). LMWH is not large enough to bind both ATIII and thrombin, and thus it is unable to bridge thrombin and ATIII. For this reason, LMWH is selective for FXa and FIXa, and has very little effect on thrombin inhibition [65]. The minimal pentasaccharide is also used as an anticoagulant, known as fondaparinux, and primarily accelerates FXa inhibition [64]. An alternative to heparins is a class of drugs derived from hirudin, a natural anticoagulant found in the saliva of leeches [66]. Hirudin directly inhibits thrombin by binding both the active site and exosite 1 (where fibrinogen binds). Other hirudin derivatives include lepirudin, a minimally modified recombinant hirudin, and bivalirudin, a synthetic peptidyl hirudin analog. Finally, argatroban is a small molecule that inhibits only the active site of thrombin [67]. It is not orally bioavailable, and is thus not suitable for oral anticoagulation.

Oral Anticoagulants

For more than 60 years, the only option available for oral anticoagulation was an indirect clotting factor antagonist, warfarin [63]. Warfarin inhibits VKOR, thereby preventing proper γ-carboxylation of Gla residues. While warfarin has excellent efficacy as an anticoagulant, its use has been plagued by complex pharmacogenetics, common drug interactions, and slow onset and offset. These characteristics necessitate routine

monitoring of clinical coagulation parameters to maintain patients within a narrow therapeutic range of anticoagulation [63, 68].

In the last decade, target specific oral anticoagulants (TSOACs), which directly inhibit thrombin [69] or FXa [70-72], have been developed. Three direct FXa inhibitors, rivaroxaban, apixaban, and edoxaban, have gained FDA approval in the last 5 years. Biochemically, these agents are reversible active site inhibitors of FXa. Thus, they display competitive inhibition kinetics with respect to small peptide substrates, but are noncompetitive inhibitors with respect to prothrombin, the natural macromolecular substrate [70-72].

Pharmacologic Properties of Direct FXa Inhibitors

The three approved direct FXa inhibitors are all administered as the active agent and do not require metabolism for *in vivo* efficacy. They have good oral bioavailability and reach peak plasma concentration within 1-4 hours [73-75]. Since they directly inhibit FXa, their activity correlates well with plasma concentration. This is in contrast to warfarin, whose onset of action is much slower and is primarily determined by the half-lives of the vitamin K-dependent factors [63]. Direct FXa inhibitors also have relatively short plasma half-lives (6-14 hours) [73, 76, 77] compared to warfarin. They are extensively plasma protein bound (55% for edoxaban [78], 87% for apixaban [79], and 95% for rivaroxaban [80]), which has important implications for reversal strategies (discussed below). Rivaroxaban, apixaban, and edoxaban are all excreted via both the kidneys and the feces, with the renal pathway being major (66%) for rivaroxaban [77] and substantial but minor for apixaban [73] and edoxaban [76] (25% and 36-45%, respectively).

Clinical Efficacy of Direct FXa Inhibitors

The efficacy of rivaroxaban, apixaban, and edoxaban has been evaluated in several large, multi-center clinical trials for multiple indications [81-97]. All three agents are approved for the prevention of stroke or systemic embolism in patients with nonvalvular atrial fibrillation. For this indication, the new agents are at least noninferior to warfarin [82, 95, 97], and a large meta analysis of the atrial fibrillation data from all direct FXa inhibitors indicated that the new agents were superior to warfarin [98]. Rivaroxaban and apixaban have also been studied and approved for the initial treatment of DVT and PE, as well as for prevention of DVT or PE recurrence in these patients following initial therapy [83, 85, 88, 89]. Edoxaban was also compared to warfarin for treatment of DVT or PE by the Hokusai VTE group following at least 5 days of LMWH or UFH therapy [96]. It was found to be noninferior to warfarin, and therefore was approved with the caveat that a parenteral anticoagulant should be used for the first 5-10 days. Edoxaban has not been evaluated for reduction of DVT or PE risk recurrence. Finally, both apixaban and rivaroxaban are approved for DVT prophylaxis as an alternative to LMWH in patients following hip or knee replacement surgery [81, 87, 92, 93].

Patients with prosthetic heart valves are at extremely high risk for stroke or systemic embolism, and these patients are routinely anticoagulated with warfarin. No data is available, however, on the efficacy of direct FXa inhibitors in this patient population. The only TSOAC for which there is clinical data is dabigatran, a direct thrombin inhibitor, but this study was terminated prematurely because of excessive stroke as well as bleeding in the dabigatran arm [99].

Safety of Direct FXa Inhibitors

Like most anticoagulants, the major safety concern with direct FXa inhibitors is bleeding, with special emphasis placed on intracranial hemorrhage and gastrointestinal bleeding. A Cochrane review of the safety endpoints from the atrial fibrillation studies concluded that direct FXa inhibitors have a favorable safety profile compared to warfarin in these patients [98]. Specifically, direct FXa inhibitors showed an impressive reduction in rates of intracranial hemorrhage (OR 0.56, 95% CI 0.45 to 0.70) compared to patients receiving warfarin. The analysis also revealed that major bleeding (as defined by the ISTH criteria [100]) was less frequent in patients taking direct FXa inhibitors, but that the improvement over warfarin was less pronounced than for intracranial hemorrhage (OR 0.90, 95% CI 0.82 to 0.98). In a similar meta analysis in patients undergoing treatment for acute DVT or PE, where standard treatment is a combination of LMWH and warfarin, anticoagulation with direct FXa inhibitors resulted in fewer major bleeding episodes (OR 0.57, 95% CI 0.43 to 0.76) [101]. No safety benefit was seen in the setting of postoperative anticoagulation in orthopedic patients, with a trend towards increased major bleeding compared to the LMWH standard therapy (OR 1.27, 95% CI 0.98 to 1.65).

Monitoring of Direct FXa Inhibitors

One of the primary drawbacks to warfarin therapy is the need for routine INR monitoring [62, 63, 68]. This is especially important for due to the numerous food-drug interactions and substantial pharmacogenetic variation in warfarin metabolism [68]. Direct FXa inhibitors, on the other hand, do not appear to require regular coagulation

monitoring in most patients [102-104]. Nonetheless, there are certain clinical scenarios where knowing the extent of anticoagulation may be beneficial. This is especially true of patients on pharmacokinetic extremes such as individuals with renal disease or obese patients [105, 106]. In addition, understanding the degree to which a bleeding patient is anticoagulated is critical if a pharmacologic reversal strategy is to be employed.

In principle, direct FXa inhibitors should affect both PT and aPTT measurements because FXa lies in the common pathway. However, these assays are only sensitive to direct FXa inhibitors at higher concentrations, near the upper limits of the therapeutic range. Thus, they are not ideal for making precise determinations of anticoagulant effect. A more useful assay is the anti-Xa assay, which uses a chromogenic FXa substrate to determine the degree to which FXa is inhibited. It is sensitive to and, importantly, linear within, the therapeutic and supratherapeutic range of direct FXa inhibitors [106]. Therefore, this assay is probably the best choice for *in vitro* monitoring of the anticoagulant effects of direct FXa inhibitors.

Reversal of Direct FXa Inhibitors

Despite widespread enthusiasm surrounding clinical trial results and subsequent approval of TSOACs, concerns about the risk of bleeding still remain. This is particularly important because there are no FDA approved countermeasures to the anticoagulant effects of TSOACs in the event of bleeding or prior to an invasive procedure [107]. This is in contrast to warfarin, for which multiple reversal strategies exist [63]. Administration of vitamin K allows for sufficient γ-carboxylation of newly synthesized clotting factors and reversal of warfarin's anticoagulant effects within 4-24 hours, depending on the

degree of anticoagulation. If more rapid reversal is required, patients can be given fully carboxylated vitamin-K dependent factors, either in the form of fresh frozen plasma (FFP) or prothrombin complex concentrates (PCCs), which contain FIX, FX, prothrombin, and, depending on the preparation, FVII [108].

Given the many years of clinical experience with an agent like warfarin that has a specific reversal strategy, the lack of a reversal strategy for TSOACs has been the subject of much discussion. Some have argued that the short half-lives of TSOACs, which typically , in conjunction with a potentially lower risk of bleeding compared to warfarin, renders reversal agents unnecessary. Nevertheless, there are several reversal agents in various stages of development [107, 109].

Proposed reversal strategies for direct FXa inhibitors fall into one of three categories. A relatively straightforward approach is to remove the drug, either by preventing absorption or by active removal from circulation. A similar but distinct strategy is to sequester the anticoagulant and relieve inhibition of FXa using a specific, noncatalytic antidote. Finally, several groups have proposed bypassing the inhibitor using a pro-hemostatic agent to generate thrombin without relieving inhibition of FXa. Of these three, specific antidotes and bypassing agents have received the most attention (**Fig. 5**).

Drug Removal Approaches

In the event of overdose, it has been suggested that activated charcoal might be effective at preventing absorption of the anticoagulant if administered shortly after ingestion. However, direct FXa inhibitors are rapidly absorbed and reach peak efficacy within 1-4 hours. Thus, even if this approach were effective, it would only be useful during a narrow

window prior to drug absorption [109]. After absorption, removing the anticoagulant by hemodialysis is also not expected to be efficient due to the high degree of anticoagulant binding to plasma proteins [78-80]. Indeed, studies in anticoagulated patients undergoing hemodialysis support the idea that direct FXa inhibitors are effectively not dialyzable [78, 110]. Even edoxaban, which has the lowest plasma protein binding of the currently approved agents (~55%) [111] was only minimally cleared after 4 hours of hemodialysis [78]. Thus, removal of the anticoagulant or prevention of absorption is unlikely to be a widely used method of anticoagulant reversal.

Anticoagulant-Sequestering Antidotes

Generally speaking, drug-sequestering antidotes bind the anticoagulant and prevent it from inhibiting FXa. Such antidotes could theoretically take multiple forms including decoy enzymes, synthetic small molecules, DNA or RNA aptamers, or antibodies. Unlike bypassing agents, they typically have no intrinsic catalytic activity, and therefore, a substantial fraction of the anticoagulant must be bound to restore normal hemostasis [112, 113]. For this reason, antidotes are usually administered at much higher concentrations than bypassing agents to effectively deplete the anticoagulant.

Of the possible antidote approaches, a monoclonal antibody (or antibody fragment) against each inhibitor would likely afford the greatest specificity. Indeed, in the direct thrombin inhibitor class of TSOACs, a F_{ab} fragment that specifically binds the direct thrombin inhibitor dabigatran is currently being developed [114, 115]. However, an antibody-based approach has not yet been reported, possibly due to the fact that there are multiple drugs in the direct FXa inhibitor class and each drug would require its own

specific antibody. Instead, all antidotes currently under development appear to have broad specificity for the entire direct FXa inhibitor class, with some also targeting indirect FXa inhibitors [112, 113].

Gla-domainless FXa$^{S[195]A}$

Gla-domainless FXa$^{S[195]A}$ (GD-FXa$^{S[195]A}$) is a recombinant variant of FXa that is being developed as a drug-sequestering antidote for direct FXa inhibitors [112]. It is conformationally similar to wt-FXa in its ability to bind active site probes, but its catalytic active site Ser$^{[195]}$ is mutated to an alanine to render the molecule catalytically inactive. It also lacks the membrane-binding Gla-domain, since FXa$^{S[195]A}$ would otherwise be able to assemble in prothrombinase and result in a dead-end complex. Thus, GD-FXa$^{S[195]A}$ binds direct FXa inhibitors with high affinity, sequestering the anticoagulant to relieve inhibition of prothrombinase. GD-FXa$^{S[195]A}$ has been shown to be highly effective at reversing the anticoagulant effects of rivaroxaban in rat and rabbit bleeding models. The 1:1 stoichiometry of GD-FXa$^{S[195]A}$ necessitates the use of high concentrations (high nanomolar) to effectively sequester the anticoagulant. This will likely mean that hundreds of milligrams of this recombinant protein will be needed to reverse anticoagulation in a single patient.

Aripazine

Aripazine is a small molecule derived from two arginine moieties attached to a piperazine core. It was designed using an *in silico* approach to non-covalently and specifically hydrogen bond not only to direct FXa inhibitors, but also direct thrombin

inhibitors and heparins [113]. It has been reported to reverse the anticoagulant effects of rivaroxaban, apixaban, edoxaban, and dabigatran *in vitro* [113] and in a rat tail transection model *in vivo* [116]. In an ongoing phase II study, aripazine was shown to restore *ex vivo* whole blood clotting time to baseline in healthy volunteers treated with a single 60 mg oral dose of edoxaban with no evidence of prothrombotic side effects [117].

Unfortunately, there is limited evidence surrounding the specificity of aripazine for its targets. Indeed, it is quite surprising that such a molecule could have high specificity for the diverse molecules in the direct FXa class, and even more unclear how it also has specificity for direct thrombin inhibitors and heparin, but nothing else. While such properties are certainly plausible, much more preclinical evidence is needed to assess this. This is particularly true in light of a study directly comparing GD-FXa$^{S[195]A}$ and aripazine *in vitro* in a purified system which indicated that aripazine may enhance FIXa activation of FX [118].

Pro-Hemostatic Bypassing Agents

Biochemical Requirements

Bypassing agents have been used for many years to prevent and treat bleeding in hemophilia patients with inhibitory alloantibodies [119]. In hemophilia, deficiency of factor VIII or FIX results in insufficient activation of FXa [119], so enhancing the activity of the other FXa-generating pathway, the extrinsic pathway, is a logical therapeutic approach. Bypassing the effects of direct FXa inhibitors is much more problematic. FXa is the last step in the coagulation cascade before prothrombin. Since there is no

intervening protease between the FXa and prothrombin, the only way to enhance prothrombin activation would be to somehow modulate FXa's activity. However, doing so by increasing the amount of FXa present (either by direct administration of FXa or by enhancing its upstream activation) has seemed illogical since the anticoagulant has high affinity for the enzyme [71, 72, 120] and is in vast excess [121]. Nonetheless, there have been several studies exploring the repurposing of hemophilia bypassing agents and warfarin reversal agents for direct FXa inhibitor reversal.

Prothrombin Complex Concentrates

PCCs are a heterogeneous group of plasma-derived products that were originally developed to treat severe FIX deficiency (hemophilia B) before purified FIX was available [122]. It is now typically used for warfarin reversal, especially in volume-sensitive patients, and are also used to treat patients with rare vitamin K-dependent factor deficiencies. There are 3-factor and 4-factor forms, with both containing FIX, FX, and prothrombin, but with 4-factor PCCs also containing FVII [122]. Most preparations also contain PC and protein S [108]. Their dosage is normalized to FIX concentration, and can vary in the concentration of the other factors [122]. In a small study of healthy volunteers given 20 mg rivaroxaban twice daily for 2.5 days, 4-factor PCC administration corrected the mildly prolonged PT, and overcorrected the endogenous thrombin potential (ETP) in thrombin generation (TG) studies [123]. Two other studies reported a similar improvement in ETP following *ex vivo* addition of PCC to blood from rivaroxaban-treated individuals [124, 125]. However, the effect of PCC on peak height in these TG studies was mixed, with only one of the studies reporting correction of peak height [124].

Animal injury studies have also yielded variable results with respect to the efficacy of PCC as a rivaroxaban reversal agent. At high doses, PCC (50 U/kg) normalized bleeding time in a rat mesenteric artery bleeding model [126]. When evaluated in a rabbit ear immersion bleeding assay, however, PCC had no effect on bleeding time and blood loss [127]. In a recent study in humans using a punch biopsy model in healthy volunteers given a single 60 mg dose of edoxaban, 50 U/kg of a 4-factor PCC reversed edoxaban's effect on bleeding duration after injury as well as the ETP parameter in *ex vivo* TG studies [128]. No effect was seen on, however, on bleeding volume in these subjects. There was also a great deal of variability within the control groups in this study, suggesting that the injury may not have been sensitive enough to the effects of the anticoagulant to reliably see large differences in bleeding.

The results from these PCC studies are difficult to interpret, in part, because the products used vary greatly. Most studies used 4-factor PCCs, but each preparation varies in terms of its exact composition. In addition, studies that measured TG parameters almost universally showed improvement in the ETP, but were less consistent with respect to other parameters [123-125]. This might reflect a dependence of the ETP on prothrombin levels, which are increased during PCC therapy, and which have been shown to correlate well with an improvement in ETP in other settings (i.e. hemophilia) [129].

Recombinant FVIIa

Recombinant FVIIa concentrate (rFVIIa) is a widely used bypassing agent for hemophilia patients with inhibitors to enhance generation of FXa through the extrinsic

pathway [119]. rFVIIa added to plasma obtained from patients anticoagulated with rivaroxaban normalized only the lag time parameter in TG experiments, with little to no effect on peak height or ETP [124]. rFVIIa also corrected the rivaroxaban-prolonged PT in this study and partially corrected the PT in animal studies in rats and baboons [126]. rFVIIa also partially normalized bleeding time in a rat mesenteric artery bleeding model, but did not statistically significantly reduce bleeding time in a skin bleeding model in baboons [126]. Like PCCs, rFVIIa also had no effect on ear bleeding in rivaroxaban-treated rabbits [127].

Activated Prothrombin Complex Concentrates

Activated prothrombin concentrates (aPCCs) are a plasma-derived product used, like rFVIIa, to treat bleeding in hemophilia patients with inhibitors [119]. Despite their name, aPCCs only contain a significant concentration of one activated factor, FVIIa [130, 131]. The other components are zymogens FIX, FX, and prothrombin, with very little FIXa, FXa, or thrombin present. The mechanism of action of aPCCs has not been fully elucidated, although some studies have suggested that the prothrombin present in the product is critical to its function [132-134].

Unlike rFVIIa and PCCs, aPCCs corrected all aspects of TG profiles, including lag time, peak height, and ETP, when added to plasma from rivaroxaban-treated individuals *ex vivo* [124]. High dose aPCCs also partially corrected the rivaroxaban-induced increased bleeding time in the rat mesenteric artery bleeding model, and, in contrast to rFVIIa, also completely normalized the skin bleeding time in baboons [126]. aPCCs also partially normalized bleeding time in edoxaban-treated rats [135].

Summary

Blood coagulation is responsible for maintenance of vascular integrity, and therefore plays a significant role in human health. For this reason, there is a great deal of interest in selective pharmacologic modulation of hemostasis to prevent or treat bleeding or thrombosis. The serine proteases of coagulation are an attractive target for such interventions, since they are essential to clot formation and since they are "druggable" enzymes. Historically, antagonizing an enzyme via an active site-directed ligand has been quite appealing since such probes tend to be specific to their targets and prevent formation of the enzymatic product. However, the enzymatic function of coagulation serine proteases is highly complex. In addition to catalyzing substrate hydrolysis, these enzymes are also subject to irreversible active site dependent inhibition by plasma protease inhibitors. This regulatory mechanism is critical to the prevention of thrombosis, and it is unclear how it might be affected by pharmacologic active site antagonism.

Nonetheless, several new molecules that directly inhibit the active site of FXa have been developed for oral anticoagulation. While they are clearly effective at prevention of thrombosis, they also increase the risk of bleeding. Although they may represent a substantial improvement in oral anticoagulant therapy compared to warfarin, there is concern about the lack of an approved antidote or countermeasure to the effects of direct FXa inhibitors.

This dissertation begins with the observation that FXa can reverse the anticoagulant effects of direct FXa inhibitors (**Chapter 2**). To understand this unexpected finding, we

explore the kinetic consequences of active site antagonism of FXa (**Chapter 3**), with a special emphasis on the effect of these anticoagulants on plasma protease inhibitors of FXa. Through this work, we demonstrate that inhibition of FXa can paradoxically lead to increased levels of uninhibited FXa. This remarkable increase in FXa may have implications for the development of FXa inhibitor reversal agents, on the clinical efficacy of FXa inhibitors, and possibly on the safety of these new oral anticoagulants.

Figure Legends

Figure 1. The coagulation cascade. The coagulation cascade of serine proteases and cofactors is shown, culminating in generation of thrombin, the effector protease of coagulation. Image courtesy of Dr. Sriram Krishnaswamy.

Figure 2. Catalytic mechanism of chymotrypsin-like serine proteases. The catalytic triad consisting of Asp[102], His[57], and Ser[195] in the chymotrypsin numbering system. A representative peptide is shown, with the first residue amino-terminal to the scissile bond denoted with P_1, and the first residue on the carboxy-terminal end of the scissile bond denoted with P_1'. The two acyl substitution reactions are shown. Each substitution step has a nucleophilic attack step, with the nucleophilicity enhanced by His[57], which generates a tetrahedral oxyanionic intermediate, followed by leaving of the leaving group and restoration of the planar carbonyl. The oxyanion hole is not shown.

Figure 3. Crystal structure of FXa. The heavy chain of FXa which contains the catalytic domain of the protease is shown in the standard orientation. The catalytic triad is shown as red sticks. Ile[16] and Asp[194] which form an internal salt bridge are also shown. DX-9065a, a bound inhibitor, is in pink. The activation domain is labeled green and a proposed FVa binding site, the "162 helix," is shown in orange. The calcium loop is in light purple and the yellow region is an acidic exosite corresponding to exosite 1 in thrombin. The light chain is not shown, but would be directly behind the heavy chain in this orientation [24].

Figure 4. Zymogen-like FXa variants. In wt-FXa, the protease conformation is favored because amino-terminal insertion (H_2N-IVGG-) stabilizes the protease.

Mutations in the conserved amino-terminus disrupt insertion and alter the equilibrium position, making a more zymogen-like variant. FVa binds the protease conformation of FXa much more tightly than the zymogen-like state, so saturating amounts of FVa in the presence of membranes can rescue activity of zymogen-like FXa.

Figure 5. General strategies for reversal of direct FXa inhibitors. The prothrombinase complex, made up of FXa and FVa on an acidic phospholipid membrane, is shown, subject to inhibition by a direct, active site inhibitor (gray circles, "In"). A drug-sequestering antidote (bottom left, large red circles, "antidote") binds the anticoagulant to relieve inhibition of the target enzyme. A bypassing agent does not relieve inhibition of the target enzyme, and instead, generates the product in spite of the inhibitor.

Figures

Figure 1

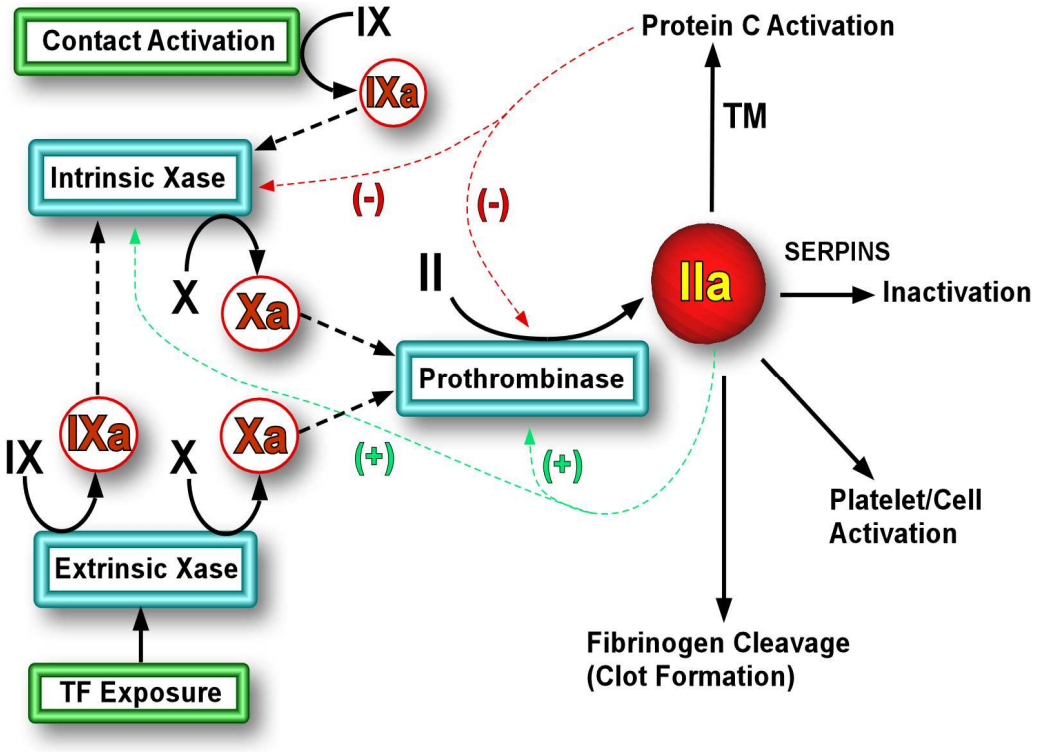


Figure 2

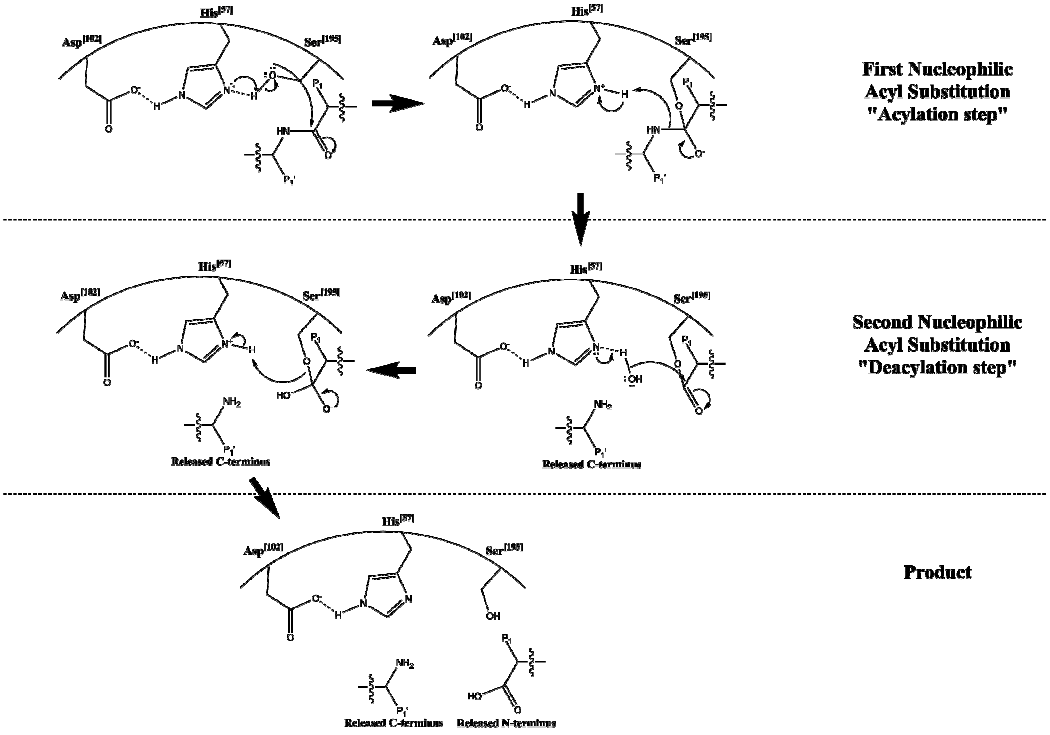


Figure 3

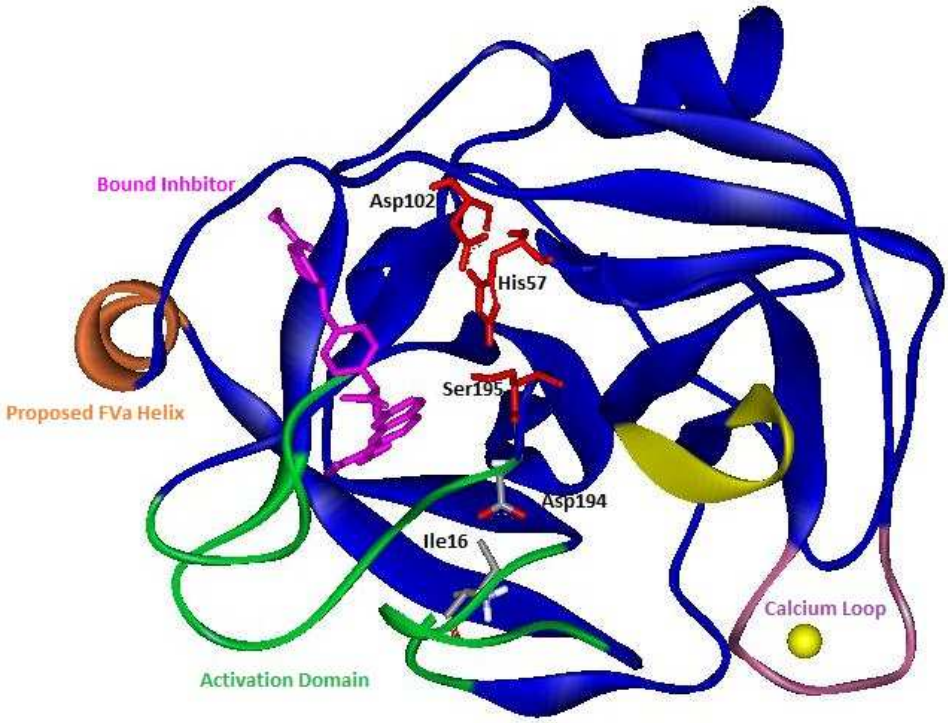


Figure 4

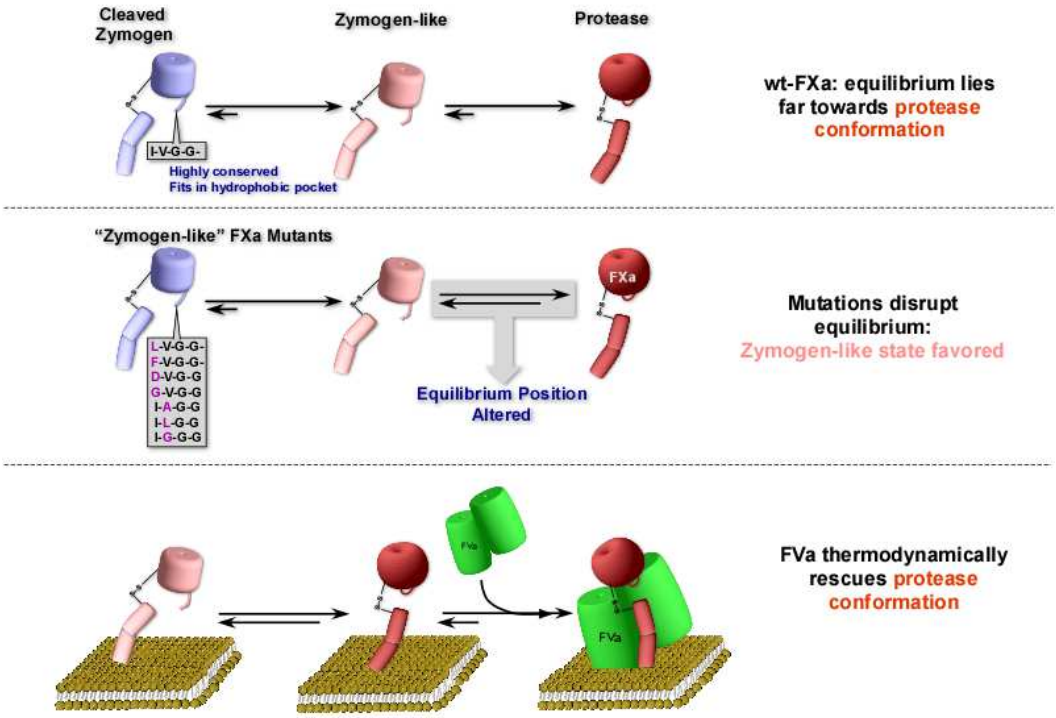
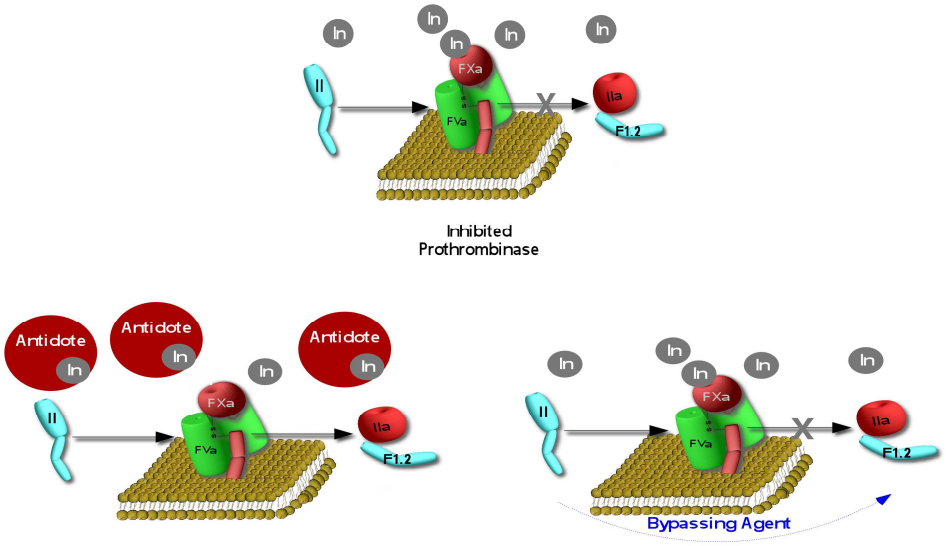


Figure 5



CHAPTER 2:

A rapid reversal strategy for oral factor Xa anticoagulants

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Abstract

The pharmacokinetic challenges of warfarin therapy have led to the development of target-specific oral anticoagulants (TSOACs) that directly inhibit coagulation factor Xa (FXa). While these new drugs have many benefits over warfarin, no approved strategy exists to reverse their anticoagulant effects in the event of life-threatening bleeding or emergent need for surgery. We hypothesized that a pro-hemostatic bypassing agent might be able to reverse the effects of direct FXa inhibitors with high potency. To evaluate this, we used a variant of FXa ($FXa^{I[16]L}$) that is more zymogen-like than wild-type (wt)-FXa. This variant is resistant to active site inhibitors and has a long plasma half-life, but is catalytically active *in vivo* at the site of vascular injury. *In vitro*, $FXa^{I[16]L}$ dose-dependently restored thrombin generation in the face of the direct FXa inhibitor rivaroxaban. $FXa^{I[16]L}$ also reversed the effects of rivaroxaban in both a large vessel and a microcirculatory injury model in mice. Importantly, $FXa^{I[16]L}$ was more potent (>50-fold) in these models than a noncatalytic antidote in clinical development. These data highlight that differences in mechanism of action between a pro-hemostatic bypassing agent and a noncatalytic antidote will have a huge impact on amounts of protein needed to revive thrombin generation. Furthermore, these data provide strong support for the *in vivo* efficacy and potency of $FXa^{I[16]L}$ as a potential pro-hemostatic bypass agent to reverse direct FXa inhibitors.

Introduction

Clot formation is a homeostatic mechanism that prevents blood loss following vascular injury. Its dysregulation can lead to the pathological activation of coagulation and thrombosis. Pharmacologic oral anticoagulation by warfarin is a mainstay of care for thrombosis and has been in clinical use for over six decades[63]. However, limitations of warfarin therapy have prompted the development of target-specific oral anticoagulants (TSOACs)[63, 136]. These small molecules reversibly bind the active site of coagulation factor Xa (FXa)[70-72] or thrombin[69] and inhibit protease function. Clinically, TSOACs are as or more effective than warfarin and are approved in Europe and the United States for stroke prevention in patients with atrial fibrillation, and for thromboprophylaxis following orthopedic surgery[87, 94-96, 137]. However, like warfarin, there is a clinically significant risk of bleeding[102, 103]. Bleeding complications with warfarin are managed by anticoagulant reversal with vitamin K, fresh frozen plasma, or prothrombin complex concentrates (PCCs)[63]. In contrast, TSOACs lack clinically approved countermeasures[107].

Proposed reversal strategies for direct FXa inhibitors fall into two categories: antidotes that bind and sequester the inhibitor[112, 117], or bypassing approaches that enhance thrombin formation and hemostasis in the face of the inhibitor[123, 124, 126, 127, 138]. An example of the former is a catalytically inactive recombinant FXa variant lacking the membrane-binding 4-carboxyglutamic acid domain (GD-FXa$^{S[195]A}$; Andexanet alfa, Portola Pharmaceuticals) currently in Phase III clinical trials[112]. This variant cannot contribute directly to thrombin formation as it lacks catalytic activity and does not bind membranes, precluding it from assembling in the prothrombinase complex. However,

because it retains binding interactions at the active site, it specifically depletes FXa inhibitors in blood. Indeed, experiments in rabbits and rats support GD-FXa$^{S[195]A}$ as a pre-injury reversal agent[112, 139]. However, because of its mechanism of action, hundreds of milligrams of GD-FXa$^{S[195]A}$ are required for a therapeutic effect[140]. Moreover, at these high concentrations, GD-FXa$^{S[195]A}$ is expected to bind and deplete the endogenous serine protease inhibitor antithrombin III (ATIII)[112] and tissue factor pathway inhibitor (TFPI), both essential for the negative regulation of coagulation[141, 142]. Since these two natural inhibitors are protective against thrombosis, their depletion could paradoxically increase thrombotic risk, especially in hypercoagulable patients. Consequently, a procoagulant hemostatic agent that is effective at catalytic concentrations may be a better choice to stop bleeding induced by FXa inhibitors.

Existing bypassing agents developed for hemophilia treatment (e.g. PCCs, activated prothrombin complex concentrates (aPCCs), and recombinant factor VIIa (rFVIIa)), are being evaluated for reversal of direct FXa inhibitors with mixed results[123, 126, 127]. Here we tested a bypassing strategy based on a FXa variant developed in our group[44, 59, 60]. The substitution of isoleucine at position 16 (numbered after chymotrypsinogen[23]) with leucine to yield FXa$^{I[16]L}$ results in an impaired conformational transition from zymogen to protease and yields a zymogen-like FXa species. Zymogen-like variants like FXa$^{I[16]L}$ have impaired active site function and are thus resistant to active site inhibitors and have longer plasma half-lives than wild-type (wt)-FXa. Notably, their impaired activity is rescued upon binding the cofactor, FVa, on membranes to form the prothrombinase complex. These properties allow zymogen-like FXa variants to effectively bypass the clotting defect in hemophilic mice without increasing risk for thrombosis or consumptive coagulopathy[60]. We hypothesized these

features would also enable them to avoid inhibition and restore hemostasis in the presence of direct FXa inhibitors. In this study, we demonstrate that $FXa^{I[16]L}$ is an effective bypassing agent to counteract direct FXa inhibitors *in vitro* and *in vivo* using established mouse hemostasis models.

Methods

Reagents. Z-Gly-Gly-Arg-AMC was from Bachem Bioscience Inc. Technothrombin thrombin calibrator and reagent RB were from Diapharma Group Inc. Pooled normal human plasma (NHP) was obtained from George King Biomedical, Inc. Rivaroxaban was from Selleck Chemicals. All tissue culture reagents were from Invitrogen except insulin-transferrin-sodium selenite, which was from Roche. Ferric chloride ($FeCl_3$) was from Sigma-Aldrich. Corn trypsin inhibitor (CTI) was from Haematologic Technologies. Activated prothrombin complex concentrates (aPCCs, FEIBA NF) were purchased from Baxter International, Inc. Innovin was from Dade Behring. Rat anti-mouse CD41 antibody prepared as a $F_{(ab)2}$ fragment was from BD Bioscience. Mouse anti-human fibrin monoclonal antibody (clone 59D8), which cross-reacts with mouse fibrin, has been previously described[143, 144]. These antibodies were conjugated with $Alexa^{555}$ or $Alexa^{488}$ using the Alexa Fluor Protein Labeling Kit according to the manufacturer's instructions (Molecular Probes/Invitrogen).

Proteins. The FX activator from Russell's viper venom, $RVV_{X\text{-}CP}$ was purified as previously described[145]. Recombinant $hFX^{I[16]L}$ and $mFX^{I[16]L}$ were expressed in human embryonic kidney 293 (HEK293) cells, purified from media, and activated using $RVV_{X\text{-}CP}$ as previously described[146, 147]. $hFX^{S[195]A}$ was expressed in HEK293 cells and purified

from the media using a protocol identical to that used for $hFX^{I[16]L}$. $hFX^{S[195]A}$ was then activated to $hFXa^{S[195]A}$ with $RVV_{X\text{-}CP}$ and purified in the same way as the other recombinant FXa molecules. To generate GD-$FXa^{S[195]A}$, $hFXa^{S[195]A}$ was digested with chymotrypsin as previously described[148] and purified using a Poros HQ/20 column, eluting with a NaCl gradient in 20 mM Tris, pH 8.3, then dialyzing against 20 mM HEPES, 150 mM NaCl, pH 7.4.

Mice. Wild-type (wt) male C57BL/6J mice purchased from Jackson Laboratory were used in all experiments. For *ex vivo* ROTEM studies and all ferric chloride carotid artery injury experiments, blood was collected from 8-10-week-old mice weighing 20-30 g. For intravital imaging studies, mice were 11-12 weeks old and weighed 25-35 g. Experimental approval was obtained from the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Clotting Assays. One μM rivaroxaban was incubated in NHP along with various concentrations of GD-$FXa^{S[195]A}$ for 30 minutes at room temperature[112]. After incubation, 50 μL of each plasma sample was incubated at 37°C for 60 s. Coagulation was then initiated by addition of 100 μL of Innovin, and time to clot formation was measured with a Start4 coagulation machine (Diagnostica Stago).

Thrombin Generation Assays. Thrombin generation assays (TGA) in NHP were performed as previously described[59] with a slight modification to accommodate the addition of rivaroxaban and reversal agents as appropriate. 40 μL NHP was added to a microtiter plate (Nunc; F16 black Maxisorp) along with 10 μL Technothrombin RB (2 pM TF, 4.0 μM phospholipid). 3 μL rivaroxaban dissolved in 20 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, pH 7.4 (HBS-PEG) was added to NHP in a black microtiter plate

(Nunc; F16 black Maxisorp) along with the 2 μL of reversal agent (hFXa$^{I[16]L}$, GD-FXa$^{S[195]A}$, or aPCCs). The reaction was initiated immediately by adding Z-Gly-Gly-Arg-AMC in 15 mM $CaCl_2$ (50 μL; 0.5 mM final). Fluorescence (λ_{ex}=360 nm, λ_{em}=460 nm) was measured at one minute intervals for 90 minutes at 37°C using a Spectramax M2^e (Molecular Devices) plate reader. The Technothrombin calibrator kit was used to convert raw fluorescence intensity to thrombin concentration. Thrombograms (nM thrombin vs. time) were made to determine the lag time, peak height, and endogenous thrombin potential (ETP).

Rotational Thromboelastography (ROTEM). The institutional review board of the Children's Hospital of Philadelphia Research Institute approved the human phlebotomy and blood use performed in this study. Informed consent was obtained from all subjects. For human studies, whole blood from 5 healthy donors was collected into CTI (final concentration 25 μg/mL) and one-tenth volume of 3.2% sodium citrate. 300 μL of blood was added to a pre-warmed (37°C) ROTEM cup along with rivaroxaban and hFXa$^{I[16]L}$ at the indicated concentrations. The reaction was initiated with 11.8 mM $CaCl_2$ and a 1:17,300 dilution of innovin. Data was exported using the manufacturer's export tool and plotted as time versus elasticity. The clot time (CT) was determined as the time to reach an amplitude of 2 mm of elasticity.

For murine *ex vivo* ROTEM studies, rivaroxaban was formulated for intravenous injection using a mixture of polyethylene glycol 400, H_2O, and glycerol (968g, 590 g, and 58 g, respectively) that was a modification to a previously reported protocol[149]. The DMSO stock of rivaroxaban was diluted in the injection solution to a concentration of 0.25 mg/mL such that the DMSO concentration in the final solution was 0.5%.

Rivaroxaban was infused via the lateral tail vein prior to anesthetizing the mouse (pentobarbital, intraperitoneal injection). The right jugular vein and infrahepatic inferior vena cava (IVC) were exposed. Proteins ($mFXa^{I[16]L}$ or $GD\text{-}FXa^{S[195]A}$) diluted in HBS-PEG were infused directly into the jugular vein using a needle crossing the pectoral muscle getting into the jugular vein. One minute after protein infusion, blood (400 µL final volume including anticoagulants) was withdrawn from the infrahepatic IVC using a 22G needle pre-loaded with one-tenth volume 3.2% sodium citrate and CTI (100 µg/mL final). The blood samples were analyzed by ROTEM as described above.

Ferric Chloride Carotid Artery Injury Model. Ferric chloride-induced carotid artery injury was performed with minor modifications to previously established protocols[60]. Rivaroxaban or vehicle was infused via lateral tail vein injection using the same protocol used in the *ex vivo* studies described above. After anesthesia with intraperitoneal pentobarbital, the right jugular vein was exposed to allow for direct jugular vein injection of proteins. The right common carotid artery from the sternal origin of the sternocleidomastoid muscle to the carotid bifurcation was exposed. Baseline carotid blood flow was recorded with a miniature Doppler flow probe (Model 0.5PSB; Transonic Systems) positioned around the carotid artery. A 2 mm x 1 mm piece of Whatman #1 filter paper soaked in 7.5% $FeCl_3$ (0.46 M) was applied to the adventitial surface of the artery for 2 minutes. The paper was then removed, the area flushed with PBS, and blood flow monitored continuously for 30 minutes. Time to complete occlusion was defined as the time from the end of the injury period to the time when blood flow had decreased by >90% of the baseline level for at least 5 continuous minutes. After the 30 minute monitoring period, reversal of rivaroxaban was attempted by infusing protein ($mFXa^{I[16]L}$ or $GD\text{-}FXa^{S[195]A}$) or vehicle via direct jugular vein injection. Following

protein injection, blood flow was monitored for an additional 30 minutes, and time to complete occlusion was recorded as the time between protein infusion and the beginning of a 5-minute-sustained occlusive event as defined above. In some experiments, protein was injected via direct jugular vein injection 1 minute prior to $FeCl_3$ injury. In all experiments, complete occlusion was verified at the site of the injury by visually observing an opaque clot.

Intravital Imaging of Thrombus Formation. Evaluation of hemostasis following laser injury to mouse cremasteric arterioles has been previously described[150] and our specific experimental system has been detailed[60, 151-153]. Rivaroxaban and proteins were formulated as described for *ex vivo* ROTEM experiments and $FeCl_3$ injury experiments, but were injected via jugular vein cannulus instead of the tail vein. After allowing rivaroxaban to distribute for 5 minutes, $Alexa^{555}$-labled rat anti- CD41 $F_{(ab)2}$ and $Alexa^{488}$-labeled anti-fibrin antibodies to detect platelets and fibrin, respectively, were infused and laser-injury was performed to the vessel wall of the cremasteric arterioles. In some experiments, rivaroxaban-treated mice were infused with the reversal agent (either $mFXa^{I[16]L}$ or $GD\text{-}FXa^{S[195]A}$, dissolved to the appropriate concentration in 20 mM HEPES, 150 mM NaCl, pH 7.4). Cremasteric arterioles of 30-50 μm were injured using a pulse-nitrogen dye laser applied through the microscope objective. Brightfield and fluorescence images were collected over 3-4 minutes at 4 frames/second and the data were analyzed with Slidebook 6 software (Intelligent Imaging Innovations). The kinetics of clot formation were analyzed by determining median fluorescence intensity over time in ~14-22 thrombi from three animals/group. For the 50 mg/kg $GD\text{-}FXa^{S[195]A}$ group, one animal was used and 5 injuries were made.

Results

FXa$^{I[16]L}$ reverses FXa inhibitors in vitro: Standard clinical clotting assays are not particularly sensitive to the effects of direct FXa inhibitors[154, 155]. Instead, we used thrombin generation assays (TGA) to determine if FXa$^{I[16]L}$ could mitigate the effects of rivaroxaban in normal human plasma (NHP). Rivaroxaban, within the therapeutically useful range (170-830 nM)[121], dose-dependently inhibited thrombin generation in NHP (**Fig. 1a-b**), reducing peak thrombin levels (**Fig. 1b**). At a fixed concentration of rivaroxaban (500 nM), FXa$^{I[16]L}$ improved peak thrombin generation with full normalization achieved at 3 nM FXa$^{I[16]L}$ (**Fig. 1c**). Even in the presence of 2.5 μM rivaroxaban to simulate over-anticoagulation, FXa$^{I[16]L}$ increased peak thrombin generation to near-normal levels (**Fig. 1d**). Similar results were obtained with apixaban, another direct FXa inhibitor (**Supplementary Fig. 1**). We also studied the effects of rivaroxaban and FXa$^{I[16]L}$ in whole blood from normal human donors using rotational thromboelastography (ROTEM) to evaluate whether additional blood components influence the results. Rivaroxaban prolonged the time to initial clot formation (CT) when added at therapeutic (**Supplementary Fig. 2a-b**) and supratherapeutic (**Supplementary Fig. 2c-d**) concentrations. The addition of FXa$^{I[16]L}$ (0.3 or 3 nM) improved the ROTEM profile in rivaroxaban-treated blood. Whether assessed in plasma or whole blood, these results illustrate the effectiveness of FXa$^{I[16]L}$ in counteracting direct FXa inhibitors.

To compare the relative *in vitro* efficacy and potency of FXa$^{I[16]L}$ to other reversal strategies, we expressed and purified GD-FXa$^{S[195]A}$. Characterization of GD-FXa$^{S[195]A}$ revealed that our preparation was comparable to those used in previous reports[112]

(**Supplementary Fig. 3**). Titration of GD-FXa$^{S[195]A}$ into NHP anticoagulated with rivaroxaban (500 nM (**Fig. 1e**) or 2.5 μM (**Supplementary Fig. 4**)) confirmed it completely restores thrombin generation when present at concentrations equal to or higher than the inhibitor. However, compared to FXa$^{I[16]L}$, GD-FXa$^{S[195]A}$ was approximately 300-fold less potent (**Fig. 1f**). We also evaluated aPCCs as reversal agents, but they did not substantially restore thrombin generation (**Supplementary Fig. 5**).

FXa$^{I[16]L}$ restores hemostasis in anticoagulated mice: For these studies, we expressed and purified mouse-(m)FXa$^{I[16]L}$ to avoid the possibility of inter-species incompatibility[60] and employed *ex vivo* ROTEM to determine the dosing range. Rivaroxaban (1 mg/kg) substantially increased the ROTEM CT compared to controls, and addition of mFXa$^{I[16]L}$ (1 mg/kg) normalized all parameters (**Fig. 2a**). As expected, GD-FXa$^{S[195]A}$ was mostly ineffective at 5 mg/kg and a much higher dose (25 mg/kg) was needed to normalize the CT.

To extend the work *in vivo,* we employed the $FeCl_3$ carotid arterial thrombosis model[60, 156] (**Fig. 2b-f** and **Supplementary Fig. 6**). Rivaroxaban (0.5 mg/kg) prolonged the time to occlusion, and a higher dose (1 mg/kg) prevented carotid artery occlusion (**Fig. 2b**). Administration of mFXa$^{I[16]L}$ (0.25 mg/kg or 1 mg/kg) 30 minutes after injury (**Fig. 2c**) rapidly restored occlusion (**Fig. 2d**). This experimental design was advantageous because both the anticoagulant effects of rivaroxaban and the pro-hemostatic effects of mFXa$^{I[16]L}$ could be observed in the same animal. The mean time to occlusion with mFXa$^{I[16]L}$ was faster than untreated controls, consistent with the fact that mFXa$^{I[16]L}$ generates thrombin by bypassing the intrinsic and extrinsic pathways. Surprisingly,

administration of GD-FXa$^{S[195]A}$ (25 mg/kg) did not restore clot formation (**Fig. 2d**), suggesting that it might not be as effective when administered after an injury has occurred. As a control, 30 minutes after administration of GD-FXa$^{S[195]A}$, infusion of mFXa$^{I[16]L}$ (1 mg/kg) led to occlusion at the site of injury (data not shown), confirming the injury was sufficient to produce carotid thrombosis.

Reversal of anticoagulation is sometimes necessary before invasive procedures. To test the effects of mFXa$^{I[16]L}$ in such a scenario, rivaroxaban-treated mice were injected with mFXa$^{I[16]L}$ 1 minute prior to $FeCl_3$ injury (**Fig. 2e**). Administration of mFXa$^{I[16]L}$ (0.5 mg/kg) resulted in complete occlusion of the carotid artery (**Fig. 2f**) with occlusion times comparable to those of controls. GD-FXa$^{S[195]A}$ also restored vascular occlusion in this model at the dose (25 mg/kg) predicted by *ex vivo* experiments while a lower dose (5 mg/kg) was ineffective. Together, these results indicate that FXa$^{I[16]L}$ is a potent hemostatic agent for rivaroxaban and suggest that it could be effective to prevent bleeding as well as to stop a bleeding episode that has already begun.

To further evaluate this reversal strategy *in vivo*, we employed a microcirculatory model of hemostasis following laser injury to mouse cremasteric arterioles[60, 156]. Compared to wild-type mice, infusion of rivaroxaban (1 mg/kg) prior to vessel injury prevented fibrin deposition and considerably decreased platelet accumulation at the site of laser injury (**Fig. 3**). Infusion of 25 or 50 mg/kg GD-FXa$^{S[195]A}$ into rivaroxaban-treated mice resulted in a small but dose-dependent increase in platelets and fibrin (**Fig. 3a-c**). In contrast, a much lower dose of mFXa$^{I[16]L}$ (1 mg/kg) following rivaroxaban administration produced a greater increase in both platelet and fibrin accumulation (**Fig. 3a-c**). Together with the $FeCl_3$ injury data, this indicates that FXa$^{I[16]L}$ can restore

thrombin generation *in vivo* in diverse vascular beds.

Discussion

Development of an effective reversal strategy for direct FXa inhibitors is imperative as their use becomes more widespread. In this study, we tested a bypassing approach for oral FXa anticoagulants with a zymogen-like variant of FXa. Administration of $FXa^{I[16]L}$ reversed the effects of rivaroxaban *in vivo* in both a mouse microcirculatory injury model as well as in a large vessel thrombosis model. Notably, $FXa^{I[16]L}$ was >50-fold more potent than GD-$FXa^{S[195]A}$, an antidote that sequesters rivaroxaban by molecular engagement. The higher potency of $FXa^{I[16]L}$ indicated that its mechanism of action must be distinct from that of GD-$FXa^{S[195]A}$.

$FXa^{I[16]L}$ has numerous features that make it an attractive agent for alleviating the anticoagulant effects of rivaroxaban. *In vivo* characterization of $FXa^{I[16]L}$ revealed that it is substantially more potent and possibly more effective than antidote approaches. Its high potency is advantageous because low quantities of the protein can be used. From a safety perspective, catalytic amounts of $FXa^{I[16]L}$ are unlikely to deplete ATIII or other endogenous protease inhibitors. Further, even though both $FXa^{I[16]L}$ and GD-$FXa^{S[195]A}$ are variants of FXa and immunogenic risk cannot be ruled out without empirical human data, lower doses of $FXa^{I[16]L}$ may decrease its likelihood. Finally, since $FXa^{I[16]L}$ is dependent on FVa to rescue its protease activity, this may localize its activity to the site of vascular injury, possibly decreasing its thrombogenic potential.

When infused after injury, GD-$FXa^{S[195]A}$ did not restore carotid artery occlusion in the $FeCl_3$ model. These results are somewhat surprising since administration of GD-

$FXa^{S[195]A}$ should render the animal hemostatically normal. This may indicate that although a scavenging antidote alone is sufficient to sequester the anticoagulant, it may not be as effective at stopping a bleeding episode that has already ensued. This could also mean that small molecule antidotes, such as PER977[117, 139], which directly binds FXa inhibitors, may only be useful in certain clinical situations. Our findings also raise concerns that similar antidote approaches for direct thrombin inhibitors (i.e. the F_{ab} antidote for dabigatran[115]) may suffer the same limitations. Most importantly, these results underscore the need for empiric efficacy data in clinical trials.

The mechanism by which $FXa^{I[16]L}$ reverses the effects of rivaroxaban is somewhat less clear. While $FXa^{I[16]L}$ is certainly an effective pro-hemostatic agent, it is not apparent how susceptible it is to inhibition by direct FXa inhibitors. The extensive literature surrounding zymogen-like FXa variants has shown that they are less inhibited by active site probes than wt-FXa when free in solution but comparable to wt-FXa when assembled in prothrombinase. Thus, it is plausible that, despite their zymogenicity and possible resistance to rivaroxaban when free in solution, $FXa^{I[16]L}$ may be just as inhibited as endogenous FXa at the site of vascular injury. If this is the case, ability of $FXa^{I[16]L}$ to reverse rivaroxaban might have a more complex and nuanced mechanism.

In conclusion, we demonstrate the efficacy of $FXa^{I[16]L}$ as a procoagulant-bypassing agent for direct FXa inhibitors. Through comparisons of $FXa^{I[16]L}$ with a potential antidote we gained insight into the fundamental efficacy and potency differences between antidotes and bypassing strategies for direct FXa inhibitors. Perhaps most importantly, we now have a better understanding of the challenges remaining to address the urgent unmet clinical need for a reversal agent for direct FXa inhibitors.

Figure Legends

Figure 1. Effect of FXa$^{I[16]L}$ or GD-FXa^{S195A} on thrombin generation in rivaroxaban-treated plasma. (***a***) Representative thrombin generation tracing in platelet poor normal human plasma (NHP) with different rivaroxaban concentrations (black, 0; red, 200 nM; blue, 400 nM; cyan, 800 nM). (***b***) Peak thrombin generation at different rivaroxaban concentrations as a percentage of NHP. (***c, d***) Normalized peak thrombin generation was measured after FXa$^{I[16]L}$ was titrated into NHP supplemented with (***c***) 500 nM or (***d***) 2.5 μM rivaroxaban. (***e***) Peak thrombin generation following GD-FXa^{S195A} titration into NHP containing 500 nM rivaroxaban. (***f***) Peak thrombin generation in the presence of 500 nM rivaroxaban and FXa$^{I[16]L}$ (blue) or GD-FXa^{S195A} (red) plotted on a logarithmic scale. All experiments in panels ***b-f*** were performed in quadruplicate, and all measurements are shown as mean ± SD.

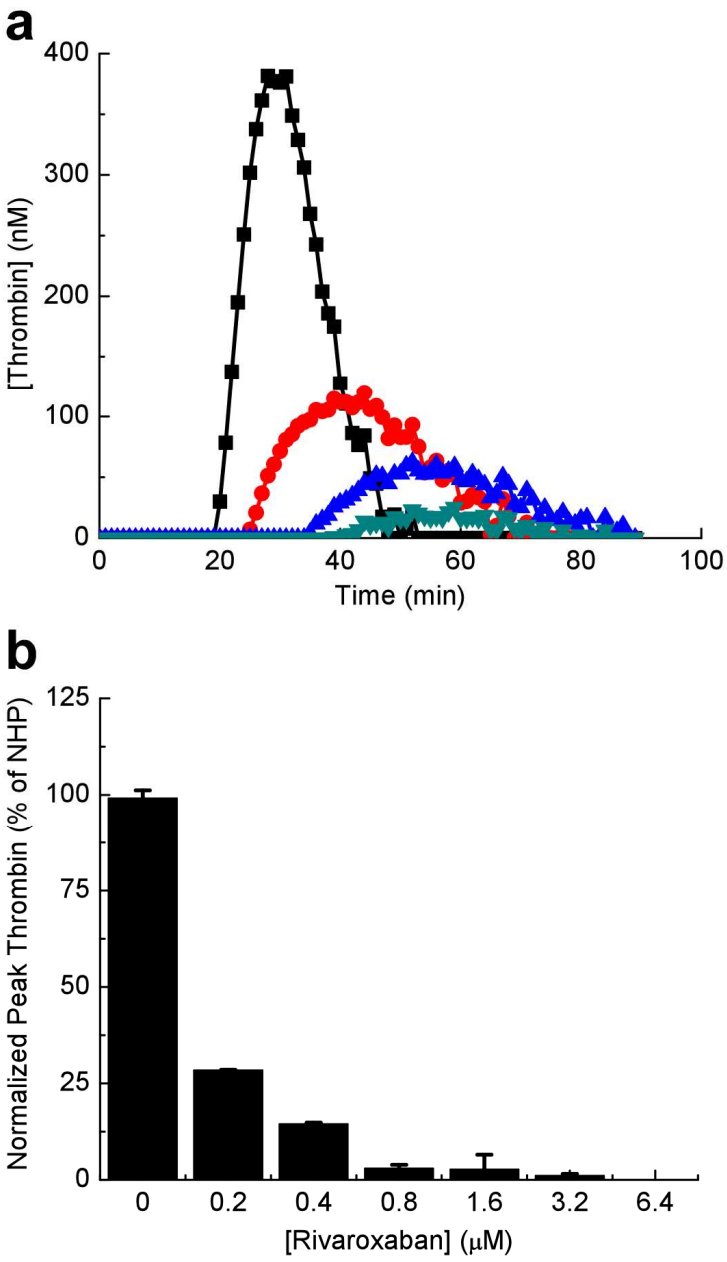
Figure 2. Reversal of rivaroxaban by FXa$^{I[16]L}$ or GD-FXa^{S195A} in anticoagulated mice. (***a***) ROTEM clot times (CT) after administration of 1 mg/kg rivaroxaban (riva) and mFXa$^{I[16]L}$ or GD-FXa^{S195A} to hemostatically normal C57BL/6 mice. (***b***) Effect of rivaroxaban dose on time to complete occlusion of the mouse carotid artery following $FeCl_3$ injury. Blood flow was monitored for 30 minutes after the injury. (***c, d***) 30 minutes after $FeCl_3$ injury, animals treated with 1 mg/kg rivaroxaban were injected with either mFXa$^{I[16]L}$ or GD-FXa^{S195A} and time to carotid occlusion was measured for another 30 minutes. (***e, f***) animals treated with 1 mg/kg rivaroxaban were infused with mFXa$^{I[16]L}$ or GD-FXa^{S195A} prior to $FeCl_3$ injury and carotid artery occlusion times were measured. In all plots, horizontal black lines represent the mean for each

group.

Figure 3. Reversal of rivaroxaban by $FXa^{I[16]L}$ or GD-FXa^{S195A} in a laser injury model. (***a***) Digital composite fluorescence and brightfield images of representative thrombi in WT mice treated with either vehicle and HBS, rivaroxaban (1 mg/kg) and HBS, rivaroxaban (1 mg/kg) and GD-FXa^{S195A} (50 mg/kg), or rivaroxaban (1 mg/kg) and $mFXa^{I[16]L}$ (1 mg/kg) before (0 s) and 30, 90, and 150 s after laser-induced injury of the cremasteric blood vessel wall. Platelets (red) were detected by an $Alexa_{555}$-labled rat anti-CD41 $F(ab)_2$ and fibrin (green) with $Alexa_{488}$-labled anti-fibrin antibody; areas of overlap are yellow. (***b, c***) Platelet and fibrin accumulation was quantified over time following laser injury in vehicle + HBS-treated mice (black; 3 mice, 22 injuries), rivaroxaban + HBS-treated mice (green; 3 mice, 15 injuries), rivaroxaban + 25 mg/kg GD-FXa^{S195A}-treated mice (purple; 3 mice, 14 injuries), rivaroxaban + 50 mg/kg GD-FXa^{S195A}-treated mice (dark red; 1 mouse, 5 injuries), and rivaroxaban + 1 mg/kg $mFXa^{S195A}$ (blue; 3 mice, 15 injuries). Median fluorescence intensity (MFI) for (***b***) platelet and (***c***) fibrin fluorescence are plotted versus time.

Figures

Figure 1



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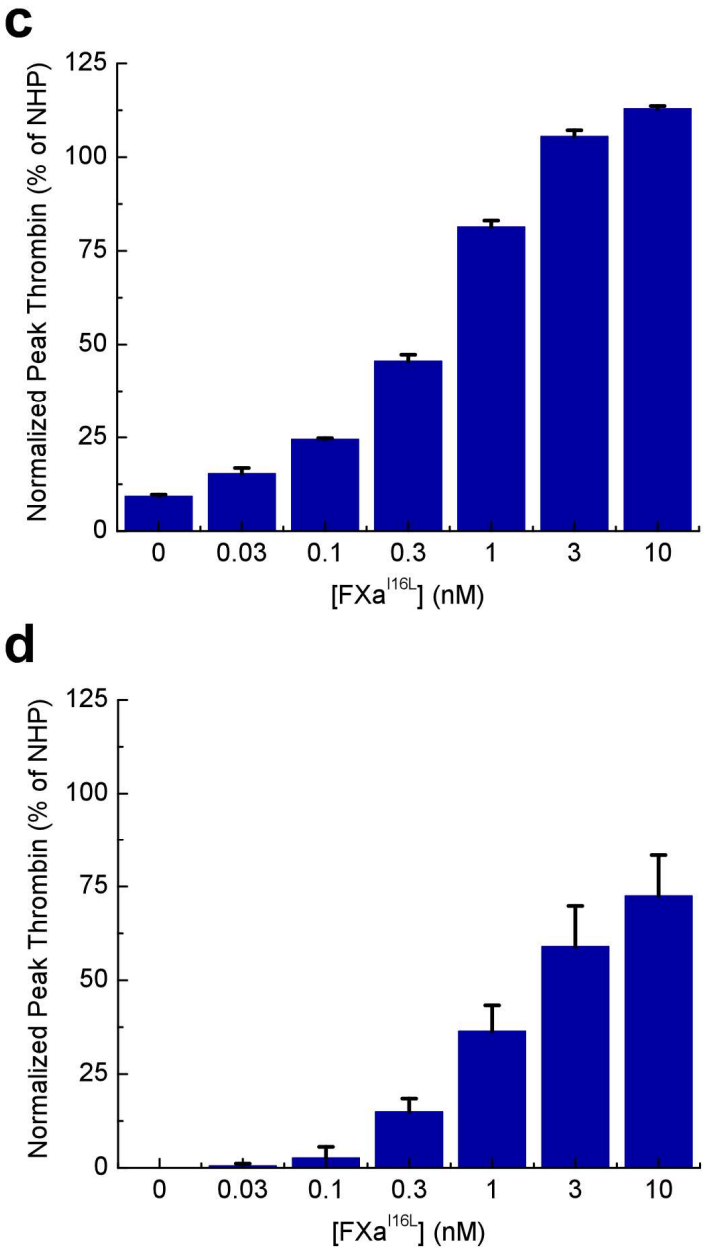

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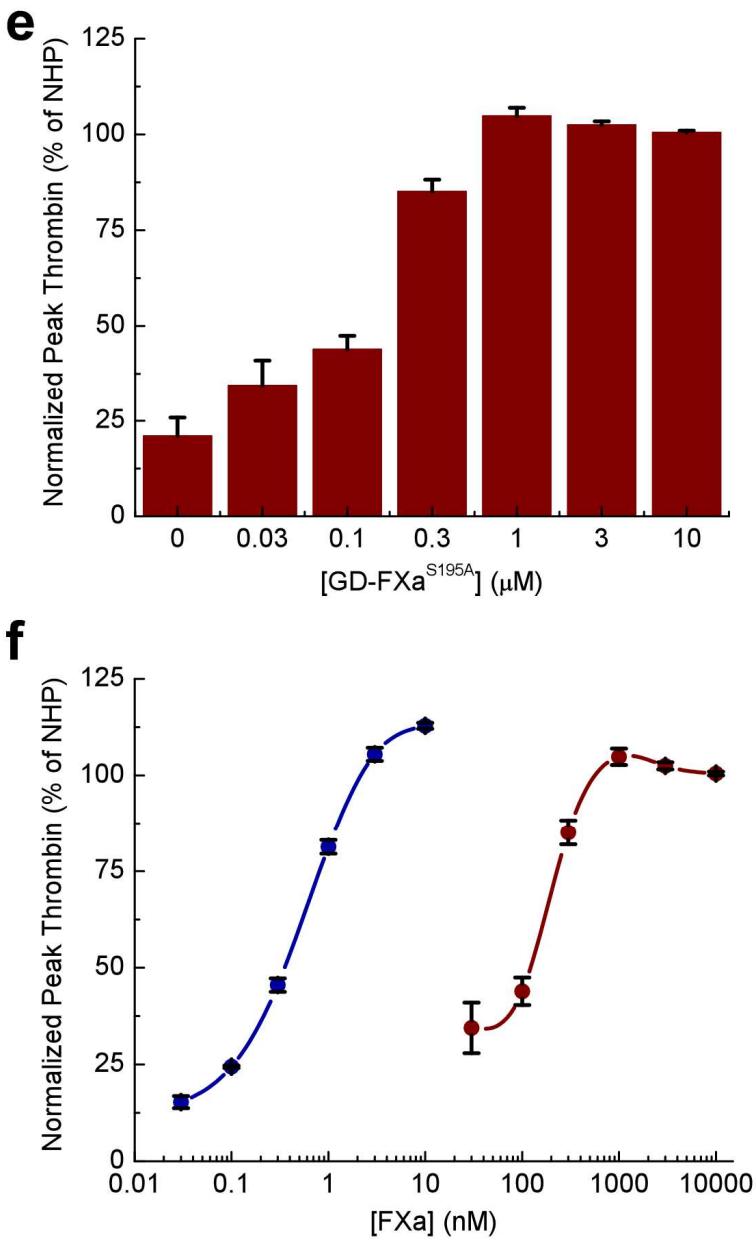
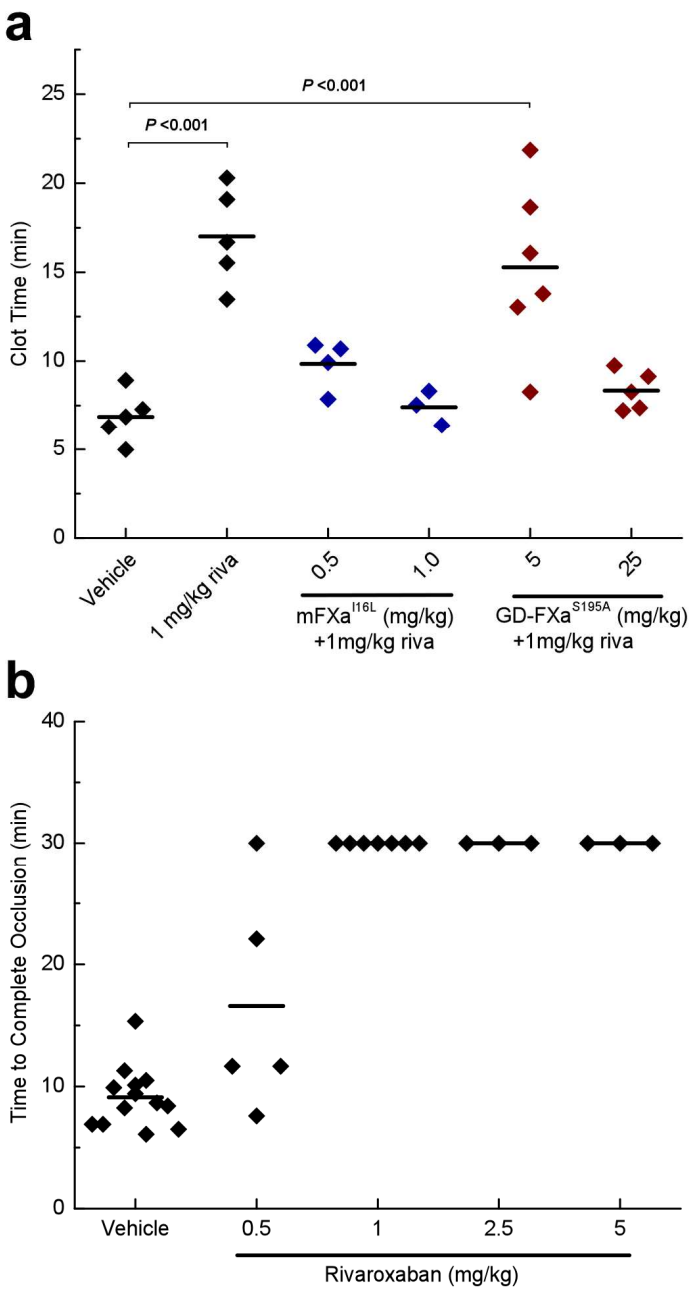


Figure 2



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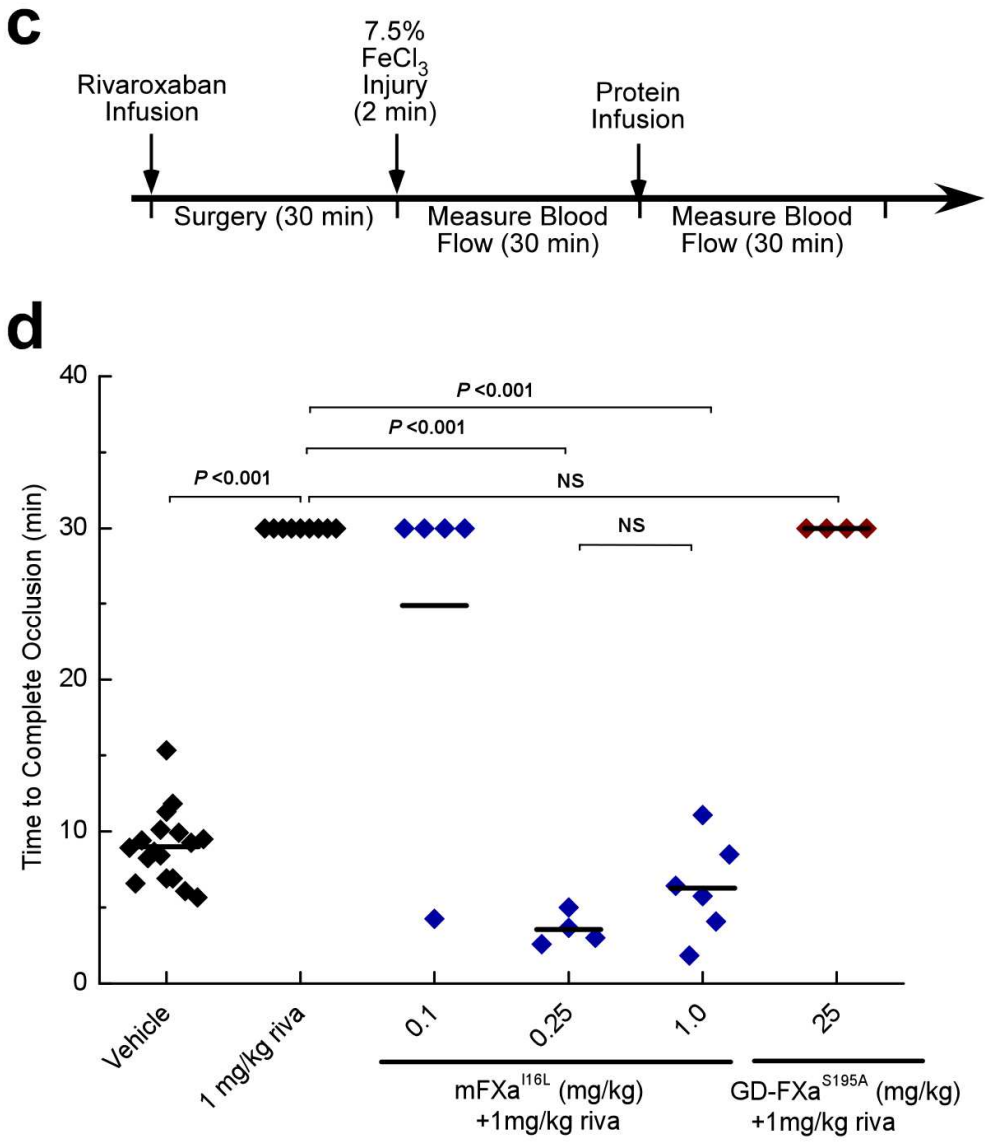

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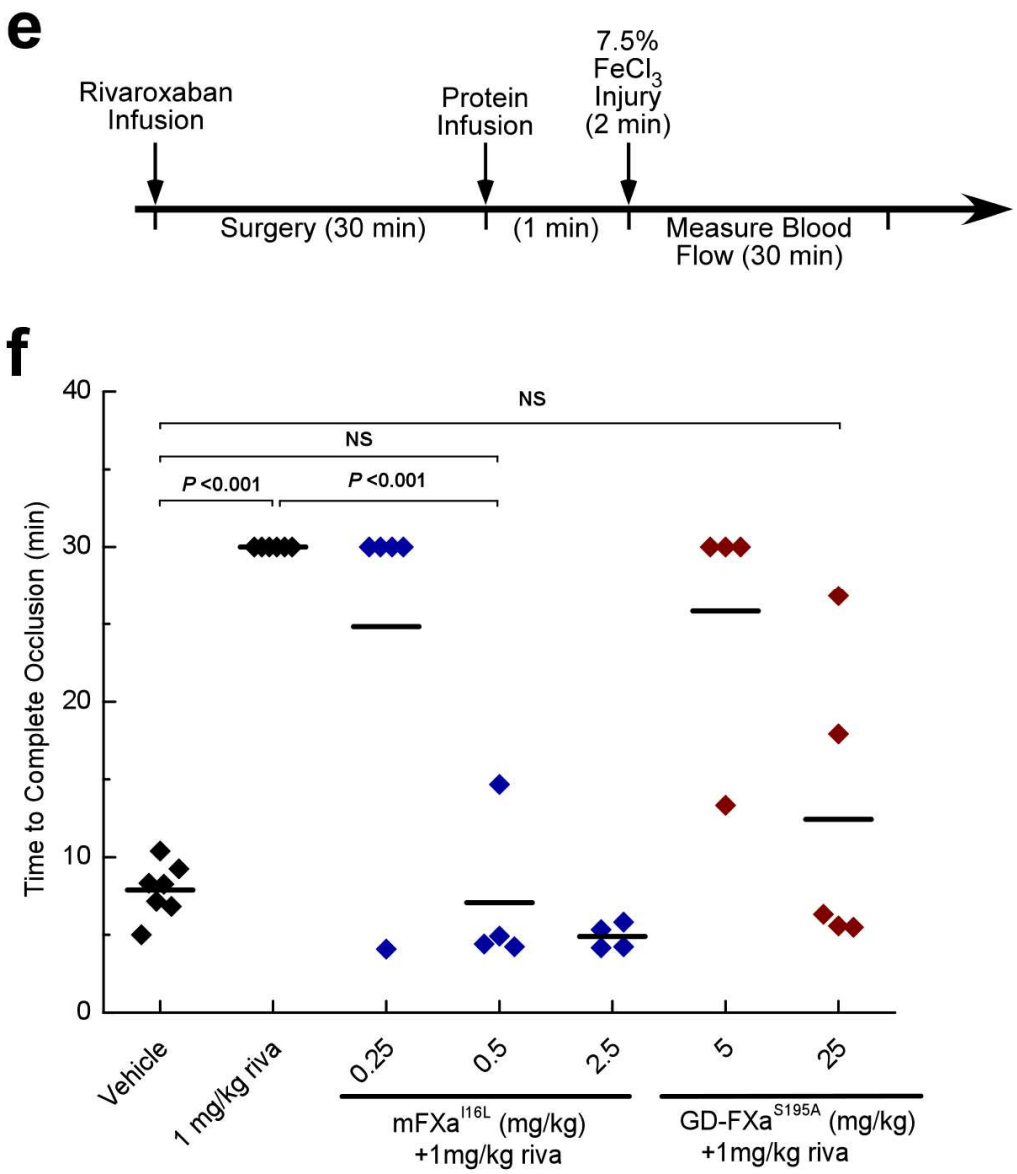
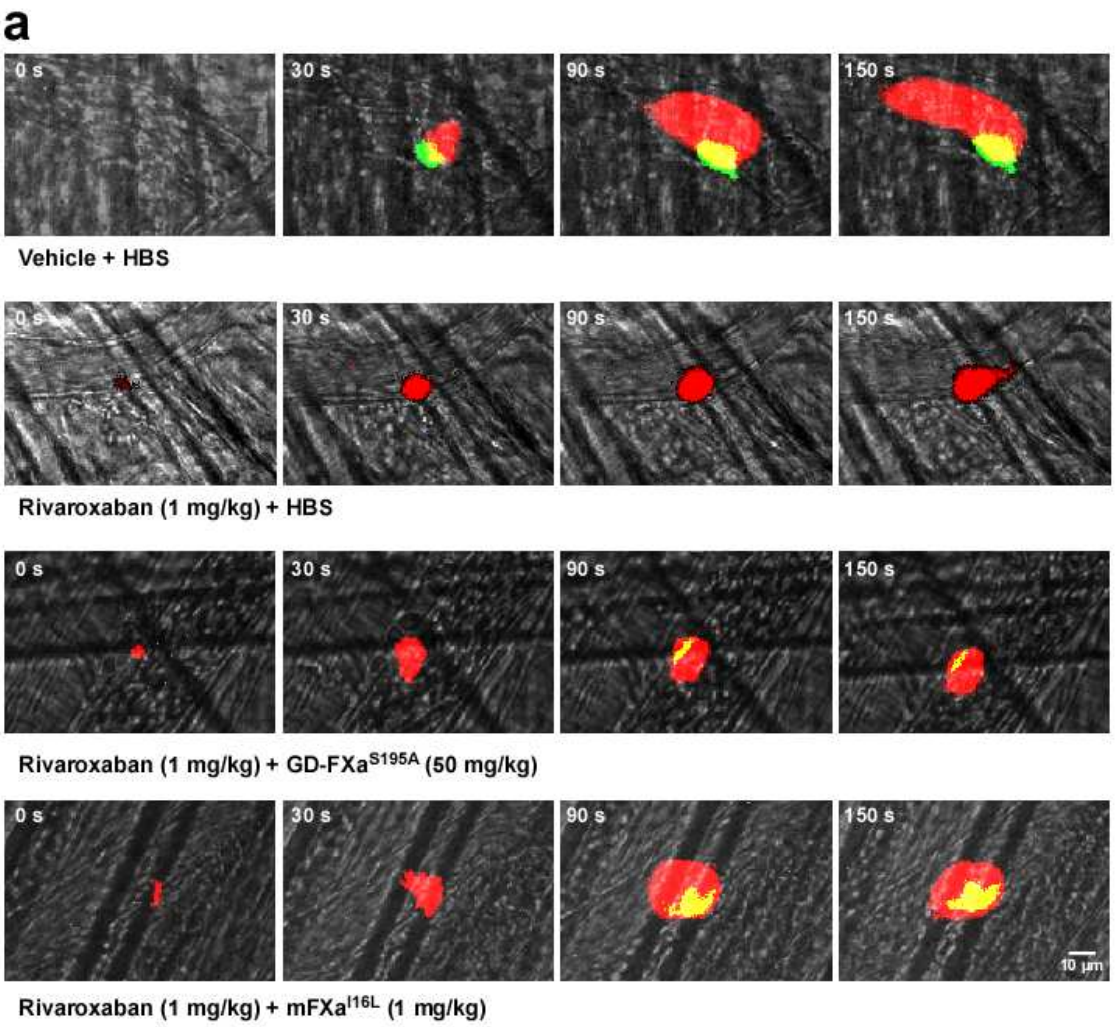


Figure 3



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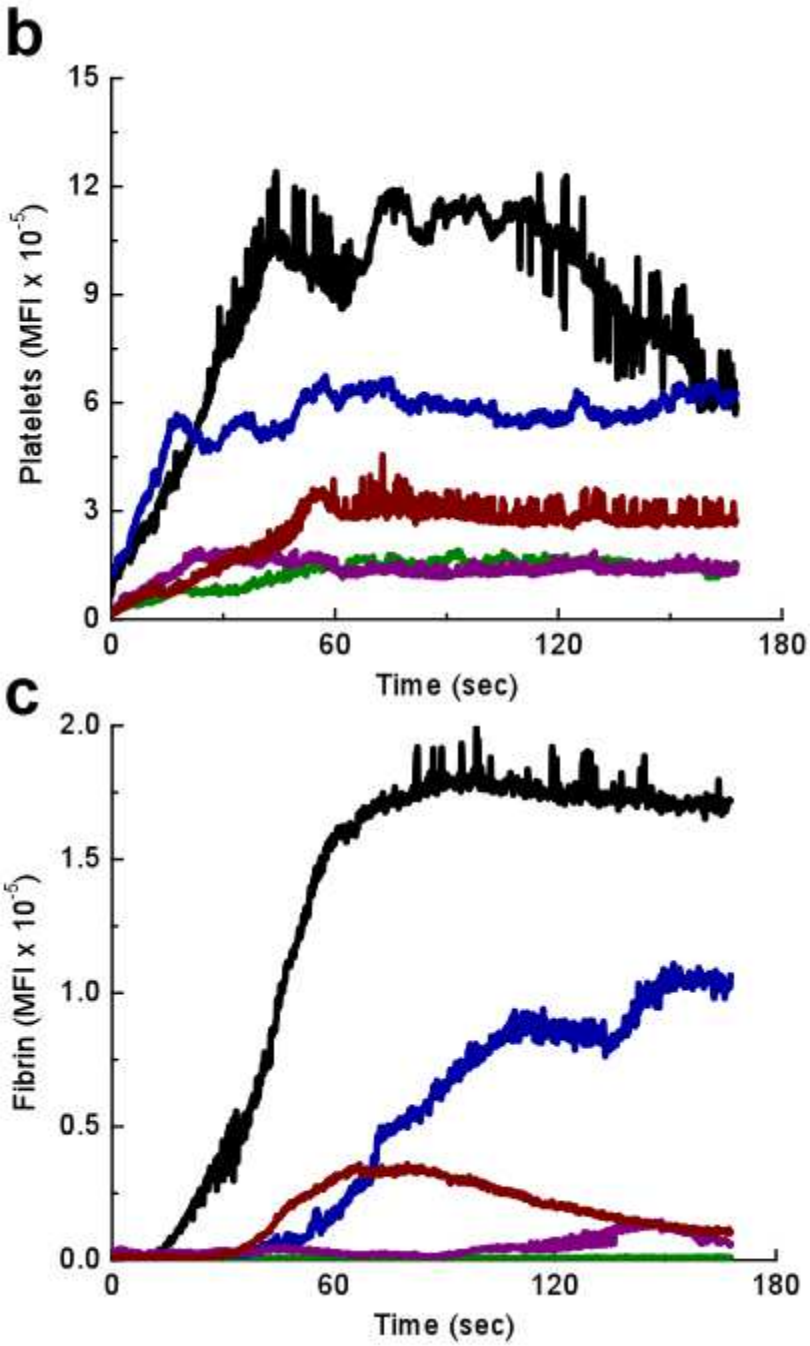


Supplementary Figure Legends

Supplementary Figure 1. Effect of FXa$^{I[16]L}$ on thrombin generation in apixaban-treated plasma. (***a***) Representative thrombin generation tracing in NHP with different apixaban concentrations (black, 0; red, 200 nM; blue, 400 nM; cyan, 800 nM). (***b***) Quantification of peak thrombin generation at different apixaban concentrations is shown as a percentage of normal peak thrombin generation. (***c, d***) Normalized peak thrombin generation was measured after FXa$^{I[16]L}$ was titrated into normal plasma supplemented with (***c***) 250 nM (***d***) 2.0 μM apixaban. All experiments in panels ***b-d*** were performed in quadruplicate, and all measurements are shown as mean ± SD.

Supplementary Figure 2. Reversal of rivaroxaban by hFXa$^{I[16]L}$ in human whole blood. (***a, b***) 500 nM or (***c, d***) 2.5 μM rivaroxaban (riva) and different concentrations of hFXa$^{I[16]L}$ were added to normal human whole blood collected in citrate and 25 μg/mL corn trypsin inhibitor (CTI). Representative ROTEM tracings (panels ***a*** and ***c***: black, dilution buffer only; red, rivaroxaban only; blue, rivaroxaban plus 0.3 nM hFXa$^{I[16]L}$; green, rivaroxaban plus 3.0 nM hFXa$^{I[16]L}$) and clot times (CT; panels ***b*** and ***d***) are shown.

Supplementary Figure 3. Characterization of GD-FXa^{S195A}. One μM rivaroxaban and different concentrations of GD-FXa^{S195A} (black bars) were incubated in platelet-poor NHP at room temperature for 30 min. Prothrombin times (PT) were then measured and

compared to NHP alone (gray bar) and NHP incubated with GD-FXa^{S195A} alone (open bar). Experiments were performed in quadruplicate and plotted as the mean ± SEM.

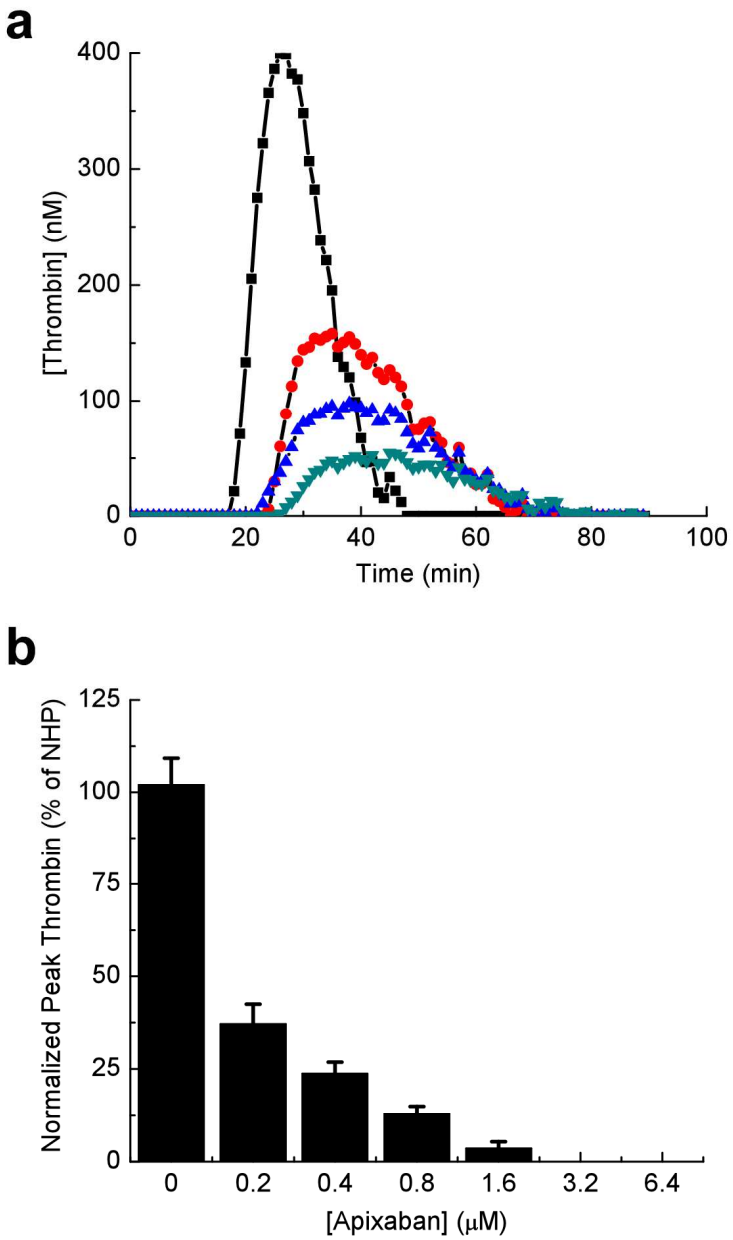
Supplementary Figure 4. Effect of GD-FXa^{S195A} on thrombin generation in high-dose-rivaroxaban-treated plasma. Increasing concentrations of GD-FXa^{S195A} were added to NHP containing 2.5 μM rivaroxaban and thrombin generation at 37°C was measured. Peak thrombin generation was normalized to that of NHP and plotted against GD-FXa^{S195A} concentration. Experiments were performed in quadruplicate, and all measurements are shown as mean ± SD.

Supplementary Figure 5. Effect of aPCCs on thrombin generation in rivaroxaban-treated plasma. Increasing concentrations of aPCC were added to NHP containing 500 nM rivaroxaban and thrombin generation at 37°C was measured. Peak thrombin generation was normalized to that of NHP and plotted against aPCC concentration. Experiments were performed in quadruplicate, and all measurements are shown as mean ± SD.

Supplementary Figure 6. Representative carotid artery Doppler flow tracings following $FeCl_3$-injury. Rivaroxaban was administered to WT C57BL/6J mice and $FeCl_3$ (7.5%) injury was performed. Blood flow is plotted against time after the 2 minute $FeCl_3$ injury period. Where shown (red arrow), protein (mFXa$^{I[16]L}$ or GD-FXa^{S195A}) was infused 30 minutes later and blood flow was monitored for another 30 minutes.

Supplementary Figures

Supplementary Figure 1



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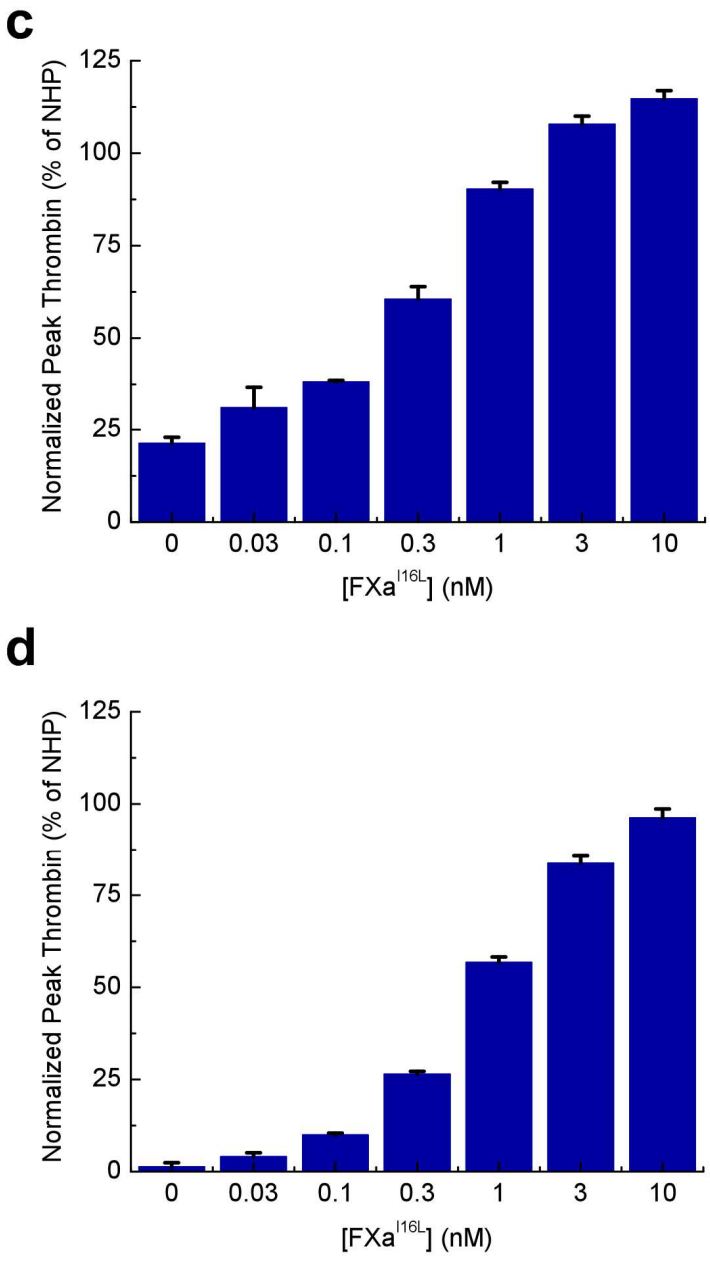
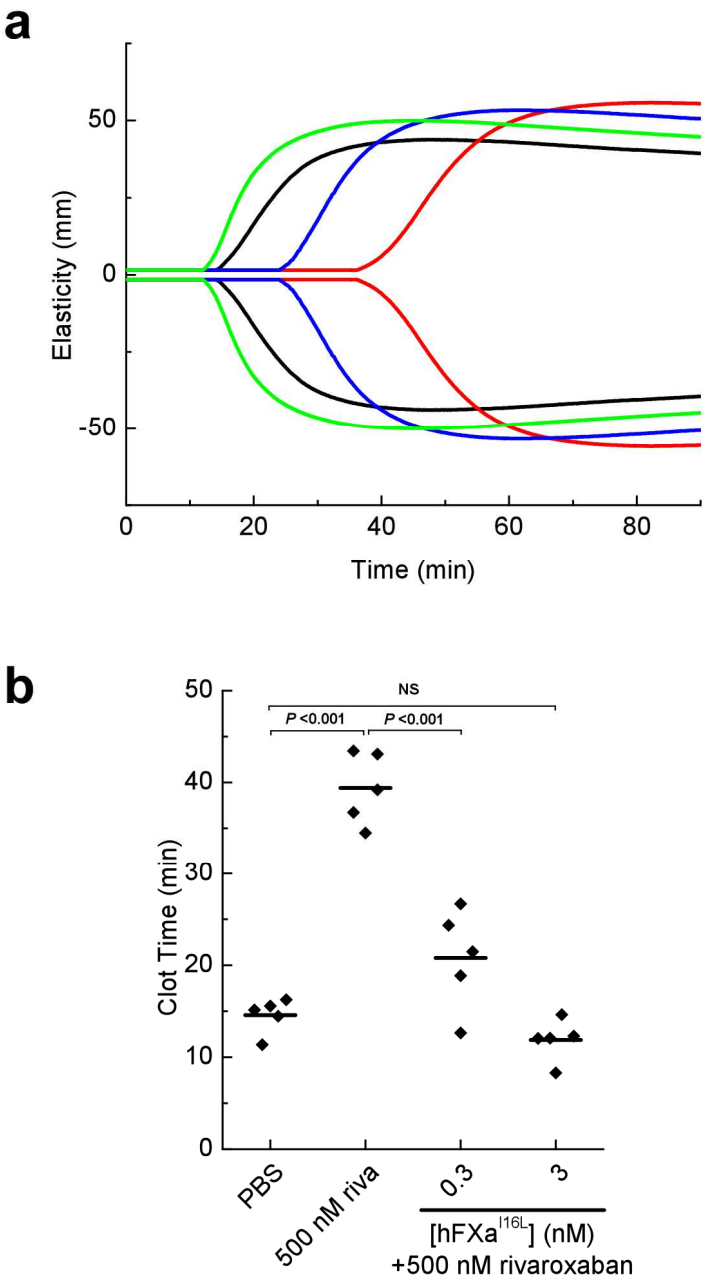


Supplementary Figure 2



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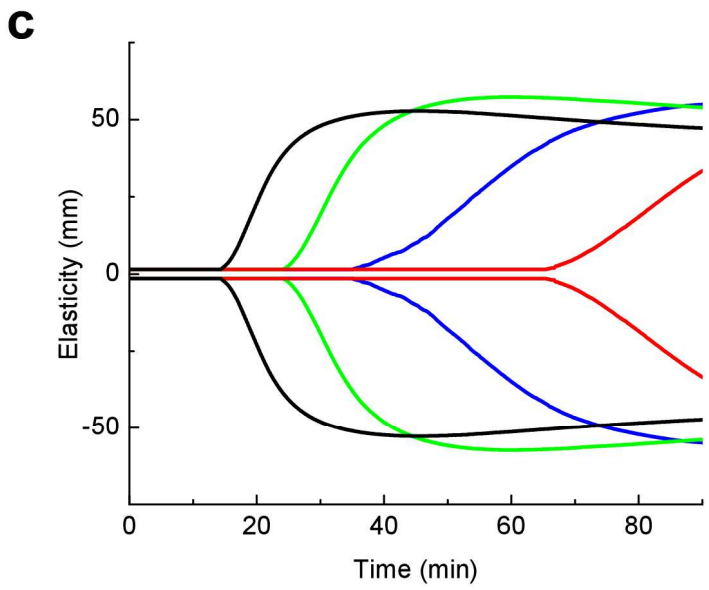
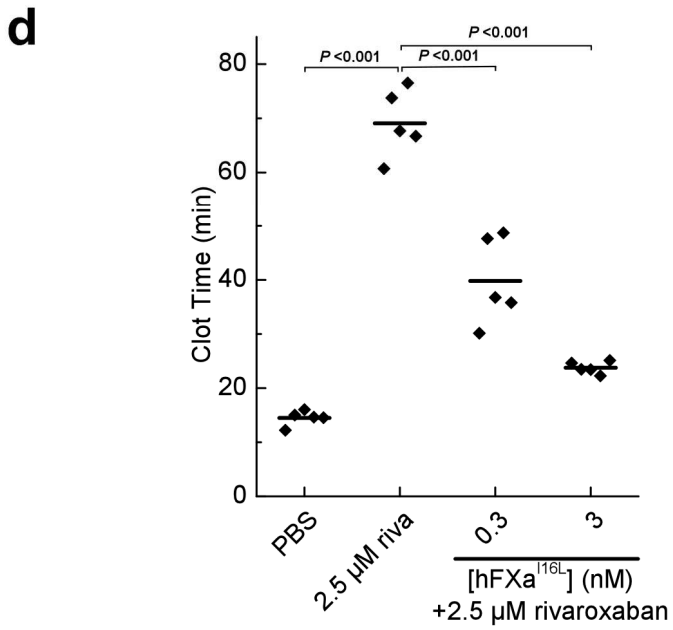
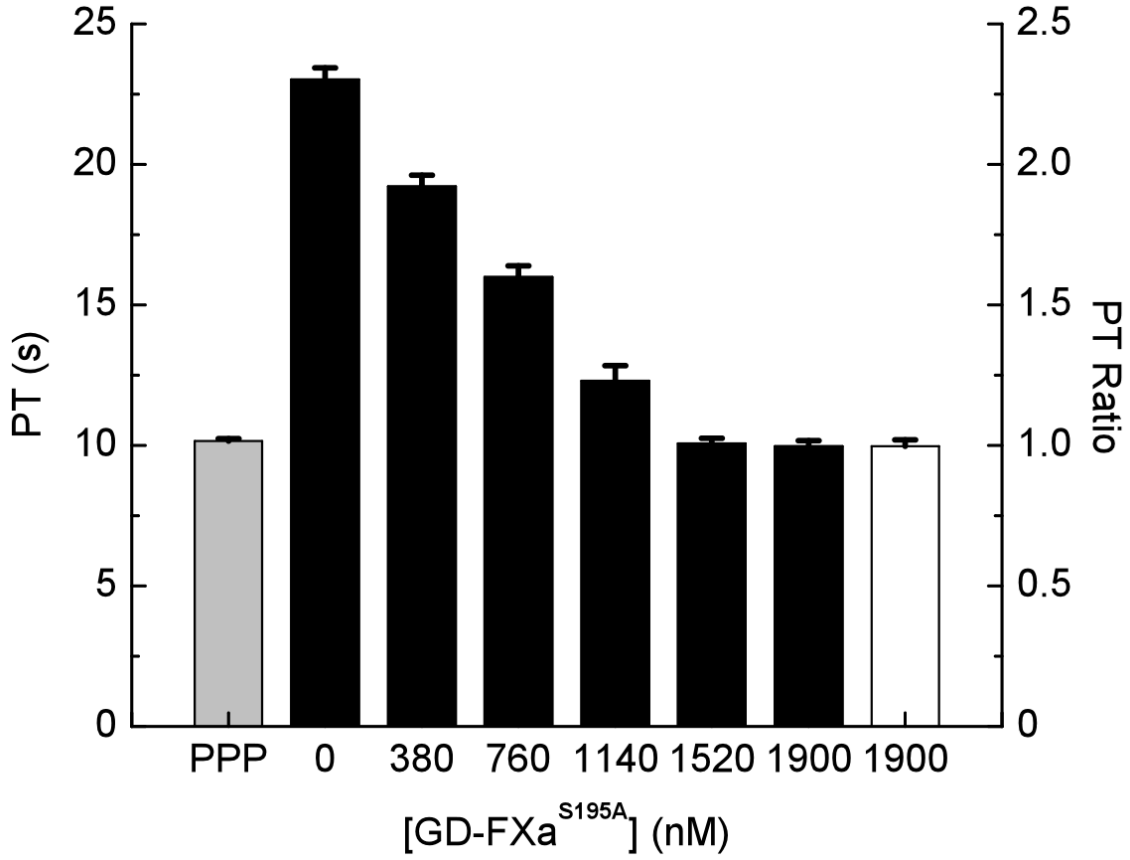




Supplementary Figure 3



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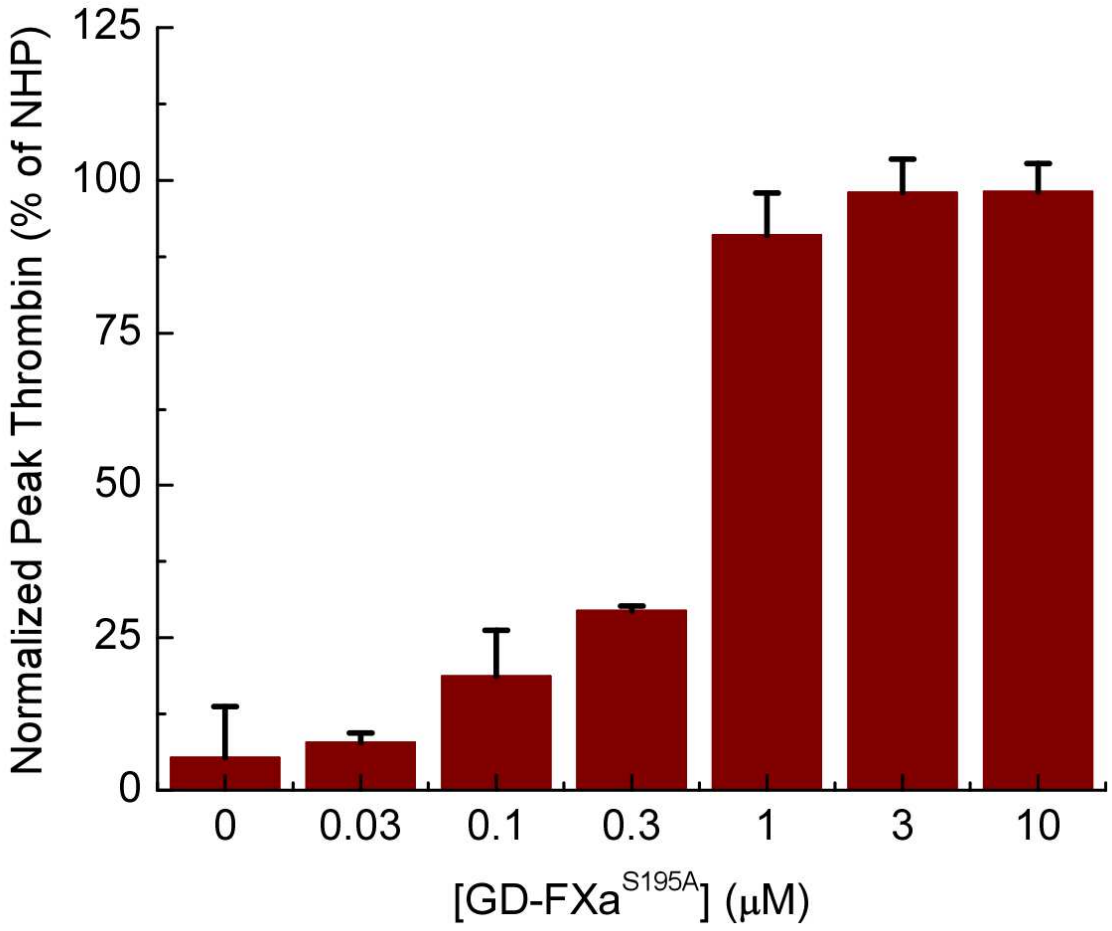
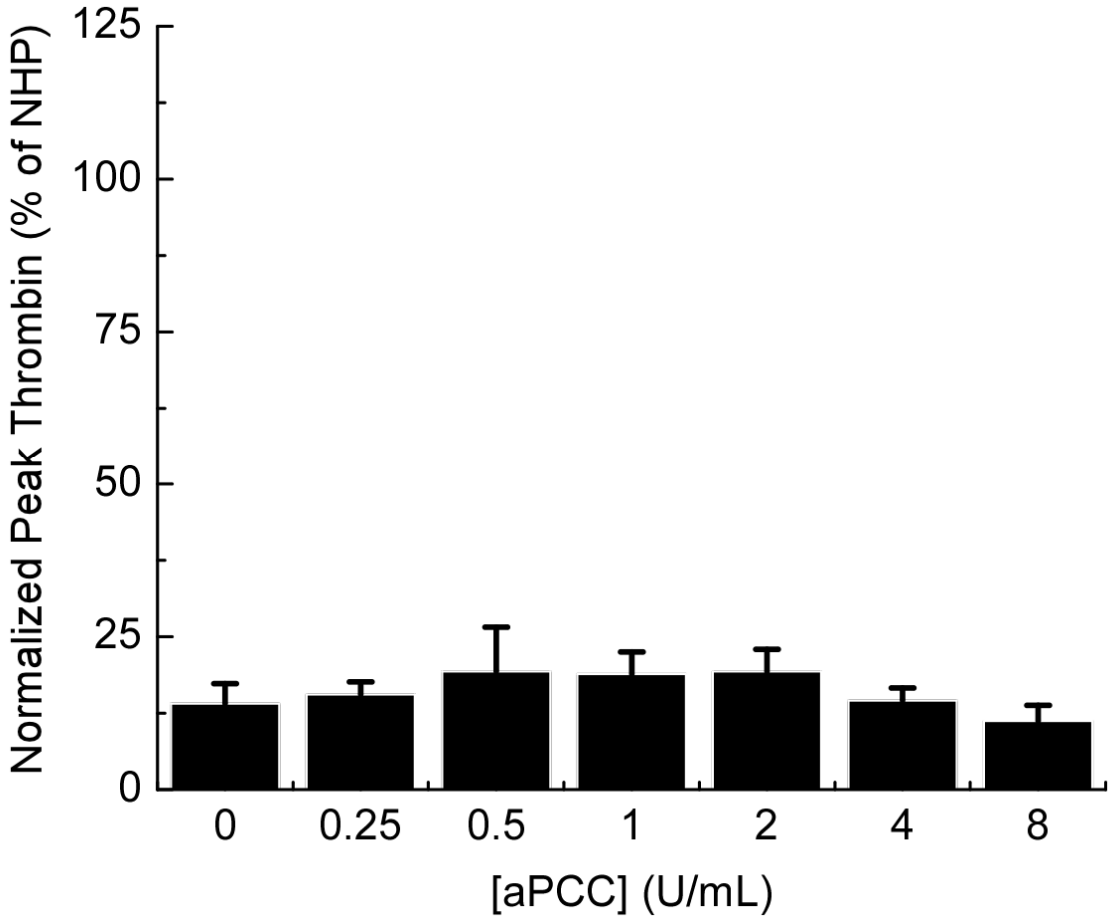
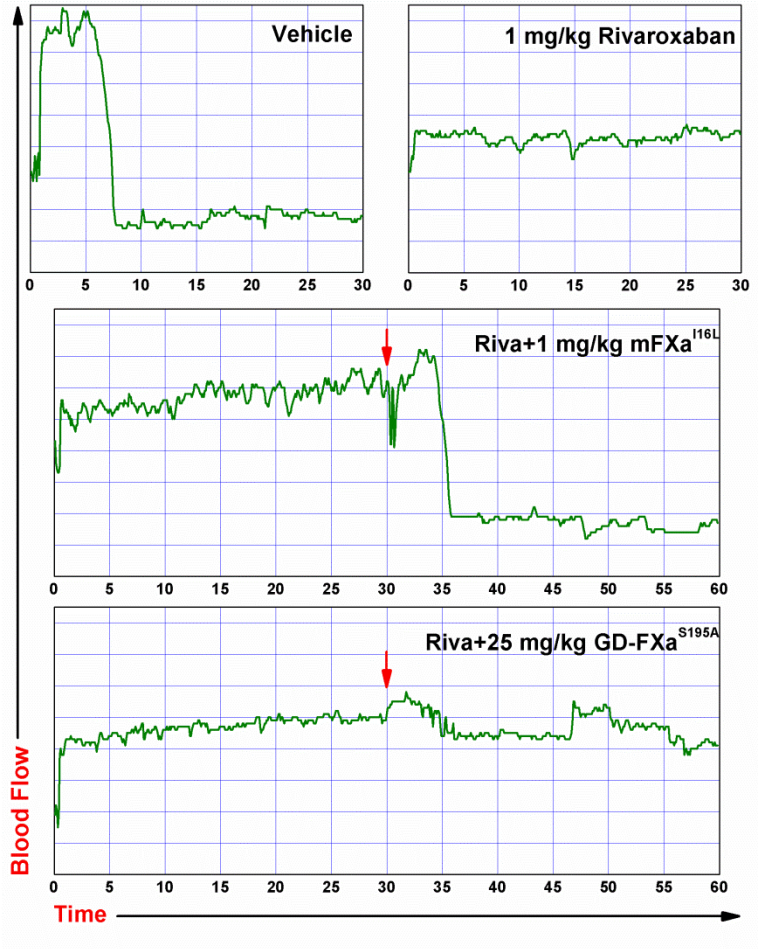


Supplementary Figure 5



Supplementary Figure 6



CHAPTER 3

The mechanism of rivaroxaban reversal by FXa reveals a paradoxical property of direct FXa inhibitors

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Abstract

Direct inhibitors of coagulation factor Xa (FXa) are a promising oral anticoagulant therapy. However, there is no approved strategy to reverse their anticoagulant effects in the event of bleeding or urgent medical procedures. We previously demonstrated that a variant of FXa, $FXa^{I[16]L}$, can reverse the effects of the direct FXa inhibitor rivaroxaban *in vivo*. Because $FXa^{I[16]L}$ is poorly inhibited active site inhibitors when free in plasma but susceptible to inhibition at the site of vascular injury when bound to factor Va (FVa), we hypothesized that reversal of rivaroxaban by $FXa^{I[16]L}$ could not be a result of the resistance of $FXa^{I[16]L}$ to the inhibitor. Inhibition studies revealed that both wild type (wt)-FXa and $FXa^{I[16]L}$ were both highly inhibited by rivaroxaban at therapeutic concentrations. Thrombin generation studies also indicated that both wt-FXa and $FXa^{I[16]L}$ could reverse the effects of rivaroxaban despite being highly inhibited. By measuring the kinetics of FXa inhibition by antithrombin III (ATIII), we found that rivaroxaban protects FXa from irreversible inactivation by ATIII, and creates a pool of FXa reversibly bound to the anticoagulant. *In silico* kinetic simulations demonstrated that, because ATIII-inhibited FXa and rivaroxaban inhibited FXa are separated by free FXa, a steady state of free FXa is formed that persists with an extended half-life. This results in a paradoxical increase in free FXa in the presence of the anticoagulant, and explains why administration of $FXa^{I[16]L}$ can restore normal hemostasis in the face of the anticoagulant. In addition to explaining the mechanism of action of a novel bypassing agent for direct FXa inhibitors, these results also indicate that active site inhibition of FXa may not be as straightforward as previously thought.

Introduction

Small molecule active site antagonists of coagulation factor Xa (FXa) and thrombin have been approved in the last 10 years in the United States, Canada, and Europe as oral anticoagulants to treat patients at risk for thrombosis. Compared to the widely used oral anticoagulant warfarin, these target-specific oral anticoagulants (TSOACs) are at least as effective at prevention of thrombosis [87, 94-96, 137]. Because TSOACs also have more straightforward pharmacokinetics and fewer food-drug interactions than warfarin, most patients taking these new agents do not need routine coagulation monitoring [107]. Unfortunately, while some studies have shown that TSOACs have a lower bleeding risk than warfarin, bleeding episodes still occur [102, 103]. In the event of a major bleeding episode or the need for an urgent invasive procedure, there are no approved approaches to reverse the anticoagulant effects of TSOACs [107]. However, there are several strategies currently being explored. These include engineered drug-specific antidotes [112, 117] as well as nonspecific bypassing agents [123, 124, 126-128, 135].

We have recently shown that a variant of FXa, $FXa^{I[16]L}$, can potently reverse the anticoagulant effects of the direct FXa inhibitors *in vitro* and *in vivo*. This "zymogen-like" variant of FXa has a single amino acid substitution that disrupts the conformational transition from inactive zymogen to active serine protease [44]. Thus, despite $FXa^{I[16]L}$ being fully proteolytically activated, it retains many of the properties of the zymogen, including poor active site function. This makes $FXa^{I[16]L}$ resistant to active site inhibitors and giving the variant a longer plasma half-life than wild-type (wt)-FXa [59]. Importantly, the impaired function of $FXa^{I[16]L}$ can be rescued by binding to the cofactor, FVa, on membranes and assembly in the prothrombinase complex [44, 59]. These

characteristics allow $FXa^{I[16]L}$ to circulate in plasma in a low-activity zymogen-like state but rapidly generate thrombin at the site of vascular injury.

Early studies with $FXa^{I[16]L}$ demonstrated that it was able to bypass the intrinsic pathway defect in animals deficient in factor VIII (FVIII) or factor IX (FIX) and restore normal hemostasis [60]. The mechanism for this is relatively straightforward, since $FXa^{I[16]L}$ acts downstream of the missing factors in these animals. On the other hand, the mechanism by which $FXa^{I[16]L}$ could reverse the effects of direct FXa inhibitors is less obvious, since $FXa^{I[16]L}$ acts at the same position in the coagulation cascade as the anticoagulant. In our previous study, we observed $FXa^{I[16]L}$ to be effective, not at stoichiometric quantities, but at catalytic concentrations compared to the anticoagulant. This indicates that $FXa^{I[16]L}$ cannot be acting as a drug-sequestering antidote, and instead must somehow bypass the effects of the anticoagulant. One possibility is that $FXa^{I[16]L}$, because of its resistance to active site probes, might also be resistant to direct FXa inhibitors and therefore be able to generate thrombin in the presence of the anticoagulant. However, $FXa^{I[16]L}$ can only generate thrombin at a high rate when assembled in the prothrombinase complex [44, 59]. Under these conditions, active site function, and, accordingly, susceptibility to inhibition, would likely be restored. If this is the case, $FXa^{I[16]L}$ must generate thrombin in the presence of a direct FXa inhibitor by some alternative mechanism.

In this study, we describe a detailed kinetic characterization of the mechanism by which $FXa^{I[16]L}$ reverses the anticoagulant effects of direct FXa inhibitors. Surprisingly, our findings suggest an unexpected mechanism by which reversal is achieved through competition between the pharmacologic inhibitor (rivaroxaban) and endogenous plasma proteinase inhibitors (ATIII) for binding to FXa. These may have broad implications for

better understanding the mechanism by which the seemingly simple direct oral anticoagulants function.

Methods

Reagents. Z-Gly-Gly-Arg-AMC was from Bachem Bioscience Inc. Technothrombin thrombin calibrator and reagent RB were from Diapharma Group Inc. Pooled normal human plasma (NHP) and human factor X-deficient plasma were obtained from George King Biomedical, Inc. Rivaroxaban and dabigatran were from Selleck Chemicals. All tissue culture reagents were from Invitrogen except insulin-transferrin-sodium selenite, which was from Roche. *o*-phenylenediamine dihyrdochloride (OPD), Gly-Pro-Arg-Pro-Amide (GPRP), and hexadimethrine bromide (polybrene) were from Sigma-Aldrich. Biotinylated Glu-Gly-Arg-chloromethylketone (B-EGRCK) and biotinylated Phe-Pro-Arg-chloromethylketone (B-FPRCK) were from Haematologic Technologies. Spectrozyme FXa (SpecXa) was from American Diagnostica, Inc. Fondaparinux sodium was from Apotex Corp. Innovin was from Dade Behring. Affinity-purified goat anti-human FX polyclonal IgG (catalog no. GAFX-AP) and peroxidase-conjugated affinity-purified sheep anti-human antithrombin III polyclonal IgG (catalog no. SAAT-APHRP) were obtained from Enzyme Research Laboratories. Horseradish peroxidase conjugated streptavidin was purchased from Life Technologies. Enzygnost TAT micro ELISA kit was purchased from Siemens.

Proteins. The FX activator from Russell's viper venom, $RVV_{X\text{-}CP}$ was purified as previously described[145]. Recombinant $hFX^{I[16]L}$ was expressed in human embryonic kidney 293 (HEK293) cells, purified from media, and activated using $RVV_{X\text{-}CP}$ as

previously described[146, 147]. Recombinant hFVa was prepared as previously described[157]. Human ATIII was purified from plasma as previously described[158].

Thrombin Generation Assays. Thrombin generation assays (TGA) in NHP were performed as previously described[59] with a slight modification to accommodate the addition of rivaroxaban and reversal agents as appropriate. 40 μL NHP was added to a microtiter plate (Nunc; F16 black Maxisorp) along with 10 μL Technothrombin RB (2 pM TF, 4.0 μM phospholipid). 3 μL rivaroxaban dissolved in 20 mM HEPES, 150 mM NaCl, 0.1% PEG-8000, pH 7.4 (HBS-PEG) was added to NHP in a black microtiter plate (Nunc; F16 black Maxisorp) along with the 2 μL of protein (wt-hFXa or $hFXa^{I[16]L}$). The reaction was initiated immediately by adding Z-Gly-Gly-Arg-AMC in 15 mM $CaCl_2$ (50 μL; 0.5 mM final). Fluorescence (λ_{ex}=360 nm, λ_{em}=460 nm) was measured at one minute intervals for 90 minutes at 37°C using a Spectramax $M2^e$ (Molecular Devices) plate reader. The Technothrombin calibrator kit was used to convert raw fluorescence intensity to thrombin concentration. Thrombograms (nM thrombin vs. time) were made to determine the lag time, peak height, and endogenous thrombin potential (ETP).

Kinetic Characterization of FXa Variant Inhibition by Rivaroxaban. Increasing concentrations of rivaroxaban were added to the chromogenic substrate SpecXa (100-300 μM depending on the enzyme used) in HBS-PEG buffer containing 2 mM $CaCl_2$. The reaction was initiated with addition of wt-hFXa or $hFXa^{I[16]L}$ and A_{405} was monitored over time to measure FXa amidolytic activity (depending on the experiment and the enzyme, 2-6 nM FXa was used to ensure sufficient signal). Initial velocities were plotted against inhibitor concentration and fit to the quadratic velocity equation for tight binding competitive inhibition to determine K_i values. To determine inhibition kinetics

of FXa in the prothrombinase complex, experiments were repeated in the presence of 30 nM FVa and 50 μM phospholipid vesicles (80% phosphatidylcholine, 20% phosphatidylserine).

Quantification of FXa-ATIII Complex Formation in Human Plasma. 0, 100 nM, or 1 μM rivaroxaban was added to recalcified (5 mM $CaCl_2$ final) human FX-deficient plasma at room temperature along with 1.33 mM GPRP and 1 μM dabigatran to prevent clotting. 25 nM wt-hFXa or $hFXa^{I[16]L}$ was added to aliquots of the plasma mixture at different time points in a reverse time course, and all samples were quenched simultaneously with 50 μM B-EGRCK. Samples were allowed to incubate with B-EGRCK for 10 minutes and then diluted 10-fold with ELISA blocking buffer (PBS, 0.1% Tween-20, 6% BSA, pH 7.4). In some experiments, 750 nM fondaparinux was added to the plasma mixture before addition of FXa, and in these studies, the reaction was quenched with 50 μg/mL polybrene (to neutralize the fondaparinux[159]) in addition to 50 μM B-EGRCK. FXa-ATIII standards were prepared by incubating 500 nM wt-FXa or $FXa^{I[16]L}$ with 5 μM hATIII, 6 μM fondaparinux, and 5 mM $CaCl_2$ in HBS-PEG for 30 minutes. The standard was then serially diluted in FX-deficient plasma to make FXa-ATIII standards ranging from 0-30 nM. For experiments with fondaparinux, standards also contained 750 nM fondaparinux and 50 μg/mL polybrene to account for matrix effects. All FXa-ATIII standards were diluted 10-fold with ELISA blocking buffer before use. FXa-ATIII levels in the samples were measured using a novel sandwich ELISA. 96-well immunoassay plates were incubated overnight at 4°C with 100 μL 10 μg/mL affinity-purified goat anti-human FX polyclonal IgG diluted in 50 mM sodium carbonate, pH 9.6. Plates were washed with PBS+ 0.1% Tween-20, pH 7.4, and then blocked with blocking buffer at room temperature for 90 minutes. Plates were then washed and incubated with

100 µL of the 10-fold diluted sample described above for 1 hour at 37°C. After washing again, plates were incubated with 100 µL of 2 µg/mL peroxidase-conjugated affinity-purified sheep anti-human antithrombin III polyclonal IgG at 37°C for 1 hour. Following a final wash step, 100 µL of freshly prepared OPD solution (1 mg/mL OPD in 10 mM sodium citrate, pH 4.5 and 0.006% hydrogen peroxide) was added to each well and allowed to incubate for 2 minutes. The reaction was stopped with addition of 50 µL 3M H_2SO_4. Plates were incubated at room temperature for 30 minutes before reading A_{490} in a SpectraMax 190 microplate reader (Molecular Devices). FXa-ATIII concentrations were determined using the generated standard curve.

Quantification of FXa-ATIII Complex Formation in a Purified System. 0, 5 nM, or 50 nM rivaroxaban was added to 2.6 µM hATIII in HBS-PEG, and 5 mM $CaCl_2$. 25 nM wt-hFXa or $hFXa^{I[16]L}$was then added and incubated as described above. Reactions were stopped with addition of 50 µM B-EGRCK and allowed to incubate with B-EGRCK for 10 minutes before 10-fold dilution with ELISA blocking buffer. FXa-ATIII complex formation was quantified using the ELISA described above. Standards were also prepared as above, but diluted into HBS-PEG instead of FX-deficient plasma.

Quantification of FXa-B-EGRCK Complex Formation. FXa-B-EGRCK standards were prepared by incubating 500 nM wt-FXa or $FXa^{I[16]L}$ with 50 µM B-EGRCK in HBS-PEG for 30 minutes and then serially diluted in FX-deficient plasma to make FXa-B-EGRCK standards ranging from 0-30 nM. For experiments with fondaparinux, standards also contained 750 nM fondaparinux and 50 µg/mL polybrene to account for matrix effects. FXa-BEGRCK labeling was measured using a novel ELISA. The ELISA was nearly identical to the FXa-ATIII ELISA described above, but with the following

modifications: Samples and standards were diluted 150-fold instead of 10-fold. Instead of the ATIII-detection antibody, 100 µL 0.25 µg/mL horseradish peroxidase-conjugated streptavidin diluted in ELISA dilution buffer was added. All other steps were identical.

Determination of FXa distribution using known rate constants. We used KinTeK Explorer (KinTeK Corp.) to calculate the concentration of the FXa-ATIII complex, the FXa-rivaroxaban complex, and free FXa over time. 25 nM free FXa, 3.4 µM ATIII, and different concentrations of rivaroxaban were used as starting conditions, and the FXa distribution was determined over time, subject to the following expressions:

$$E + R \rightleftharpoons E \cdot R$$

$$E + AT \longrightarrow E \cdot AT$$

where E represents free FXa, R represents free rivaroxaban, AT represents ATIII, E·R represents rivaroxaban-bound FXa, and E·AT represents antithrombin-bound FXa. Rivaroxaban association (1.7×10^{7} $M^{-1}s^{-1}$) and dissociation (5×10^{-3} s^{-1}) rate constants were used for the first expression[80], and the second-order rate constant for ATIII inhibition (4×10^{3} $M^{-1}s^{-1}$) of FXa was used for the second expression[160].

Determination of the kinetics of thrombin-ATIII complex formation. 0, 100 nM, or 1 µM dabigatran was added to recalcified (5 mM $CaCl_2$ final) human prothrombin-deficient plasma at room temperature along with 1.33 mM GPRP to prevent clotting. 500 pM plasma-derived human α-thrombin was added to aliquots of the plasma mixture at different time points in a reverse time course, and all samples were quenched simultaneously with 50 µM B-FGRCK. Samples were then analyzed for TAT content with the Enzygnost TAT micro kit per the manufacturer's instructions.

Results

***FXa$^{I[16]L}$ is not resistant to rivaroxaban*:** Based on its zymogen-like character, we initially presumed that FXa$^{I[16]L}$ was effective in reversing the inhibitory effect of rivaroxaban because of its lower affinity for active site inhibitors compared to wt-FXa. Indeed, we observed a ~38-fold difference in the K_i of rivaroxaban between wt-FXa and FXa$^{I[16]L}$ (**Fig. 1a**). Despite this, we were surprised to find that wt-FXa and FXa$^{I[16]L}$ were both equally effective reversing rivaroxaban in TGA experiments (**Fig. 1b**). This indicates that the ability of FXa$^{I[16]L}$ to overcome the effects of rivaroxaban cannot be explained by differences in affinity of the free enzyme for the inhibitor. Further, consistent with our previous observations that FXa$^{I[16]L}$ is rescued upon assembly in the prothrombinase complex[44], the K_i of rivaroxaban for wt-FXa and FXa$^{I[16]L}$ in prothrombinase were the same (**Fig 1a**). While this accounts for the equivalence of wt-FXa and FXa$^{I[16]L}$, it fails to explain how the proteins generate thrombin in the face of rivaroxaban considering the inhibitor concentration is more than 10-fold greater than the K_i for wt-FXa and FXa$^{I[16]L}$ (free or prothrombinase). Collectively these data suggest that other aspects of the regulation of active FXa in plasma must contribute to the bypassing effects of these enzymes.

FXa can paradoxically persist in plasma in the presence of rivaroxaban: Normally, FXa introduced into plasma is irreversibly inhibited by ATIII, resulting in rapid first-order decay with a half-life of 2-3 minutes[28, 64]. A hallmark of FXa$^{I[16]L}$ is its resistance to ATIII inhibition[59, 60]. We measured the kinetics of FXa-ATIII complex formation by ELISA after addition of FXa to plasma in the presence of rivaroxaban. Increasing concentrations of rivaroxaban inhibited FXa-ATIII complex

formation with wt-FXa (**Fig. 2a**). As expected, $FXa^{I[16]L}$ was resistant to ATIII, but rivaroxaban further decreased the rate of ATIII inhibition (**Fig. 2b**). Importantly, the rates of ATIII inhibition of wt-FXa and $FXa^{I[16]L}$, which differed by ~20-fold in the absence of rivaroxaban, were nearly identical in the presence of 1 μM rivaroxaban (**Supplementary Table 1**). This indicates that wt-FXa and $FXa^{I[16]L}$ react with ATIII in a similar way in the presence of the inhibitor. In concurrent experiments, to account for the decrease in FXa-ATIII complex formation in the presence of rivaroxaban, we used saturating amounts of biotinylated Glu-Gly-Arg-chloromethylketone (B-EGRCK) to covalently trap and label all FXa species (free and rivaroxaban-bound) not irreversibly inhibited by ATIII. B-EGRCK labeling was increased in rivaroxaban-containing samples compared to rivaroxaban-free samples (**Fig. 2c,d** and **Supplementary Table 2**). Together, measurements of FXa-ATIII and B-EGRCK labeling of FXa account for all the FXa added to the system. We also measured FXa-ATIII formation and B-EGRCK labeling kinetics in a purified system with physiologic concentrations of purified ATIII[161] and obtained comparable results to those in plasma (**Supplementary Fig. 1** and **Supplementary Tables 3-4**).

The ability of rivaroxaban to diminish FXa-ATIII complex formation over time suggests a mechanism by which both wt-FXa and $FXa^{I[16]L}$ can persist and function in the presence of rivaroxaban. Specifically, these data are consistent with formation of a pool of FXa that is irreversibly inhibited by ATIII and a pool of FXa reversibly inhibited by rivaroxaban. Importantly, since rivaroxaban and ATIII compete for binding, these two pools must be separated by free, uninhibited FXa (**Fig. 2e**). This steady-state level of free FXa is likely responsible for the thrombin generation observed in the face of rivaroxaban. Directly measuring this free FXa, however, is complicated by the fact that

any probe would perturb the equilibrium between FXa and rivaroxaban. As an alternative, we used the known rate constants for FXa inhibition by ATIII[160] and the on and off rates for rivaroxaban binding to FXa[80] to calculate the amount free FXa present at steady state. In the absence or presence of rivaroxaban, calculated free FXa levels drop rapidly after initial mixing (**Fig. 2f,g**, blue traces). In the absence of rivaroxaban, this decrease in free FXa is due to inhibition by ATIII (**Fig. 2f**, red trace). However, in the presence of rivaroxaban, the majority of FXa becomes reversibly complexed with the FXa inhibitor (**Fig. 2g**, green trace), with an associated decrease in FXa-ATIII complex formation (red trace). Plotting the kinetics of free FXa concentration on semi-logarithmic scale revealed that, without rivaroxaban, the free FXa concentration decays exponentially, such that less than 10 pM free FXa remains after 10 minutes (**Fig. 2h,** black trace). Interestingly, in the presence of rivaroxaban, following a rapid initial decrease, free FXa levels reach a nonzero steady state (**Fig. 2h**, magenta and cyan traces). Remarkably, this causes a paradoxical and persistent increase in levels of free FXa at pharmacologic rivaroxaban concentrations (**Fig. 2i**). Since we have previously shown that 30-100 pM free FXa is sufficient for normal thrombin generation in hemophilic plasma[59], these free FXa levels can account for the restoration of hemostasis in the presence of rivaroxaban.

Based on our model (**Fig. 2e**), direct FXa inhibitors establish a new equilibrium that not only diminishes the rate of FXa-ATIII complex formation but also establishes a small but important pool of free FXa. In support of this model, we experimentally tested the impact of disrupting this equilibrium. We found that addition of fondaparinux, a heparin derivative that accelerates ATIII inhibition of FXa, markedly enhanced the kinetics of wt-FXa-ATIII complex formation (**Fig. 3a** and **Supplementary Table 5**),

and B-EGRCK labeling was correspondingly reduced (**Fig. 3b** and **Supplementary Table 2**). The addition of increasing amounts of rivaroxaban blunted the effect of fondaparinux and reduced FXa-ATIII complex formation. Similar results were obtained with $FXa^{I[16]L}$ (**Supplementary Fig. 2**). This redistribution of FXa away from ATIII, even in the presence of fondaparinux, is not specific to rivaroxaban as p-aminobenzamidine, a FXa active site inhibitor with fast dissociation kinetics[162], also inhibits FXa-ATIII complex formation (data not shown). Together these data show that any active site directed FXa inhibitor will disrupt FXa-ATIII complex formation and establish a small pool of free FXa. The size of the pool will depend on how much FXa is produced or how much is added exogenously.

Finally, we hypothesized that active site inhibitors of other serine proteases should affect their inhibition by serpins. To test this, we quantified the kinetics of thrombin inhibition by ATIII in the presence of dabigatran, a direct thrombin inhibitor, using an ELISA specific to the thrombin-ATIII complex (TAT). Dabigatran markedly inhibited TAT formation at concentrations within the therapeutic range of the anticoagulant (**Figure 4)**, suggesting that competition between reversible active site inhibitors and serpins may not be unique to FXa, but rather, a common property of serine proteases.

Discussion

The observation that $FXa^{I[16]L}$, a zymogen-like variant of FXa, was an effective bypassing agent for direct FXa inhibitors prompted us to explore its mechanism of action. Initially, we hypothesized that $FXa^{I[16]L}$ would be resistant to active site inhibition and therefore could generate thrombin in the presence of rivaroxaban. However, wt-FXa and $FXa^{I[16]L}$

were both highly inhibited by rivaroxaban yet comparably effective at restoring thrombin generation in the face of this inhibition. As a possible explanation for these seemingly contradictory findings, we discovered that rivaroxaban shifts the distribution of FXa from an irreversible complex with ATIII to a reversible complex with rivaroxaban (**Fig. 2**). Because the pathway for conversion of the rivaroxaban-inhibited enzyme to the complex with ATIII requires formation of free Xa, a steady-state amount of free FXa is formed that is not seen in the absence of rivaroxaban. Thus, pharmacologically relevant concentrations of rivaroxaban produce a paradoxical increase in free FXa. It is important to note that the increase in free FXa is most relevant when FXa is administered exogenously. Normally, the overall result of rivaroxaban therapy is a net decrease in endogenous FXa activity. In this context, a pool of FXa will exist, but its magnitude and significance will depend on the rate of FXa formation.

The fact that rivaroxaban competes with ATIII for FXa has major implications for the use of active site directed anticoagulants and their antidotes. *In vivo*, prevention of FXa inactivation by ATIII may result in abnormal levels of FXa. While this FXa will circulate predominantly in complex with the anticoagulant, as the anticoagulant is metabolized, this FXa would be released gradually and could contribute to thrombosis. Although most reports of rebound hypercoagulability following rivaroxaban cessation have been attributed to under-anticoagulation in high-risk patients, it is plausible that release of free FXa may contribute to this [163, 164]. This could be particularly important in the setting of reversal of direct FXa inhibitors with an antidote like GD-FXa$^{S[195]A}$. Instead of FXa being released from the rivaroxaban-FXa complex slowly, administration of these antidotes could result in rapid liberation of FXa. In a patient population already enriched for underlying thrombophilia, this could result in paradoxical thrombosis.

Our findings reveal that rivaroxaban-mediated inhibition of FXa-ATIII complex formation is not a result of the kinetics of rivaroxaban binding, but instead, a consequence of the kinetics of ATIII inactivation of FXa. Specifically, this is likely related to the binding of ATIII to FXa, which is normally extremely weak but enhanced by addition of fondaparinux [160]. The important implication is that it is not possible to engineer an active site antagonist of FXa that does not substantially disrupt the FXa-ATIII interaction, and this suggests that the approach of inhibiting the FXa active site may not be as effective as hoped. On the other hand, the ability of fondaparinux to rapidly eliminate the FXa-rivaroxaban pool suggests a specific mechanistic countermeasure to this problem, should it arise.

This work demonstrates that inhibition of the active site of a serine protease is not as straightforward once thought. The active site is not only responsible for substrate cleavage. Its kinetic activity also governs the enzyme's susceptibility to irreversible plasma protease inhibitors. Thus, a small molecule antagonist must disrupt both kinetic processes. This is not unique to rivaroxaban, nor is it unique to FXa. Our results provide a provocative mechanism for the ability of FXa to generate thrombin in the presence of a reversible active site inhibitor.

Figure Legends

Figure 1. Reversal of the anticoagulant effect of rivaroxaban by wt-FXa and FXa$^{I[16]L}$. (***a***) Inhibition kinetics of rivaroxaban for wt-hFXa and hFXa$^{I[16]L}$ were measured using the FXa peptidyl substrate, SpecXa. Studies were performed with free FXa as well as with FXa assembled in prothrombinase by addition of 50 μM PCPS and 30 nM FVa. Initial velocity measurements were fit to the quadratic velocity equation for tight binding competitive inhibition to determine K_i values. Error values are reported as ± 2 SD of the fit. All experiments were performed in duplicate. (***b***) Peak thrombin generation was measured in NHP supplemented with 500 nM rivaroxaban and increasing concentrations of wt-hFXa (black) or hFXa$^{I[16]L}$ (blue). Experiments were performed in quadruplicate and peak thrombin ± SD versus FXa concentration is shown on a semi-logarithmic plot.

Figure 2. Distribution of FXa in plasma in the presence or absence of rivaroxaban. Kinetics of FXa-ATIII complex formation after addition of 25 nM (***a***) wt-hFXa or (***b***) hFXa$^{I[16]L}$ to FX-deficient plasma containing 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. Solid lines represent the fit of the points to a single exponential rise. B-EGRCK labeling of (***c***) wt-hFXa or (***d***) hFXa$^{I[16]L}$ was quantified by ELISA in the presence of 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. Solid lines in ***c*** represent fitting to a single exponential decay with the exception of the 1 μM line (which could not be fit well and represents a smoothed connection of the points). The solid lines in ***d*** also represent a smoothed connection of the data points. Data points in (***a***-***d***) are plotted as the mean of 3 separate experiments ± SEM. (***e***) Scheme depicts ATIII-inhibited FXa and

rivaroxaban-inhibited FXa, separated by free FXa. Known rate constants for each kinetic step are indicated. Concentration of different FXa species in the (***f***) absence or (***g***) presence of 50 nM rivaroxaban were calculated using known rate constants and an initial (t=0) concentration of 25 nM free FXa. FXa-ATIII complex levels (red traces), free-FXa levels (blue traces), and FXa-rivaroxaban complex levels (green trace in ***g***) are shown. (***h***) Free FXa levels (calculated as in ***f*** and ***g***) at different rivaroxaban concentrations (0, black; 5 nM, magenta; 50 nM, cyan) are plotted versus time on a semi-logarithmic scale. (***i***) Calculated free FXa levels from (***h***) at 10 or 30 minutes are plotted versus rivaroxaban concentration. The gray box indicates the therapeutic range of rivaroxaban concentrations.

Figure 3. Effect of fondaparinux on rivaroxaban inhibition of FXa-ATIII complex formation. Kinetics of (***a***) FXa-ATIII complex formation and (***b***) B-EGRCK-labeling after addition of 25 nM wt-hFXa to FX-deficient plasma containing 750 nM fondaparinux and 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. The solid lines represent the fit of the points to a single exponential rise (for the 0 rivaroxaban data), and a two-exponential rise (for the 100 nM and 1 μM data). Data are shown as the mean of 3 separate experiments ± SEM.

Figure 4. Effect of dabigatran on the kinetics of thrombin inhibition by ATIII. Kinetics of thrombin-ATIII (TAT) complex formation after addition of 500 pM thrombin to human prothrombin deficient plasma containing 0 (-■-), 100 nM (-●-) or 1

μM (-▲-) dabigatran. The solid lines are arbitrarily drawn. Data are shown as the mean of 2 separate experiments ± SEM.

Figures

Figure 1

a

Enzyme	K_i (nM)
Free FXa	
wt-FXa	0.15 ± 0.04
FXa^{I16L}	5.7 ± 0.6
Prothrombinase	
wt-FXa	3.0 ± 0.7
FXa^{I16L}	2.2 ± 0.4

b

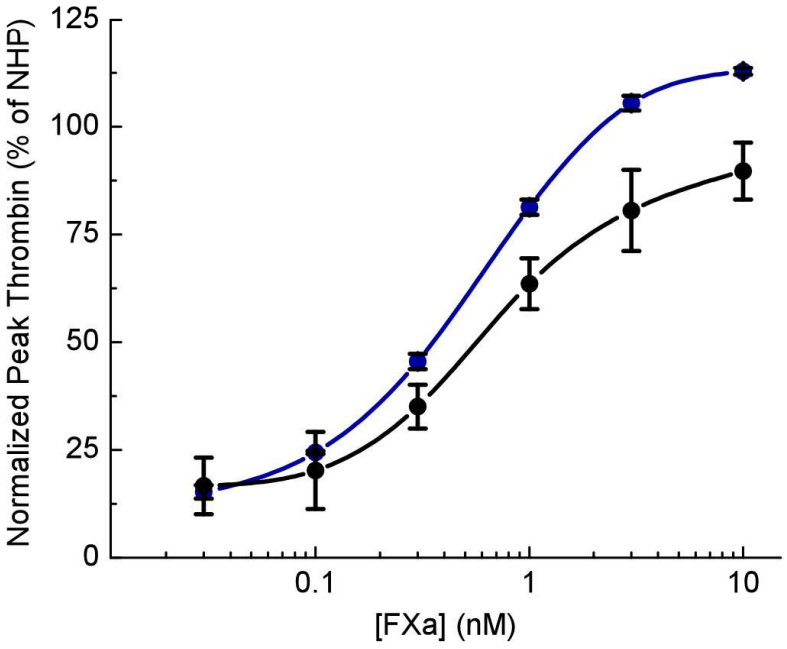
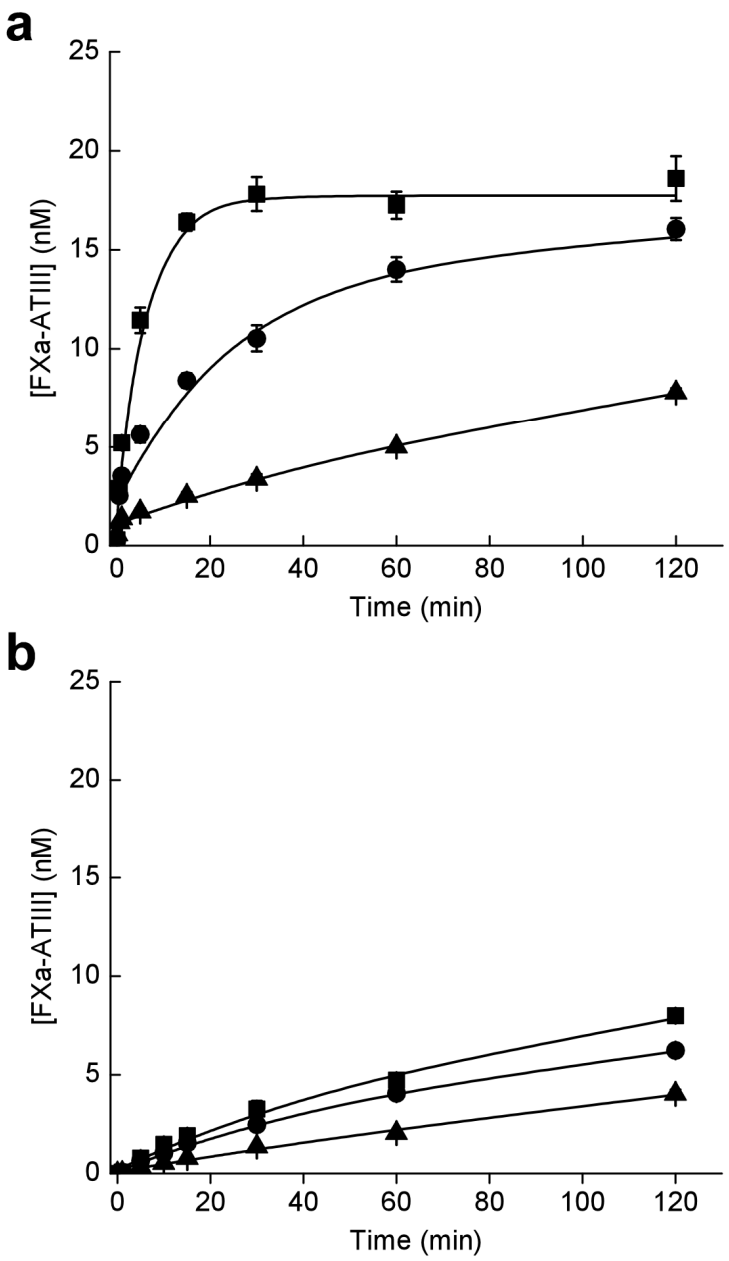


Figure 2



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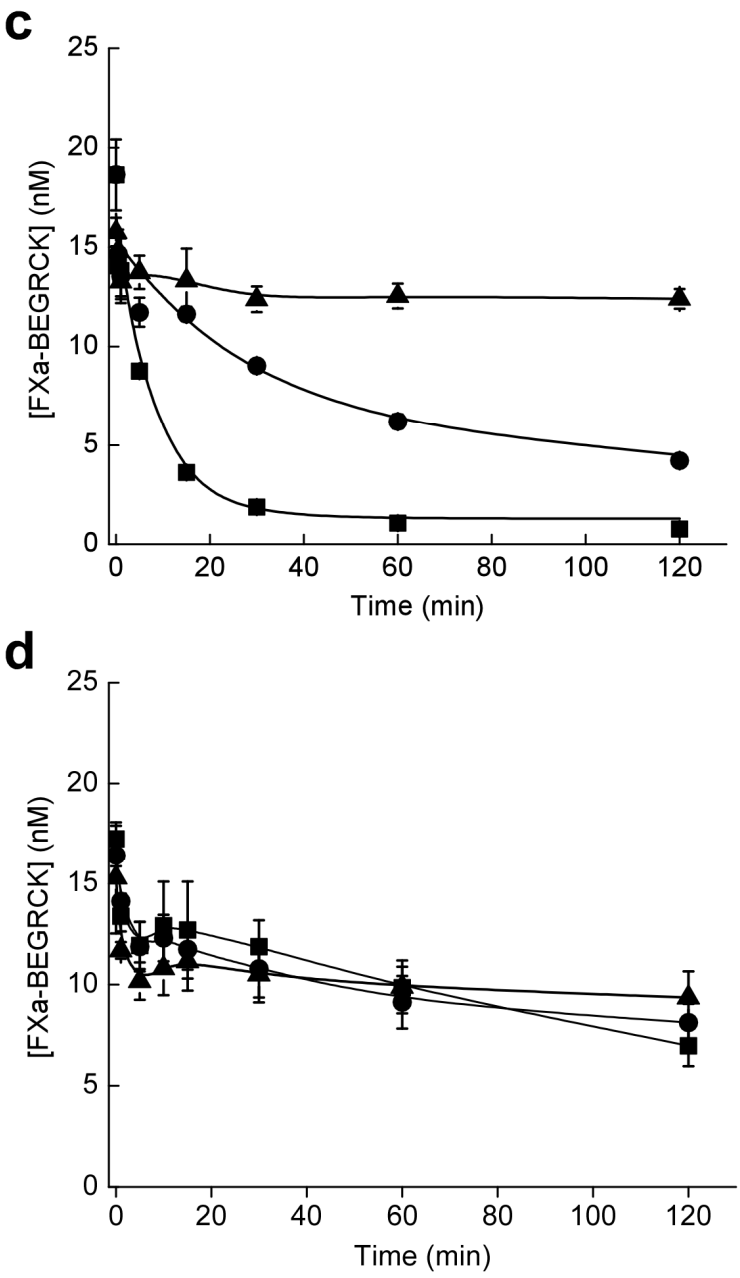

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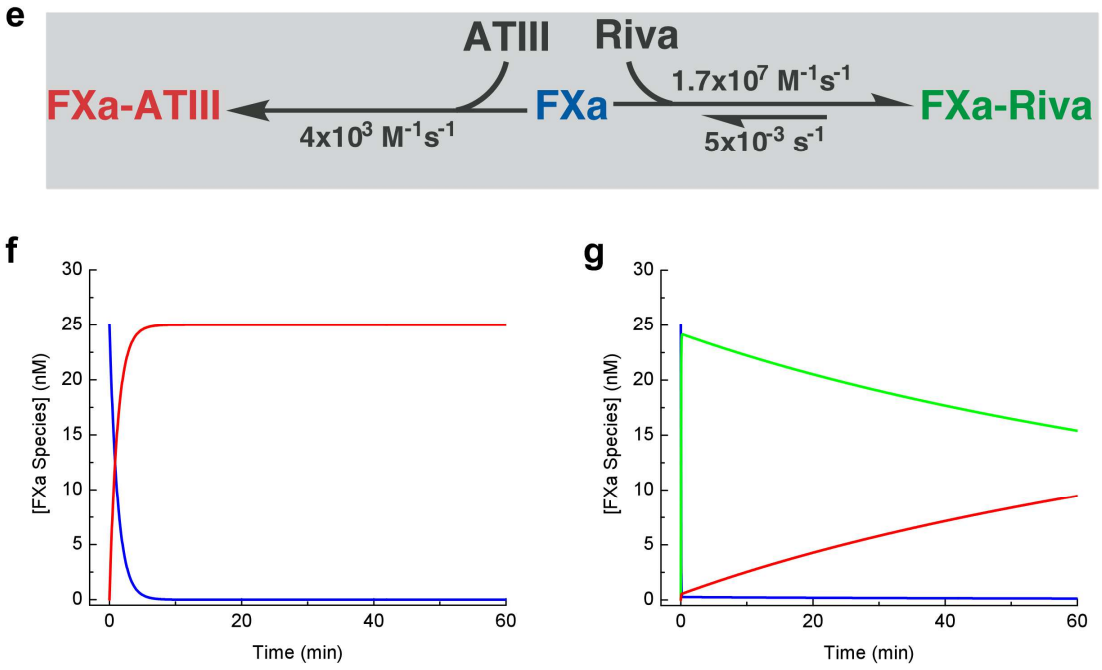

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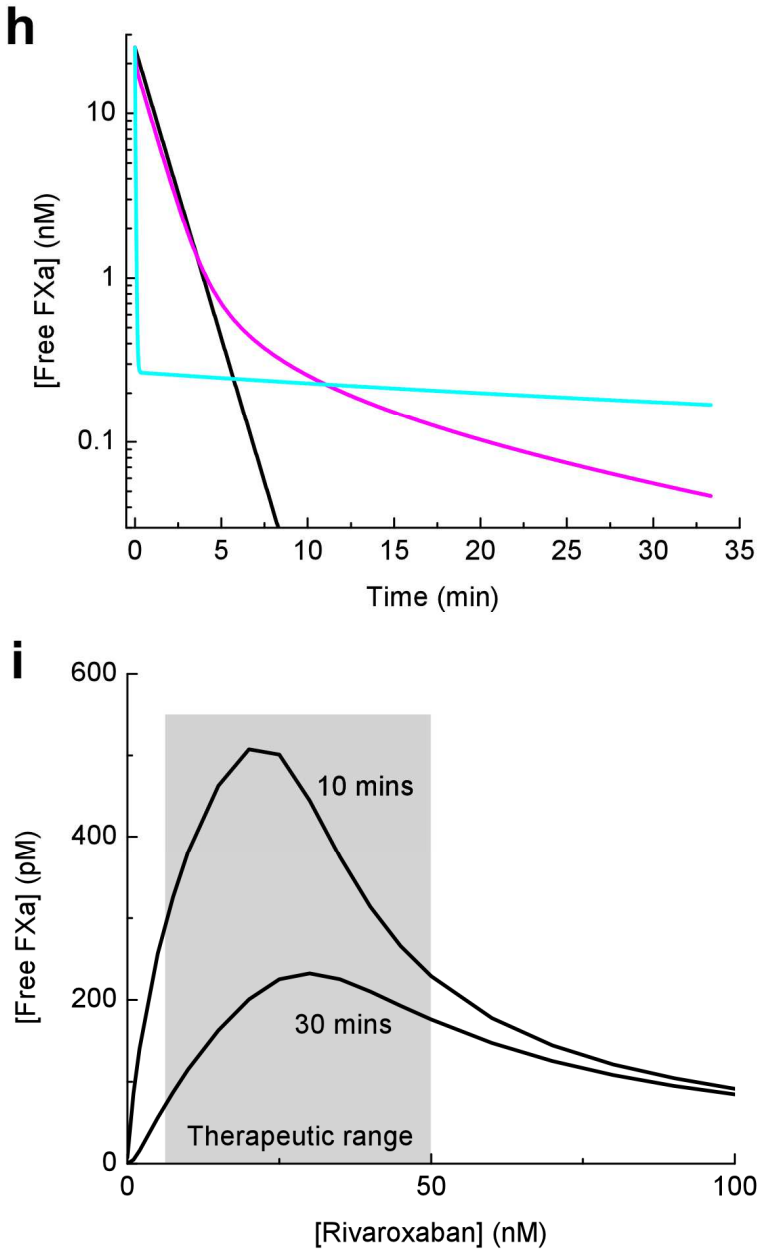


Figure 3

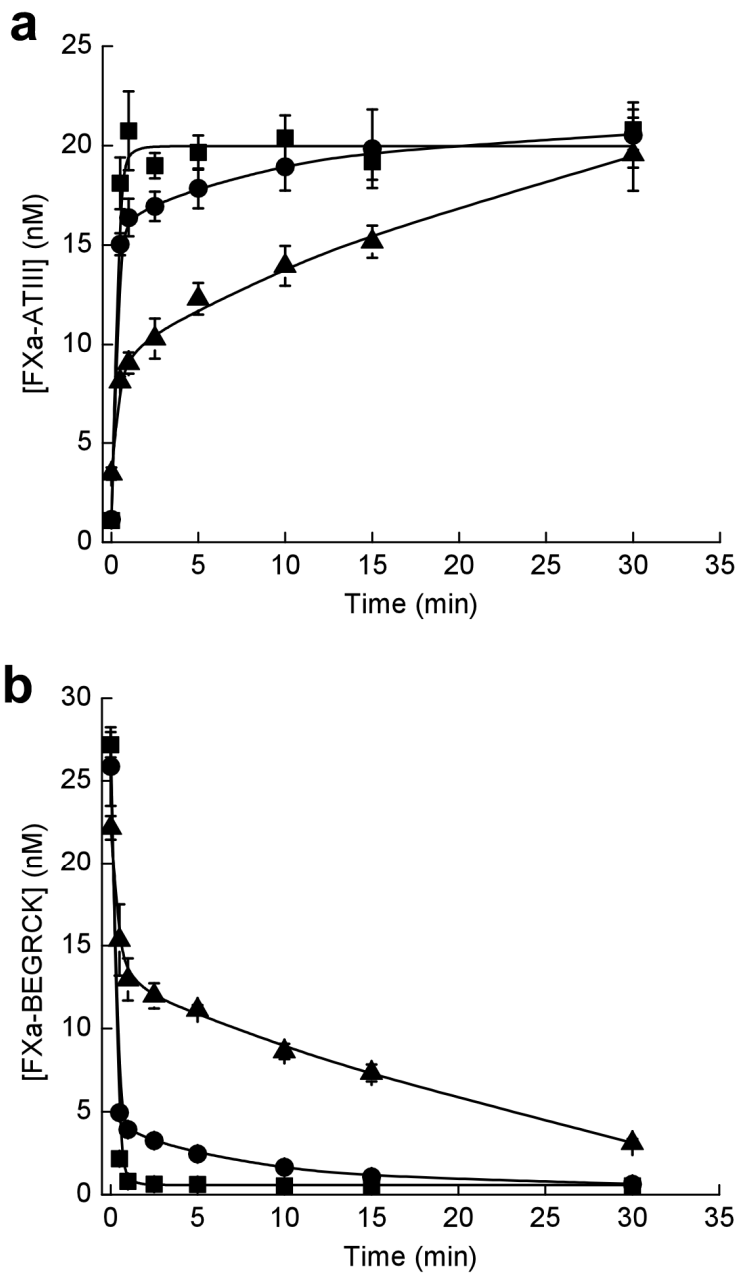
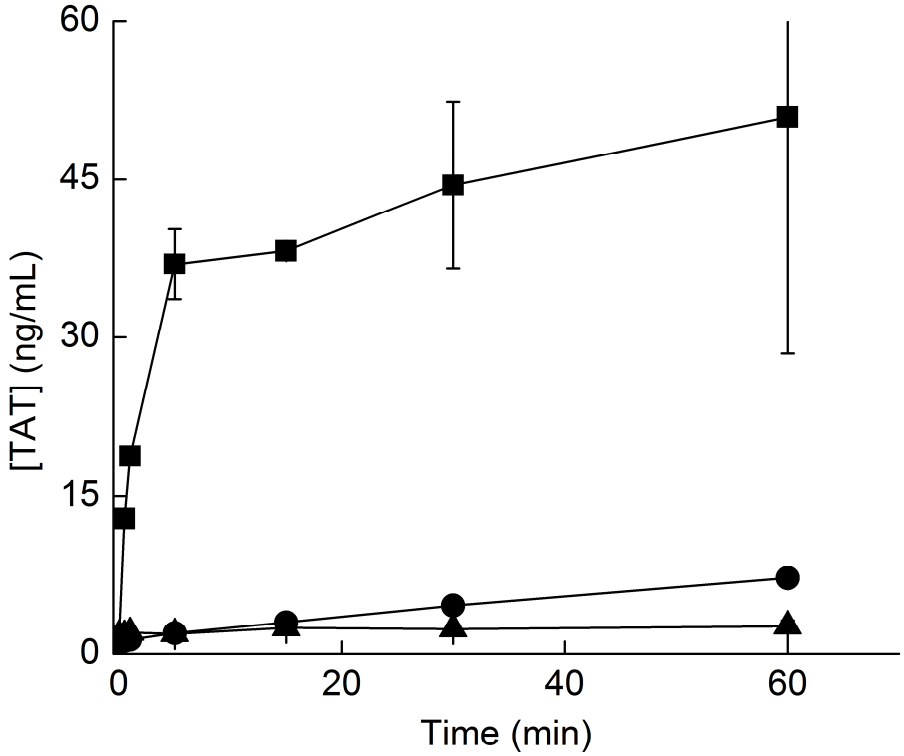


Figure 4



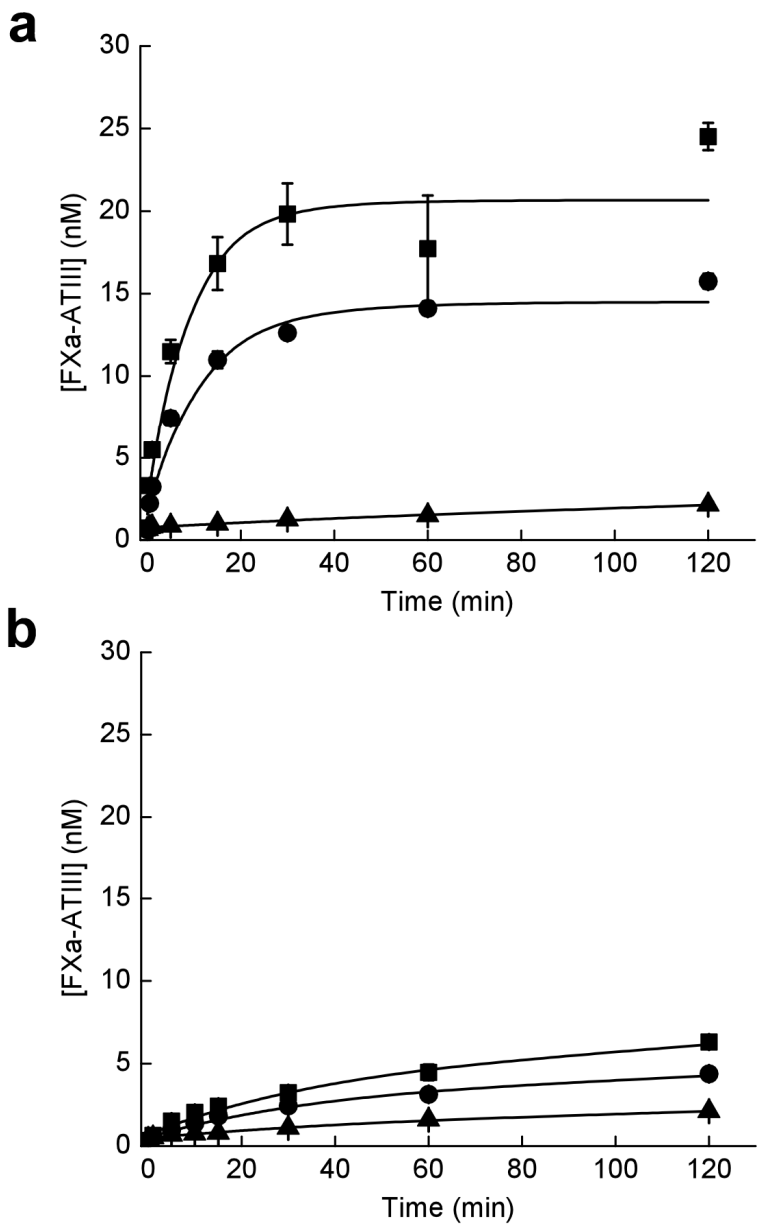
Supplementary Figure Legends

Supplementary Figure 1. Kinetics of FXa-ATIII and FXa-B-EGRCK complex formation in a purified system. Kinetics of FXa-ATIII complex formation were measured after mixing of (***a***) 25 nM wt-hFXa or (***b***) hFXa$^{I[16]L}$ with 2.6 μM hATIII in the presence of 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. Solid lines represent the fit of the points to a single exponential rise with offset. B-EGRCK labeling of (***c***) wt-hFXa or (***d***) hFXa$^{I[16]L}$ not inhibited by ATIII was quantified by ELISA in the presence of 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. The solid lines for the 0 and 100 nM rivaroxaban experiments in panel ***c*** represent the fit of the points to a single exponential rise with offset. All other drawn lines (in panel ***c*** and ***d***) are a smoothed connection of the points due to poor fit. Error bars indicate the SEM.

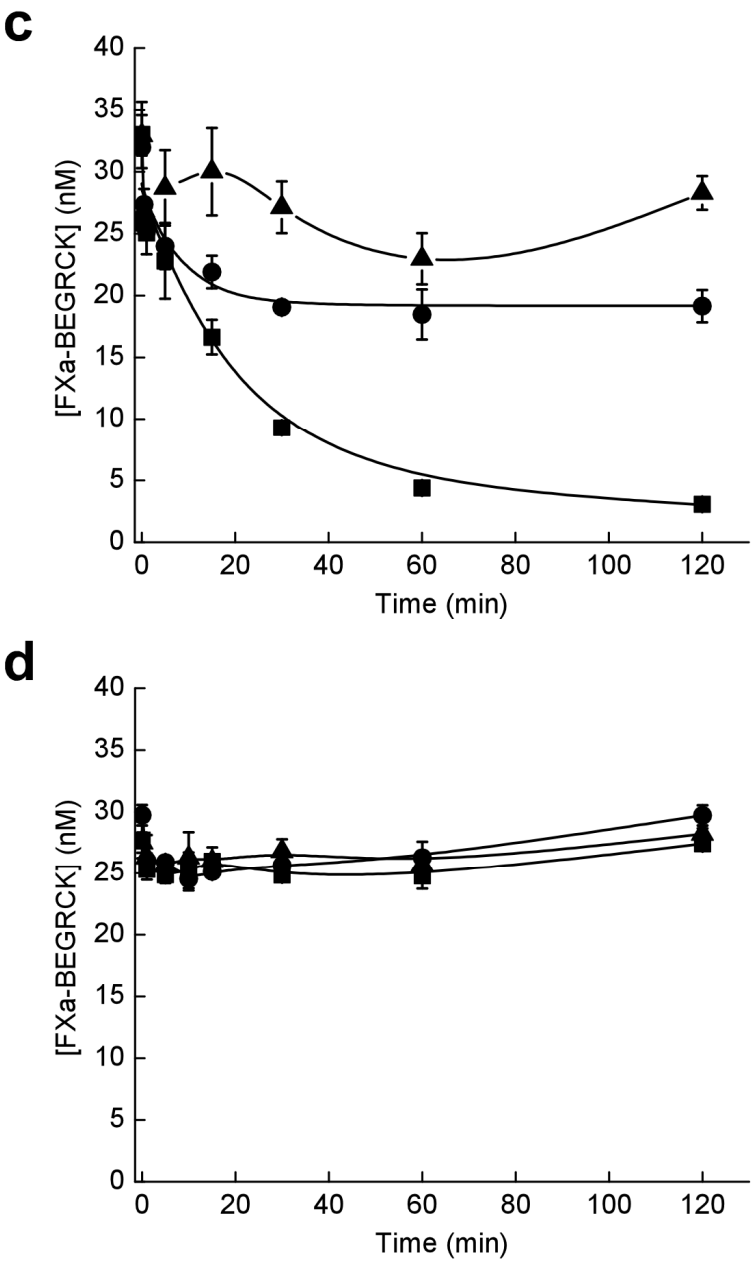
Supplementary Figure 2. Effect of fondaparinux and rivaroxaban on FXa-ATIII complex formation and B-EGRCK labeling of FXa$^{I[16]L}$. Kinetics of FXa-ATIII complex formation (***a***) and FXa labeling by B-EGRCK (***b***) after addition of 25 nM hFXa$^{I[16]L}$ to FX-deficient plasma containing 750 nM fondaparinux and 0 (-■-), 100 nM (-●-) or 1 μM (-▲-) rivaroxaban. The solid lines represent the fit of the points to a single exponential rise except for the 1 μM data in ***a*** and the 100 nM data in ***b***, which are fit to a two-exponential rise. Error bars indicate the SEM.

Supplementary Figures

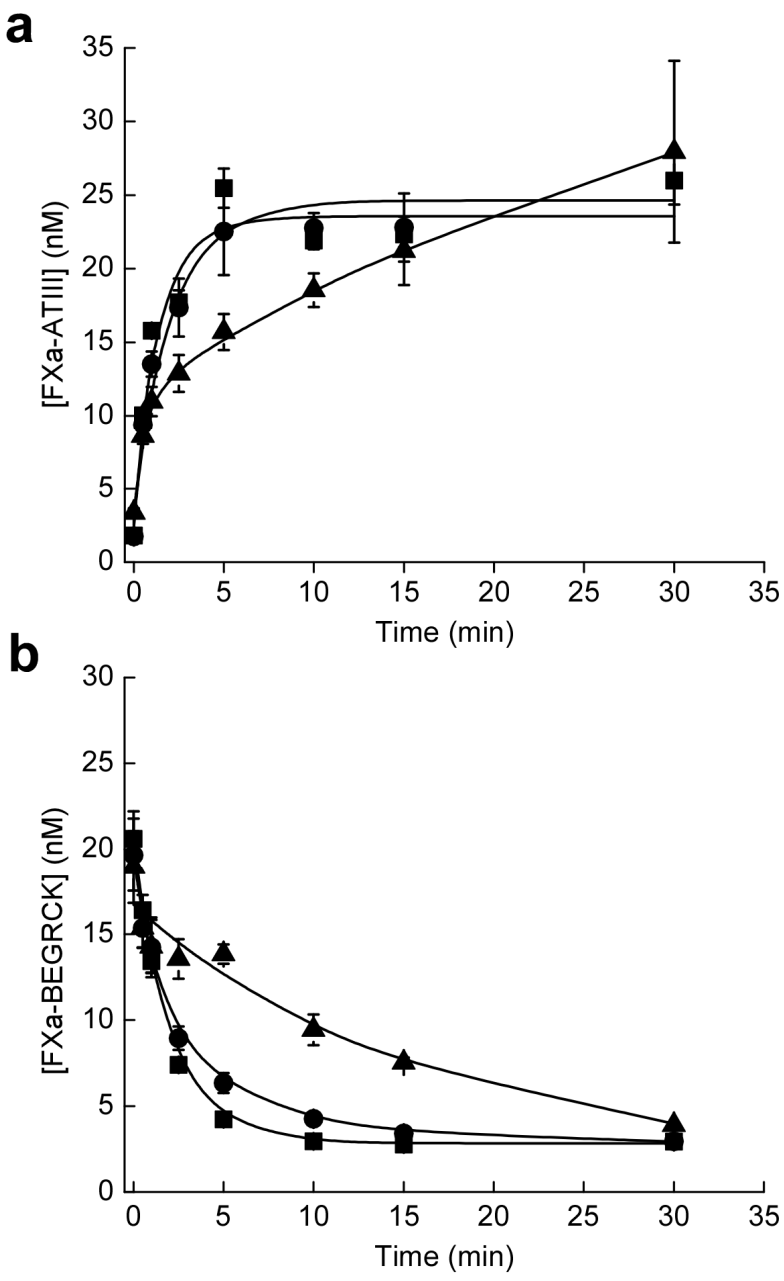
Supplementary Figure 1



Supplementary Figure 1 (cont.)



Supplementary Figure 2



Supplementary Tables

Supplementary Table 1.

Effect of rivaroxaban on pseudo-first-order rate constants of FXa-ATIII complex formation in plasma.

	wt-FXa	**FXa$^{I[16]L}$**
Rivaroxaban	**k_{obs}**	**k_{obs}**
nM	**s^{-1} ($x10^{-4}$)**	**s^{-1} ($x10^{-4}$)**
0	33.9±4.8	1.7±0.4
100	6.2±1.6	2.0±0.2
1000	1.3±0.5	0.6±0.4

k_{obs} values were determined by fitting the data in **Figures 2a** and **2b** to a single exponential rise expression with offset. The data are reported as the fitted values ± 2 SD.

Supplementary Table 2.

Effect of rivaroxaban and fondaparinux on pseudo-first-order rate constants for the decrease in B-EGRCK labeling of wt-FXa in plasma.

		- Fondaparinux	+Fondaparinux
Protein	**Rivaroxaban**	**k_{obs}**	**k_{obs}**
	nM	**s^{-1} ($x10^{-4}$)**	**s^{-1} ($x10^{-4}$)**
wt-FXa	0	24.4±5.7	938.8±18.8
wt-FXa	100	5.2±2.8	913.8±29.5*
wt-FXa	1000	ND	145.3±19.0*

k_{obs} values were determined by fitting the data in **Figures 2c** and **3b** to a single exponential decay expression with offset. The data are reported as the fitted values ± 2 SD.
*Better fit was obtained with a two-exponential decay expression with offset. k_{obs} represents the average of the two fitted rate constants, weighted for amplitude of each exponential term ± 2 SD.
ND, not determined.

Supplementary Table 3.

Effect of rivaroxaban on pseudo-first-order rate constants of FXa-ATIII complex formation in a purified system

Protein	Rivaroxaban	k_{obs}
	nM	**s^{-1} ($x10^{-4}$)**
wt-FXa	0	21.3±7.9
wt-FXa	5	15.9±3.5
wt-FXa	50	0.9±0.5
$FXa^{I[16]L}$	0	3.1±0.8
$FXa^{I[16]L}$	5	3.5±0.7
$FXa^{I[16]L}$	50	2.0±0.3

k_{obs} values were determined by fitting the data in **Supplementary Figures 1a** and **1b** to a single exponential rise expression with offset. The data are reported as the fitted values ± 2 SD.

Supplementary Table 4.

Effect of rivaroxaban on pseudo-first-order rate constants for the decrease in B-EGRCK labeling in a purified system.

Protein	Rivaroxaban	k_{obs}
	nM	**s^{-1} ($x10^{-4}$)**
wt-FXa	0	7.7±2.1
wt-FXa	5	24.0±13.9
wt-FXa	50	ND
$FXa^{I[16]L}$	0	ND
$FXa^{I[16]L}$	5	ND
$FXa^{I[16]L}$	50	ND

k_{obs} values were determined by fitting the data in **Supplementary Figures 1c** and **1d** to a single exponential decay expression with offset. The data are reported as the fitted values ± 2 SD.
ND, not determined.

Supplementary Table 5.

Effect of rivaroxaban on pseudo-first-order rate constants of FXa-ATIII complex formation of wt-FXa in plasma in the presence of 750 nM fondaparinux.

Protein	Rivaroxaban	k_{obs}
	nM	s^{-1} (x10^{-4})
wt-FXa	0	804.8±177.7
wt-FXa	100	650.0±33.3*
wt-FXa	1000	116.4±38.8*

k_{obs} values were determined by fitting the data in **Figure 3a** to a single exponential decay expression with offset. The data are reported as the fitted values ± 2 SD.
*Better fit was obtained with a two-exponential decay expression with offset. k_{obs} represents the average of the two fitted rate constants, weighted for amplitude of each exponential term ± 2 SD.

CHAPTER 4: Discussion

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Reversal of direct FXa inhibitors using zymogen-like FXa

The emergence of new oral anticoagulants that directly inhibit the active sites of coagulation serine proteases has marked a substantial advance in therapy for thrombosis [107]. They have good efficacy [98, 101] and several clinical indications [81, 83, 85, 87-89, 92, 93], but the lack of an approved reversal agent in the event of bleeding or need for emergency surgery is of great concern [107, 109]. Based on the biochemistry and pharmacokinetics of the oral direct FXa inhibitors, two broad strategies could be used to reverse the effects of these anticoagulants: sequestration of the drug using a specific stoichiometric antidote, or bypassing the anticoagulant using a catalytic, pro-hemostatic agent. Recently, drug-binding antidotes (i.e. GD-FXa$^{S[195]A}$ and aripazine) have received a great deal of attention and are in various stages of clinical development [112, 113]. The advantage of these agents is that they are relatively specific to the anticoagulant. However, because they must bind the anticoagulant in a 1:1 ratio, high doses are needed. In addition to the obvious difficulty of producing such large amounts of a protein product, these agents have the potential to deplete endogenous regulators of coagulation when present at high concentrations. A pro-hemostatic agent with intrinsic catalytic activity might be effective at lower concentrations and thereby avoid the potential pitfalls associated with direct antidote-based reversal. Moreover, the development of a strategy that reverses the effects of direct FXa inhibitors by a different mechanism of action may be useful, since it is not clear which general approach will have the best clinical efficacy. Prior to our work, many early studies explored the possibility of repurposing existing bypassing agents to reverse the new anticoagulants [123-128, 135], but the results have been mixed and devoid of any rational mechanistic explanation for their effects.

We have developed a pro-hemostatic bypassing strategy for direct FXa inhibitors using a zymogen-like variant of FXa, $FXa^{I[16]L}$. When free in plasma, this variant is resistant to active site inhibitors, has low catalytic activity, and has a long half-life [44, 59]. However, it is also fully functional when assembled in prothrombinase, and is thus an effective pro-hemostatic molecule [44, 59, 60]. *In vitro*, we demonstrated that $FXa^{I[16]L}$ can, at catalytic concentrations, reverse the anticoagulant effect of the direct FXa inhibitor rivaroxaban. This was in stark contrast to GD-$FXa^{S[195]A}$, which was also very effective, but with much poorer potency. $FXa^{I[16]L}$ was also effective in two *in vivo* hemostasis models and, again, more potent than GD-$FXa^{S[195]A}$. The potency difference we observed between $FXa^{I[16]L}$ and GD-$FXa^{S[195]A}$ was consistent with the fact that they work by different mechanisms of action. Thus, our results suggest that zymogen-like FXa may be a promising bypassing agent for direct FXa inhibitors.

Determining the efficacy of a reversal strategy

Given the numerous potential reversal agents for direct FXa inhibitors, it is crucial to identify which approaches are the most effective clinically. Ultimately, a large randomized trial examining clinically relevant endpoints in bleeding patients or in those in need of an invasive procedure is necessary to definitively determine which agent is best. However, this is complicated in part because of the time-sensitive nature of bleeding, where efficacy of a reversal agent might depend on how rapidly it can be administered. Moreover, the relative infrequency of bleeding episodes with these new agents [98, 101] makes a large controlled trial difficult. For these reasons, the initial clinical trials studying reversal agents for direct FXa inhibitors have been performed in healthy volunteers using surrogate markers of coagulation [117, 123]. While this is

certainly understandable, it also limits the conclusions that can be drawn about these new agents with respect to efficacy and safety. This is particularly true of the risk of thrombosis, which cannot be adequately evaluated in healthy volunteers since patients taking anticoagulants typically have some degree of underlying hypercoagulability that might be exacerbated by the reversal agent.

Nonetheless, understanding the biochemical mechanism by which each agent works and the nature of the surrogate marker being used can give a context in which to interpret these clinical trial results. For example, for specific drug-sequestering antidotes, measurement of anti-FXa activity is a strong indicator of the degree to which the antidote has relieved inhibition of FXa, since these agents reduce the unbound fraction of the drug [112]. Thus, in a setting where anticoagulation needs to be reversed before a procedure, a normalized anti-FXa activity following administration of a drug-sequestering antidote almost certainly suggests that the individual is hemostatically normal (or at least, at baseline). In contrast, using the anti-FXa activity as an indicator of the ability to stop bleeding and improve outcomes is less straightforward. In this scenario, the anti-FXa assay still reflects the extent of anticoagulant sequestration by the antidote, but does not take into account other factors that affect the outcome of a bleeding episode, such as how soon the antidote was administered as well as the specific vascular bed that was injured.

The anti-FXa assay is a poor marker of reversal using a pro-hemostatic bypassing agent, since the lower, catalytic quantities of the agent used are heavily diluted to the point of being ineffective in this assay. While other assays, such as TG studies or thromboelastography (TEG/ROTEM) are certainly responsive to bypassing agents [123-

126, 128], these assays, unlike the anti-FXa assay, are global coagulation studies that do not reflect any specific aspect of coagulation. Therefore, it is impossible to make clinical conclusions from these results without a corresponding empirical clinical study.

Competition between direct FXa inhibitors and endogenous protease inhibitors

Despite the numerous bypassing agents being studied for reversal of direct FXa inhibitors, there have been no mechanistic studies exploring how these products could overcome the effects of a noncompetitive inhibitor of FXa. Since their biochemistry dictates that they do not sequester the inhibitor, these agents could only generate thrombin if they allow catalytically active and uninhibited FXa to persist in the presence of the inhibitor. However, prior to this work, there has been no plausible explanation for how this might occur.

By studying the mechanism of rivaroxaban reversal by $FXa^{I[16]L}$, we have discovered a characteristic of direct FXa inhibitors that allows a paradoxical increase in the amount of uninhibited enzyme in the presence of the inhibitor. In our study, we observed that both $FXa^{I[16]L}$ and wt-FXa normalize thrombin generation in the face of rivaroxaban, suggesting that the ability of $FXa^{I[16]L}$ to reverse rivaroxaban's effects was not related to its zymogenicity. However, the high level of thrombin generation seen in our experiments was inconsistent with kinetic studies showing that, when assembled in prothrombinase, $FXa^{I[16]L}$ and wt-FXa are both highly inhibited by rivaroxaban. This indicated that some previously unreported phenomenon must allow for more uninhibited FXa than our kinetic studies predicted. To reconcile these discordant

findings, we evaluated rivaroxaban's effect on ATIII, a key regulator of FXa activity in plasma. We found that rivaroxaban dose-dependently impaired ATIII-dependent FXa inhibition. We also observed a corresponding rise in reversibly inhibited FXa, consistent with formation of a rivaroxaban-FXa complex. Together, these observations suggested a model wherein FXa can form either a reversible complex with FXa, or else an irreversible complex with ATIII. Formation of these complexes is mutually exclusive, and, critically, the two complexes are separated by free, uninhibited FXa. Using an *in silico* approach, we determined that a steady-state of uninhibited FXa is generated in the presence of rivaroxaban that decays very slowly. This pool of FXa does not exist to a high degree in the absence of rivaroxaban, and thus it likely explains the thrombin generation we observed.

This paradoxical increase in free FXa illustrates the importance, and the complexity, of the plasma protease inhibitors of the coagulation serine proteases. In the absence of a pharmacologic active site inhibitor of FXa, a bolus of FXa would be rapidly inactivated in a pseudo-first-order fashion by endogenous protease inhibitors [28, 29]. Within 8 minutes, less than 1% of the administered FXa would remain. In the presence of a direct FXa inhibitor, exogenously administered FXa would become rapidly but reversibly bound to the anticoagulant, dramatically slowing the rate of irreversible inactivation of the protease and prolonging the half-life of the bolus. While it is certainly true that the majority of the bolus would remain inhibited by the anticoagulant, the reversibility of the interaction ensures that a small but relevant fraction of uninhibited FXa remains. Crucially, the concentration of this small pool of FXa is greater than it would be if rivaroxaban were not present.

The efficacy of direct FXa inhibitors as anticoagulants suggests that this paradoxical increase in free FXa is probably less relevant to endogenously generated FXa. While rivaroxaban almost certainly decreases the rate at which endogenously generated FXa is inactivated, it is likely that FXa generation *in vivo* is quite slow compared to exogenous bolus administration of FXa. Under these conditions, the pool of FXa reversibly bound to the drug is small, and the pool of free FXa is smaller than it would be in the absence of the anticoagulant.

It is possible that some disease states may result in much higher levels of protease activation. For example, patients with mechanical heart valves are typically more aggressively anticoagulated with warfarin to prevent prosthesis-associated thrombosis [165]. Dabigatran, a direct thrombin inhibitor, was surprisingly less effective than warfarin for anticoagulation in these patients, despite its good efficacy for other types of thromboprophylaxis [99]. A possible explanation for failure of dabigatran in this setting is that there is a high rate of prothrombin activation driven by the indwelling contact-activating valve. Since we observed that dabigatran prevents inhibition of thrombin by ATIII, a high rate of thrombin generation could lead to a paradoxical increase in free thrombin in the presence of the anticoagulant, thereby reducing its effectiveness.

In addition to explaining the mechanism of action of FXa-dependent reversal of rivaroxaban, our results provide insight into the fundamental rationale for development new anticoagulants. At first glance, active site inhibition of the coagulation serine proteases seems to be a logical way to achieve anticoagulation, especially when compared to the indirect inhibition of coagulation that warfarin provides. Enzymatic active sites are routinely targeted with small, orally bioavailable compounds that can easily disrupt

substrate binding to the enzyme. However, the substrates of serine proteases are large macromolecules that form a broad interface with the enzyme at exosites distinct from the site of catalysis [4]. Moreover, serine proteases are regulated by endogenous inhibitors that primarily engage the active site [25-27]. For these reasons, an active site directed small molecule will have no effect on substrate binding, but will block both catalysis and regulatory inhibition. Thus, these "target-specific" anticoagulants are really nonspecific with respect to how they affect the kinetics of their targets. Depending on the rate of activation of the protease, they might have anticoagulant effects or procoagulant effects. Obviously, clinical trials have shown that the anticoagulant effect predominates under most conditions. Nonetheless, our results demonstrate that active site antagonism affords less precise control over anticoagulation than was initially hoped. These findings also suggest that targeting macromolecular complex assembly might provide better control over anticoagulation, since this would have no effect on the active site function of the protease and thus not interfere with endogenous regulators of protease activity. Development of such an agent is difficult, however, since it would require disruption of large protein-protein interfaces.

Future directions

This work raises several new questions that need to be addressed through future studies. Although we have shown that direct FXa inhibitors compete with ATIII for binding and inhibition of FXa, ATIII is not the only plasma protease inhibitor of FXa. α_2M is likely to be as important, and perhaps even more important, than ATIII for the regulation of FXa activity *in vivo*. The mechanism of action of α_2M inhibition requires proteolysis of α_2M by FXa [27]. Thus, we expect that direct FXa inhibitors to interfere with α_2M-mediated

FXa inactivation. This can best be evaluated in an *in vitro* system using purified FXa and α_2M, since the ATIII present in plasma would interfere with this determination. At different time points, ATIII and heparin (to accelerate the reaction) would then be added to inactivate any FXa not in complex with α_2M. Since FXa trapped within α_2M can still hydrolyze oligopeptide substrates [27], we can use FXa chromogenic activity as an indicator of the degree to which FXa is "protected" from ATIII by α_2M. Performing such experiments in the presence and absence of a direct FXa inhibitor will then allow us to determine how the anticoagulant affects the α_2M/FXa interaction.

Our kinetic studies with rivaroxaban and ATIII also raise the question of how much FXa persists *in vivo* reversibly bound to the anticoagulant. Determining this is crucial to understanding the potential implications of the competition between direct FXa inhibitors and endogenous plasma protease inhibitors. It will also allow us to predict how much FXa could be liberated upon reversal of anticoagulation with a drug-sequestering antidote such as GD-FXa$^{S[195]A}$ [112] or aripazine [113]. This could be studied by collecting blood samples from anticoagulated patients in a tube containing a high concentration of B-EGRCK to trap any FXa in the sample that has not been irreversibly inhibited. We could then use an ELISA approach similar to the one used in our current studies to measure the amount of FXa reversibly bound to the anticoagulant. Unfortunately, our current assay has poor sensitivity below the high picomolar range, which will likely limit our ability to detect the trapped FXa in these samples. Thus, it will be important to optimize the assay to improve its sensitivity, since even low picomolar concentrations of FXa could have clinical significance. Another alternative approach would be to perform similar experiments in patients anticoagulated with dabigatran, which give information about the persistence of reversibly-inhibited thrombin. This

strategy might be better, since thrombin is generated at much higher concentrations than FXa and therefore may be easier to detect.

A counterintuitive but potentially quite interesting implication of this work is that direct FXa inhibitors could be used to extend the half-life of FXa-based pro-hemostatic agents. wt-FXa is a poor therapeutic agent in part because of its extremely short half-life [28, 166]. One of the major reasons $FXa^{I[16]L}$ and other zymogen-like FXa variants are effective procoagulants *in vivo* is due to their extended half-lives [60, 167]. Because direct FXa inhibitors impair the inhibition of FXa by plasma protease inhibitors, they are capable of dramatically extending the half-life of wt-FXa in a concentration-dependent manner. Since we have also shown that a small portion of the reversibly-inhibited FXa remains completely uninhibited, a direct FXa inhibitor could work in conjunction with wt-FXa to make it an effective, pro-hemostatic therapeutic with a long half-life. This could be evaluated by administration of wt-FXa alone or wt-FXa combined with rivaroxaban to hemophilic mice. We have previously shown (unpublished data) that wt-FXa does not restore hemostasis in murine bleeding or thrombosis models in hemophilic animals. If the combination of wt-FXa and rivaroxaban were to restore hemostasis, this would suggest that rivaroxaban can paradoxically enhance the pro-hemostatic potential of FXa by extending its half-life.

Conclusions

Chymotrypsin-like serine proteases have numerous biological roles, from digestion to allergy and inflammation [3]. While the catalytic function of these enzymes is important, regulation of these enzymes to prevent unwanted proteolysis is of equal significance, and

dysregulation can lead to disease [1]. This is readily apparent in hemostasis, where serine proteases are the machines of the clotting cascade, allowing for rapid, localized formation of a protein thrombus following vascular injury. Coagulation has a tremendous impact on human health, and disordered hemostasis is responsible for many diseases. Consequently, several pharmacologic agents designed to modulate blood coagulation have been developed. However, despite the placement of serine proteases at the heart of hemostasis, direct antagonists of these enzymes have only recently been approved for use as anticoagulants. Although these new agents are intended to block the catalytic function of their serine protease targets, little is known about their effect on the regulatory processes of coagulation.

In this dissertation, we determined that direct inhibitors of coagulation FXa and thrombin impair the action of the key regulators of coagulation serine protease activity in plasma. For FXa, we showed that this phenomenon is true of any active site inhibitor of the enzyme, meaning that there is no way to engineer an anticoagulant that inhibits the active site without also disrupting the action of plasma protease inhibitors. By combining these results with an *in silico* simulation of the kinetics of FXa inhibition, we found that, under conditions where FXa is either rapidly generated or else exogenously administered, there is a paradoxical increase of free, uninhibited FXa in the presence of the target-specific anticoagulant. Critically, these observations were made at pharmacologically relevant concentrations of these drugs, suggesting that such a phenomenon could occur during anticoagulant therapy. These results also explain our observation that a zymogen-like variant of FXa was effective *in vivo* to reverse the anticoagulant effects of direct FXa inhibitors. Such a reversal agent could be useful in emergency settings to either stop bleeding or restore normal hemostasis in an

anticoagulated patient. Together, these studies provide evidence for the first mechanism-driven bypassing strategy for direct FXa inhibitors. Perhaps more importantly, this work highlights the importance of both catalysis and regulation in serine protease biology and demonstrates that both aspects must be considered when developing a targeted therapy.

APPENDIX:

Correction of human hemophilia A whole blood abnormalities with a novel bypass agent: zymogen-like FXa$^{I[16]L}$

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Summary

Background: Approximately 30% of hemophilia A (HA) and 5% of hemophilia B patients develop inhibitors to protein replacement therapy which is the major cause of disease-related morbidity in the developed world. We previously developed zymogen-like factor Xa (FXa) molecules with impaired active site maturation enabling a greater half-life than wild-type FXa while maintaining full procoagulant function in the prothrombinase complex. Here we evaluated the ability of zymogen-like $FXa^{I[16]L}$ to correct whole blood thromboelastometry abnormalities of severe HA subjects with and without inhibitors.

***Methods*:** 14 severe HA subjects without and 5 with inhibitors were enrolled at baseline (FVIII:C <1%) >5 half lives from factor or bypass therapy. Subjects' whole blood was evaluated by thromboelastography (ROTEM®) using INTEM analysis with two concentrations of $FXa^{I[16]L}$ or recombinant factor VIIa (rFVIIa).

***Results*:** With 0.1 nM $FXa^{I[16]L}$, CT in minutes (min) among HA subjects without and with inhibitors (mean=2.87 min, 95% CI=2.58-3.15 min and mean=2.9 min, 95% CI=2.07-3.73 min, respectively) did not significantly differ from control CT (mean=2.73 min, 95% CI=2.62-2.85 min). Addition of 20nM rFVIIa, simulating a 90µg/kg dose, resulted in significantly prolonged CTs for HA subjects without and with inhibitors (mean=5.43 min, 95% CI=4.53-6.35 min and mean=4.25 min, 95% CI=3.32-5.17 min, respectively) relative to controls.

***Conclusions*:** $FXa^{I[16]L}$ restored thromboelastometry CT to control values in severe HA subjects with and without inhibitors. Findings corroborate previous animal data and

demonstrate the first evidence of zymogen-like $FXa^{I[16]L}$ to correct human HA subject whole blood abnormalities and support the use of $FXa^{I[16]L}$ as a novel hemostatic agent.

Introduction

Congenital hemophilia is characterized by deficiency of factor VIII (hemophilia A [HA]) or factor IX (hemophilia B [HB]). Patients incur sequelae of bleeding resulting from inadequate production of activated factor X (FXa) and ultimately thrombin. Specifically, factor VIII (FVIII) and factor IX (FIX) maintain hemostasis through their essential role in the intrinsic pathway converting zymogen FX to the active protease FXa. Thereafter, the assembled prothrombinase complex (membrane-bound FXa and its cofactor factor Va [FVa]) converts prothrombin to thrombin, which then activates platelets and converts fibrinogen to insoluble fibrin, yielding a hemostatic plug [4, 168]. Therapeutically, this aberration in hemophilia patients is restored by peripheral administration of plasma derived or recombinant protein products [169, 170].

Despite the overwhelming positive impact of protein replacement therapy, up to 30% of patients with HA and 5% of patients with HB develop neutralizing alloantibodies (inhibitors) to protein replacement which now accounts for the major cause of disease related morbidity and mortality in developed nations [171, 172]. Currently available bypassing agents (*i.e.* recombinant activated factor VII [rFVIIa] and activated prothrombin complex concentrates [aPCCs]) are directed at FXa production and thereby thrombin formation [130, 173]. These strategies to bypass the defective intrinsic pathway are viable to achieve hemostasis in most patients with inhibitors, but are not universally effective and do not completely normalize thrombin generation [173-175]. Additionally, bypass therapies have associated prothrombotic risk (particularly if management requires tandem bypassing agents), in the case of aPCCs, are plasma-derived with intendant risks of blood borne disease, and need for frequent infusions results in costly

treatment [173, 176-178].

As an alternative, administration of FXa to increase prothrombinase complex formation would represent a direct approach for thrombin formation. However, wild-type (wt)-FXa is limited by its rapid inactivation by physiologic inhibitors resulting in a short half-life (<1-2 minutes). Further, the ability of the free protease to activate a range of procoagulant clotting factors with possible pathological activation of coagulation could also be problematic [28, 179, 180]. Collectively, these realities preclude the use of wt-FXa as a bypassing therapeutic.

Drawing from the known biochemical properties common to all chymotrypsin-like serine proteases, we previously developed FXa variants (*e.g.* $FXa^{I[16]L}$, chymotrypsinogen numbering system [23]) with impaired conformational transition from zymogen to active protease [44, 59, 60]. For FX, zymogen cleavage between Arg^{15}-Ile^{16} results in a new N-terminus consisting of the conserved amino acid sequence Ile^{16}-Val-Gly-Gly. The insertion of the nascent N-terminus into a binding pocket followed by salt bridge formation with Asp^{194} confers a conformational change driving the zymogen to the active protease state, critical for full enzymatic function. Modification of FXa at Ile^{16} and Val^{17} results in an immature active site, thereby altering the protein to adopt a zymogen-like state. This effectively causes a redistribution of the zymogen-protease equilibrium that normally lies towards the mature protease. As a consequence of this altered conformation, $FXa^{I[16]L}$ is less susceptible to plasma protease inhibitors and therefore has a prolonged half-life (>30 minutes). Importantly however, FVa preferentially binds the protease conformation of zymogen-like FXa effectively 'rescuing' the active protease through the principle of mass action. Evaluation of $FXa^{I[16]L}$ in murine hemophilia

models has not demonstrated evidence of systemic activation of coagulation or undesired thrombus formation [59, 60]. The net effect is full procoagulant function and normal thrombin generation demonstrated in both *ex vivo* and *in vivo* murine HB and HA models [44, 59, 60].

Here we examined whether these FXa variants may be effective procoagulants for hemostatic management of hemophilia patients using an *ex vivo* approach. This work follows demonstrated efficacy in animal studies and is the first human data. We evaluated if zymogen-like $FXa^{I[16]L}$ corrects whole blood thromboelastometry hemostatic abnormalities observed in HA subjects with and without inhibitors and compared the results to the most widely used bypassing agent, rFVIIa.

Methods

The Children's Hospital of Philadelphia Institutional Review Board approved participant recruitment for this study. Signed informed consent (parent with child assent where appropriate) was obtained prior to participation. Severe HA subjects without inhibitors (HA) and with inhibitors (HA-I) were prospectively and consecutively recruited during outpatient visits to the Hemophilia and Thrombosis Center at CHOP. Subjects were enrolled at hemostatic baseline (FVIII:C <1%). Factor VIII activity and inhibitor values (Bethesda assay) were determined from the same blood draw at sample collection. Patients were excluded from this study if they were <1 year, within 5 half lives of factor replacement or bypass therapy, and/or had a known or suspected secondary hemostatic abnormality.

Blood was collected via peripheral venipuncture into a 3.8% sodium citrate vacutainer.

Subject whole blood hemostatic abnormalities were assessed by ROTEM® thromboelastometry using the INTEM® assay. The INTEM reagent, an intrinsic pathway activator comprised of kaolin, in the presence of calcium chloride, initiated coagulation. Experimental conditions varied only with respect to the addition of supplemental protein, which included either: 0.05 or 0.1nM $FXa^{I[16]L}$, 20 nM rFVIIa, FVa with or without 2 nM $FXa^{I[16]L}$, or no supplemental protein (buffer). Thromboelastometry assay analysis was uniformly initiated one hour following peripheral venipuncture. Control samples from five hemostatically normal subjects on no medications were collected and analyzed under the same conditions as study subjects. Differences between HA and HA-I subjects without and with addition of $FXa^{I[16]L}$ or rFVIIa were analyzed using a paired t-test. One-way analysis of variance (one-way ANOVA) was used to evaluate INTEM clot time differences among controls, HA subjects and HA-I subjects for each experimental condition. Adjusted *p*-values were computed using the Tukey-Kramer procedure, *p*-values <0.05 were considered significant. Equality of HA, HA-I and control group variances were evaluated by Levene's test.

Results/Discussion

Fourteen HA subjects and 5 HA-I subjects were enrolled. All subjects were male ages 1 to 11 years with confirmed FVIII:C <1% at the time of blood draw. Among the 5 subjects with inhibitors, ages ranged from 3 to 11 years with inhibitor values at time of enrollment of 26-96 Bethesda Units (B.U.). Five control subjects were enrolled. All control subjects were adults. Control INTEM assay parameters were consistent with published normal adult values. Of note, adult INTEM clot time (CT) normal values, minimally, if at all, differ from published >6 months pediatric normal values [181, 182].

As expected, CT was the most abnormal parameter in HA and HA-I subjects and thus, the focus of our analysis under varying experimental conditions. Figure 1 presents CT times for the control group at baseline as well as HA and HA-I groups at baseline and in the presence of 0.1nM $FXa^{I[16]L}$ and 20nM rFVIIa. Among HA and the HA-I subjects, pre-treatment INTEM CTs were significantly greater than control CT (Figure 1; p=0.006, HA mean=6.35 min, 95% CI=5.13-7.57 min; and p=0.001, HA-I mean=4.82 min, 95% CI=4.82-11.15 min; control mean=2.73 min, 95% CI=2.62-2.85 min). HA and HA-I baseline CTs were not significantly different (p=0.278). There was no significant difference in group variances (Levene's Test, p=0.254).

Addition of $FXa^{I[16]L}$ dose dependently decreased INTEM CT in both HA and HA-I subjects. Specifically, after the addition of 0.1 nM $FXa^{I[16]L}$ to HA and HA-I subject samples, there was no significant difference between HA and HA-I CT from control CT (F-Statistic 1.36, p=0.841; HA mean=2.87 min, 95% CI=2.58-3.15 min; HA-I mean=2.90 min, 95% CI=2.07-3.73 min. Further, Levene's test for equality of variances was not significant, p=0.277. There was no significant difference among CT changes in HA and HA-I subjects at baseline and in the presence of 0.1nM $FXa^{I[16]L}$ (p=0.726; HA mean=3.49 min, 95% CI=2.35-4.62 min; HA-I mean= 5.10 min, 95% CI=2.44-7.75 min). Thus, irrespective of the presence or absence of inhibitors, addition of 0.1 nM $FXa^{I[16]L}$ corrected hemophilic subject CT to control findings.

Next the procoagulant effect of rFVIIa was assessed in 9 of the 14 HA subjects and the 5 HA-I subjects. A concentration of 20 nM rFVIIa was chosen for comparison to approximate plasma concentration achieved after administration of a dose of 90 μg/kg, which is commonly employed for bleeding manifestations in hemophilia patients with

inhibitors. Although the addition of 20 nM rFVIIa decreased CT in all subjects, CT remained significantly prolonged relative to controls for both HA and HA-I subjects (p=0.002, HA mean=5.43 min, 95% CI=4.53-6.35 min; and p=0.046, HA-I mean=4.23 min, 95% CI=3.32-5.17 min; control mean=2.73 min, 95% CI=2.62-2.85 min). As expected, in the presence of 20nM rFVIIa, the post-treatment CT did not significantly differ between HA and HA-I subjects (p=0.0761) and Levene's test for equality of variances was not significant (p=0.123).

Figure 2a shows representative tracings from an HA subject with inhibitor, with the addition of 0.5 and 0.1 nM $FXa^{I[16]L}$ compared to 20 nM rFVIIa. The delayed and blunted tracing profile of the HA-I patient's blood was essentially restored with 0.1 nM $FXa^{I[16]L}$ and was nearly indistinguishable from that of normal control blood. The incomplete response with 20 nM rFVIIa highlights the superior effectiveness of $rFXa^{I[16]L}$ on a molar basis; however, the data must be interpreted with caution, as conditions in the assay do not simulate *in vivo* conditions such as drug volume of distribution, complete phospholipid membrane binding surfaces or tissue factor availability. Nevertheless, the data are generally consistent with our prior *in vivo* mouse studies demonstrating the enhanced effectiveness of murine $rFXa^{I[16]L}$ relative to rFVIIa [60].

Traditional clotting assays employed in medical practice may predict some measure of clinical outcome but neither adequately captures the hemostatic effect of bypassing agents nor allow for comparison of the hemostatic effect of various bypassing agents. Within the hemophilia community, there is growing interest in the potential use of global viscoelastic assays (*e.g.* thromboelastography/thromboelastometry) for monitoring therapeutic interventions in hemophilia, particularly those on bypassing therapy [183].

As such, we felt that the ROTEM® INTEM assay would optimize *ex vivo* capacity to both observe the hemostatic effect of $FXa^{I[16]L}$ and allow for comparison to other bypass strategies, *i.e.* rFVIIa. Additionally the use of a kaolin based coagulation initiator (INTEM reagent) appears better than tissue factor at discriminating the effects of rFVIIa in hemophilia thromboelastography analysis; however, notably these findings are generally limited to single center studies [184, 185]. Thromboelastography clot time (CT), like thromboelastogram R time, is thought to be a measure specific to alterations in coagulation factor protein function and quantity and least influenced (relative to other parameters) by fibrinogen, platelet quantity or function [186].

Although limited by *ex vivo* analysis, our findings provide initial evidence in human whole blood and corroborate animal data demonstrating $FXa^{I[16]L}$ is able to correct hemostatic abnormalities observed in murine models of hemophilia [59, 60]. Additionally *ex vivo* findings of this work are consistent with our previous *in vivo* observations supporting that $FXa^{I[16]L}$ is able to restore hemostasis at much lower concentrations than rFVIIa [60]. A clear difference between rFVIIa and $rFXa^{I[16]L}$ are their half-lives (2-3 hours vs. 30 minutes, respectively). While this is an apparent limitation, at present it is unclear how half-life, when coupled to hemostatic effectiveness of the product, would actually impact clinical outcome.

Lastly, due to the requirement of FVa to 'rescue' the protease conformation of $FXa^{I[16]L}$, we speculate the procoagulant function of zymogen-like FXa will be limited by the availability of FVa. Therefore, FVa is thought to impart procoagulant injury site specificity and potentially protect against off target thrombosis. To probe this further, in concurrent but separate experiments, we titrated increasing concentrations of $FXa^{I[16]L}$ in

HA patient whole blood. At $FXa^{I[16]L}$ concentrations above 2 nM, CT no longer shortened (data not shown) suggesting $FXa^{I[16]L}$ saturation with the *in situ* generated FVa. To test this further, additional FVa was added to the system. As shown in Figure 2b, the addition of 2 nM $FXa^{I[16]L}$ and 5 nM FVa shortened the CT beyond that observed with 2 nM $FXa^{I[16]L}$ alone and controls. The addition of 10 nM FVa with 2 nM $FXa^{I[16]L}$ further shortened CT beyond that observed with 2 nM $FXa^{I[16]L}$ and 5 nM FVa and controls. The addition of FVa (5 nM or 10 nM) without $FXa^{I[16]L}$ had no appreciable effect on CT relative to HA patient whole blood analysis without added FVa. These results were recapitulated in three separate patient samples; however, small sample size precluded statistical analysis and further validation of findings. Nonetheless, these observations suggest that, through the requirement of FVa to bind and thereby rescue the $FXa^{I[16]L}$ protease, available FVa may limit $FXa^{I[16]L}$ procoagulant potential and thereby prevent undesired thrombosis. Given the inherent limitations of *ex vivo* modeling and artificial circumstances in which FVa quantity may be limited in this assay system, caution must be used in interpreting results. Nonetheless our findings support what is known about the underlying mechanism of $FXa^{I[16]L}$ protease conversion. Specifically, zymogen-like FXa molecules may have limited off target prothrombotic potential since available FVa dictates their activity. If true, zymogen-like FXa molecules may have less thrombotic risk than current bypass therapies.

Conclusion

At much lower concentrations than rFVIIa, $FXa^{I[16]L}$ normalized INTEM CT in HA subjects both with and without inhibitors. These findings are congruent with prior pre-clinical hemophilic animal studies and further support the use of $FXa^{I[16]L}$ as an

alternative bypassing therapy for hemophilia patients with inhibitors. Further, the requirement of FVa to rescue $FXa^{I[16]L}$ protease function suggests procoagulant injury site specificity and limited off target thrombotic potential. A Phase I study is currently evaluating the safety of $FXa^{I[16]L}$ in healthy human volunteers (ClinicalTrials.gov; NCT01897142).

Figure Legends

Figure 1. Comparison of HA and HA-I subject findings in the presence of $FXa^{I[16]L}$ and 20 nM rFVIIa relative to control subjects. Box plots represent median, 25% and 75% interquartile ranges; whiskers represent ± 2 standard deviations from the mean. Reported p-values are adjusted using the Tukey-Kramer procedures after a one-way analysis of variance to evaluate differences from control clot time findings.

Figure 2. ROTEM tracings comparing various bypassing strategies and studying the dependence of $FXa^{I[16]L}$ on FVa. (***a***) Sample ROTEM tracings demonstrate $FXa^{I[16]L}$ dose response relative to rFVIIa. (***b***) HA subject with an inhibitor: ROTEM tracings in the presence of $FXa^{I[16]L}$ and FVa.

Figures

Figure 1

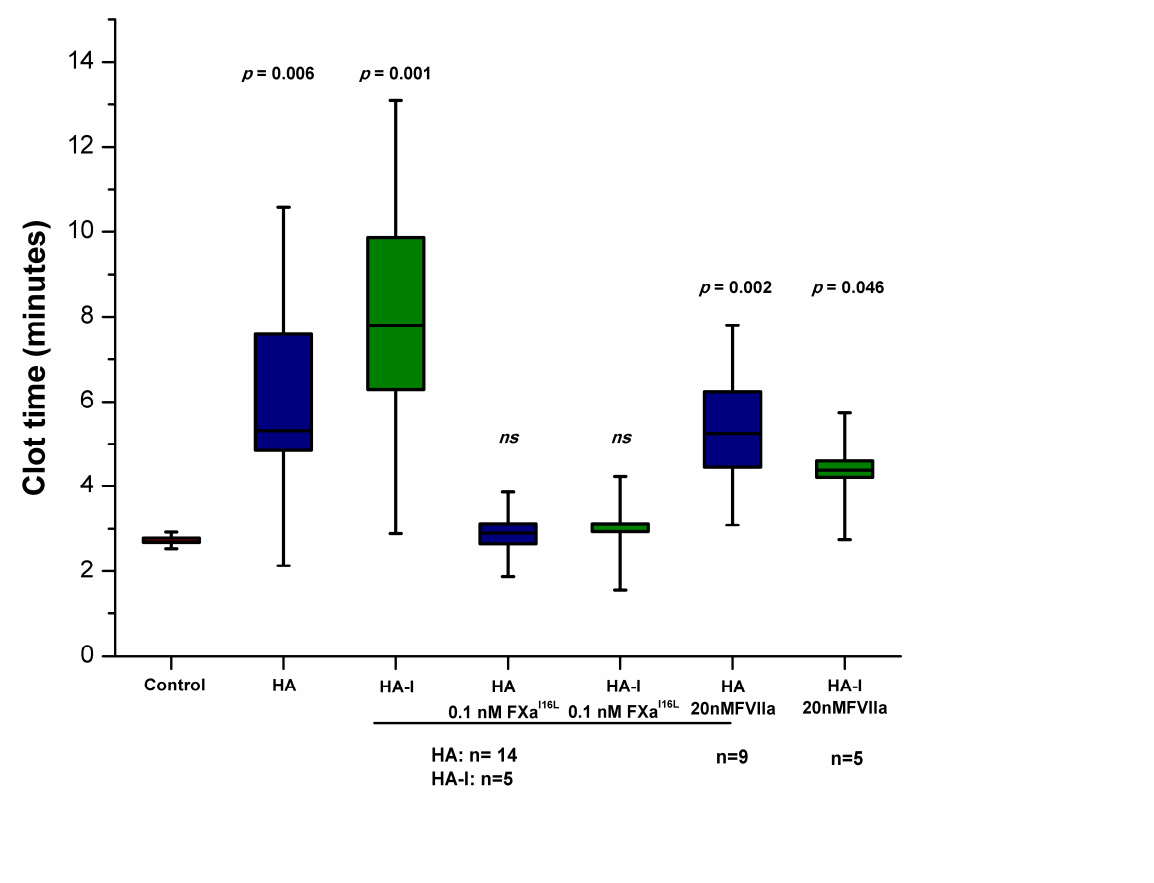
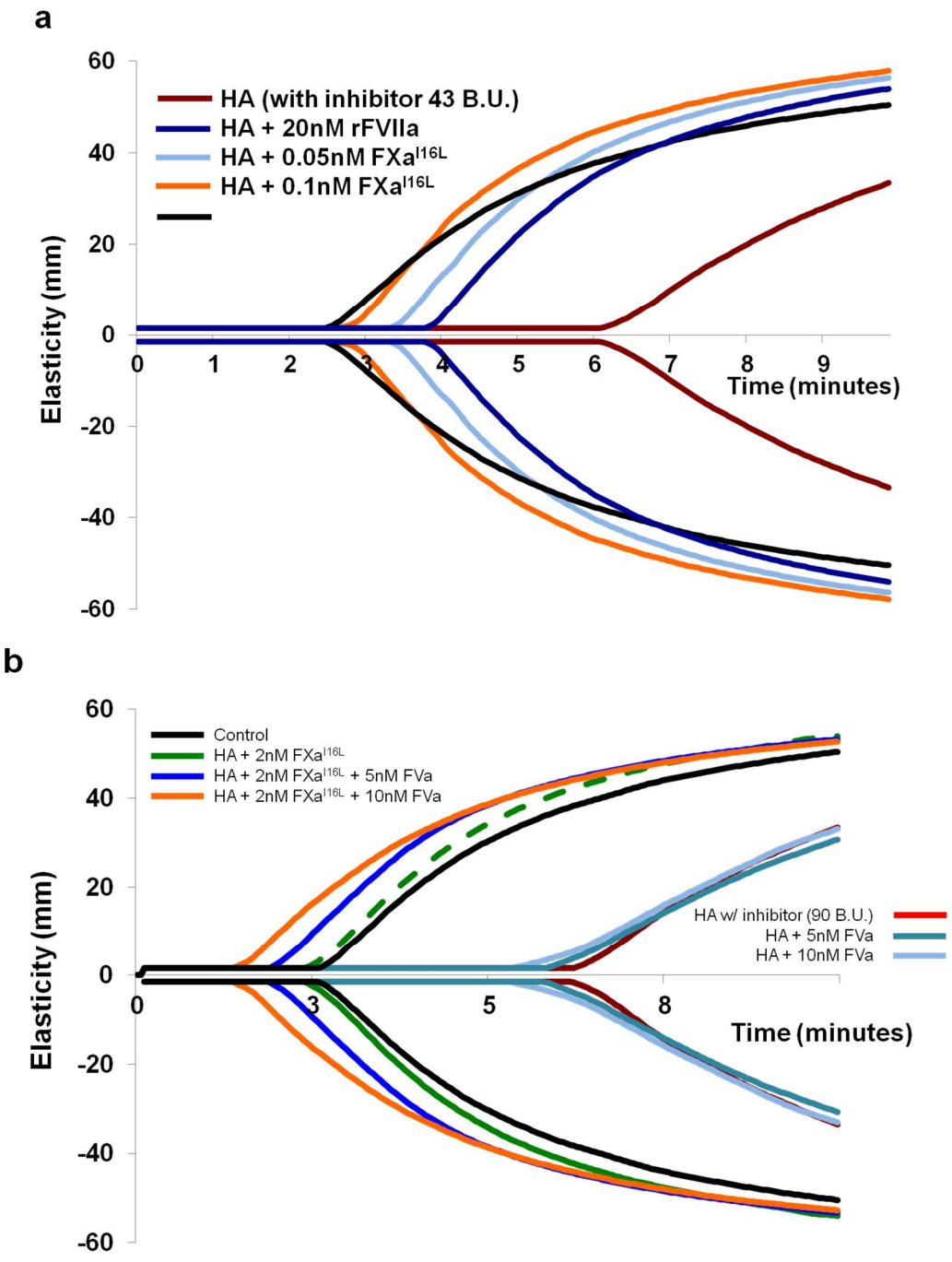


Figure 2



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