HHS Public Access

Author manuscript

Curr Opin Hematol. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

Curr Opin Hematol. 2017 September ; 24(5): 453–459. doi:10.1097/MOH.0000000000000369.

Rendering Factor Xa Zymogen-like as a Therapeutic Strategy to Treat Bleeding

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Abstract

Purpose of review—New therapies are needed to control bleeding in a range of clinical conditions. This review will discuss the biochemical properties of zymogen-like FXa, its pre-clinical assessment in different model systems, and future development prospects.

Recent findings—Underlying many procoagulant therapeutic approaches is the rapid generation of thrombin to promote robust clot formation. Clinically tested prohemostatic agents (e.g factor VIIa) can provide effective hemostasis to mitigate bleeding in hemophilia and other clinical situations. Over the past decade, we explored the possibility of using zymogen-like FXa variants to rapidly improve clot formation for the treatment of bleeding conditions. Compared to the wild-type enzyme, these variants adopt an altered, low activity, conformation which enables them to resist plasma protease inhibitors. However, zymogen-like FXa variants are conformationally dynamic and ligands such as its cofactor, FVa, stabilize the molecule rescuing procoagulant activity. At the site of vascular injury, the variants in the presence of FVa serve as effective prohemostatic agents. Pre-clinical data support their use to stop bleeding in a variety of clinical settings. Phase 1 studies suggest that zymogen-like FXa is safe and well-tolerated, and a phase 1b is ongoing to assess safety in patients with intracerebral hemorrhage.

Summary—Zymogen-like FXa is a unique prohemostatic agent for the treatment of a range of bleeding conditions.

Keywords

factor Xa; hemophilia; procoagulant; zymogen; bleeding; hemostatic agent

Introduction

Hemostasis is a key component of vascular homeostasis that prevents blood loss following vessel injury while also maintaining vascular patency. It is achieved, in part, through a

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Conflicts of Interest

RMC receives research support from Pfizer, Novo Nordisk and Bayer and license fees from Pfizer for technology related to FXa. RMC also serves as a consultant to Pfizer, Bayer and Spark Therapeutics.

cascade of serine proteases that are sequentially activated from precursors called zymogens, culminating in formation of the effector protease, thrombin, and ultimately in the formation of a blood clot [1]. Normally, this process is tightly regulated, however, a failure of the system to respond appropriately due to a host of factors can lead to bleeding or thrombotic complications. Currently, there are highly effective strategies for the treatment and prevention of thrombosis [2], but hemostatic management of patients with excessive bleeding remains a major challenge. Bleeding can occur in a variety of clinical scenarios, including trauma, surgery, anticoagulation, and coagulation factor deficiencies such has hemophilia. In some situations, where bleeding is occurring from a single, accessible source, direct endoscopic, surgical, or vascular approaches can be very effective at arresting bleeding [3–6]. However, when bleeding occurs more diffusely, when the bleeding source is difficult to access, or when traditional treatments for inherited bleeding disorders fail, alternative therapies may be more appropriate. In this review, we summarize a new technology, zymogen-like FXa, that has the potential to serve as a rapid and versatile hemostatic agent for the treatment of bleeding conditions. We will discuss the preclinical and early clinical development of zymogen-like FXa and its potential applications.

Prohemostatic Molecules-Overview

There is a great deal of interest in the development of protein therapeutics to mitigate bleeding in certain clinical settings. Over the past two to three decades, largely as a result of work aimed at preventing or stopping hemophilia-associated bleeding, several prohemostatic agents have been developed. Examples include prothrombin complex concentrates (PCCs or activated PCCs) and recombinant factor VIIa (rFVIIa), which are used to varying degrees to improve hemostasis and control bleeding [7–9].

PCCs are a heterogeneous group of plasma-derived products that were originally developed to treat severe FIX deficiency (hemophilia B; HB) before purified FIX was available [10]. It is now typically used for warfarin reversal, especially in patients who may not be able to tolerate large volumes of fresh frozen plasma (FFP), and is 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 [10]. Most preparations also contain the anticoagulant proteins C and S [11]. Recombinant FVIIa (rFVIIa) is a widely used bypassing agent for hemophilia A (HA) and HB patients with inhibitors to enhance FXa generation through the extrinsic pathway [9, 12]. Activated PCCs are a plasma-derived product used, like rFVIIa, to treat bleeding in hemophilia patients with inhibitors [12, 13]. Despite their name, aPCCs only contain a significant concentration of one activated factor, FVIIa [14]. 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 [15–17].

A common feature of these direct prohemostatic approaches for hemophilia is that they work by accelerating the formation of FXa. The outcome of these strategies is to boost thrombin generation to provide adequate hemostasis. In principle, a more straightforward approach is direct infusion of FXa. FXa is strategically positioned as the ideal enzyme to bypass

defective clotting in hemophilia and potentially other clinical scenarios. However, early attempts to document the usefulness of FXa were disappointing largely due to its very short half-life (<1 min) caused by rapid inactivation by endogenous plasma inhibitors, such as antithrombin (AT) and tissue factor pathway inhibitor (TFPI) [18].

Zymogen-like FXa-Biochemical perspectives

The problems associated with wild-type FXa essentially eliminate the possibility that it could be an effective pro-hemostatic agent. To address these issues, we bioengineered a FXa that could overcome protective inhibitory mechanisms without compromising hemostatic benefit. Specifically, proteolytic activation of FXa from its precursor FX at a highly conserved site (e.g., R^{15}–I^{16}-...) [19] liberates a new amino-terminus (I^{16}-VGG). This new amino terminus inserts into a hydrophobic pocket within the catalytic domain forming a salt-bridge with Asp^{194} [20–22]. This leads to a series of conformational changes that yield the mature protease, FXa. The relative abundance of these conformations and their equilibrium distribution is determined, in part, by the degree to which the new amino terminus is stabilized within the hydrophobic pocket [23]. To alter this process and the equilibrium of conformations that normally exist, we changed the amino acid at position 16 or 17 to make the ensuing structural change sub-optimal and create a “zymogen-like state”. Biochemical characterization of a range of zymogen-like variants revealed they have varying degrees of catalytic impairment (5 to 1,000-fold; see Table 1) [24]. They also have partial resistance to circulating inhibitors (e.g. AT and TFPI) and, consequently a prolonged half-life in plasma. Interestingly, variants with lowest catalytic activity (the most “zymogen-like”) were the most resistant to inhibitors [24, 25]*. Importantly, since the zymogen-like state has plasticity and is highly dynamic, ligands that engage the enzyme can readily alter its conformational equilibrium. An important biological consequence of this is the activity of zymogen-like FXa can be thermodynamically rescued by binding FVa, resulting in a prothrombinase enzyme with near-normal procoagulant activity (Table 1) [24–26]*.

Zymogen-like FXa for Use in Hemophilia

Hemophilia is an inherited, X-linked bleeding disorder caused by deficiency of FVIII or FIX. Replacement of the deficient factor with recombinant or plasma-derived protein is the standard of care. 5–30% of patients develop neutralizing autoantibodies to FVIII or FIX, necessitating use of prohemostatic bypass agents such rFVIIa or aPCCs [27]. These agents can achieve hemostasis in most patients with inhibitors, but are not always effective and do not completely normalize thrombin generation [9, 28, 29]. There is also an inherent prothrombotic risk associated with bypass therapies. Finally, aPCCs are plasma-derived, which increases the risk of blood-borne disease [9, 30–32].

We hypothesized that zymogen-like FXa might be an effective alternative to existing bypassing agents due to its unique properties and position in the coagulation cascade. *In vitro* and *ex vivo* studies of increasing complexity showed that FXa^{I16L}, a prototypical zymogen-like FXa species, was highly effective in promoting thrombin generation in hemophilic plasma with or without inhibitors and in hemophilic blood [25, 26, 33]*. As expected from previous biochemical studies, these effects were FVa-dependent. Initial

preclinical studies in hemophilic mice indicate that FXa^{I16L} appears safe and effective after a single dose when administered either before or after injury in multiple models [34]. Importantly, repeated infusions at the effective dose of FXa^{I16L} did not result in systemic activation of coagulation, with no significant changes in levels of fibrinogen, platelets, thrombin-antithrombin (TAT) or D-dimer over an extended time course in either hemophilic or wild-type mice. Further, histopathological evaluation in different tissues revealed no evidence of treatment related increases in fibrin deposition [34].

Despite its zymogen-like character, FXa^{I16L} has a relatively short half-life in mouse plasma (<10 min), limiting its use as a prophylactic agent [25, 26]. However, it may be ideal for acute bleeding, where the desired effect is to stop blood loss quickly without prolonged activation of coagulation. Since zymogen-like variants have a wide range of activities and half-lives, we explored the possibility that variants with prolonged half-lives in plasma may be more appropriate in other clinical scenarios. Indeed, *in vivo*, we found that long half-life variants (e.g. FXa^{I16T}; >1.5 hr) were most effective when given prior to injury (a prophylaxis strategy), whereas short-to-intermediate half-life variants (e.g. $FXa^{I16L;}$ <10 min; Table 1) were best when given after injury (an on-demand strategy) [25]*. Together these studies underscore that the tunability of the FX/Xa zymogen-to-protease transition allows for the development of therapeutics with broad applicability, and prompted us to explore other clinical applications outside of hemophilia.

Zymogen-like FXa Can Overcome Direct Oral Anticoagulants

In 2010, dabigatran, the first orally bioavailable direct thrombin inhibitor, was approved as an anticoagulant for several indications including primary prevention of stroke in patients with nonvalvular atrial fibrillation, and prevention and treatment of venous thromboembolism [35, 36]. Since then, several orally administered direct FXa inhibitors, including rivaroxaban, apixaban, and edoxaban, were approved for similar indications [37–45]. Collectively, the direct thrombin and direct FXa inhibitors are known as direct oral anticoagulants (DOACs), and are all active site inhibitors of their respective proteases [46, 47]. Following their introduction, DOACs have gained a tremendous share of an anticoagulant market that was once dominated by the vitamin K antagonist warfarin. By the end of 2014, office visits for the DOACs had nearly surpassed office visits for warfarin [48]. Their rapid adoption is due to several successful phase III clinical trials in thousands of patients showing efficacy equal to warfarin but a significantly reduced risk of bleeding [2, 49]. Further, they have improved pharmacokinetics compared to warfarin which allowed for simpler dosing schemes and decreased or eliminated the need for anticoagulant monitoring [50]. However, like all anticoagulants, DOACs do carry a risk of bleeding [51]. The effects of older anticoagulants like warfarin and heparin can be reversed pharmacologically in the event of bleeding or in the event that patients require urgent or emergent surgery [52]. In contrast, when they were developed, DOACs lacked a reversal agent, which has caused concern [50]. PCCs, aPCCs, or rFVIIa have been shown to have limited to no efficacy as reversal agents for DOACs. In 2015, the FDA approved idarucizumab, a monoclonal antibody fragment that binds and neutralizes dabigatran, and can reverse its anticoagulant effects [53]*. To date, there are no FDA approved reversal agents for direct FXa inhibitors. The most promising reversal approach in development is andexanet alfa (Gla-domainless

FXa^{S195A}, GD-FXa^{S195A}), a recombinant FXa molecule that lacks catalytic activity as well as the ability to bind membranes, making it a scavenger for all direct FXa inhibitors [54].

Neither idarucizumab nor andexanet alfa have catalytic activity, meaning they function as drug-sequestering antidotes. We explored the hypothesis that zymogen-like FXa could mitigate the effects of DOACs. We found FXa^{I16L} to be highly effective at reversing the effects of anticoagulation in mice treated with rivaroxaban [55]*. This was true in multiple injury models. FXa^{I16L} was also between 50 and 300 times more potent than GD-FXa^{S195A}, an andexanet biosimilar, supporting the idea that the mechanism by which FXa^{I16L} reverses the effects of rivaroxaban is related to its intrinsic catalytic activity. FXa^{I16L} was also able to reverse the effects of dabigatran in a mouse tail bleeding model [55]*. Together, these data suggest that FXa^{I16L} may fill an unmet clinical need for a universal rapid countermeasure to the effects of DOACs.

Zymogen-like FXa for Acute Warfarin Reversal

As mentioned previously, warfarin can be reversed if needed by replacing the affected clotting factors. Typically, this is achieved with fresh frozen plasma (FFP) or PCCs. In addition, vitamin K can be administered if reversal is less urgent. However, none of these agents are prohemostatic [52]. Moreover, since these agents either replenish vitamin K or the affected factors, patients who require temporary reversal of anticoagulation (i.e. for invasive procedures) must wait 2–4 days after resumption of warfarin to become therapeutically anticoagulated. Since zymogen-like FXa has a relatively short half-life, we investigated whether it might be effective as a quick-acting temporary prohemostatic reversal agent for warfarin. Conceptually, this idea is bolstered by recent biochemical work that suggests that prothrombin carboxylation (which is impaired by warfarin treatment) is not crucial for prothrombin activation by prothrombinase [56]. Administering FXa^{I16L} to warfarin-treated mice in a large-vessel thrombosis model restored clot formation, indicating that, *in vivo*, FXa^{I16L} is able to overcome the effects of warfarin [57].

Clinical development of Zymogen-like FXa

Given the promising *in vivo* animal studies establishing FXa^{I16L} as a rapid prohemostatic, its clinical development is underway as PF-05230907. In preclinical pharmacokinetic studies in cynomolgus monkeys, its half-life was found to be ~3 minutes. A dose-dependent decrease in the activated partial thromboplastin time (aPTT) and increase in TAT levels was also observed, consistent with FXa^{I16L} functioning as a prohemostatic [58]*. As expected, in nonclinical toxicology studies in monkeys at doses exceeding the projected efficacious dose, changes in markers of coagulation were observed as were thrombi in different tissues [59]*.

In a first-in-human phase 1 dose escalation study in 49 healthy adults (NCT01897142), FXa^{I16L} was well-tolerated up to 5 mg/kg, with a half-life of about 4 minutes. There were no serious adverse events, and no dose-limiting toxicity up to the maximal dose. aPTT and thrombin generation assay parameters changed in a dose-dependent manner, as did prothrombin fragment 1.2 and D-dimer levels. One patient developed a weakly positive, transient, non-neutralizing antibody to FXa^{I16L} that did not cross-react with FX or FXa; no

other patients exhibited an immune response [60]*. These early results support the further clinical development of FXa^{I16L}.

Future Clinical Assessment -Intracerebral Hemorrhage

Perhaps the most exciting potential application of zymogen-like FXa is in the setting of intracerebral hemorrhage (ICH). Spontaneous ICH (bleeding in the brain) is a major cause of death and disability accounting for approximately 8–25% of strokes worldwide [61–63]. ICH continues to be associated with poor clinical outcomes. Approximately 40% of patients die within one month, 1-year mortality is over 50% [64], and a large number of individuals fail to regain functional independence by six months [65]. The current management of ICH is largely supportive and the lack of effective treatment for ICH remains a critical and unmet need [3]. Unfortunately, recent trials exploring mechanism-driven interventions, including surgical hematoma evacuation and intensive blood pressure lowering, have failed to show a clinical benefit [66–69]*. For these reasons, there has not been an improvement in ICH mortality rate for decades [70].

Many factors predict ICH outcomes, including age, Glasgow Coma Scale (GCS), location of the hemorrhage, and hematoma volume [71]. In particular, hematoma volume has emerged as a therapeutic target because it is a strong predictor of mortality and disability. Furthermore, nearly 40% of ICH patients experience a 33% or greater increase in hematoma volume, and every 10% increase in hematoma volume results in a 5% increased risk of death [72]. Soon after approval of rFVIIa for hemophilia, there was a great deal of interest in exploiting its prohemostatic potential to prevent ICH hematoma volume expansion. A phase II study of rFVIIa for ICH (399 patients randomized) demonstrated a more than 50% decrease in hematoma growth and an absolute mortality decrease of 11% in the 80 μg/kg group [73]. Unfortunately, in a subsequent phase 3 trial (Factor Seven for Acute Hemorrhagic Stroke, FAST) of 841 patients, despite a significant decrease in hematoma growth in patients receiving rFVIIa, there was no significant mortality benefit [74]. The results of this trial effectively ended consideration of rFVIIa as a therapeutic agent for ICH. Nonetheless, analysis of the data from the FAST trial has given insight into why rFVIIa decreased hematoma growth without improving outcomes. In particular, one of the major challenges of the FAST trial was the mismatched rate of intraventricular hemorrhage (IVH) between the high-dose 80 μg/kg rFVIIa group (41%) and the placebo group (29%). Post-hoc analysis of the data indicates that, for a subset of patients, particularly younger patients with low levels of IVH who receive treatment rapidly may benefit from rFVIIa [75]. These considerations likely translate to any prohemostatic approach that targets hematoma expansion.

Zymogen-like FXa, therefore, may have the potential to improve outcomes in certain patients with ICH. Since FXa is a more downstream activator of coagulation than FVIIa, zymogen-like FXa may be even more effective than rFVIIa at arresting hematoma growth. In addition, as described above, zymogen-like FXa but not rFVIIa can overcome the effects of both DOACs and warfarin [55, 57]. Since anticoagulation is an important cause of ICH [76], a prohemostatic that can treat ICH regardless of anticoagulation status is desirable. In

preclinical models of ICH in rats and mice, FXa^{I16L} dose-dependently reduced final hematoma volume by as much as 47% [77]*.

Armed with the lessons learned from the FAST trial, FXa^{I16L} (PF-05230907) is currently being studied as a therapeutic for ICH. A phase 1b trial of approximately 50 patients with ICH is currently underway (NCT02687191). Although this is primarily a safety and tolerability study, secondary endpoints will include hematoma growth and neurologic function.

Conclusion

Excessive bleeding derived from a range of clinical conditions continues to present a management challenge. While physical control of the bleeding source is always ideal, in many cases this cannot be achieved, and therefore there is a need for new therapeutic approaches including prohemostatics. Due to the mechanism of action of prohemostatics, there is always a risk of thrombotic complications with their use. However, through bioengineering, molecules that minimize this risk can be developed. Early preclinical work with zymogen-like FXa demonstrated its ability to enhance clot formation and thereby control bleeding in a localized manner. These studies illustrated that such FXa variants can serve as versatile, rapid, pan-hemostatic agents for a variety of bleeding conditions. Ongoing clinical trials will shed more light on the efficacy and safety of this new approach.

Acknowledgments

Financial Support and Sponsorship

This work was supported in part by National Institutes of Health Grant F30 HL-120487 and T32 HL-GM-007170 to NKT and P01 HL-74124, R01 HL-131833, and research funding from Pfizer to RMC.

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Key Points

- New therapeutic approaches are needed to control excessive bleeding.
- Engineering coagulation factor Xa has allowed for development of “zymogen-like” factor Xa variants that are biologically active only at the site where coagulation is needed and can overcome the inherent regulatory mechanisms of coagulation.
- Preclinical development of zymogen-like FXa shows that it is efficacious in numerous bleeding scenarios, and in early clinical testing, it appears safe and well tolerated.

Table 1

Biologic Assessment of Zymogen-like FXa Variants[a]

Substrate/Inhibitor	Spec Xa		ATIII		Prothrombin	
	K_m μM	k_{cat} s^{-1}	$k_2 \pm SD \times 10^3$ $M^{-1}s^{-1}$	K_m μM	k_{cat} s^{-1}	$t_{1/2}$ [b] min
Free FXa						
wt-FXa	86 ± 7	75 ± 1.5	1.67 ± 0.07	--	--	0.23 ± 0.01
FXa-V17M	600 ± 60	62 ± 3.2	0.1 ± 0.008	--	--	2.2 ± 0.08
FXa-I16L	1200 ± 270	38 ± 8.0	0.03 ± 0.002	--	--	8.2 ± 0.7
FXa-V17T	> 1mM	<35	0.02 ± 0.001	--	--	16 ± 1.1
FXa-I16M	> 1mM	<30	0.02 ± 0.0008	--	--	23 ± 2.0
FXa-V17S	> 1mM	<15	0.01 ± 0.0007	--	--	61 ± 2.5
FXa-I16T	> 1mM	<1	ND	--	--	89 ± 5.8
Prothrombinase						
wt-FXa	160 ± 20	115 ± 4.0	--	0.40 ± 0.04	45 ± 1.3	--
FXa-V17M	260 ± 50	64 ± 4.9	--	0.36 ± 0.04	38 ± 1.2	--
FXa-I16L	370 ± 30	47 ± 1.9	--	0.39 ± 0.03	42 ± 1.4	--
FXa-V17T	540 ± 60	60 ± 3.8	--	0.38 ± 0.05	33 ± 1.3	--
FXa-I16M	500 ± 80	55 ± 5.1	--	0.38 ± 0.05	42 ± 2.0	--
FXa-V17S	1100 ± 130	41 ± 8.5	--	0.20 ± 0.02	20 ± 0.5	--
FXa-I16T	500 ± 63	5.3 ± 0.38	--	0.12 ± 0.02	5.7 ± 0.2	--

[a] Data taken from [25]. ND, not able to determine a value.

[b] These values represent the activity half-life of the variants in HB mouse plasma. Half-life values taken from [25] were adjusted to correct for mouse plasma dilution (5X).