Development of selective FXIa inhibitors based on cyclic peptides and their application for safe anticoagulation

Short title: Peptide FXI inhibitors for safe anticoagulation

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ABSTRACT

Coagulation factor XI (FXI) has emerged as a promising target for the development of safer anticoagulation drugs that limit the risk of severe and life-threatening bleeding. Herein, we report the first cyclic peptide-based FXI inhibitor that selectively and potently inhibits activated FXI (FXIa) in human and animal blood. The cyclic peptide inhibitor (K_i = 2.8 ± 0.5 nM) achieved anticoagulation effects that are comparable to that of the gold-standard heparin applied at a therapeutic dose (0.3–0.7 IU/ml in plasma) but with a substantially broader estimated therapeutic range. We extended the plasma half-life of the peptide via PEGylation and demonstrated effective FXIa inhibition over extended periods *in vivo*. We validated the anticoagulant effects of the PEGylated inhibitor in an *ex vivo* hemodialysis model with human blood. Our work shows that FXI can be selectively targeted with peptides and provides a promising candidate for the development of a safe anticoagulation therapy.

INTRODUCTION

Thrombotic diseases, being one of the primary causes of morbidity and mortality worldwide[1], can be treated with anticoagulant drugs, but existing therapies can lead to severe bleeding, which is life-threatening. To limit the risk of bleeding, anticoagulants are applied cautiously at a narrow therapeutic dose range, which does not prevent coagulation in all patients[2,3]. An ideal anticoagulant would target a protein only involved in thrombosis and not normal hemostasis. A novel strategy for the development of safer anticoagulants with limited risk of severe bleeding involves targeting coagulation factor XI (FXI)[4,5]. Epidemiological and clinical data show that elevated levels of FXI are associated with a greater risk of venous thromboembolism (VTE) and ischemic stroke, while reduced plasma levels of FXI offer some protection from these complications[6]. Studies in mice, rabbits, and baboons targeting FXI have demonstrated its importance in thrombosis with limited contribution to hemostasis, indicating that targeting FXI could prevent pathological clot formation without affecting normal coagulation[7–11]. Based on such evidence, prophylactic FXI inhibition has been predicted to be beneficial in VTE, thrombosis in end-stage renal disease, and for anticoagulation in patients undergoing extracorporeal membrane oxygenation or hemodialysis[12]. Thus, FXI is a promising target for the development of safer anticoagulants.

FXI-targeting molecules currently in clinical development[13] include small molecule inhibitors[14,15], antisense oligonucleotides[16], and monoclonal antibodies[17–19]. Further FXI-targeting molecules based on direct small molecule inhibitors[13,20,21], allosteric small molecule inhibitors[22,23], DNA or RNA aptamers[24,25] and proteins are developed pre-clinically[26–28]. Thus far, no peptide-based FXI inhibitor drug development program has

been reported, despite the attractive properties of peptides as a drug format such as the good binding qualities, fast development by in vitro evolution techniques, predictable and tunable pharmacokinetic properties, and low development risk due to non-toxic metabolic products. In our experience, the absence of existent FXI peptide-based inhibitor programs may be due to the challenging nature of the target. For example, we previously generated inhibitors with single-digit nM K_is to FXIa. However, they bound human FXIa and not animal homologs, which hindered their evaluation *in vivo* for further development[29]. Additionally, prior attempts to isolate peptide FXIa inhibitors from one-bead-one-compound or phage display peptide libraries yielded binders to the apple domains but not the catalytic domain, which neither inhibited the enzyme's activity, nor prolonged the coagulation time (e.g. aPTT)[30,31].

In this work, we developed cyclic peptide inhibitors of FXIa for anticoagulation applications. To do this, we screened a new phage display library comprising over 500 billion ($> 5\times10^{11}$) unique cyclic peptides and extensive structural diversity not found in other peptide libraries of any format. From this library, we identified peptides that inhibited human FXIa with high affinity and selectivity that were stable and active in blood. We improved the plasma half-life of the best peptide through PEGylation. The engineered peptide displayed anticoagulation activity *in vivo* in rabbits and in a human *ex vivo* model of hemodialysis, thus demonstrating the applicability of peptides for safe anticoagulation.

RESULTS

Screening a 500-billion cyclic peptide library yields FXIa binders

We generated a library that is larger and more structurally diverse than any previously reported peptide phage display library. This large phage display library encoding 30 billion linear peptides was recently obtained with a new cloning procedure based on whole-vector PCR[32] using a phagemid system that allowed efficient production and bacterial transformation of circular DNA[31]. The library comprises more than 500 billion unique elements and contains both cyclic and bicyclic architectures (Fig. 1a). The library was prepared for screening by cyclizing 30 billion random peptides of the form $XC(X)_mC(X)_nC(X)_oCX$ (where C = cysteine, X = any random amino acid, m + n + o = 12) through reacting pairs of two cysteines with eight distinct bis-electrophile chemical linkers (Fig. 1b)[33]. The four cysteine residues of the linear peptides could be connected by the linkers in three different ways, allowing for three bicyclic peptide structures per linear peptide. While the cyclization reactions yield bicyclic peptides, the library is also suited to screen for monocyclic peptides; for example, the first format shown in Fig. 1a displays two monocyclic peptide rings that can independently engage with the target protein. The combinatorial variation of the formula $XC(X)_mC(X)_nC(X)_oCX$ yielded 273 types of bicyclic peptide backbones (91 different cysteine-spacing patterns that are cyclized in three different ways), as presented comprehensively in Supplementary Fig. 1, and 12 different monocyclic backbones (two cysteines connected by linkers, the cysteines being spaced by 1 – 12 random amino acids).

To find FXI binders, we performed three rounds of phage selection in which we iteratively produced phage, cyclized peptides on the tip of phage with the eight bis-electrophile

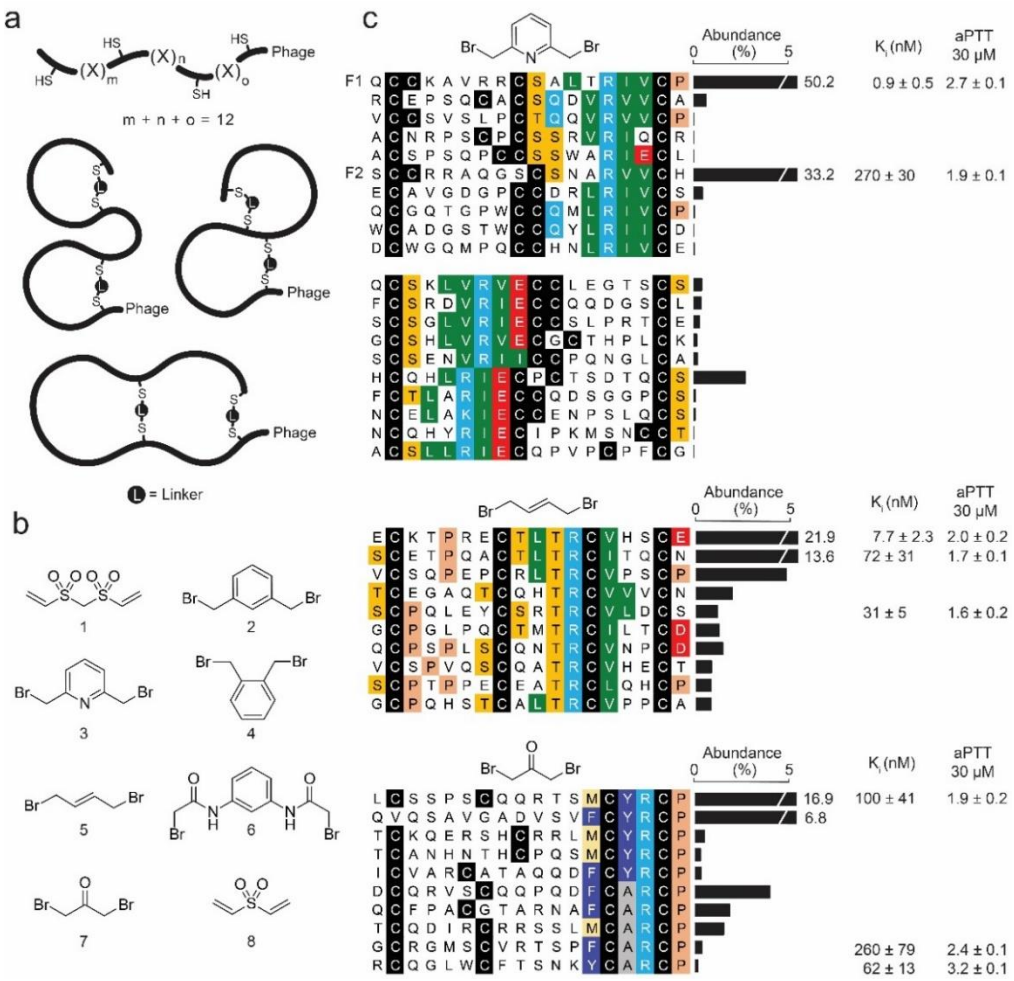

Figure 1. Phage display selection of cyclic peptides against FXIa. (**a**) Library format, and subsequent peptide structures in the library. (**b**) Bis-electrophiles for peptide cyclization. (**c**) Peptide sequences enriched after three rounds of phage selection against FXIa. The ten most abundant peptides for each consensus group selected with linkers **3**, **5**, and **7** are shown. Sequence homology for each consensus group is indicated by color-coding. The K_i and prolongation of aPTT at 30 μM (relative to plasma without inhibitor) are indicated for the most active isomer of each peptide. A full list of peptide sequences is provided in Supplementary Fig. 3. Values are means and SD of at least three measurements.

chemical linkers, panned the sub-libraries for each chemical linker against immobilized human FXIa (5 μg in rounds 1 and 2, 0.5 μg in round 3), and propagated phage by bacterial infection. In order to isolate inhibitory peptides binding to the catalytic domain of FXIa, we eluted phage using the covalent active site FXIa inhibitor PPACK (D-phenylalanyl-prolyl-arginyl chloromethyl ketone), which would compete off only those phage binding to the inhibitor binding site. In the third round of selection, the number of captured phage increased more than 100-fold compared to the first round in the selections with the cyclization reagents **3**, **4**, **5**, **7**, and **8**, suggesting that target selective binders were enriched (Supplementary Fig. 2). High-throughput sequencing and comparison of the sequences with a recently developed peptide sequence alignment tool[34] identified strong consensus sequences between the peptides identified from the selections with the chemical linkers **3**, **5**, and **7** (Fig. 1c and Supplementary Fig. 3). For each linker, the consensus sequences were localized to one region of the three randomized peptide segments [$C(X)_mC$, $C(X)_nC$, or $C(X)_oC$]. An identical consensus sequence was found in the first and third peptide segment when screening the library with linker **3**, which suggests that these regions bind as monocyclic peptides (Fig. 1c).

Cyclic peptide inhibits FXIa with high affinity and selectivity

To assess the inhibition of these identified peptides, we synthesized representative linear peptides of each consensus sequence, cyclized them with linkers **3**, **5**, or **7** and purified the three regioisomer products that are formed by HPLC. We assessed the inhibitory potency of the peptides through assays with a chromogenic FXIa substrate. Additionally, we assessed the prolongation of activated partial thromboplastin time (aPTT) in human

plasma, a parameter that indicates inhibition of the intrinsic coagulation pathway (Fig. 1c). The most active peptide in terms of both inhibition (K_i = 0.9 ± 0.5 nM) and aPTT prolongation (2.7-fold prolongation of aPTT at 30 μM) was isomer 1 of F1 (Fig. 2a and Supplementary Fig. 4). A peptide with the same consensus sequence, F2, showed a 200-fold weaker K_i and prolonged aPTT to a smaller extent (1.9-fold at 30 μM; Fig. 1c and Supplementary Fig. 5).

We next assessed whether isomer 1 of peptide F1, termed F1 in the following, fulfills the key parameters for potential therapeutic application as a safe anticoagulant. These requirements include inhibition of the intrinsic coagulation pathway at single-digit micromolar concentrations; selectivity, stability, and activity in blood; and inhibition of FXI animal homologs. F1 prolonged the aPTT in human plasma in a dose-dependent manner and at low micromolar concentrations (EC_{2x} = 2.9 ± 1 μM; Fig. 2b), indicating efficient inhibition of the intrinsic coagulation pathway. At the same time, the peptide did not affect the prothrombin time (PT), even at a high concentration of 150 μM (Fig. 2c), showing that serine proteases of the extrinsic and common pathway were not inhibited and thus suggesting a high target selectivity. Indeed, a specificity profiling of F1 with a panel of seven homologous trypsin-like serine proteases revealed a high selectivity of the peptide (> 1000-fold), with trypsin being the only protease inhibited at low micromolar concentrations (Fig. 2d).

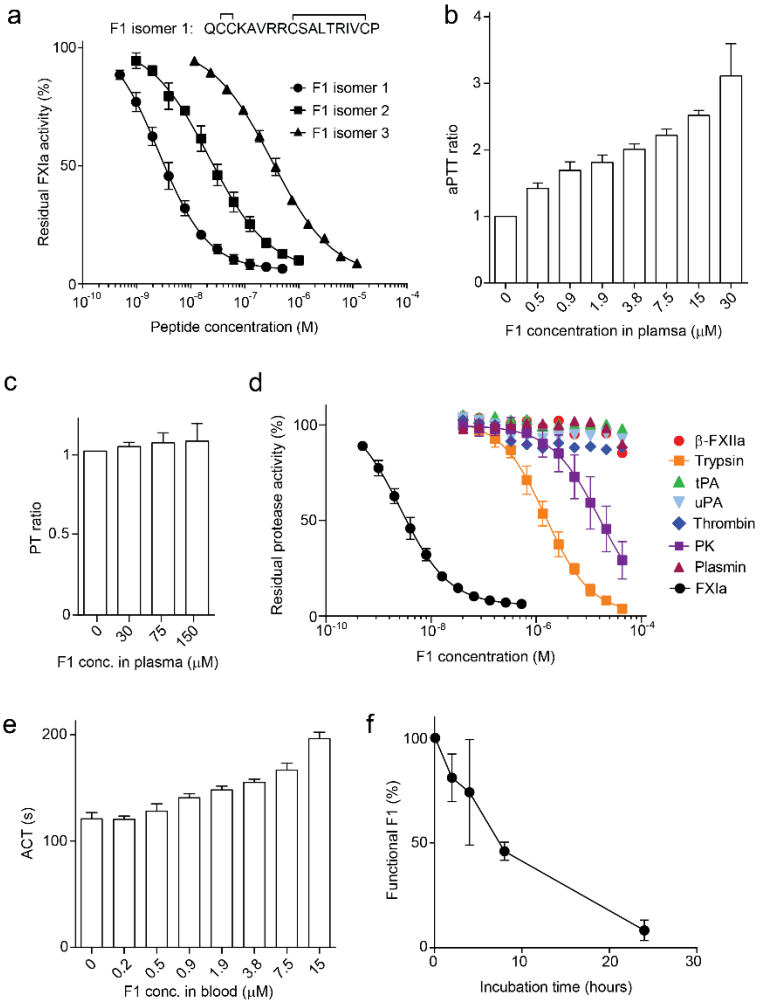

Figure 2. Activity, selectivity and stability of double-bridged peptide F1. (**a**) Inhibition of human FXIa by three regioisomers of F1. Means and SD of three independent measurements. (**b**) Prolongation of aPTT by F1 in human plasma. Data show mean and SD of at least three independent measurements. (**c**) Prolongation of PT by F1 in human plasma. Data show mean and SD of three independent measurements. (**d**) Inhibition of homologous serine proteases. Data show mean and SD of three independent measurements for trypsin, PK, and FXIa. (**e**) Prolongation of ACT by F1 in human blood. Data show mean and SD of at least three independent measurements. (**f**) Stability of F1 in human plasma at 37°C. The amount of F1 remaining functional was quantified in an

FXIa inhibition assay. Data show mean and SD of three independent measurements.

Assessment of the activated clotting time (ACT), a parameter used to characterize intrinsic pathway-driven coagulation in whole blood, showed that F1 was also active in this more complex and clinically relevant environment ($EC_{1.5x}$ in blood = 12 ± 3 μM; Fig. 2e). The peptide stability was tested by incubation in human plasma at 37°C followed by quantification of residual FXIa inhibitory activity over time. F1 remained functional for over 8 hours ($t_{1/2}$ = 7.6 ± 2.9 hours; Fig. 2f). Finally, we tested whether F1 inhibits animal homologs of FXIa. While F1 did not inhibit mouse FXIa, it prolonged aPTT in rabbit plasma (EC_{2x} = 2 μM), which provides a suitable model for *in vivo* studies.

Peptide engineering affords inhibitor with long plasma half-life

We next aimed to i) reduce the synthetic complexity of the peptide, ii) improve its stability, and iii) prevent rapid elimination *in vivo* through renal filtration, which is typical for peptides[35]. First, to reduce the overall synthetic complexity, we tested whether the Cys2-Cys3 chemical bridge in F1 was essential for its activity, or if a monocyclic peptide with the same sequence would similarly inhibit FXIa. Peptide F3, which contains the modifications Cys2→Ser and Cys3→Ser, displayed a K_i and activity in plasma and whole blood that was comparable to F1 (Fig. 3a and Supplementary Fig. 6). This provided valuable insight, as synthesis of monocyclic peptides is simpler than multicyclic structures. For the facile conjugation of PEG polymers to the N-terminal amine of the peptide – required for prolonging the peptide's *in vivo* half-life – we mutated Lys4 to arginine to retain a similar group while removing nucleophilicity (F4; Fig. 3a and Supplementary Fig. 6). We

also found that deleting the first three amino acids did not negatively impact activity and yielded the minimized peptide F5 (Fig. 3a and Supplementary Fig. 7).

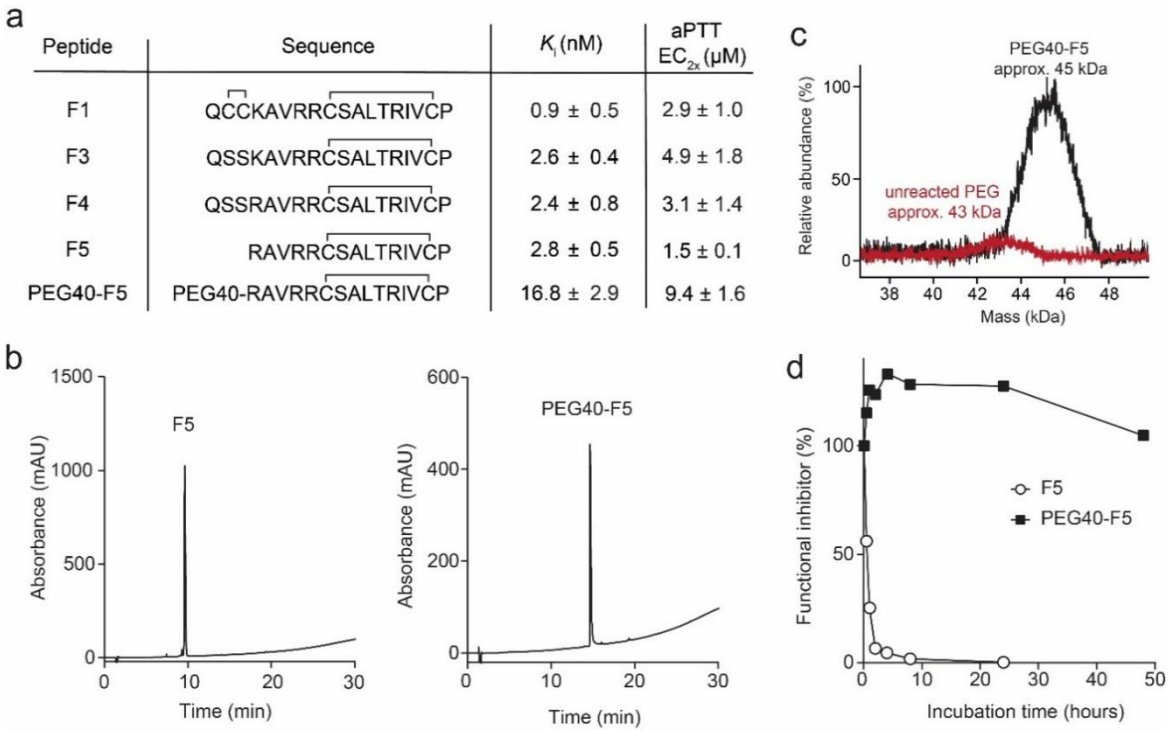

Figure 3. PEGylated cyclic peptide FXIa inhibitor. (**a**) Structure and activity of cyclic peptide variants based on F1. Mean and SD of at least three measurements are shown. (**b**) Analytical HPLC traces of cyclic peptide F5 and PEG40-F5. (**c**) MALDI-TOF analysis of PEG40-F5 (1 nmol) compared to unreacted PEG (1 nmol). (**d**) Stability of F5 in human plasma at 37°C relative to PEG40-F5. Residual fraction of functional inhibitor was assessed by FXIa inhibition assays.

To next address the stability and half-life in plasma, we conjugated polyethylene glycol (PEGylation) to F5, as this method has been shown to prolong plasma half-life from hours to days by preventing renal filtration, depending on the size of the PEG[36,37]. In addition,

PEGylation has been shown to improve the stability of proteins and peptides[38,39]. We conjugated a linear 40 kDa PEG polymer (PEG40-*N*-hydroxysuccinimide ester) to the N-terminal amino group of F5 to yield PEG40-F5 (Fig. 3b and 3c). The PEGylated peptide could be obtained in high purity by sequential purification by cation exchange that removed non-conjugated PEG but retained the positively charged F5 and PEG40-F5, and RP-HPLC that separated PEGylated from non-PEGylated F5. MALDI-TOF analysis revealed that exactly one 40 kDa PEG was conjugated to one peptide (Fig. 3b). Analytical RP-HPLC analysis showed that the PEG40-F5 eluted as a single, sharp peak and thus had the same high purity as the non-conjugated peptide F5 (Fig. 3c). We found that PEG40-F5 inhibited FXIa with a K_i of 17 ± 3 nM and doubled the aPTT with an EC_{2x} of 9.4 ± 1.6 μM. This corresponded to a ~6-fold lower potency in PEG40-F5 relative to the F5 peptide alone (Fig. 3a and Supplementary Fig. 7). While PEGylation reduced inhibitory activity, it substantially improved the stability of F5, as the PEG40-F5 conjugate retained 100% of its initial activity after 48 hours in human plasma (Fig. 3d). A specificity profiling with homologous trypsin-like serine proteases showed that the PEGylated inhibitor had a high selectivity too, though slightly lower (30-fold over trypsin, > 500 fold over all other six proteases tested; Supplementary Fig. 7c).

Cyclic peptide has a much broader estimated therapeutic range than heparin

We next compared the activity of F5 and PEG40-F5 with unfractionated heparin (UFH), the gold standard in many anticoagulation therapies, to estimate the therapeutic range for the cyclic peptide FXIa inhibitor. The main challenge with heparin is its narrow therapeutic range of 0.3–0.7 IU/ml plasma (factor = 2.3)[40,41]. Higher doses increase the risk of

bleeding and lower doses do not suppress pathologic coagulation. With this extremely narrow range, it is hard to slowly add more heparin to keep the patient in an anticoagulated state over the course of a treatment, as even small additions could put patients over the safe upper range. It is likely that many patients treated with heparin are not optimally anticoagulated, at least not over the entire time period targeted[2,42–45].

We first assessed the concentration of F5 required to achieve the same prolongation of aPTT as with 0.3–0.7 IU/ml UFH (Fig. 4a; dotted lines). At 0.3 IU/ml UFH, there was a 1.6-fold prolonged aPTT, which could be achieved with 0.34 ± 0.04 μM of F5; at 0.7 IU/ml UFH, there was a 5.1-fold prolonged aPTT, which could be achieved with 45 ± 3 μM of F5 (Fig. 4b; dotted lines). The F5 concentration range needed to achieve the same aPTT prolongation as with UFH at a therapeutic dose was thus between 0.3 and 60 μM, which is an over 100-fold broader range than that of UFH, giving likely much more leeway and safety margin in the possible treatment doses. The minimal concentration of PEG40-F5 required to reach a 1.6-fold prolongation of the aPTT, and thus estimated to be required for therapeutic anticoagulation, was 1.3 ± 1.1 μM due to its slightly lower activity than unconjugated F5 (Fig. 4b). We further estimated the minimal concentration required for therapeutic anticoagulation by testing the inhibitor in whole blood *ex vivo*. We found that the ACT was prolonged by 10% at the lower end of the therapeutic range of UFH (0.15 IU/ml blood; Fig. 4c). F5 and PEG40-F5 concentrations in blood of 0.77 ± 0.29 and 3.14 ± 0.93 μM, respectively, prolonged the ACT to the same extent (Fig. 4d).

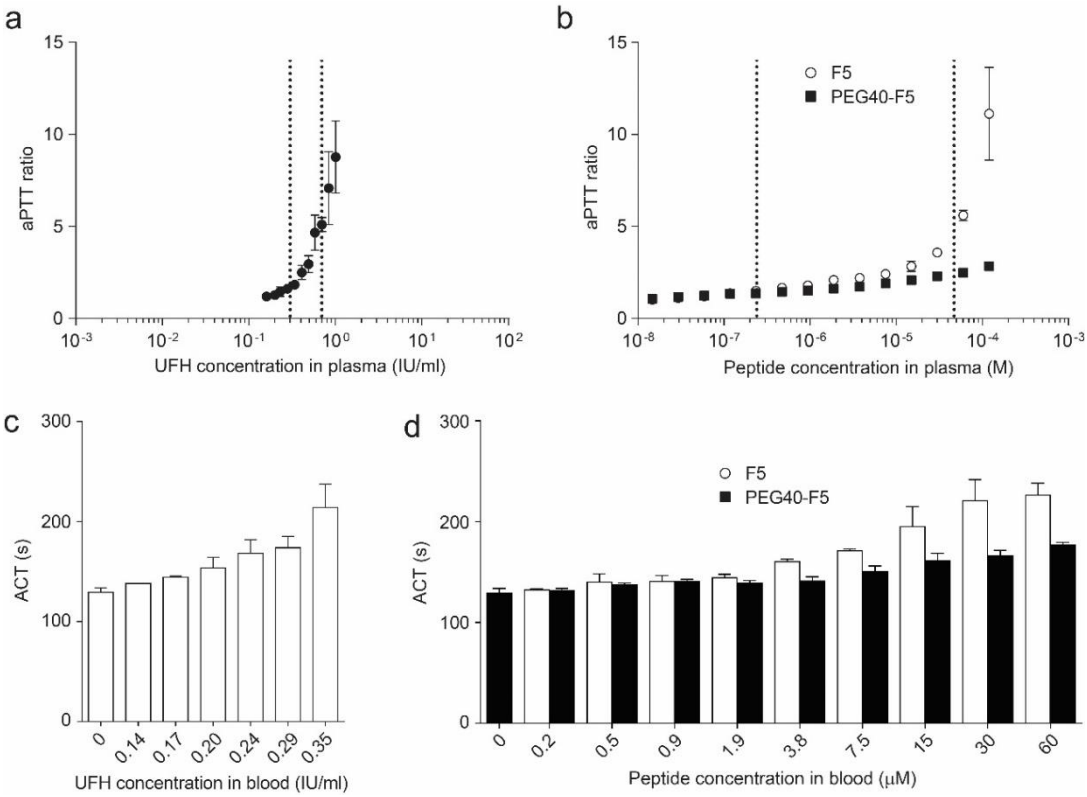

Figure 4. Comparison of cyclic peptide inhibitor with heparin and estimation of therapeutic range. (**a**) Prolongation of aPTT by different unfractionated heparin (UFH) concentrations in human plasma. The therapeutic range of 0.3 to 0.7 IU/ml of UFH in plasma is indicated by dotted lines. (**b**) Prolongation of aPTT at increasing cyclic peptide concentrations. The F5 concentrations at which the same aPTT prolongations are achieved within 0.3 to 0.7 IU/ml of UFH are indicated by dotted lines. **(c)** ACT at different concentrations of UFH in human blood. (**d**) ACT at different concentrations of F5 and PEG40-F5 in human blood. Data points in all figure panels are mean values of at least three measurements, and the SDs are indicated.

Inhibiting the intrinsic coagulation pathway in rabbits for hours

We next tested the function of PEG40-F5 in rabbit plasma, and found that it prolonged the aPTT in a dose dependent manner and with an EC_{2x} of 5.4 ± 3.3 μM, which is similar to in human plasma (Fig. 5a). We assessed the pharmacokinetic properties of the inhibitor and its activity after circulation *in vivo* by administering PEG40-F5 to New Zealand White rabbits (n = 3) at a dose of 60 mg/kg. We conducted a time-course study and found that the inhibitor prolonged aPTT in rabbit plasma 1.7 to 2.5-fold over the first eight hours and retained activity up to 24 hours (1.2-fold) (Fig. 5b). The anticoagulation activities measured in the first 24 hours correspond to plasma concentrations of 2.5 to 10.6 μM. Based on the activity reduction over time, a half-life of around one day was estimated for PEG40-F5 in rabbits.

Cyclic peptide reduces coagulation in a hemodialysis model

The efficient anticoagulation activity observed in the whole blood assay suggested that PEG40-F5 may be used for safe anticoagulation in hemodialysis, an essential medical procedure for purifying the blood of patients with impaired kidney function[46]. A challenge in hemodialysis is the suppression of coagulation induced by the blood contacting the tubing and the filtration membranes. Heparin is used as efficient anticoagulant in these procedures, but it can cause bleeding complications[47,48]. We tested PEG40-F5 in an established *ex vivo* hemodialysis model[49] in which citrated human whole blood from healthy donors is recirculated in a dialysis circuit until total clotting occurs (Fig. 5c). Citrate was efficiently removed after one passage of blood through the dialyzer and thus in less

than around two minutes, as verified by measuring free ionized calcium levels in four test runs (1.04–1.29 mM).

In a preliminary experiment, we tested if PEG40-F5 remained in the circulation as expected based on the large size and hydrodynamic radius of PEG40, or if it was cleared through the dialyzer membrane. We applied the peptide together with a high dose of UFH (1.75 IU/ml blood) that prevented coagulation, took samples and determined the plasma concentration of PEG40-F5 by measuring FXIa inhibition. Control experiments showed that UFH did not interfere with the FXIa inhibition assay (Supplementary Fig. 8). PEG40-F5 was detected at similar plasma concentrations (15.3 μM to 22.2 μM) over one hour of hemodialysis, showing that the inhibitor was efficiently retained in the system and therefore indicating that this system would work well for measuring the activity of PEG40-F5 (Fig. 5d).

We then tested the anticoagulation effect of PEG40-F5 compared to heparin in parallel hemodialysis experiments. We obtained blood from single donors, split the blood into two portions to have an anticoagulated sample (either heparin or PEG40-F5) and a negative control (saline), and conducted parallel hemodialysis experiments on each sample. The heparin-treated bag of blood was injected with UFH to reach a concentration in blood of 0.35 IU/ml, and thus a concentration at the upper limit of the therapeutic range, whereas a second bag of blood was treated with saline as a negative control. In the absence of heparin, blood clotted after 2.7 ± 1.5 minutes (n = 3). UFH delayed coagulation by nearly 6.8-fold to 18.3 ± 9.2 min (n = 3; Fig. 5e). The clotting of blood after a relatively short time

was expected, as the volume of blood in the *ex vivo* hemodialysis model (230 ml) is much smaller than that of a human, leading to greater circulation of the entire blood sample during the experiment (10 times/1.5 minutes). We then evaluated the efficacy of PEG40-F5 anticoagulation under similar conditions; we added the inhibitor as a bolus to reach a concentration in blood of 15 μM. We performed this experiment six times with blood from single donors (n = 6), and as with the heparin control, treated half of the blood from the same donor with saline as a negative control (n = 6). Blood without inhibitor clotted after 4.7 ± 2.7 minutes, whereas blood treated with PEG40-F5 clotted after 13.7 ± 4.5 min ($p < 0.001$). The 2.9-fold delay in clotting clearly indicates the anticoagulation effect of the FXIa inhibitor (Fig. 5f). The delay in coagulation afforded by PEG40-F5 approaches that of heparin and suggests that the new peptidic inhibitor has potential as a new anticoagulation agent.

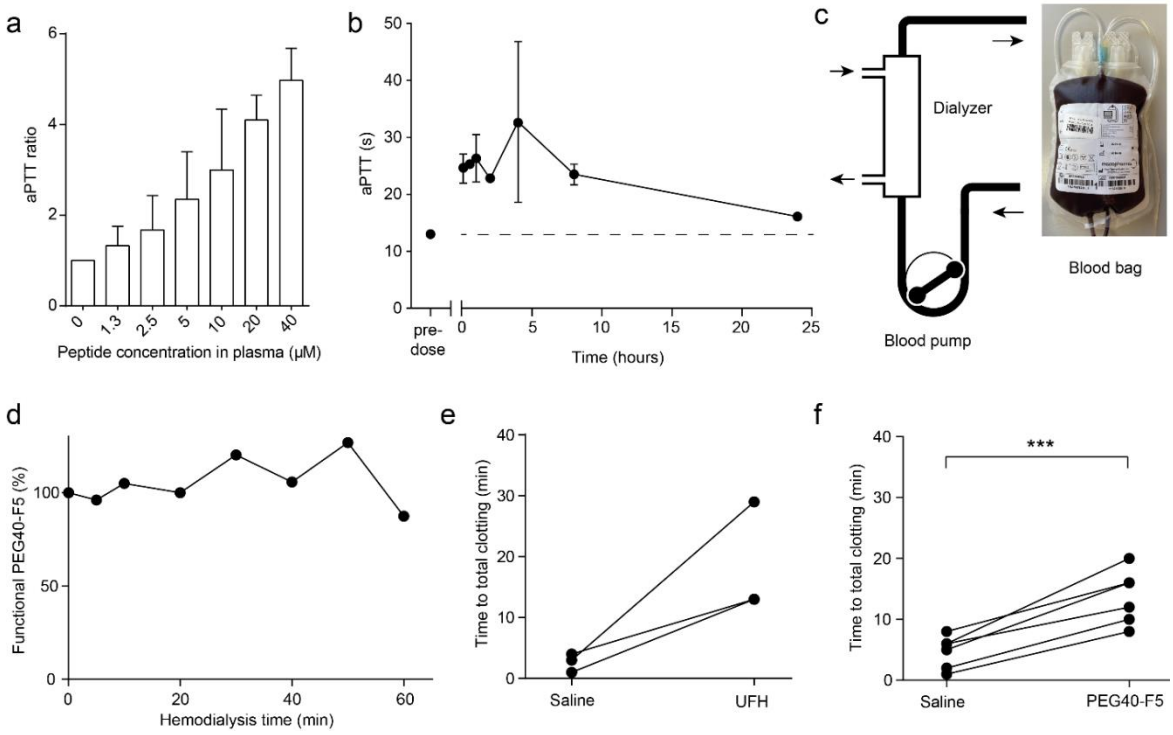

Figure 5. Inhibition of FXIa in rabbits and in a human ex vivo hemodialysis model. (**a**) Prolongation of aPTT by PEG40-F5 in rabbit plasma. Mean values and SD of at least three measurements are shown. (**b**) Prolongation of aPTT in plasma samples taken from New Zealand White rabbits (n = 3) injected intravenously (i.v.) with 60 mg/kg of PEG40-F5 at time = 0. The first blood sample was taken after 5 min. The aPTT of each sample was measured twice. (**c**) *Ex vivo* hemodialysis setup. (**d**) Residual amount of functional PEG40-F5 in hemodialysis circuit at different time points. Blood coagulation was suppressed by co-application of a high dose of UFH. The inhibitor plasma concentration in blood samples was determined in a FXIa inhibition assay. **(e)** Prolongation of the time to total clotting within the hemodialysis circuit by UFH (n = 3) and **(f)** PEG40-F5 (n = 6) relative to saline controls. For each hemodialysis experiment, blood units from a single donor were divided into two bags to which either inhibitor or saline was added. Statistical analyses were performed using the paired t-test to compare the results within the different series (PEG40-F5 vs. saline and UFH vs. saline). ***$p < 0.001$.

DISCUSSION

Existent anticoagulant drug therapies to prevent or treat thrombotic diseases can cause life-threatening bleeding. Targeting FXI is a novel strategy for the development of safer anticoagulants. In this work, we identified a cyclic peptide that inhibits FXIa as a potential anticoagulant using a novel combinatorial cyclic peptide library that is larger and more structurally diverse than any previously reported phage-encoded peptide repertoire. The newly discovered peptide, F5, binds to FXIa with single-digit nanomolar affinity and high selectivity over homologous proteases. Unlike a previously reported peptide-based FXIa inhibitor, the newly generated peptide F5 inhibits rabbit FXIa, which allows for *in vivo* evaluation of its efficacy in animals. Further, the peptide F5 is active in plasma and whole blood, which is a requisite for therapeutic application as a potential anticoagulant. We refined our peptide through structure-activity relationship analysis and demonstrated that a monocyclic peptide is sufficient for binding and inhibition. Synthetic simplification from a double-bridged peptide to a monocyclic structure allows for easier manufacturing of a potential therapeutic. Our work showed that peptide-based inhibitors with a high affinity and selectivity can be developed to FXIa.

A common issue with peptide therapeutics is an incredibly short half-life, often less than minutes, in circulation. We discovered that the modulation of F5 through PEGylation extended this plasma half-life of the cyclic peptide, which creates synthetic flexibility in tuning pharmacokinetic properties. Conjugation to PEG40 yielded a peptide with a half-life of several hours in rabbits, which correlates based on allometric scaling to potentially several days in humans. Depending on the specific therapeutic application, smaller PEG chains may be used to achieve shorter half-lives. For example, for the suppression of

contact activation in extracorporeal circulation or in hemodialysis, procedures that usually do not take longer than a few hours, a smaller PEG chain could yield a shorter half-life—and potentially a higher inhibitory activity. Conversely, for preventing thrombosis for several days after surgery, a longer exposure is desired, and an inhibitor with a longer PEG chain may be used. Overall, with this extended half-life, we were pleased to see that the PEGylated peptide could also still remain functional 24 hours after i.v. administration to rabbits, showing that the peptide is feasible for use *in vivo*.

With the potent and specific FXIa inhibitor in hand, we queried whether the peptide could inhibit anticoagulation therapeutically and if its inhibitory potency was comparable to a gold standard anticoagulant such as heparin in the potential application of suppressing contact activation in hemodialysis. Specifically, we asked the question if contact activation occurring by exposure of human blood to plastic tubes and membranes, used in medical devices for extracorporeal circulation, can be efficiently suppressed by the peptide FXIa inhibitor. In assays measuring ACT, we found that the cyclic peptide can delay the clotting of whole blood to the same extent as heparin (UFH) applied at a therapeutic dose. The absolute concentrations of the peptide in blood required to reach the effects achieved with the minimal therapeutic dose of UFH were 0.77 ± 0.29 μM for the peptide F5 and 3.14 ± 0.93 μM for the PEGylated peptide PEG40-F5. These concentrations are relatively high for therapeutic application, but the potency of the inhibitor may be improved in the future by increasing the binding affinity for FXIa, for example by optimizing the amino acid sequence with natural or unnatural amino acids, a strategy that is routinely applied to improve phage-selected peptides[50–52], and that was not yet applied to F5.

Inhibition of FXIa could potentially prevent coagulation as efficiently as heparin, but is expected to be associated with a much smaller bleeding risk. Furthermore, FXIa inhibition could potentially be of interest to hemodialysis patients who cannot be anticoagulated by heparin due to heparin-induced thrombocytopenia (HIT). The ability of PEG40-F5 to efficiently delay blood clotting in an *ex vivo* model of hemodialysis to a similar extent as heparin applied at the maximal recommended dose of 0.35 IU UFH per ml blood means it is likely to be an efficient anticoagulant. Therefore, the properties of this molecule make it a good candidate for indications of thrombosis prevention such as hemodialysis and post-operative anticoagulation, where there is a clear unmet need for safer anticoagulation strategies.

EXPERIMENTAL SECTION

Phage selections

A phage display library comprising peptides of the format $XC(X)_mC(X)_nC(X)_oCX$ (C = cysteine, X = any amino acid, m + n + o = 12) was used[31]. Phage selections were performed as previously reported[33], with a few modifications. Library glycerol stocks were diluted to OD_{600} = 0.1 in 1 L of 2YT/ampicillin (100 μg/ml) with 100 mM glucose and grown at 37°C until OD_{600} = 0.5. The culture was subsequently infected with hyperphage M13 K07ΔpIII (Progen Biotechnik GmbH) at a multiplicity of infection of 10 for 20 min at 37°C without shaking. The culture was then incubated for 45 min at 37°C with 200 rpm shaking. Cells were then pelleted and resuspended in 1 L of 2YT/ampicillin (100 μg/ml) + kanamycin (50 μg/ml). The culture was grown overnight at 30°C and then pelleted. Phage found in the supernatant were precipitated and the cysteine residues of the peptides were reduced and cyclized with reagents **1** – **8** as described previously[29].

Human FXIa (Molecular Innovations) was biotinylated as previously described. 5 μg of biotinylated target protein was immobilized on 50 μl of magnetic streptavidin beads (ThermoFisher Scientific) and biopanning was performed. Active site binders were selectively eluted by incubating beads with 100 μl of 1 mM PPACK (D-phenylalanyl-prolyl-arginyl chloromethyl ketone) for 30 min on the rotating wheel. Eluted phage were added to 10 ml of TG1 *E. coli* cells (OD_{600} = 0.5). After 30 min incubation at 37°C, the bacteria were plated on large 2YT/ampicillin (100 μg/ml) plates. Bacterial cells were recovered the next day and stored as glycerol stocks at -80°C. In the second round of selection

neutravidin-coated magnetic beads were used. In the third round of selection, the amount of biotinylated FXIa immobilized on beads was reduced to 0.5 μg.

Next Generation Sequencing (NGS)

The DNA of phage selected in the third round was isolated with a commercial plasmid purification kit (Macherey-Nagel). The DNA sequences encoding the peptides were amplified in a first PCR using an equimolar mixture of five forward primers and five reverse primers:

NGS forward hyper 1–5

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG$(N)_x$CTGCTGGCAGCTCAGC-3'

x = 0–4

NGS reverse hyper 1–5

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG$(N)_x$CAGTTTCAGCGCCAGAACC-3'

x = 0–4

For the reaction, primers (40 nM final conc. for each one of the five forward and reverse primers), dNTP mix (250 μM each, final conc.), 100 ng of phagemid DNA as template, 0.9 μl of DMSO, 6 μl of 5× HF buffer, and 0.6 units of Phusion High-Fidelity Polymerase (New England Biolabs) were used in a 30-μl PCR reaction. Twenty-five PCR cycles were performed (initial denaturation at 98°C for 2 min, then 25 cycles of 98°C for 15 sec, 55°C for 30 sec, and 72°C for 15 sec, and final elongation at 72°C for 5 min). Product formation was assessed by agarose (UltraPure agarose, Invitrogen) gel electrophoresis (2.5% agarose gel). A second PCR was performed with primers containing the adapter and index sequences.

The following primers were used:

Condition: linker **1**

NGS 2nd Forward S506

5’-AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC-3’

NGS 2nd Reverse N721

5’-CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCGG-2’

Condition: linker **2**

NGS 2nd Forward S505

5’-AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC-3’

NGS 2nd Reverse N705

5’-CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG-3’

Condition: linker **3**

NGS 2nd Forward S505

NGS 2nd Reverse N706

5’-CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG-3’

Condition: linker **4**

NGS 2nd Forward S505

NGS 2nd Reverse N707

5’-CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG-3’

Condition: linker **5**

NGS 2nd Forward S505

NGS 2nd Reverse N714

5’-CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCGTGGGCTCGG-3’

Condition: linker **6**

NGS 2nd Forward S506

NGS 2nd Reverse N715

5’-CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCGG-3’

Condition: linker **7**

NGS 2nd Forward S506

NGS 2nd Reverse N716

5’-CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGG-3’

Condition: linker **8**

NGS 2nd Forward S506

NGS 2nd Reverse N719

5’-CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCGTGGGCTCGG-3’

A PCR reaction per condition with a total volume of 60 μl was set up, which contained primers (400 nM final conc. for each one of the two primers), dNTP mix (250 μM each, final conc.), 2 μl of product of the first PCR, 1.8 μl of DMSO, 12 μl of 5× HF buffer, and 1.2 units of Phusion High-Fidelity Polymerase. The PCR was performed by applying the same program as described above. PCR products were run on a 2.5% agarose gel (UltraPure agarose) and purified using a commercial gel extraction kit (QIAquick Gel Extraction Kit, Qiagen). The PCR products were subsequently loaded at 1.5 pM on a Mid Output flow cell (Illumina) and sequenced with a NextSeq 500 instrument (Illumina) according to the manufacturer’s instructions, yielding paired-end reads of 75 nucleotides. Overlapping paired end reads were merged using CLC Genomics Workbench v9.5 (Qiagen). The DNA was sequenced along with at least 30% of unrelated non-amplicon-

based libraries to avoid low-diversity problems. Results from sequencing were analyzed with MatLab scripts developed in our group as previously described[34,53].

Peptide synthesis and characterization

Peptides were synthesized by standard solid-phase peptide synthesis using Fmoc-protected amino acids at a 0.05 mmol scale and subsequently cyclized in solution as described previously[33]. In the cyclization reaction, 2 equivalents (monocyclic peptides) or 4 equivalents (double-bridged peptides) of linker reagents were used. The purity of all peptides was > 95% except for F3 that had a purity of around 70%. Peptides were characterized in terms of inhibitory activity, coagulation activity (aPTT, PT, and ACT), plasma stability, and specificity.

Determination of inhibitory activity of selected peptides

For the determination of inhibitory constants (K_i) of peptides, the residual enzymatic activity of coagulation factor XIa in presence of serial dilutions of inhibitor (ranging from 10 μM to 900 pM final concentration) was assessed using the commercially available substrate Pyr-Pro-Arg-pNA (Bachem). Activity curves were determined at 25°C in activity-assay buffer containing 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% (w/v) BSA, and 0.01% (v/v) Triton-X100. Reactions were started by adding the substrate (400 μM final concentration) to 0.5 nM FXIa (full size, Molecular Innovations) in the presence or absence of peptides. The release of pNA (p-nitroaniline) following hydrolysis of the substrate was monitored over at least 20 min by measuring the increase in absorbance per minute at 405 nm using an Infinite M200 Pro plate reader (Tecan). The

rate of substrate cleavage is proportional to enzyme activity. The IC_{50} values were determined by fitting sigmoidal curves to the data using the following four-parameter logistic equation:

$$y = \frac{100}{1 + 10^{(logIC_{50} - x)p}},$$

wherein y is the residual activity (%) of protease, x is the logarithm of peptide concentration, IC_{50} is the concentration of inhibitor that produces 50% inhibition, and p is the Hill coefficient. The K_i values were calculated based on the IC_{50} using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]_0}{K_m}}$$

wherein $[S]_0$ is the initial concentration of substrate, and K_m is the Michaelis-Menten constant for the enzyme and substrate. The K_m of FXIa for Pyr-Pro-Arg-pNA was determined to be 260 ± 10 μM.

Inhibitor specificity profiling

For specificity profiling, the residual activities of several serine proteases homologous to FXIa were determined similarly as described above. The final concentrations of the proteases were: 2 nM β-FXIIa, 0.1 nM trypsin, 7.5 nM tPA, 1.5 nM uPA, 2 nM thrombin, 0.25 nM plasma kallikrein, 2.5 nM plasmin (all from Molecular Innovations, PK from

Innovative research). Fluorogenic substrates (Bachem) were used at 50 μM: Z-Phe-Arg-AMC for PK, Z-Gly-Gly- Arg-AMC for thrombin, trypsin, tPA, and uPA, H-D-Val-Leu-Lys-AMC for plasmin. Fluorescence (AMC ex. 368 nm, em. 467 nm) was measured at 25 °C over time using a fluorescence microtiter plate reader (Infinite M200Pro, Tecan).

Plasma stability assays

Plasma stability assays were performed as described previously[50]. Briefly, 18 μl of 2 mM peptide in ddH_2O were added to 892 μl of single donor human citrated plasma (Innovative Research), obtaining a final plasma peptide concentration of 40 μM. Samples were incubated at 37°C in a water bath, and 30 μl samples were taken at different time points (0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h), diluted to 400 μl with activity assay buffer without BSA (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.01% [v/v] Triton-X100), and incubated for 20 min at 65°C in order to inactivate plasma proteases. The peptide activity in the samples was determined as follows. Samples were centrifuged for 5 min at 16,000 g, and the supernatant was transferred to a fresh tube. Samples were serially diluted, obtaining final peptide concentrations ranging from 1 μM to 900 pM, and the residual activity of FXIa was measured as described above. Residual inhibitory activity over time (as a measure of stability) was calculated as a fraction of the activity at time 0.

aPTT and PT coagulation activity measurements

aPTT and PT coagulation assays were performed to characterize the anticoagulation activity of the peptide and were determined in human or rabbit plasma using a STAGO

STart4 coagulation analyzer (Diagnostica Stago). For PT, 50 μl of citrated human single donor plasma (with or without peptide dilutions) were incubated in the dedicated chamber of the instrument for 2 min at 37°C. Then, 100 μl of Innovin (recombinant human tissue factor, synthetic phospholipids, and calcium in stabilized HEPES buffer system; Dade Behring/Siemens) were added using the instrument's pipette. The movement of a steel ball in plasma (induced electromagnetically) was monitored. The coagulation time was defined as the time after addition of Innovin until the ball stopped moving. For aPTT with citrated human single donor plasma (Innovative Research), 100 μl of plasma (with or without peptide dilutions) were incubated with 100 μl of Pathromtin SL (silicon dioxide particles, plant phospholipids in HEPES buffer system, Siemens) for 2 min at 37°C, or with Dade Actin activated cephaloplastin (cephalin in ellagic acid, buffered and preserved, Siemens) for 3 min at 37°C for experiments determining the therapeutic range. Coagulation was triggered by adding 100 μl of $CaCl_2$ solution (25 mM, Siemens) with the dedicated pipette, and the movement of the steel ball was monitored as described above. For aPTT with citrated rabbit plasma (Innovative Research), the same procedure was followed, using Dade Actin activated cephaloplastin (cephalin in ellagic acid, buffered and preserved) as activator and with an incubation time of 3 min.

Activated clotting time (ACT)

ACT tests were performed to characterize the anticoagulation activity of peptides in whole blood. Briefly, 7 μl of peptide (final concentrations in blood ranging from 0.2 to 60 μM), or unfractionated heparin (final concentrations in blood ranging from 0.14 to 0.35 IU/ml), or PBS (negative control) were added to 213 μl of citrated human single donor whole blood

freshly drawn a maximum of 7 days before the experiment (Cambridge Bioscience). Samples of 200 μl were added to Recalcified Activated Clotting Time (RACT) cartridges (Medtronic) containing 100 μl of 2.2% (w/v) kaolin, 50 μM $CaCl_2$ in HEPES buffer, and sodium azide, and the ACT clotting time was recorded using an automated ACT Plus machine (Medtronic).

PEGylation of cyclic peptides and purification

We conjugated a linear 40 kDa PEG polymer functionalized with N-hydroxysuccinimide ester (PEG40-NHS) to F5 by reacting the peptide with a 2-fold molar excess of PEG40-NHS. Purification of the PEG40-F5 conjugate was facilitated by the high net positive charge of F5 (+5) that allowed for separation of PEG40-F5 (+4) from unreacted PEG by cation exchange chromatography. A subsequent desalting step removed salts used for elution in the cation exchange and allowed for separation of PEG40-F5 and unconjugated peptide. The 40 kDa linear PEG-NHS (SUNBRIGHT ME-400HS, NOF Europe) was conjugated to the peptide via the N-terminal amine group. The reaction was performed in 15 mM HEPES buffer, pH 7, using a peptide concentration of 800 μM and a PEG-NHS concentration of 1.6 mM for 2 h at room temperature. The reaction was monitored by analytical RP-HPLC (1260 HPLC system, Agilent), with a C8 column (Aeris™ 3.6 μm WIDEPORE XB-C8 200 Å, 150 × 2.1 mm, Phenomenex), with a linear gradient of 0–100% solvent B (MeCN, 0.1% TFA v/v) in solvent A (ddH_2O, 5% MeCN v/v, 0.1% TFA v/v) in 30 minutes. The conjugate was separated from unreacted PEG and peptide via cation exchange chromatography using a 100-ml column packed with Capto SP ImpRes resin (GE Healthcare) and 15 mM HEPES, pH 7, and 1 M NaCl as solvents. The mass of the

conjugate was checked with a MALDI-TOF mass spectrometer (AutoFlex Speed, Bruker). Pure fractions were desalted by size exclusion chromatography using ddH_2O as solvent. The conjugate was lyophilized and dissolved in ddH_2O. The purity was assessed by analytical RP-HPLC as described above and was > 95%.

Approval for animal experiments

All experiments in rabbits have been approved by the Ethics Affairs department of EPFL. The experiments were performed by Washington Biotechnology, Inc., in AAALAC-accredited facilities with IACUC-approved standard protocols. Office of Laboratory Animal Welfare (OLAW) Assurance No. A4192-01.

Pharmacokinetics/pharmacodynamics study in rabbits

New Zealand White (NZW) rabbits (female, 2–3 kg) were injected with 60 mg/kg of PEG40-F5 in 4.2 ml PBS via the marginal ear vein. Blood samples were collected from each animal via the auricular artery in sodium citrate Vacutainer tubes (BD) 10 min before and 5, 30, 60, 120, 240, 480, and 1,440 min after PEG40-F5 injection. Blood samples were processed to plasma by centrifugation at 2,000 g for 15 minutes at 4°C and frozen immediately.

Ex vivo hemodialysis study

The study was performed similarly as described previously[49]. A bag of freshly drawn blood (400 ml per donor) containing 63 ml citrate phosphate dextrose (CPD) solution (Cambridge Bioscience) was split equally into two bags, and two dialysis circuits were built using the same tubing system. Two FX50 CorDiax dialyzers (steam sterilized, surface

area 1.0 sqm, priming volume 53 ml, high-flux membrane; Fresenius Medical Care) were run in parallel with two Fresenius 4008 S dialysis machines. Peptide (final concentration in blood of 15 μM), unfractionated heparin (final concentration in blood of 0.35 IU/ml), or saline were injected into the blood bag. In each series, either peptide or heparin-containing blood was dialyzed in parallel with saline-containing blood (negative control). The blood was recirculated at 150 ml/min and dialyzed until total clotting. Time until total clotting was recorded. The concentration of ionized calcium was determined to estimate the time for sodium citrate removal. No unexpected or unusually high safety hazards were encountered.

Determination of peptide elimination from the hemodialysis system

To assess the extent of peptide elimination from the system, PEG40-F5 was injected to reach a concentration in blood of 15 μM, together with 1.75 IU/ml of UFH. The hemodialysis run was performed as described in the main text. Blood samples were collected at 5, 10, 20, 30, 40, 50, and 60 min and processed to plasma. Residual FXIa inhibitory activity of the peptide was tested as described above (plasma stability assays).

Statistical analysis

Statistical analysis of the results of the *ex vivo* hemodialysis study was performed with a two-tailed paired t-test using the software Prism 5 (GraphPad). Significance threshold was defined as $p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at XYZ

The supporting information contains data about the structural diversity of the peptide phage display library, phage titers obtained throughout the rounds of phage display, complete consensus sequences, analytical data for the peptides (HPLC traces and MS spectra), activity data for the peptides (dose-response curves of FXIa inhibition, aPTT, PT, and ACT), and control experiments for the HD study.

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Author contributions

V.C. and C.H. conceived the strategy, designed experiments and analyzed data. V.C. cloned the libraries, performed the phage selections against FXIa, and supervised the *in vivo* experiment in rabbits. B.S., V.C., Y.W., J.G., and C.H. designed and performed the

ex vivo hemodialysis studies. R.M. performed the large-scale synthesis of PEG40-F5. X.K. contributed in defining the library design strategy. V.C., C.R., Q.L., E.C., M.K., and C.D. synthesized, purified, and characterized the peptides. V.C., X.K., and C.V. performed the NGS and analyzed the results. T.M. contributed in characterizing the PK profile of the PEGylated peptide. C.L. contributed to developing the PEGylation strategy. V.C., K.D., and C.H. wrote the manuscript.

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Notes

V.C. and C.H. are inventors of a patent application covering the synthetic FXI inhibitor. All other authors have no potential competing interest.

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ABBREVIATIONS USED

ACT, activated clotting time; aPTT, activated partial thromboplastin time; FXI, coagulation factor XI; FXIa, activated FXI; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography-mass spectrometry; MeCN, acetonitrile; PEG, polyethylene glycol; PPACK, D-phenylalanyl-prolyl-arginyl chloromethyl ketone; PT, prothrombin time; RP-HPLC, reversed phase HPLC; TFA, trifluoroacetic acid; UFH, unfractionated heparin; VTE, venous thromboembolism

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