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Brief Article

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Development of a Selective Peptide Macrocycle Inhibitor of Coagulation Factor XII toward the Generation of a Safe Antithrombotic Therapy

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Supporting Information

ABSTRACT: Inhibition of coagulation factor XII (FXII) activity represents an attractive approach for the treatment and prevention of thrombotic diseases. The few existing FXII inhibitors suffer from low selectivity. Using phage display combined to rational design, we developed a potent inhibitor of FXII with more than 100-fold selectivity over related proteases. The highly selective peptide macrocycle is a promising candidate for the control of FXII activity in antithrombotic therapy and a valuable tool in hematology research.

INTRODUCTION

The formation of a fibrin clot upon vascular injury is a vital process to prevent excessive blood loss. However, under certain circumstances, pathological thrombus formation can occur and lead to vessel obstruction. Fibrin formation can be triggered by two distinct pathways, by the exposure of blood to the ruptured subendothelial layer (extrinsic) and by the activation of FXII (intrinsic). The discovery that FXII-deficient mice are profoundly protected from pathological thrombus formation while presenting a normal hemostatic capacity highlighted the specific implication of the intrinsic pathway in pathological coagulation.[1] Inhibition of FXII activity may thus represent an attractive approach for the treatment and prevention of thrombotic diseases without the associated bleeding complications that come along with the currently used anticoagulants.[2]

Activation of FXII occurs by contact with negatively charged surfaces.[3] This contact induces a conformational change in the zymogen leading to activation of a small amount of FXII. Activated FXII (FXIIa) in turn activates plasma kallikrein (PK) by proteolytic cleavage, and active plasma kallikrein reciprocally activates larger amounts of FXII. Reduction of FXII activity by lowering expression with antisense oligonucleotides, by inhibition of zymogen activation, or by inhibiting the activated protease abolishes occlusive thrombus formation in vivo.[4−8] A limitation of existing FXII inhibitors[9−17] is the relatively low selectivity over paralogous proteases. Even the most selective one, the hematophagous insect protein domain infesin 4 (K_i = 0.128 nM), inhibits four out of the five tested proteases, including the physiologically important plasmin (K_i = 2.1 nM, only 16-fold selectivity over FXII).[9] Additionally, the exogenous origin of the protein-based inhibitors (tick, cabbage seed, pumpkin seed, corn, bacteria)[4,9−12,14−16] might be a hindrance toward their clinical use.

The de novo development of highly selective serine protease inhibitors has been challenging because of the large structural similarity among the over 70 trypsin-like serine proteases in human.[17] Bicyclic peptides have proven to be a valuable binding molecule format for developing potent and specific serine protease inhibitors.[18−20] Most bicyclic peptide inhibitors isolated from large phage display libraries showed high selectivity over homologues.[19−21] With the aim of generating a safe antithrombotic drug, we developed a bicyclic peptide-based inhibitor of FXII and assessed the inhibition of the intrinsic and extrinsic coagulation pathways. In addition, we combined the FXII inhibitor with a previously developed selective bicyclic peptide inhibitor of PK to test for synergistic effects by selectively inhibiting the two proteases.

RESULTS AND DISCUSSION

Two combinatorial libraries of jointly >4 billion different bicyclic peptides were screened for inhibitors of human FXIIa by phage display. The libraries were generated, as previously described,[20,21] by displaying peptide of the format Cys-$(Xaa)_n$-Cys-$(Xaa)_n$-Cys (n = 4, 6) on phage and subsequent cyclization with compound 1,3,5-tris(bromomethyl)benzene (TBMB) that reacts with the three cysteine side chains. FXII is expressed as a zymogen and activated by proteolytic cleavage in a two-step process. A first cleavage gives rise to the α-FXIIa being composed of a heavy and a catalytic light chain held together by a disulfide bond. Further cleavage yields the smaller β-FXIIa, which comprises only the catalytic domain. Bicyclic peptides isolated after three rounds of phage selection against α-FXIIa yielded binders to many epitopes, as evidenced by 10 different consensus sequences. Peptides of several consensus sequences bound to FXIIa with high affinity, but none of them efficiently inhibited the protease (supplementary Figure 1 in Supporting information). Bicyclic peptides isolated in panning experiments

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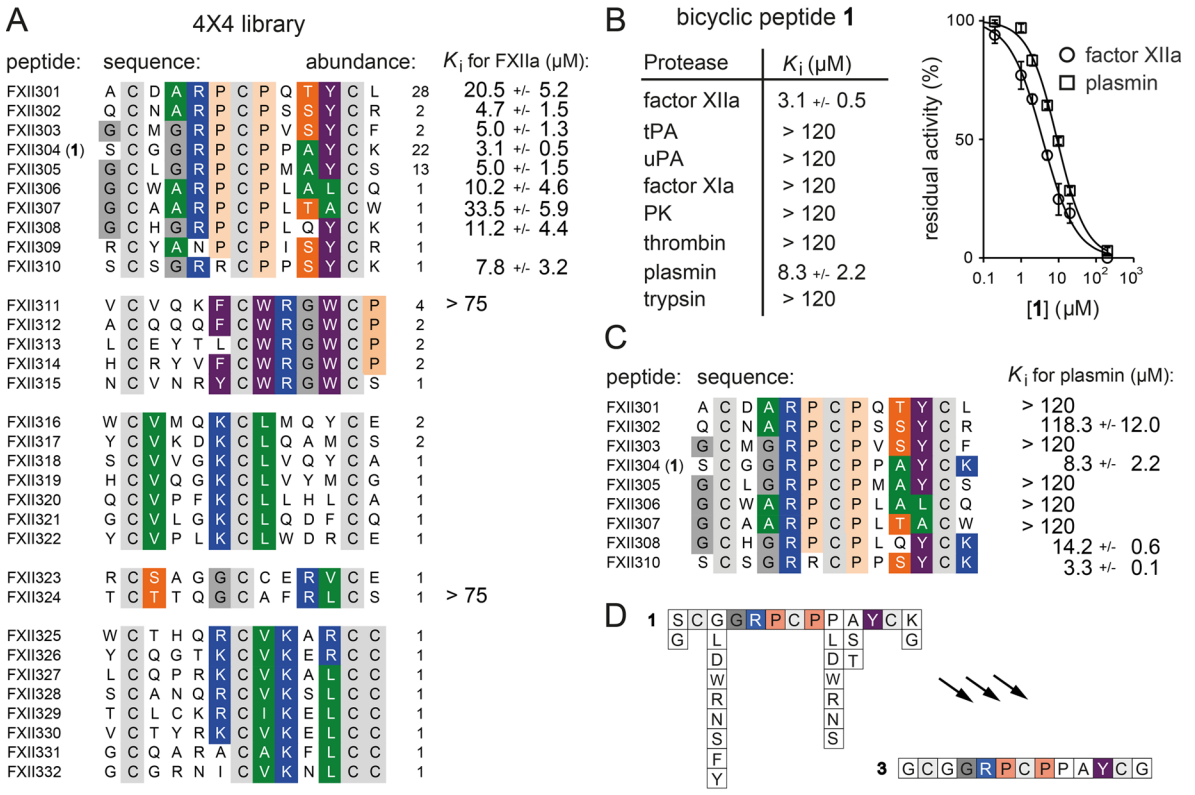

Figure 1. Development of a bicyclic peptide inhibitor of FXII. All indicated inhibitory constants (K_i) are averages of at least three measurements. Standard deviations are indicated. Amino acid similarities are highlighted in Rasmol color code. (A) Peptide sequences isolated after three rounds of phage panning against β-FXIIa from a 4 × 4 bicyclic peptide library (library format Xaa-Cys-$(Xaa)_4$-Cys-$(Xaa)_4$-Cys-Xaa; "Cys" stands for cysteine and "Xaa" for any random amino acid). (B) Inhibitory activity of bicyclic peptide **1** (clone FXII304) toward FXIIa and a panel of human proteases. (C) Inhibition of plasmin by FXII inhibitors. All peptides inhibiting plasmin efficiently contain a lysine residue at the C-terminus (highlighted in blue). (D) Affinity maturation of bicyclic peptide **1**. A range of bicyclic peptides with amino acid substitutions in the indicated positions were synthesized and tested and led to bicyclic peptide **3** (clone FXII402) having a 3-fold improved K_i (see also supplementary Figure 3).

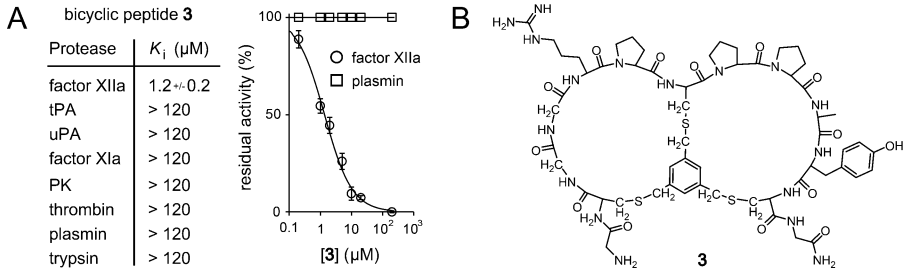

Figure 2. Bicyclic peptide **3**, a potent and selective inhibitor of FXII. (A) Inhibitory activity of bicyclic peptide **3** toward FXIIa and a panel of human proteases. All indicated inhibitory constants (K_i) are averages of at least three measurements. Standard deviations are indicated. (B) Chemical structure of bicyclic peptide **3**.

against β-FXIIa showed two new consensus sequences of which one contained peptides efficiently inhibiting activated FXII (Figure 1A; supplementary Figure 2). Inhibitory activity of bicyclic peptides was determined by incubation of the protease with various peptide concentrations and quantification of the residual activity with a fluorogenic FXII substrate. The best inhibitor, bicyclic peptide **1** (clone FXII304), blocked β-FXIIa with a K_i of 3.1 ± 0.5 μM (Figure 1A) and α-FXIIa with a comparable activity. Compared to bicyclic peptides that we isolated against other targets, the binding of the FXII inhibitors was not as strong. The reason for this is that the 4 × 4 bicyclic peptides have only four amino acids per ring to interact and thus fewer than most of the previously isolated bicyclic peptides. Selections with this target were particularly challenging because most isolated peptides bound to exosites. Only 4 × 4 peptides gave rise to the active site binders.

Specificity profiling of bicyclic peptide **1** showed that structurally and functionally related proteases were not inhibited at high inhibitor concentrations (Figure 1B). An exception was plasmin, which was inhibited at low micromolar concentrations (K_i = 8.3 ± 2.2 μM). Inhibitory assays with bicyclic peptides of the same consensus sequence revealed several FXII inhibitors that did not affect plasmin at all. Peptides inhibiting plasmin presented a lysine at the last amino acid position, indicating that this residue could mediate the unspecific inhibition (Figure 1C). In fact, lysine and lysine analogues such as the antifibrinolytics tranexamic acid and ε-aminocaproic acid inhibit plasmin.[22] Substitution of lysine in **1**

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to glycine completely abolished plasmin inhibition, while the activity toward FXII was essentially retained (bicyclic peptide **2** (clone FXII401), K_i of 4.3 ± 1 μM; supplementary Figure 3). The inhibitory activity of bicyclic peptide **1** was improved 3-fold in an affinity maturation effort sampling various amino acids in four positions being not part of the consensus sequence (Figure 1D, supplementary Figure 3). The resulting bicyclic peptide **3** (clone FXII402) inhibited FXIIa with a K_i of 1.2 ± 0.2 μM while sparing all seven other proteases tested (K_i > 120 μM) (Figure 2). With a >100-fold selectivity over related trypsin-like proteases, bicyclic peptide **3** displays a significantly improved specificity profile compared to previously reported FXII inhibitors.

The effect of bicyclic peptide **3** on intrinsic and extrinsic coagulation was determined by measuring the coagulation times aPTT (activated partial thromboplastin time) and PT (prothrombin time) and the residual activity of FXII in human plasma in the presence of different inhibitor concentrations. aPTT and PT measure coagulation specifically initiated via the intrinsic and extrinsic pathway of coagulation, respectively. Physiological values for aPTT range from 26 to 37 s and for PT from 70% to 120% (Service and Central Laboratory of Hematology, Lausanne University Hospital, Switzerland). Coagulation times expected in the case of complete FXII inhibition are similar to the ones obtained for FXII-deficient plasma (aPTT > 150 s and physiologic PT; Service and Central Laboratory of Hematology, Lausanne University Hospital, Switzerland). Bicyclic peptide **3** inhibited the intrinsic pathway and FXII activity in a concentration dependent manner, as shown by increasing aPTT times and decreasing FXII activity upon increasing concentration of **3** (Figure 3A). Up to 200 μM, the extrinsic pathway was not strongly affected as the PT values remained physiologic (≥70%) (Figure 3A). Bicyclic peptide **3** was next combined with bicyclic peptide **4** (clone PK128),[19] a potent bicyclic peptide inhibitor of human plasma kallikrein (K_i = 0.3 nM) to block simultaneously FXII zymogen activation. Combining the two selective bicyclic peptide inhibitors potently inhibited intrinsic coagulation wherein concentrations as low as 50 μM for bicyclic peptides **3** and **4** were sufficient to entirely block the intrinsic cascade (aPTT > 150 s, FXII remaining activity <5%, Figure 3B). At these concentrations, the extrinsic cascade was not significantly affected (Figure 3B). The observed synergistic effect is likely based on the inhibition of reciprocal activation of FXII and PK. This finding suggests that dual targeting of the two proteases with selective inhibitors is an attractive avenue to inhibit intrinsic coagulation. At the same time, bicyclic peptide **3** may also be applied to potentiate PK antagonists used in the treatment of hereditary angioedema patients.[23]

CONCLUSION

In summary, we report the development of a potent and selective inhibitor of coagulation FXII. Combined with PK blockade, FXII inhibition leads to the complete inactivation of the intrinsic cascade implicated in pathological coagulation while not interfering with the extrinsic cascade implicated in physiological coagulation. On the basis of its high selectivity, being superior to existing FXII inhibitors, the bicyclic peptide is a valuable tool in hematology research, as it allows inhibition of FXII without interfering with the activity of other proteases. In addition, the FXII alone or in combination with the PK inhibitors is an attractive clinical lead candidate for prevention of thrombosis or the treatment of other diseases potentially benefiting from control of FXII and/or PK activity, like hereditary angioedema.

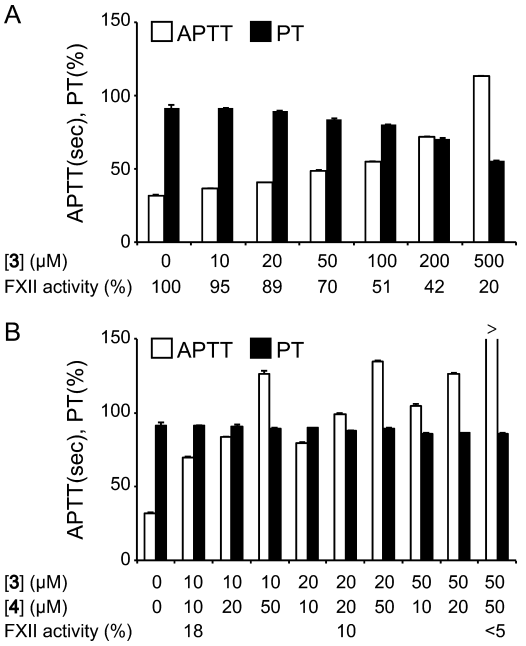

Figure 3. Coagulation parameters (aPTT, PT, and FXII activity) in the presence of the engineered FXII inhibitor bicyclic peptide **3** alone or in combination with the plasma kallikrein inhibitor bicyclic peptide **4**. aPTT is given in seconds and PT in percentage of normal. Assays were performed and measured at least in duplicate, and the average and standard deviation are indicated. (A) Measurement of aPTT, PT, and FXII activity in human plasma with increasing concentrations of bicyclic peptide **3**. (B) aPTT and PT values obtained in plasma in the presence of various concentrations of both inhibitors. Remaining FXII activity is indicated for three different inhibitor mix concentrations. ">" corresponds to aPTT > 150 s and thus to a complete inhibition of the intrinsic pathway.

EXPERIMENTAL SECTION

Phage Selections against Activated Human FXII. Phage (from library 6×6^{20} and 4×4^{24}) were separately expressed, purified, and chemically modified as previously described.[20,21] Biotinylated human FXIIa (5 μg; Molecular Innovations, Novi, MI, U.S.), α- or β-FXIIa, was incubated with 50 μL of prewashed magnetic streptavidin beads (Dynal, M-280 from Life Technologies, Carlsbad, CA, U.S.) for 30 min at room temperature. Beads were washed prior to blocking with 0.5 mL of buffer A (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) containing 1% BSA and 0.1% Tween 20 for 30 min at room temperature. Chemically modified phage (10^{10}–10^{11} t.u. dissolved in 2 mL of buffer A) were concomitantly blocked by addition of 1 mL of buffer A containing 3% BSA and 0.3% Tween 20. Blocked beads with target were then mixed with the blocked phage and incubated for 30 min on a rotating wheel at room temperature. Beads were washed 8× with buffer A containing 0.1% Tween 20 and 2× with buffer A before incubation with 100 μL of 50 mM glycine, pH 2.2, for 5 min. Eluted phage was neutralized with 50 μL of 1 M Tris-Cl, pH 8, incubated with 30 mL of exponentially growing TG1 cells for 90 min at 37 °C, and the cells were plated on 2YT/chloramphenicol plates. The next day, bacteria were collected. Two additional rounds of panning were performed using the same procedures. In the second round, neutravidin-coated magnetic beads were used to prevent the enrichment of streptavidin-specific peptides. The neutravidin beads were prepared by reacting 0.8 mg of neutravidin (Pierce, Rockford, IL, U.S.) with 0.5 mL of tosyl-activated magnetic beads (Dynal, M-280 from Invitrogen, Paisley, U.K.) according to the supplier's instructions. Minipreps were performed, and DNA retrieved from isolated phage

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particles was sequenced by Macrogen Europe (Amsterdam, The Netherlands).

Bicyclic Peptide Synthesis. Peptides were synthesized in house by standard solid-phase peptide synthesis using Fmoc-protected amino acids (scale, 0.03 mmol). As solid support, Rink amide AM resin was used to obtain peptides with a free N-terminus and an amidated C-terminus. Peptides were cleaved from the resin under reducing conditions (90% TFA, 2.5% H_2O, 2,5% thioanisol, 2,5% phenol, 2.5% 1.2-ethanedithiol) and partially purified by precipitation. Crude peptide at 0.5 mM was reacted with 1 mM TBMB in 80% aqueous buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0) and 20% acetonitrile for 1 h at 30 °C. The product was purified by reversed-phase chromatography on a C18 column, and H_2O/0.1% TFA and 95% acetonitrile/5% H_2O/0.1% TFA were used as solvents. Pure bicyclic peptides were lyophilized and dissolved in water. The purity was assessed by RP-HPLC and was >95% for all peptides. The identity was confirmed by ESI or MALDI-TOF spectrometry.

Protease Inhibition Assays. Inhibitory activity of bicyclic peptides was determined by incubation of each protease with various peptide concentrations and quantification of the residual activity with a fluorogenic substrate. Residual enzymatic activities were measured in 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, 0.01% v/v Triton-X100, and 5% v/v DMSO in a volume of 150 μL. Final concentrations of human serine proteases were the following: FXIIa (HFXIIA and HFXIIAB; Molecular Innovations) 100 nM, tPA (Molecular Innovations) 7.5 nM, uPA (Molecular Innovations) 1.5 nM, factor XIa (Innovative Research, Novi, MI, U.S.) 6 nM, PK (Innovative Research) 0.25 nM, thrombin (Innovative Research) 10 nM, plasmin (Molecular Innovations) 2.5 nM, trypsin (Molecular Innovations) 0.05 nM. Dilutions of peptides were prepared ranging from 0.2 to 200 μM. For the determination of the IC_{50} inhibitory constants, the following fluorogenic substrates were used at a final concentration of 50 μM: Z-Phe-Arg-AMC (for PK; Bachem, Bubendorf, Switzerland), Boc-Phe-Ser-Arg-AMC (for factor XIa; Bachem), Z-Gly-Gly-Arg-AMC (for FXIIa, tPA, uPA, thrombin, and trypsin; Bachem), and H-D-Val-Leu-Lys-AMC (for plasmin; Bachem). Fluorescence intensity was measured with a Spectramax Gemini fluorescence plate reader (excitation at 355 nm, emission at 460 nm; Molecular Devices, Sunnyvale, CA, U.S.). The reactions were performed at 25 °C. IC_{50} values were calculated using Excel software. Fitting curves were generated with Matlab software (MathWorks, Natick, MA, U.S.). The inhibitory constant K_i was calculated according to the equation of Cheng and Prusoff[25] $K_i = IC_{50}/(1 + [S]_0/K_m)$ wherein IC_{50} is the functional strength of the inhibitor, $[S]_0$ is the total substrate concentration, and K_m is the Michaelis–Menten constant. Inhibitory constants were measured at least in triplicate. Average and standard deviations are reported.

Measurement of Coagulation Times and FXII Activity in Human Plasma. Coagulation times (activated partial thromboplastin time, aPTT, and prothrombin time, PT) and FXII activity were determined, at least in duplicate, in human plasma using an automated blood coagulation analyzer (Sysmex CA-7000 or BCS XP system) with standard reagents according to the manufacturer's instructions (Siemens Healthcare, Eschborn, Germany). Average and standard deviations are reported. Human plasma used in this study resulted from a pool of fresh frozen plasma units provided by the Service Régional Vaudois de Transfusion Sanguine, Switzerland.

■ ASSOCIATED CONTENT

Supporting Information

Figures documenting the sequencing results obtained after affinity selection against α-FXIIa (supplementary Figure 1) and β-FXIIa (supplementary Figure 2), and the step-by-step rational design leading to the final bicyclic peptide inhibitor **3** (supplementary Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

FXII, coagulation factor XII; FXIIa, activated coagulation factor XII; PK, plasma kallikrein; TBMB, 1,3,5-tris(bromomethyl)-benzene; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator

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