Design, Synthesis, and Biological Evaluation of Peptidomimetic Inhibitors of Factor XIa as Novel Anticoagulants

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Human coagulation factor XIa (FXIa), a serine protease activated by site-specific cleavage of factor XI by thrombin, FXIIa, or autoactivation, is a critical enzyme in the amplification phase of the coagulation cascade. To investigate the potential of FXIa inhibitors as safe anticoagulants, a series of potent, selective peptidomimetic inhibitors of FXIa were designed and synthesized. Some of these inhibitors showed low nanomolar FXIa inhibitory activity with >1000-fold FXa selectivity and >100-fold thrombin selectivity. The X-ray structure of one of these inhibitors, **36**, demonstrates its unique binding interactions with FXIa. Compound **32** caused a doubling of the activated partial thromboplastin time in human plasma at 2.4 μM and was efficacious in a rat model of venous thrombosis. These data suggest that factor XIa plays a significant role in venous thrombosis and may be a suitable target for the development of antithrombotic therapy.

Introduction

A well-balanced and highly regulated hemostatic process is essential to normal physiology. The overproduction of thrombin may lead to vascular occlusion as a consequence of many cardiovascular diseases such as acute myocardial infarction (AMI), stroke, pulmonary embolism (PE), and deep vein thrombosis (DVT). The inappropriate down-regulation of thrombin generation may also cause hemorrhagic problems.[1,2] Thus far, none of the available therapies for the prevention and treatment of thromboembolic disorders are ideal. The most commonly used anticoagulants, heparin and warfarin, require very careful and costly monitoring to ensure safe therapeutic drug levels for the duration of treatment because of the high risk of bleeding. Warfarin and other coumarins, the only orally administered anticoagulants, often exhibit drug−drug and food−drug interactions due to their erratic plasma concentration and anticoagulant effects.[3] Heparin also has variable effects due to its nonspecific binding with multiple coagulation factors in the system.[4] In addition, a small population of patients can develop an autoimmune syndrome known as heparin-induced thrombocytopenia (HIT).[5] Clearly, there is a strong medical need for a well-tolerated, efficacious anticoagulant with a wide therapeutic window. To address this need, a significant focus within the pharmaceutical industry for decades has been the development of potent and selective inhibitors of the coagulation enzymes to replace heparin and warfarin. Among the coagulation enzymes, thrombin (factor IIa) and factor Xa (FXa) have received the most attention due to their multiple functions and key roles in the final common pathway in the blood coagulation cascade. Some direct thrombin inhibitors and FXa inhibitors have been evaluated in phase III clinical trials or have been licensed in North America or Europe for limited indications. However, all of these new anticoagulants are still associated with bleeding problems and have no significant improvement in efficacy-to-safety index compared with heparin or warfarin in many studies (reviewed in refs 6 and 7). More recently, a new trend in the development of antithrombotic agents is to explore inhibitors targeting the factor VIIa/tissue factor complex,[8,9] factor IXa,[10] factor XIa,[11,12] carboxypeptidase U (thrombin activatable fibrinolysis inhibitor, TAFIa),[13] or agents that enhance activated protein C that in turn inactivates factor Va and VIIIa.[14]

The roles of factor XI in the intrinsic pathway of coagulation and its thrombin-mediated feedback activation were not fully understood until the early 1990s.[15] Thrombin formation can occur via activation of the extrinsic or intrinsic pathway of coagulation. In the extrinsic pathway, thrombin generation is initiated by exposure of blood to the tissue factor/activated factor VII (FVIIa) complex. This complex activates clotting factors X and IX (proteolysis rate of FX ≫ FIX), leading to the generation of thrombin and the formation of fibrin.[16] This pathway is usually rapidly shut down by tissue factor pathway inhibitor (TFPI) and anti-thrombin III. To maintain normal hemostasis, thrombin-mediated activation of FXI initiates an amplification phase via the intrinsic pathway. Activated factor XI (FXIa) continues to activate factor IX and then factor X, resulting in the formation of additional thrombin, which takes place inside the fibrin clot. This additional thrombin may be required for the activation of thrombin activatable fibrinolysis inhibitor (TAFI) to protect fibrin clots against lysis.[17] Therefore, the role of FXI in hemostasis can be considered as a combination of procoagulant and antifibrinolytic actions, which is critical to the consolidation of blood coagulation when tissue factor is not available.[18]

Hypothetically, a specific inhibition of FXIa, which is involved only in the amplification phase via the intrinsic pathway, represents an attractive strategy for novel antithrombotic agents. Specific inhibitors of FXIa might inhibit thrombosis without completely interrupting the function of normal hemostasis and, thus, might prevent or minimize the risk of hemostasis complication. Deficiencies in factors VIII and IX can cause hemophilia A and B, respectively, whereas genetic FXI deficiency results in only a mild-to-moderate bleeding disorder.[19] High levels of FXI have been demonstrated as risk factors for venous thrombosis[20] and acute myocardial infarction.[21] Recently, our hypothesis has been supported by a ferric chloride ($FeCl_3$)

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Chart 1. Factor XIa Inhibitor Design

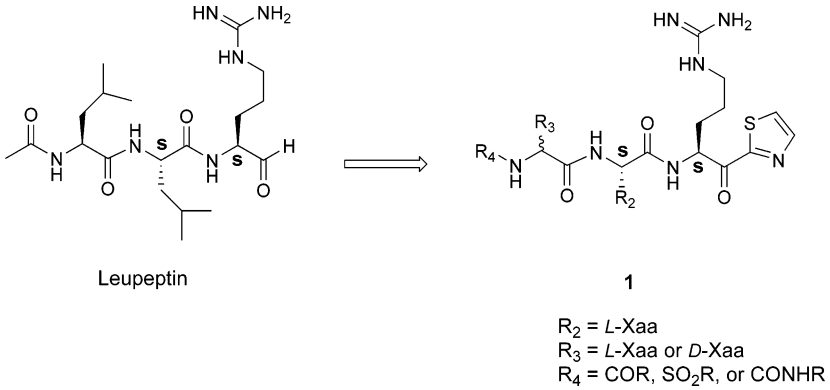


Chart 2. Natural Cleavage Sites on FIX Performed by FXIa

Prioritized sublibraries:

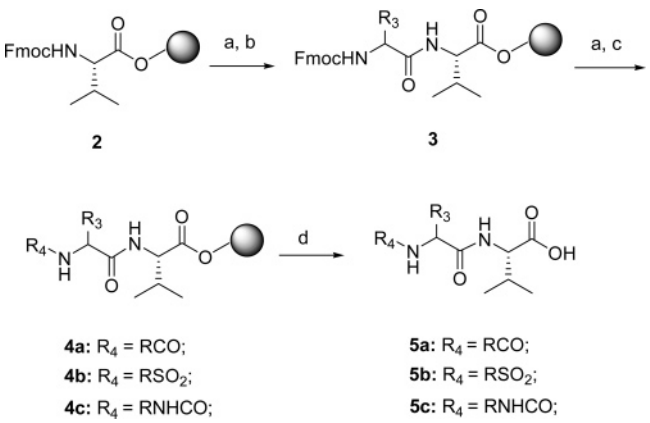
1. P4-Phe-Val-Arg-CO-(2-thiazole)
2. P4-Leu-Val-Arg-CO-(2-thiazole)

induced arterial injury mouse model,[22] where both FXI- and FIX-deficient mice were fully or partially protected from occlusion depending on the concentration of $FeCl_3$. However, FXI-deficient and wild-type mice were shown to have similar bleeding time, whereas FIX deficiency was associated with severely prolonged bleeding time. Furthermore, anti-FXI antibodies were shown to reduce both clot size and bleeding time in a baboon model of thrombosis[23] and to enhance fibrinolysis in a rabbit model.[17b] Interestingly, therapy using recombinant VIIa on a patient with a FXI/FXIa inhibitor has been found to be effective and safe, ensuring adequate hemostasis without thrombotic complication.[24]

Inhibitor Design

Due to the lack of literature precedents of FXIa inhibitors, the starting point of our search for potent and selective inhibitors of FXIa was initially based on two known thrombin inhibitors, leupeptin (L-Leu-Leu-ArgCHO)[25] and PPACK (D-Phe-Pro-ArgCH_2Cl).[26] We used tripeptides as templates to interact with the S_1, S_2, and S_3 pockets of the enzyme and further elaborated the C terminus and N terminus of the tripeptides to gain more specific interactions with our target enzyme. We first chose to synthesize a number of arginine derivatives, **1,** as shown in Chart 1, with activated electrophilic carbonyl groups such as an α-ketothiazole that has a well-characterized covalent interaction with Ser195,[27] in which the Arg-α-ketothiazole component served as the P_1-P_1' template. P_2 and P_3 components were initially selected from hydrophobic residues, including Phe, Val (as replacement of Thr), Leu, Ile, Pro, Ala, and Tyr, as the corresponding non-prime sequences of the two scissile FIX substrate sites contain hydrophobic P_2 and P_3 subunits (Chart 2). The X-ray crystal structure of a FXIa−ecotin complex[28a] also provided us with a comprehensive understanding of the FXIa active site to guide our inhibitor design.

Scheme 1.[a] Method A



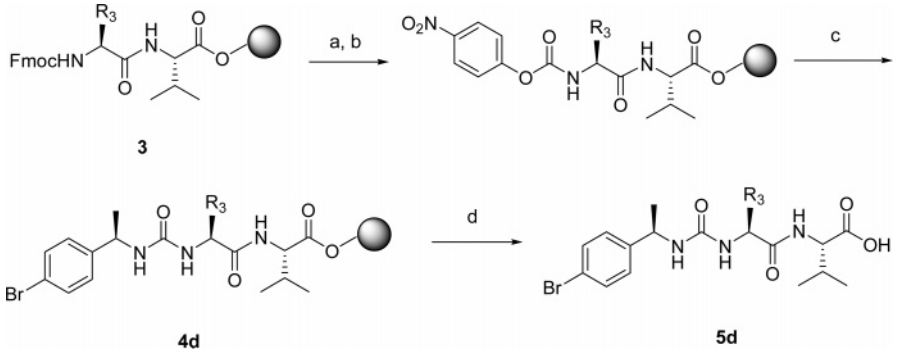
[a] Reagents and conditions: (a) 20% piperidine/DMF; (b) EDCI, HOBt, DIEA, (L)-(Fmoc)NH-Xaa-OH or (D)-(Fmoc)NH-Xaa-OH; (c) pyridine or triethylamine, RCOCl, RSO_2Cl, RNCO; (d) 50% TFA/CH_2Cl_2.

Chemistry

A combination of solid- and solution-phase synthesis was utilized to prepare a series of peptidomimetic inhibitors as shown in Schemes 1−4. The dipeptide cores, designed as P_2-P_3 components, were readily constructed on Wang resin by employing a 9-fluorenylmethoxycarbonyl (Fmoc) coupling strategy. The elaboration of the N terminus with various acylating reagents such as acid chlorides, sulfonyl chlorides and isocyanates was carried out in the final step on solid support, followed by a standard TFA cleavage protocol to yield a variety of dipeptido precursors, **5a**−**5c** (Scheme 1). As an alternative to the isocyanate approach (method A), N-terminal ureas were also synthesized by reaction of the amine terminus of the resin with *p*-nitrophenylchloroformate (5−10 equiv) in the presence of diisopropylethylamine (DIEA), followed by the addition of excess (5−10 equiv) primary or secondary amine. In one example, the resin-supported *p*-nitrophenylcarbonate dipeptide was treated with (*R*)-1-(4-bromophenyl)ethylamine to provide resin-supported compound **4d**. Optically pure isomer **5d** was readily obtained after cleavage with 50% TFA in dichloromethane (method B shown in Scheme 2).

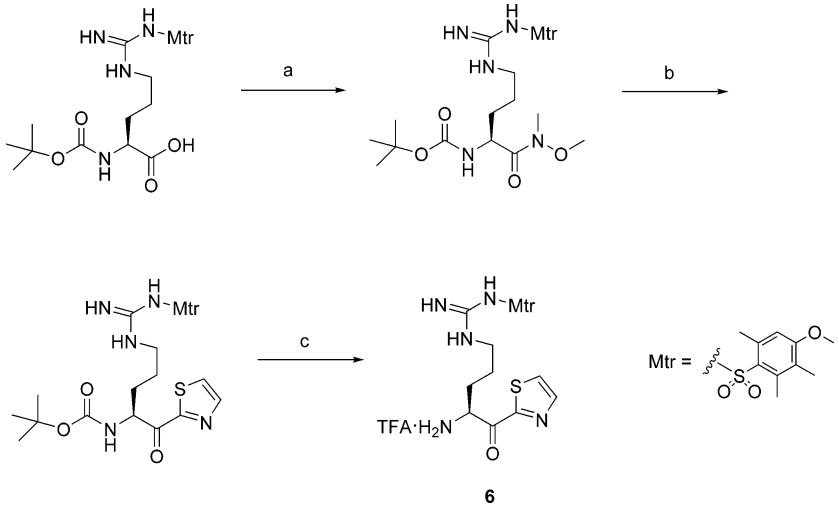
The α-ketothiazole derivative, 6, was prepared in three steps from commercial Boc-NH-Arg(Mtr)-OH as described by Berryman *et al.*[29] (Scheme 3). Coupling of acids **5a**−**d** with α-ketothiazole, **6**, using 1-hydroxybenzotriazole (HOBt)/1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) chemistry and subsequent deprotection of the 2,3,6-trimethyl-4-methoxybenzenesulfonyl (Mtr) group under strong acidic conditions (95% TFA/2.5% thioanisole/2.5% water) yielded peptidomimetics **1a**−**d** (Scheme 4).

Scheme 2. Method B[a]



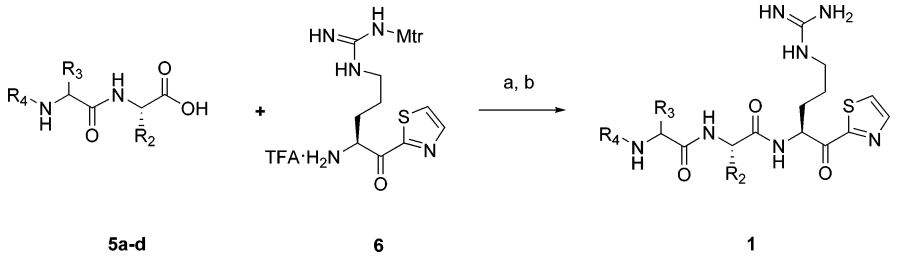
[a] Reagents and conditions: (a) 20% piperidine/DMF; (b) 4-nitrophenyl chloroformate, DIEA, THF/DCM (1:1); (c) triethylamine, (*R*)-1-(4-bromophenyl) ethylamine (5 equiv), DMF; (d) 50% TFA/CH_2Cl_2.

Scheme 3[a]



[a] Reagents and conditions: (a) Me(MeO)NH·HCl, EDCI, HOBt, DIEA, THF; (b) thiazole, n-BuLi, TMEDA, −78 to −40 °C, then −78 °C; (c) 25% TFA/CH_2Cl_2.

Scheme 4[a]



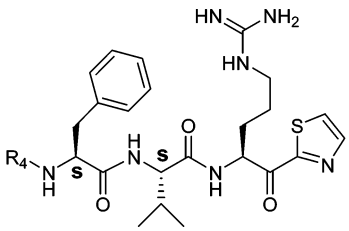
[a] Reagents and conditions: (a) EDCI, HOBt, DIEA, THF; (b) TFA/thioanisole/water (95:2.5:2.5), 1−3 h.

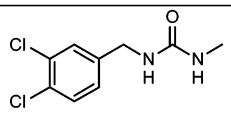
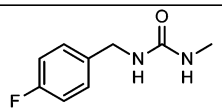
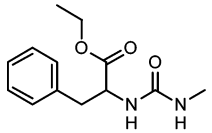
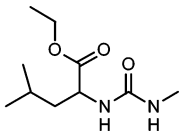
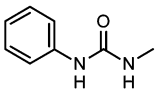
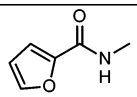
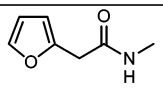
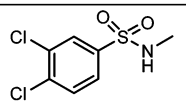
Results and Discussion

The lead compound, **7**, emerged from the early exploration of the P_4 component using Phe-Val-Arg-α-ketothiazole as $P_3-P_2-P_1-P_1'$ template (Table 1). Incorporation of various isocyanates, acid chlorides, and sulfonyl chlorides on the N terminus generated urea-linked, amide-linked, and sulfonamide-linked R_4 substitutions, respectively. Compounds with urea-linked R_4 substitutions such as compounds **7**−**10** showed superior potency against FXIa, compared with those compounds with amide- or sulfonamide-linked R_4 substitutions (**12**−**14**). Compound **7**, with a 3,4-dichlorobenzyl urea link, exhibits potent FXIa activity with an IC_{50} of 90 nM and good selectivity over thrombin (72-fold) and FXa (153-fold). The potency loss of compound **11**, which has a rigid phenyl urea R_4 component, indicates that a flexible spacer group such as $-CH_2-$ or $-CH_2CH_2-$ in urea-linked R_4 elements is needed for FXIa binding affinity.

Another lead compound, **15**, from the Leu-Val-Arg-α-ketothiazole sublibrary, also displayed potent FXIa activity with an IC_{50} of 63 nM (Table 2). Starting from lead compound **15**, we explored the SAR of P_2 groups by fixing the 3,4-dichlorobenzyl urea-linked R_4 component. As shown in Table 2, FXIa appears to prefer a relatively small, branched hydrophobic group such as Val, Leu, or Ileu in its S_2 pocket. Compounds **15** and **16** showed not only potent inhibitory properties against FXIa (IC_{50} = 63 and 76 nM, respectively) but also decent selectivity over FXa (for **16**, >1000-fold) and thrombin (for **16**, 34-fold). Highly branched, larger R_2 groups such as cyclohexyl, Phe, and ^{t}Bu (**18**, **19**, and **21**, respectively) or a very small group such as Ala (**20**) displayed weaker FXIa affinity. However, the larger Tyr residue at the P_2 position (**17**) showed unexpectedly good binding affinity for FXIa with an IC_{50} of 200 nM. The *p*-hydroxyl group in the Tyr residue of **17**

Table 1. Early Lead Exploration



Comp No.	R_4	IC_{50} FXIa (nM)	IC_{50} FXa (nM)	IC_{50} thrombin (nM)
7		93	14,200	6,700
8		160	nd[a]	4,300
9		258	14,300	2,100
10		403	15,400	2,100
11		1,920	>20,000	935
12		2,200	nd[a]	nd[a]
13		2,600	nd[a]	nd[a]
14		2,300	nd[a]	nd[a]

[a] nd, not determined.

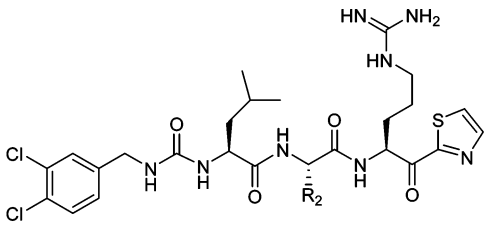
may interact with Glu 98 in the small S_2 loop of FXIa (see the detailed structure information of FXIa[28]).

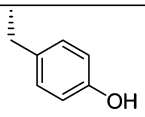
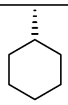
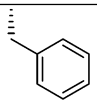
Taking advantage of solid- and solution-phase synthesis technologies, we were able to quickly optimize R_3 and R_4 elements in parallel (Tables 3−5). As shown in Table 3, the racemic 1-(4-bromophenyl)ethyl urea at the R_4 position (**22**) gave the best FXIa potency (IC_{50} = 10 nM) and good FXa (1500-fold) and thrombin (140-fold) selectivity in this series. The dichloro substitution at the benzyl phenyl ring was found to be favored by FXIa when the activities of compounds **15** and **23** were compared to those of **24** and **25**. Replacement of the urea linkage of compound **25** with carbamate analogue **26** resulted in a 2−3-fold loss in potency. Truncation of the R_4 element, such as in compound **27**, reduced the potency dramatically. The stereochemistry of the α-methyl benzyl substituent of **22** was also studied. The inhibitory activity against FXIa of the compounds with an (*R*)-configuration such as **28** and **30** is 10−20-fold enhanced compared with the corresponding (*S*)-diastereomeric isomers **29** and **31**, respectively (Table 4). The selectivity of **28** and **30** over FXa and thrombin is also 2−17-fold better than that of the (*S*)-isomers **29** and **31**.

Further efforts to explore the SAR of the R_3 elements are shown in Table 5. Either hydrophobic or hydrophilic residues at the R_3 position demonstrated excellent potency against FXIa. Hydrophobic residues had better selectivity over FXa and thrombin such as **22** and **28**. Compounds with less hydrophobic R_3 residues such as **32** and **37** showed less selectivity over FXa (∼200-fold); however, residues with the most hydrophilicity at R_3, for example, **33**, significantly reduced the selectivity against both FXa and thrombin (Table 5).

Some of the above potent compounds were selected and tested in in vitro clotting assays. The concentration needed to cause a doubling of the activated partial thromboplastin time (aPTT) and the prothrombin time (PT) in human plasma in vitro was

Table 2. SAR Exploration of R_2 Elements



Comp No.	R_2	IC_{50} FXIa (nM)	IC_{50} FXa (nM)	IC_{50} thrombin (nM)
15		63	>20,000	876
16		76	85,000	2,600
17		200	1,700	1,000
18		340	>200,000	7,700
19		710	8,500	27,000
20	CH_3	920	22,000	10,000
21		9,200	>200,000	12,000

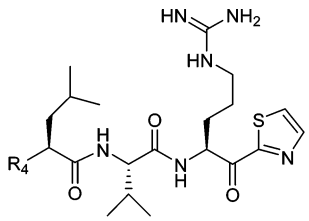
determined for each compound (see Table 6). Most compounds required only submicro- to low micromolar concentrations in coagulation (2× aPTT) assays and 10−50-fold higher concentrations in the 2× PT assay. The results indicated that potency in the aPTT versus the PT is consistent with the high level of specificity for FXIa over thrombin or FXa as determined by the biochemical assay. Compound **28** resulted in a significant loss of potency in both coagulation assays, likely due to poor solubility rendered by the lipophilic cyclohexyl group at R_3.

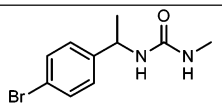
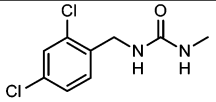
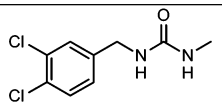
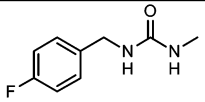
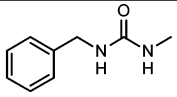
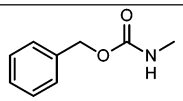
The X-ray crystal structure of **36** in complex with FXIa exemplifies how this series of potent inhibitors interact with FXIa (Figure 1). A detailed scheme of the key binding interactions of **36** with FXIa is described in Chart 3. As expected, the arginine of **36** forms a bidentate interaction with Asp189 in the S_1 pocket similar to the FXIa-ecotinM84R structure.[28a] The C-terminal thiazole binds in the S_1' pocket: the nitrogen of thiazole forms a hydrogen bond with His57; the ketocarbon is covalently bound to the hydroxyl group of Ser195. The valine residue of **36** fits in the S_2 pocket. The P_3 component, a citrulline side chain, unexpectedly binds in the S_4 pocket, instead of the S_3 site, but does not have any direct polar interactions with the S_4 pocket of FXIa. Interestingly, the (*R*)-1-(4-bromophenyl)ethyl urea substituent interacts with Lys192 of FXIa by hydrophobic packing between the phenyl group and the lysine side chain. The (*R*)-configuration of this substituent enables the right orientation for the phenyl group to make such contact. This explains why the (*R*)-isomers are substantially more potent and selective than the (*S*)-isomers (Table 4). In addition, there are hydrogen-bonding interactions between the urea C═O group, the Val C═O group (P_2), and Nζ of Lys192 (see Figure 1). These key interactions are most likely responsible for the observed improvement in potency and selectivity. Compared with other trypsin-like coagulation enzymes, only FXIa and FVIIa have a lysine at position 192.[28] As shown in the X-ray crystal structure of **36**, Lys192 may be a key selectivity element for FXIa.

The IC_{50} values of **32** versus selected serine proteases are shown in Table 7. Compound **32** showed good selectivity versus thrombin (340-fold), FXa (267-fold), FVIIa (>6000-fold), and activated protein C (APC, >3000-fold), but poor selectivity over plasma kallikrein and trypsin (both only ∼2-fold). In addition to potency and selectivity, we evaluated the pharmacokinetic profile of **32** via intravenous administration to rats. Compound **32** displayed relatively high clearance (32 mL/kg/min), a short half-life ($t_{1/2}$ of 45 min), and a low volume of distribution (V_{dss} ∼ 236 mL/kg).

To test the importance of factor XI in clot formation in vivo, we used an established rat model of venous thrombosis.[30,31] In this model, a hypotonic insult is used to induce clot formation in the inferior vena cava. To test for potential species specificity, we investigated the ability of **32** to affect the aPTT and PT in rat plasma (data in human plasma, see Table 6). Compound **32** was ∼10-fold less effective at prolonging rat aPTT than human aPTT. In rat plasma, 25 μM **32** was required to yield a doubling

Table 3. Optimization of R_4 Elements



Comp No.	R_4	IC_{50} FXIa (nM)	IC_{50} FXa (nM)	IC_{50} thrombin (nM)
22		10	15,000	1,400
23		45	13,000	386
15		63	>20,000	880
24		114	>20,000	3,100
25		136	>20,000	2,800
26		356	nd[a]	nd[a]
27		26,000	>200,000	>200,000

[a] nd, not determined.

of the aPTT, whereas >120 μM was required to double the PT. This was most likely a direct effect on factor XI as shown by the fact that the IC_{50} of this compound for recombinant rat FXI catalytic domain was also 10-fold higher than the IC_{50} for the human enzyme.[32] Despite the lower potency against the rat enzyme, continuous intravenous administration of **32** suppressed thrombus formation in the inferior vena cava induced by rapid infusion of hypotonic saline followed by partial stenosis in the rat (Figure 2). Administration of **32** commenced 5 min prior to thrombus induction, with determination of thrombus mass at 10 min postchallenge. Efficacy was observed at a total dose exposure of 0.25 mg/kg. The extent of the reduction is comparable to that obtained with heparin given as an intravenous bolus of 50 units/kg followed by continuous infusion at 25 units/kg/h. Similar results were obtained with **37** (data not shown). Compound **37** was also tested in a rat mesenteric arteriole bleeding model.[31,35,36] When administered at up to 4 times the efficacious dose (1 mg/kg), **37** did not alter the bleeding time, whereas heparin, at a dose comparable to the clinically relevant dose, significantly increased bleeding (Figure 3).

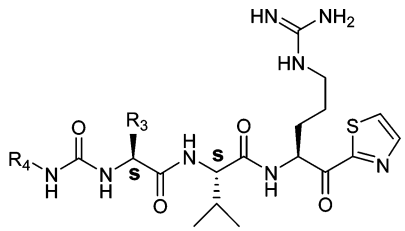
Conclusion

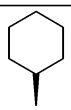
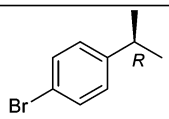
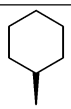
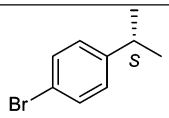
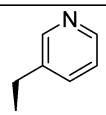
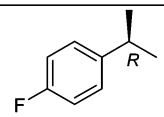
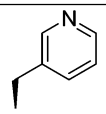
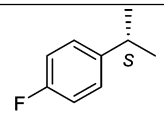
A series of potent, selective peptidomimetic inhibitors of FXIa have been designed and synthesized. Some of these inhibitors showed low nanomolar FXIa inhibitory activity with >1000-fold FXa selectivity and >100-fold thrombin selectivity. The X-ray structure of the inhibitor, **36**, demonstrates its unique binding with FXIa. In vivo efficacy of **32** was demonstrated in a rat model of venous thrombosis. The results presented in this paper clearly suggest that FXIa plays a significant role in clot formation and/or maintenance and that it is a suitable target for the development of novel antithrombotic agents.

Experimental Section

Chemistry. General Methods. The preloaded amino acid Wang resin [100−200 mesh, 1% divinylbenzene (DVB) cross-linking, 0.63 mmol/g] and Wang resin (100−200 mesh, 1% DVB cross-linking, 1.2 mmol/g) were obtained from Novabiochem Co. The amino acids were purchased either from Novabiochem Co. or from Advanced Chemtech, Inc. Other chemicals and anhydrous solvents were obtained from Aldrich, Fluka, Lancaster, and Fisher. All reagents and solvents were used without further purification. All synthetic compounds and intermediates gave satisfactory MS. Mass spectrometry analyses were performed on a Finnigan LCQduo system. HPLC analyses were recorded on a Gilson HPLC system consisting of a 170 diode array detector, a 215 liquid handler, and a 322 pump. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz on a

Table 4. Stereochemistry Studies of R_4 Elements



Comp No.	R_3	R_4	IC_{50} FXIa (nM)	IC_{50} FXa (nM)	IC_{50} thrombin (nM)
28			10	2,700	976
29			132	14,900	5,600
30			28	nd[a]	523
31			533	nd[a]	606

[a] nd, not determined.

Bruker 300 NMR spectrometer with $CDCl_3$, DMSO-d_6, and MeOH-d_4 as solvents.

General Procedure for Preparation of Resin 3. Deprotection of Fmoc Group of Resin 2. The preloaded Fmoc-Val-Wang resin **2** (10.0 g, 6.3 mmol, 0.63 mmol/g) was treated with 20% piperidine in DMF (120 mL) at room temperature for 2 h. The resin-bound amine was filtered and washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and then dried under vacuum.

Coupling with (L)-Fmoc-Xaa-OH. The above resin-bound amine (6.3 mmol) was preswelled in 30 mL of dry CH_2CH_2. HOBt (3.65 g, 27.0 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.39 g, 3.2 mmol) were then added. To this mixture was added a solution of (L)-Fmoc-Xaa-OH (10.5 g, 27.0 mmol) in dry DMF (70 mL), followed by the addition of 1,3-diisopropylcarbodiimide (DIC) (4.23 mL, 27.0 mmol). The mixture was agitated using an orbital shaker at room temperature for 16 h. The resin was filtered, washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and dried under vacuum to give resin **3**.

General Procedure for Preparation of Acid 5. Deprotection of Fmoc Group of Resin 3. The above resin (4.0 g, 2.4 mmol, 0.60 mmol/g) was treated with 20% piperidine in DMF (50 mL) at room temperature for 2 h. The resin was filtered and washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and then dried under high vacuum, yielding 3.1 g of the resin-bound amine.

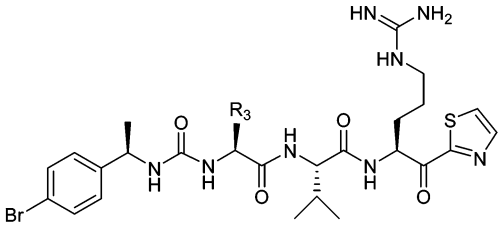
Formation of the Resin-Bound Amide 4a. The resin-bound amine from **3** (200 mg, 0.12 mmol) was treated with acid chloride (6 equiv, 0.72 mmol) in 2 mL of the mixture of pyridine/CH_2Cl_2 (1:9). The resulting slurry was agitated for 3 h at room temperature. The resin was filtered and washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and then dried under vacuum overnight to give resin **4a**.

Formation of the Resin-Bound Sulfonamide 4b. The resin-bound amine from **3** (300 mg, 0.18 mmol) was treated with sulfonyl chlorides (6 equiv, 1.2 mmol) in 3 mL of the mixture of 2,6-lutidine/CH_2Cl_2 (1:9). The resulting slurry was agitated for 3 h at room temperature. The resin was filtered and washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and then dried under vacuum overnight to give resin **4b**.

Formation of the Resin-Bound Urea 4c (Method A). The resin-bound amine from **3** (200 mg, 0.12 mmol, loading = 0.63 mmol/g) was treated with isocyanate (0.72 mmol, 6.0 equiv) and DMAP (0.5 equiv) in 2 mL of the mixture DMF/CH_2Cl_2 (2:1). The reaction mixture was shaken overnight at room temperature. The resin was filtered and washed with DMF → MeOH (four times) and CH_2Cl_2 (two times) and then dried under vacuum overnight to give resin **4c**.

Formation of the Resin-Bound Urea 4d (Method B). The resin-bound amine from **3** (3.0 g, 1.9 mmol) was treated with a freshly prepared 0.5 M solution of *p*-nitrophenyl chloroformate (3.81 g, 19 mmol, 10 equiv) and DIEA (3.3 mL, 19 mmol, 10 equiv) in 38 mL of THF/CH_2Cl_2 (1:1). The resulting slurry was agitated for 1 h at room temperature. The resin was filtered and washed with THF/CH_2Cl_2 (1:1) (three times), followed by the addition of (*R*)-1-(4-bromophenyl)ethylamine (3.8 g, 19 mmol, 0.5 M) and triethylamine (2.6 mL, 19 mmol, 0.5 M) in 38 mL of DMF. After 2 h of mixing, the resin was washed with DMF (four times) and THF/CH_2Cl_2 (1:1) (four times), then dried under vacuum overnight to give resin **4d**.

Table 5. SAR Exploration of R_3 Elements



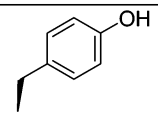
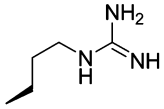
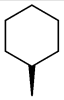
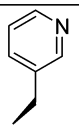
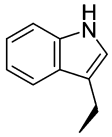
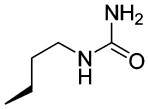
Comp No.	R_3	IC_{50} FXIa (nM)	IC_{50} FXa (nM)	IC_{50} thrombin (nM)
32		6	1,600	2,040
33		7	250	440
34		8	1,600	400
22		10	15,000	1,400
28		10	27,000	980
37		12	1,400	1,400
35		29	2,100	710
36		30	1,900	1,100

Table 6. In Vitro Anticoagulant Activity in Human Plasma

compd	IC_{50} (nM)	2× aPTT (μM)	2× PT (μM)
32	6	2.4	25.0
34	8	0.56	26.3
37	12	2.4	31.0
36	30	1.35	48.6
28	10	15.4	178.6

Cleavage. Resin **4** was swollen with 15 mL of CH_2Cl_2. The solvent was then drained. The resin was treated with 50% TFA/DCM (38 mL) at room temperature for 3 h. The resin was filtered and washed with CH_2Cl_2 (two times). The collected cleavage product was combined with washes and mixed with 1.5 mL of ^{i}PrOH. After concentration of solvents in vacuo, the collected yellow residue was triturated with Et_2O (50 mL), then filtered, and dried under vacuum, yielding a sufficiently pure product **5** for the next coupling step without further purification.

General Procedure of Coupling of Acid 5 and Amine 6 To Afford Product 1. Coupling Reaction of 5 with 6 (Scheme 3). The acid, **5**, (500 mg, 1.02 mmol), HOBt (168 mg, 1.24 mmol), and EDCI (257 mg, 1.34 mmol) were dissolved in dry THF (10 mL), followed by the addition of DIEA (0.53 mL, 3.1 mmol). Sonication of the mixture may be needed to assist solubility. After 5−10 min of stirring, a solution of the thiazolylarginol trifluoroacetic acid salt **6**[29,33] (511 mg, 0.9 mmol) in THF (4 mL) was added along with a THF (0.5 mL) rinse. The reaction mixture was stirred at room temperature for 3 h and then diluted with 100 mL of ethyl acetate. The organic layer was washed sequentially with 5% aqueous acetic acid (40 mL × 2), saturated aqueous sodium bicarbonate (40 mL × 2), water (40 mL), and brine. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo

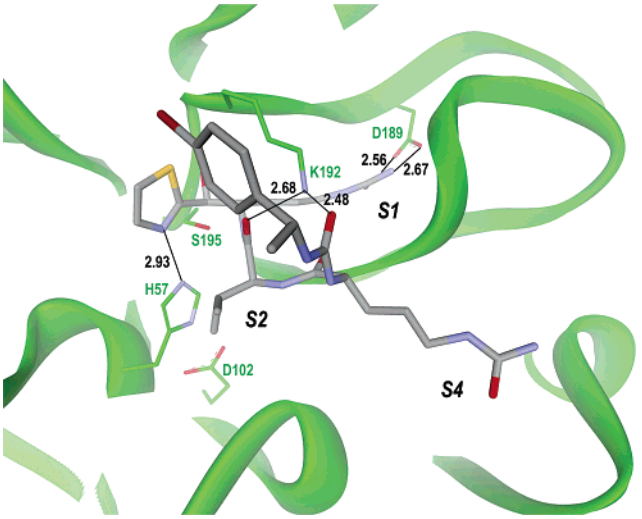


Figure 1. Active site of the crystal structure of FXIa−**36** complex. FXIa is in a green ribbon representation with S_1, S_2, and S_4 pockets labeled. Ligand **36** is in stick representation and colored according to atom type: oxygen in red, nitrogen in blue, sulfur in yellow, bromine in dark red, and carbon in gray. The catalytic triad (S195, H57, and D102) of FXIa and residues D189 and K192 are in stick representation with carbon atoms in green. Hydrogen-bonding interactions that are discussed in the text are shown in black lines with the distance indicated in angstroms

Chart 3. Schematic Representation of the Binding of **36** in the FXIa Substrate Site As Seen by X-ray Crystallography

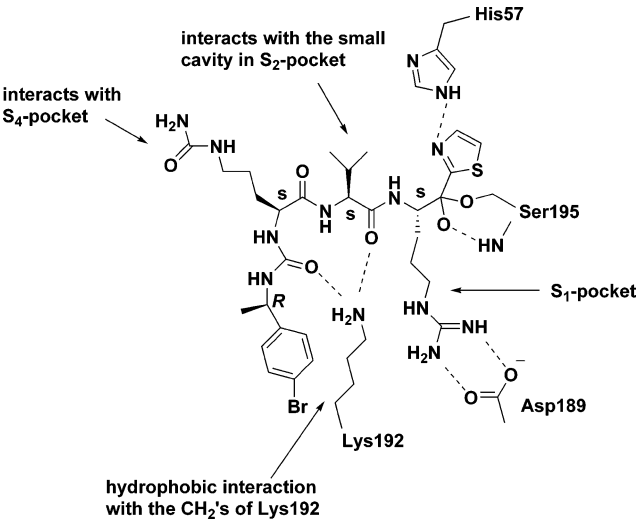
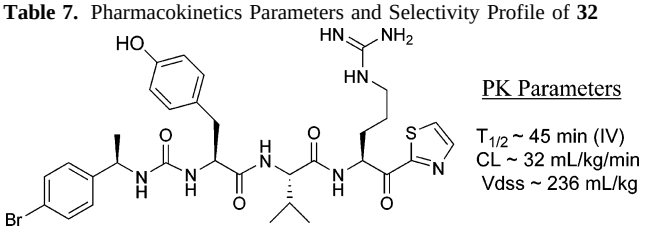


Table 7. Pharmacokinetics Parameters and Selectivity Profile of **32**



compd	FXIa	thrombin	FXa	FVIIa	plasma kallikrein	APC	trypsin
32	6[a]	2,040	1600	38000	10	20700	12

[a] All IC_{50} values are nanomolar.

to yield Mtr-protected **1** as yellow foam. LC-MS analysis indicated the presence of a trace amount of undesired diastereomeric isomers.

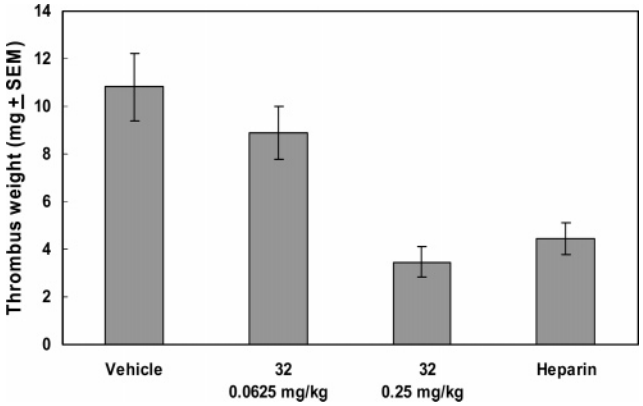


Figure 2. Efficacy of **32** in a rat model of venous thrombosis. The model used stenosis of the inferior vena cava with rapid infusion of hypotonic saline followed by partial stasis of blood flow to induce clot formation.

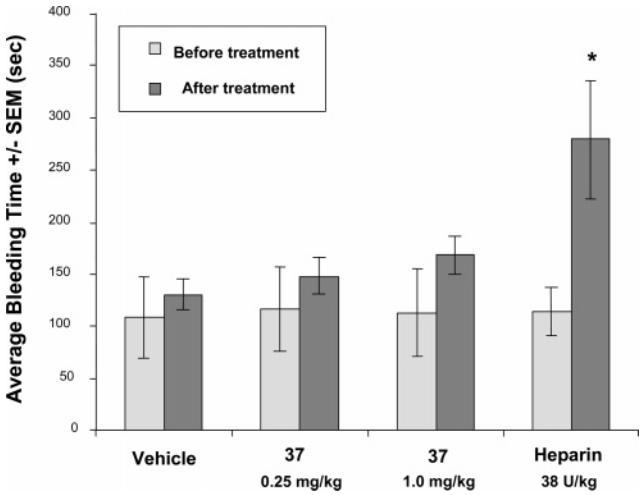


Figure 3. Effect of **37** on rat mesenteric arteriole bleeding time. The bleeding time of punctured mesenteric arterioles was determined prior to compound administration (light gray) and at 15 min after initiation of compound administration (dark gray). Compound **37** was administered as a continuous infusion into the femoral vein at 0.25 and 1 mg/kg per 15 min. Heparin was administered as a bolus injection followed by a continuous infusion into the femoral vein at a dose of 25 unit/kg + 50 units/kg/h (total dose = 38 units/kg). Results are expressed as mean bleeding times ± SEM (n = 6−8 per group: *, $p < 0.05$, Student's t test).

Deprotection of Mtr Group. The above coupling product (728 mg) was dissolved in 95% TFA (14.3 mL), 2.5% water (375 μL), and 2.5% thioanisole (375 μL). The resulting dark brown solution was stirred for 3 h at room temperature. The solution was concentrated under reduced pressure to a dark brown gum. The gum was triturated with diethyl ether. The ether supernatant was decanted. The ether trituration was repeated two more times. The yellow solid was dried under vacuum overnight to yield 720 mg of deprotected product **1** with a ratio of 2.3:1.0 mixture of the desired and undesired isomers. This crude mixture was purified by preparative HPLC to give the desired isomer with purity >95%.

Enzyme Affinity and Anticoagulant Activity. Inhibition Assay (IC_{50}). Peptidolytic activities for thrombin, trypsin, and factor XIa were measured using a fluorogenic reporter group, 7-methyl-4-aminocoumarin (AMC), attached via an amide linkage to the custom tripeptide pyroglutamic acid−proline−arginine (Glp-Pro-Arg). Commercially available substrate, H-Pro-Phe-Arg-AMC (BACHEM I-1295), was used for FXa activity. All assays were carried out in a Tris buffer (100 mM

Tris-HCl/200 mM NaCl and 0.02% Tween 20) at pH 7.4. Enzyme and substrate concentrations were 50 nM and 50 μM, respectively, for thrombin, trypsin, and factor XIa. Factor Xa required twice the concentrations. For all of the enzymes but factor Xa, the 10-point dose−response assays were run as endpoints after 15−30 min of incubation at 30 °C. Factor Xa required a longer, 60 min incubation time. Released AMC was measured at an emission wavelength of 460 nm on a Wallac 1420 Multilabel Counter excited at 355 nm using a continuous source. The data were fit to a dose−response one-site model using a nonlinear least-squares algorithm to determine the IC_{50} and Hill coefficient.

Human Plasma Based Clotting Assay. aPTT Assay. To determine the effect of compounds on coagulation pathway, activated partial thromboplastin time was determined using a Thromboscreen 400C instrument. This pathway involves FXII, kallikrein, and FXI, which activates factors IX and FVIII, leading to activation of FX and FV and then activation of factor II to form a blood clot. Plasma (50 μL) and different concentrations of compounds were added to cuvettes. After incubation for 2 min, aPTT reagent (ALEXIN, Sigma) was added (50 μL) and incubated for an additional 3 min. The cuvettes were transferred to a measuring position,and prewarmed $CaCl_2$ reagent (50 μL, 30 mM) was added. Readings were then taken over a maximum of 300 s. A dose−response curve was generated, and the concentration at which the clotting time was doubled (2× aPTT) was determined.

PT Assay. The PT assay was also used to measure inhibition of coagulation. In this assay, the factor XI dependent steps are bypassed. Hence, the assay measures inhibition of factor VIIa, factor Xa, and thrombin, but not FXI. This assay measures the ability of factor VIIa to activate factor X, which activates factor II to form a blood clot. The assay was performed using a Thromboscreen 400C instrument and thromboplatin reagent (ThromboMax with calcium, Sigma). Plasma (50 μL) and compounds were prewarmed at 37 °C for 3 min in the cuvettes. The cuvettes were transferred to the measuring position. The prewarmed thromboplastin reagent (100 μL) was then added, and readings were taken over 300 s. A dose−response curve was generated, and the concentration at which the clotting time was doubled (2× PT) was determined.

Rat Model of Venous Thrombosis. An established rat model of venous thrombosis was used to assess the antithrombotic potential of FXI inhibitors. Briefly, under anesthesia a stenosis of the inferior vena cava with rapid infusion of hypotonic saline followed by reduced blood flow was used to induce clot formation.[29,30] Rats were treated with vehicle alone (0.1 M NaOAc, pH 4.0), **32** at 0.0625 or 0.25 mg/kg intravenous infusion, or heparin (50 units/kg by intravenous bolus followed by a continuous infusion of 25 units/kg/h). Following a treatment time of 10 min, the clot was isolated and weighed. The thrombus weight in both the 0.25 mg/kg **32** and heparin-treated groups was significantly reduced compared to that found in the vehicle-treated animals ($p < 0.05$, $n = 9$, Tukey−Kramer multiple-comparison test) (see Supporting Information for complete experimental details).

Rat Mesenteric Arteriole Bleeding Time. The rat model used was modified from established techniques.[31,35,36] Briefly, male Sprague−Dawley rats were anesthetized with ketamine/xylazine. Body temperature was maintained at 37 °C. Compounds were administered through a catheter implanted in the left femoral vein. A small portion of the small intestine was exteriorized to display the mesenteric artery. Three arterioles branching from the mesenteric artery were punctured with a 30G needle while being superfused with saline at 37 °C. Bleeding time was determined as cessation of blood flow from the puncture sites. After the intestines were replaced, vehicle or heparin was administered as an intravenous bolus injection followed by continuous infusion via the femoral vein. Compound **37** was administered as a continuous infusion only. Fifteen minutes after the start of infusion, another small portion of the small intestine was exteriorized, and again three arterioles were punctured with a 30G needle and flushed with saline at 37 °C to determine bleeding time. The animals were then sacrificed by an anesthetic overdose.

Bleeding time from mesenteric arterioles was determined in each animal prior to compound administration and then after 15 min of continuous infusion. Compound infusion was continued during the determination of bleeding time. Animals with bleeding times of >210 s prior to compound administration were excluded from the study. If bleeding time was >9 min, the time was assumed to be 9 min for calculation purposes.

Protein Purification and X-ray Crystallography. The detailed expression and purification procedure of the mutated catalytic domain of FXIa (rhFXI$_{370-607}$-S434A,T475A,C482S,-K437A, hereinafter FXIac) and the crystallization method for the FXIac−benzamidine complex has been published.[28b] Compound **36** was added to a drop of crystallization solution (2.0 M ammonium sulfate, 0.1 M Tris-HCl, pH 8.5) in powder form. A FXIac−benzamidine crystal was soaked in the compound **36** containing solution overnight at 10 °C. After soaking, the crystal was briefly put into cryoprotection solution containing crystallization solution with 20% (v/v) glycerol before flash freezing in liquid nitrogen. X-ray diffraction data were collected on a RAXIS-IV^{++} image plate with an RU-H3R rotating anode X-ray source (Rigaku) equipped with blue Osmic mirrors and X-stream 2000 system (Rigaku). Data indexing, processing, and scaling were performed with HKL2000.[34] The structure of FXIac−compound **36** was solved using the structure of FXIac complexed with benzamidine (PDB code 1ZHR) with benzamidine and water molecules removed from the initial model. Refinement and difference maps were calculated by CNX (Accelrys), and ligand placement and manual rebuilding of FXIac were performed using QUANTA (Accelrys). The final model contains residues 370−606 of factor XI (residues 16−244 using chymotrypsin numbering), 128 water molecules, and the bound ligand. The crystal structure of **36** was refined to an R of 20.7% and an R_{free} of 22.6% at 2.25 Å resolution (PDB code 1ZOM).

Supporting Information Available: Detailed synthetic procedures and a table of characterization data of final α-ketothiazole products **7**−**37**. This material is available free of charge via the Internet at http://pubs.acs.org.

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