Allosteric Inhibition of Human Factor Xla: Discovery of Monosulfated Benzofurans as a Class of Promising Inhibitors

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ABSTRACT: Factor Xla (fXla) is being recognized as a prime target for developing safer anticoagulants. To discover synthetic, small, allosteric inhibitors of fXla, we screened an in-house, unique library of 65 molecules displaying many distinct scaffolds and varying levels of sulfation. Of these, monosulfated benzofurans were the only group of molecules found to inhibit fXla (~100% efficacy) and led to the identification of monosulfated trimer 24 (IC₅₀ 0.82 μM) as the most potent inhibitor. Michaelis–Menten kinetics studies revealed a classic noncompetitive mechanism of action for 24. Although monosulfated, the inhibitors did not compete with unfractionated heparin alluding to a novel site of interaction. Fluorescence quenching studies indicated that trimer 24 induces major conformational changes in the active site of fXla. Docking studies identified a site near Lys255 on the A3 domain of fXla as the most probable site of binding for 24. Factor Xla devoid of the A3 domain displayed a major defect in the inhibition potency of 24 supporting the docking prediction. Our work presents the sulfated benzofuran scaffold as a promising framework to develop allosteric fXla inhibitors that likely function through the A3 domain.

INTRODUCTION

Maintenance of hemostasis requires a delicate balance between coagulation and anticoagulation to prevent excessive bleeding while avoiding hemorrhage. Aberrant coagulation requires intervention with anticoagulants, which have primarily targeted two key proteases belonging to the common pathway of the coagulation cascade, namely, thrombin and factor Xa.1,2 Traditionally, inhibition of thrombin and factor Xa has been considered essential to induce effective anticoagulation. Yet, knocking out these proteases also eliminates hemostatic control leading to significant bleeding.3 An ideal anticoagulant would be able to parse thrombotic and hemostatic functions, and selectively modulate thrombosis. A growing paradigm in this direction is factor Xla (fXla) as a target of anticoagulant therapy.8

Structurally, fXla is a unique 160 kDa coagulation serine protease that differs from other proteases of the cascade in being a homodimer of identical subunits.9–11 Each subunit consists of four Apple domains (labeled A1, A2, A3, and A4) composed of 90–91 amino acids each at the N-terminus and a tryptophan-like catalytic domain (CD) at the C-terminus. The two subunits are held together in solution by an interchain Cys321–Cys321 bond. The active enzyme is formed from itszymogen fXI when factor Xla (fXla) cleaves the Arg269–Ile270 bond of each subunit. The fXla so formed then activates fIX to fIXa, which in turn sets up activation of the common pathway eventually amplifying clot formation. Interestingly, fXI can also be activated by the feedback action of thrombin, which is generated in early stages of coagulation.12 Factor Xla can also trigger its own formation from fXl.11 The multiple mechanisms of fXla formation and its contribution to the amplification of the procoagulant signal suggests that regulating its catalytic activity may have a cascading effect on thrombin generation with a concomitant reduction in coagulation flux. In addition, fXla also enhances activation of thrombin-activable fibrinolysis inhibitor, which is known to reduce the susceptibility of fibrin-rich clots to fibrinolytic agents.13 Thus, inhibiting fXla is expected to inhibit the generation of fibrinolysis inhibitor and help dissolve them faster through natural mechanisms, e.g., by plasmin action.

Multiple studies have highlighted fXla as a promising target for the development of safer anticoagulants. For example, fXI-null mice were much less susceptible to arterial and venous thrombosis in comparison to wild-type mice.14,15 More importantly, fXI-deficient mice grow healthy and do not suffer from bleeding.15 Studies with neutralizing antibodies against fXI in rabbits also demonstrated significant defects in thrombus formation.16 Finally, the natural deficiency of fXI, known as hemophilia C, has been reported to introduce a very benign bleeding phenotype in strong contrast to hemophiliacs associated with deficiencies of factors VIII and V.17–20 Thus, targeting this upstream protease appears to be a promising strategy for developing much safer anticoagulants than those being used in the clinic today.

We have embarked on a program to discover allosteric inhibitors of human fXla.21,22 Allosteric regulation of fXla has been demonstrated earlier through highly charged polyanions such as dextran sulfate, heparin, hypersulfated heparin, and sulfated pentagalloyl glucose (SPGG).21,22 Later work showed that synthetic molecules belonging to the monosulfated quinazolinone (QAO) scaffold were also allosteric inhibitors of...
Figure 1. Structures of the sulfated small molecules constituting the library screened for factor Xla inhibition. The group of 65 molecules displayed 1 to 8 sulfate groups per molecule, more than 12 different scaffolds, and possible three-dimensional conformation from linear to globular.
fXa. Sulfated QAOs are the only allosteric small molecule inhibitors of fXa reported to date.22 Although interesting, the molecules have shown moderate potency. We reasoned that it should be possible to discover better inhibitors by screening a library of sulfated small molecules.

We present the discovery of the class of monosulfated benzofurans as promising inhibitors of human fXa by screening an in-house library of sulfated small molecules prepared earlier. The library included 65 homogeneous molecules based on polysulfated and monosulfated scaffolds (Figure 1). These agents were synthesized in our laboratory earlier as potential modulators of coagulation, angiogenesis, and other processes.22,24–27 A specific monosulfated benzofuran trimer was identified as the most potent molecule that reduces the catalytic activity of fXa by binding at an allosteric site and inducing conformational changes in the catalytic triad. This work is expected to be especially useful in developing more potent allosteric inhibitors of fXa that are based on the sulfated small molecule scaffold.

## RESULTS

### Rationale for Screening a Library of Sulfated Small Molecules against Factor Xa.

As discussed above, our previous work led to the discovery of two classes of allosteric human fXa inhibitors including SPGG and sulfated QAO.21,22 Whereas SPGG was found to bind in the heparin-binding site of the enzyme, QAOs targeted a hydrophobic domain near the heparin-binding site. The fundamental reason why these groups of molecules appeared to recognize fXa was the presence of the sulfate group(s), which invoked interaction with one or more critical basic residue(s). Interestingly, human fXa displayed several hydrophobic domains adjacent to basic residues (see below for additional discussion on this). We reasoned that it should be possible to uncover a more potent sulfated small molecule that allosterically inhibits the enzyme by screening an in-house library (Figure 1) based on various scaffolds including sulfated flavonoids,24,25 sulfated tetrahydroisoquinoline,26,28,29 sulfated quinazolinone,22 sulfated benzofurans,30–32 and other sulfated small molecules.26,33 Each of these sulfated small molecules had been synthesized earlier in connection with attempts to discover inhibitors or activators of other coagulation proteins (antithrombin, factor Xa, or thrombin).

As a group, the library represents at least 12 distinct scaffolds and 65 unique molecules (Figure 1) possessing one to eight sulfate groups. The library contained sulfated hydrophobic molecules possessing one to several aromatic rings, except for two saccharide-based molecules 1 and 2. It included agents that are very small, e.g., 3, to the considerably large, e.g., 32, which displayed four benzofuran rings in linear sequence. A majority of the members display a projected size of ~10–20 Å. The three-dimensional shape of these molecules have not been studied as yet; however, the orientation of different aromatic rings ensures a range of structures from primarily linear (e.g., sulfated benzofurans) to significantly globular (e.g., sulfated flavonoids). The sulfated small molecules studied here are water-soluble, but their hydrophobic character spans a large range. For example, the sulfated flavonoids 51–57 are considerably less hydrophobic than the sulfated benzofurans 15–21, which in turn are less hydrophobic than the sulfated saccharides 1 and 2. Finally, while the sulfated flavonoids display less conformational flexibility, the presence of linkers connecting aromatic rings in the sulfated benzofurans induces considerable flexibility. Thus, overall, our library of sulfated small molecules presents considerable configurational, conformational, and sulfate density diversity to enhance the probability of a potent hit.

### Identification of Promising Inhibitors through Screening.

Screening of the library against fXa was performed using our earlier S-2366 hydrolysis assay21,22 adopted for medium throughput conditions. Figure 2 shows the results of the screen performed at 300 μM inhibitor concentration in 50 mM Tris-HCl buffer, pH 7.4, at 37 °C. Of the 65 compounds, 12 showed a reduction in residual fXa activity of more than 50% including 15, 21, 23–28, 30, 32, 61, and 63. These molecules belong to the sulfated benzofuran dimer, trimer, and tetramer class of compounds, except for 61 and 63. Yet, 61 and 63 were earlier synthesized to mimic the action of sulfated benzoferans suggesting a common structural pattern in the identified inhibitors. Interestingly, 10 monosulfated small molecules of the 25 present in the library were found to inhibit fXa well. In contrast, only two polysulfated agents of the 40 present in the library were active. Closely related sulfated benzoferans, e.g., 22 and 31, which are regioisomers of 16 and 27, respectively, did not inhibit fXa, suggesting a strong possibility of selective recognition. Of note was the observation that 29, an unsulfated benzofuran trimer, inhibited fXa only about 50%, while monosulfated analogues related to 29

![Figure 2. Medium-throughput screening of a library of 65 molecules against fXa in 50 mM Tris-HCl buffer, pH 7.4, at 37 °C using the chromogenic substrate hydrolysis assay. The concentration of each molecule was held constant at 300 μM. Experiments were performed at least in duplicate. The error shown represents standard deviation and was typically found to be less than 10%. The screening exercise identified not only inhibitors of factor Xa but also some activators including 33, 35, 36, and 37.](image-url)
inhibited nearly 100% suggesting a key role for the sulfate group on the benzofuran scaffold. Another interesting observation was that some sulfated small molecules enhanced Fxa catalysis by a substantial 20–30%. These included sulfated small molecules 33, 35, 36, and 37 (Figure 2). These molecules are highly sulfated and belong to the tetrahydroisoquinoline class.28,29 These factor Xa activators may be interesting from the perspective of enhancing coagulation and coagulation factor activation, especially in cases of hemophilia,34 they are not studied further in this work.

**Factor Xa Inhibition and Binding Potency of Promising Sulfated Small Molecules.** Inhibitors 15, 21, 23–28, 30, 32, 61, and 63 were studied further for Fxa inhibition by measuring S-2366 hydrolysis at concentrations spanning 3 log units (Figure 3), as described earlier.21,22 The decrease in the initial rate of Fxa activity as a function of inhibitor concentration was analyzed using the logistic dose–response equation to calculate inhibition parameters.

Each molecule studied exhibited inhibition for these interesting molecules. Typically, allosteric inhibitors display noncompetitive Michaelis–Menten kinetics, as noted for a wide range of sulfated inhibitors of coagulation enzymes.21,22,31,32 To assess the mechanism of inhibition, studies on the rate of substrate hydrolysis were performed using the traditional Michaelis–Menten equation, respectively, pH 7.4, at 37 °C. A decrease of 36 ± 1 and 52 ± 2% in the fluorescence emission at 522 nm was observed for both 21 and 24, respectively, suggesting change in electrostatics around the active site due to the interaction with the two inhibitors (Figure 4A). The equilibrium dissociation constants calculated using eq 2 were found to be 4.5 ± 0.35 μM for 21 and 1.2 ± 0.3 μM for 24. These constants are in the same range of IC50 as expected, on the basis of direct inhibition.

**Michaelis–Menten Kinetic Studies in the Presence of 21 and 24.** Considering that blocking the active site with a fluorophore (above) did not disrupt the interaction of 21 and 24 with Fxa, we suspected an allosteric mechanism of inhibition for these interesting molecules. Typically, allosteric inhibitors display noncompetitive Michaelis–Menten kinetics, as noted for a wide range of sulfated inhibitors of coagulation enzymes.21,22,31,32 To assess the mechanism of inhibition, studies on the rate of substrate hydrolysis were performed using a wide range of S-2366 concentrations (0.01–1.6 mM) in the presence of fixed concentrations of 21 (not shown) and 24 (Figure 4B). Analysis of the profiles using the traditional Michaelis–Menten kinetic equation showed that both inhibitors reduced the VMAX in a dose-dependent manner without a significant change in the KM (Table 2). This is characteristic of noncompetitive inhibition and illustrates that these inhibitors bind at a site away from the active site.

**Induction of Conformational Changes in the Active Site of Factor Xa by 24.** To further confirm the nature of the allosteric effect, we utilized collisional quenching experiments. We reasoned that if inhibitor 24 induces conformational changes in the active site, then a nonspecific, small collisional quencher, such as iodide, would reduce fluorescence emission of the active site probe at a rate different from that for the native enzyme. The phenomenon relies on direct molecular contact of sodium iodide with the fluorophore, which quenches...
fluorescence from the excited state. Typically, reduction in the molecular accessibility or the fluorophore, arising from an altered conformation or orientation, retards iodide’s quenching effect.

Figure 5A shows the iodide-induced fluorescence quenching of active-site-labeled fEGR-fXIa. For the enzyme alone, as the concentration of sodium iodide increased gradually to 0.175 M the fluorescence decreased nearly 50%, as expected. However, in the presence of saturating concentrations of 24 the rate of quenching was significantly lower (Figure 5A). Additionally, a bolus of inhibitor 24, so as to give ~33 μM concentration in fXIa solution, quenched with 0.175 M NaI resulted in a nearly full recovery of fluorescence to levels observed for the titration in the presence of 24. This suggested a fully reversible nature of interaction between 24 and fXIa.

Quantitative analysis of collisional quenching results can be performed using the Stern–Volmer theory. For dynamic quenching at low quencher concentrations, the incremental change in fluorescence (DF) as a function of the quencher concentration ([Q]) in the absence (Δ) and presence (■) of inhibitor 24. The arrow shows the recovery of fluorescence upon the addition of a bolus of 24 at 0.175 M quencher, which matches the fluorescence of the titration in the presence of 40 μM 24. (B) Stern–Volmer analysis of the quenching results presented above. Solid lines represent the linear fit to the data as predicted by the Stern–Volmer equation. The slopes of the linear fits are significantly different suggesting a change in the conformation of the active site of factor XIa upon interaction with 24.

Figure 5. (A) Profile of the decrease in fluorescence intensity of active site labeled fluorescein-EGR factor XIa (λEX = 480 nm; λEM = 522 nm) as a function of the quencher concentration [Q] in the absence (Δ) and presence (■) of inhibitor 24. The arrow shows the recovery of fluorescence upon the addition of a bolus of 24 at 0.175 M quencher, which matches the fluorescence of the titration in the presence of 40 μM 24. (B) Stern–Volmer analysis of the quenching results presented above. Solid lines represent the linear fit to the data as predicted by the Stern–Volmer equation. The slopes of the linear fits are significantly different suggesting a change in the conformation of the active site of factor XIa upon interaction with 24.

Table 2. Michaelis–Menten Kinetics of Human Factor XIa Hydrolysis of S-2366 in the Presence of 21 and 24a

| [S-2366] (mM) | VMAX (mAU/min) | KM (mM) |
|--------------|----------------|---------|
| 0 μM         | 43.1 ± 0.6c    | 0.25 ± 0.01 |
| 5 μM         | 37.4 ± 1.4     | 0.23 ± 0.02 |
| 7.25 μM      | 30.7 ± 0.6     | 0.25 ± 0.01 |
| 10 μM        | 20.3 ± 1.2     | 0.31 ± 0.04 |
| 24a          | 43.1 ± 0.6c    | 0.25 ± 0.01 |
| 0 nM         | 77.4 ± 1.7     | 0.26 ± 0.02 |
| 150 nM       | 57.1 ± 1.8     | 0.38 ± 0.03 |
| 300 nM       | 48.7 ± 1.7     | 0.35 ± 0.03 |
| 550 nM       | 34.9 ± 0.9     | 0.29 ± 0.02 |
| 900 nM       | 9.8 ± 0.5      | 0.28 ± 0.04 |

*KM and VMAX were measured by monitoring the initial rate of factor XIa hydrolysis of S-2366 from the linear increase in A245 in the presence of fixed concentrations of 21 and 24 in 50 mM Tris-HCl buffer, pH 7.4, at 37 °C. The data were fitted using the standard Michaelis–Menten equation to obtain KM and VMAX as described in Experimental Procedures. The concentrations of factor XIa and S-2366 were 0.365 nM and 0–1.6 mM. Errors represent ±standard deviation of the mean from at least two measurements. aThe concentrations of factor XIa and S-2366 were 0.765 nM and 0–1.6 mM.
predicted by the equation, and is indicative of the presence of only one type of fluorophore species in solution under the experimental conditions.

\[ \frac{F_0}{\Delta F} = \frac{1}{K_{SV} [Q]} + 1 \]

More importantly, $K_{SV}$ decreased dramatically from $8.5 \pm 0.3$ for fXIa alone to $2.3 \pm 0.1$ for the fXIa–24 complex. This implies that in the presence of 24 the active-site fluorophore is less accessible. Theoretically, such a change can be observed if either the ligand binds close to the fluorophore and sterically reduces the molecular accessibility of iodide or the ligand binds at an allosteric site and induces a change in the conformation of the active site, thereby altering fluorophore accessibility. Considering that the hydrodynamic volume of the quencher is small, which enables penetration into small cavities, the possibility of steric reduction of quenching effectiveness by 24 is less likely. In addition, Michaelis–Menten kinetics shows no $K_M$ defect, which could be expected if the fluorophore and 24 bound very close to each other. Thus, Stern–Volmer analysis predicts that inhibitor 24 induces a conformational change in the active site of fXIa. This conformational change is likely to be felt by the enzyme’s catalytic triad resulting in inhibition.

Do Sulfated Small Molecules Bind in the Heparin Binding Site of Factor Xla? Our previous work on allosteric inhibitors of fXla showed them to be either ideal or partial competitors of heparin.\(^{21–23}\) We suspected that inhibitors 21 and 24 may also display a similar feature considering their sulfated scaffold. Therefore, we performed competitive inhibition studies for both molecules in the presence of varying levels of unfractionated heparin (UFH). Heparin binds to fXla in two sites: in the A3 domain (Lys252, Lys253, and Lys255) and in the catalytic domain (Lys529, Arg530, Arg532, Lys535, and Lys539). Varying affinities have been reported for this interaction from 8.6 nM to 1.5 $\mu$M.\(^{21,22,36–39}\) Hence, we chose to use UFH concentrations in the range of 0 to 16 $\mu$M to assess competition rigorously, if any. Figure 6 shows fXIa inhibition by 21 and 24 in the presence of UFH. Surprisingly, no significant change in the IC$_{50}$ of these inhibitors was observed in the presence of UFH. This indicates that 21 and 24 do not compete with UFH for binding to fXla. Alternatively, the result indicates that the molecules probably bind to allosteric sites that do not impede simultaneous interaction with UFH.

Prediction of Site of Binding Using Molecular Docking Studies. We reasoned that molecular modeling may identify the site of binding of inhibitor 24. Docking studies have been used earlier, especially for sulfated benzofuran oligomers binding to exosite 2 of thrombin.\(^{31–33,40}\) However, docking studies with fXIa are not as straightforward considering that the crystal structure of full-length fXIa containing its four Apple domains is not available. Yet, the crystal structures of the full-length zymogen form of fXIa, i.e., fXI,\(^{41}\) and the catalytic domain of fXIa are available,\(^{17}\) which offer an interesting opportunity to generate a reasonable chimeric model of full-length fXIa from the two. The chimeric full-length fXIa was generated by replacing the inactive catalytic domain from the zymogen structure of fXI with that of fXIa. Interestingly, the chimera did not display any significant steric clashes, and minor clashes could be resolved by a simple energy minimization procedure. The catalytic domains of fXI and fXIa showed an overall RMSD of 0.44 Å for backbone atoms. Thus, the chimeric model appeared to be a reasonable model of full-length fXIa in solution.

Considering that little information was at hand on the possible site of binding, we reasoned that monosulfated benzofuran trimer 24 would bind to wild-type fXla utilizing a dual element strategy involving (1) initial attraction of its 5-OSO$_3^-$ group to one or more exposed Arg/Lys followed by (2) engagement of an adjacent hydrophobic patch to form a tight complex. We proposed this strategy earlier in the design of monosulfated QAOs and monosulfated benzofurans as inhibitors of fXIa and thrombin, respectively.\(^{22,31–33}\) Additionally, the current inhibition results also support the possibility of dual element strategy considering that the scaffold containing only one -OSO$_3^-$ group appeared to induce inhibition.

To assess the hypothesis of the dual element recognition more quantitatively, we studied the full-length fXIa model and identified eight sites on the protein surface displaying a relatively higher positive charge density and adjacent to a hydrophobic patch. These sites were centered around residues Lys8, Arg136, Gln153, Lys252, Lys255, Lys357, Asn566, and Arg584 (Figure 7A), which were stochastically selected as possible binding sites of inhibitor 24. All residues within 24 Å around the identified Arg/Lys were defined as the binding site for molecular docking purposes. This operation covered practically the entire protein surface, thus ensuring exhaustive exploration for identification of possible binding site(s).

Inhibitor 24 was docked at each of these eight sites using a genetic algorithm-based docking and scoring technique, as developed in the literature.\(^{43,44}\) We focused primarily on consistency of docking, as evident by the RMSD between docked poses, following multiple docking runs to derive...
Genetic algorithm-based docking studies to identify a putative binding site of \( 24 \) on factor \( \text{Xla} \). (A) Plausible sites of binding were identified by searching for hydrophobic subdomains in the vicinity of a basic (Arg/Lys) residue that could engage the sulfate group and aromatic scaffold of \( 24 \). Eight plausible sites in the vicinity of Lys25, Arg136, Gln153, Lys252, Lys325, Lys357, Asn566, and Arg854 (shown as blue van der Waals atom spheres) were identified by generating a chimeric model from the crystal structure of the catalytic domain (PDB ID: 1ZOM) and the heavy chain of factor XI (the zymogen, PDB ID: 2F83). (B) One specific site of docking near Lys255 was identified by GOLD as the most probable binding site of \( 24 \). GOLD predicted docking solutions within 1.6 Å RMSD suggesting highly selective recognition. Rings A, B, and C refer to the three benzofuran rings of \( 24 \). (C) Close-up of the \( 24 \)–factor \( \text{Xla} \) docked complex showing engagement of Lys255, Asn189, Tyr278, and Gln226, each of which shows ionic or hydrogen bond interactions, and plausible ionic \( \pi \)-cation interaction with Arg210 and ionic/\( \pi \)-\( \pi \) interaction with Phe206. The site is adjacent to Arg184 known to play an important role in factor IX activation by factor \( \text{Xla} \). See text for details.

The docking results suggested that the site around Lys252, present in the A3 domain of \( \text{fXla} \), was the only binding site that displayed high consistency of binding. Five out of six docked poses of \( 24 \) in this site displayed a RMSD of 1.6 Å (Figure 7B), which is much lower than the literature suggested cutoff of 2.5 Å for specific recognition.

An analysis of the atomic level interactions that appear to contribute to recognition of the site around Lys252 suggests that \( 24 \) engages Lys255, Asn189, Tyr278, and Gln226 residues. Lys255 and Asn189 are predicted to form ionic/\( \pi \)-hydrogen-bonding-type interactions with the \( 5 \)-\( \text{OSO}_3^- \) group of \( 24 \), while Gln226 and Tyr278 are predicted to hydrogen bond to benzofuran rings A and C (Figure 7C). In addition, Arg210 appears to form a ionic interaction with benzofuran ring C, and Phe206 is located within ionic/\( \pi \)-\( \pi \) stacking distance with the same benzofuran ring. The model predicts that nearly all components of the monosulfate benzofuran trimer interact with the site around Lys252, thereby favoring specific recognition.

The predicted binding geometry of \( 24 \) on the A3 domain of \( \text{fXla} \) explains the SAR of the benzosulfonamides quite well. The \( 5 \)-\( \text{OSO}_3^- \) group present on benzofuran ring A is essential for activity because it forms strong interactions with Lys255. To quantitatively evaluate its loss, we measured the inhibition potency of \( 29 \) and found it to be \( 384 \pm 38 \mu \text{M} \) (Figure 8A), a nearly 13-fold loss in activity from its sulfated analogue \( 30 \) (Table 1). The 6-ethoxy group of benzofuran ring A of \( 24 \) is predicted to favorably occupy a shallow hydrophobic subpocket (IC\( _{50} \) 0.82 \( \mu \text{M} \)). This subpocket can presumably accommodate smaller groups, e.g., the methoxy group of inhibitors \( 23 \) (IC\( _{50} \) 14.9 \( \mu \text{M} \)) and \( 27 \) (IC\( _{50} \) 6.4 \( \mu \text{M} \)) but not bulkier groups, e.g., the isoproxy group of \( 25 \) (IC\( _{50} \) 47 \( \mu \text{M} \)) (see Figure 1 and Table 1). Finally, the model also explains the weaker IC\( _{50} \) of inhibitor \( 30 \) (29.9 \( \mu \text{M} \)) reasonably well. Inhibitor \( 30 \), which contains a 3-carboxylate group in benzofuran ring C, appears to be 4.7-fold less potent than its 3-carboxyethyl ester analogue \( 27 \), possibly because of the unfavorable interactions of the anionic group within the primarily hydrophobic binding pocket.

Interestingly, the predicted site of binding of \( 24 \) involves Lys255, which is known to interact with UFH.\(^{37,38} \) As stated above, heparin binds at two sites on full-length \( \text{fXla} \). Whereas the site on the enzyme’s catalytic domain is known to be allosterically coupled to the active site, the site on the A3 domain was thought of as primarily contributing to the bridging mechanism of \( \text{fXla} \) inhibition.\(^{10,36} \) This work predicts that the A3 site may also be allosterically linked to the active site. This prediction is novel and valuable for the design of advanced analogues, yet it is important to note that the computational results will require further support from site-directed mutagenesis and/or crystallography studies. It is not clear at this time why UFH does not compete with \( 24 \) (Figure 6), although both appear to utilize Lys255. One plausible reason is that UFH binds to the A3 domain with a weaker affinity than its interaction with the catalytic domain. Comparative SPR studies have shown that full-length \( \text{fXla} \)
binds UFH with 8.6 nM affinity, which is virtually identical to that of the FXa catalytic domain alone (11.2 nM). Thus, the 16 μM UFH concentration used in competition experiments may not have saturated the A3 binding site fully resulting in a lack of competition. Another much more plausible explanation is the role of hydrophobic forces for inhibitor 24 binding to FXa. It is possible that the highly ionic UFH utilizes a polar protein interface that avoids the hydrophobic interface utilized by inhibitor 24. In fact, other residues of the heparin-binding site containing Lys255, i.e., Lys252 and Lys253, are located away from the site of predicted binding site of 24. This means that although the two molecules utilize Lys255, their orientations are likely to be completely different resulting in the absence of competition.

Inhibition of the Catalytic Domain of Factor Xla. A good avenue to test the above model of the FXa–24 complex is site-directed mutagenesis. Unfortunately, these mutants of FXa are not available immediately. However, we reasoned that if the A3 domain is involved in this process, then the 24 inhibition potential would be impaired by its removal. To test this, we studied the inhibition of the catalytic domain of factor Xla (FXa-CD), which is devoid of all Apple domains, by 24 (Figure 8B). The results show that FXa-CD was inhibited with an IC₅₀ of 49 ± 2 μM suggesting a ~40-fold loss in potency from the full-length FXa (1.2 ± 0.2 μM). This significant loss in activity supports the modeling prediction but also suggests that our monosulfate benzofuran trimer 24 may engage the catalytic domain alone with much weaker affinity. Such dual recognition is not unusual considering that UFH also displays two binding sites. Thus, we predict that inhibitor 24 (and possibly other monosulfated benzofuran) bind to FXa primarily in the A3 domain near Lys255 and induce the conformational disruption of its catalytic site, resulting in inhibition.

**DISCUSSION**

This work presents the discovery of a class of monosulfated benzofurans as human FXa inhibitors. Specifically, a monosulfated benzofuran trimer (24) was found to be the most promising inhibitor. In comparison to our earlier studies, 24 is nearly 50-fold more potent than the best sulfated QAO designed earlier. In fact, a monosulfated benzofuran dimer (21) also shows 10-fold improvement from earlier work, and several other analogues were moderately potent. In addition to the improvement in inhibition realized through screening, this work presents a small group of molecules that enhance the activity of factor Xla. This observation is likely to be considerably important to developing procoagulants that may have application in the field of hemophilia. Also, this is the first time a library of this size has been studied with regard to sulfated GAG mimetics. Traditionally, it has been assumed that interactions of sulfated molecules with proteins depend only on the presence of sulfate group(s). This work shows that this assumption should be questioned because several scaffolds with higher sulfate density were completely inactive. Likewise, the structural diversity represented by the library highlights the specificity of interaction arising form the monosulfated benzofurans.

Homogenous monosulfated benzofuran dimers and trimers were designed earlier as human thrombin inhibitors based on results with oligomeric sulfated low molecular weight lignins. Interestingly, the inhibitors target a heparin-binding site on thrombin, i.e., exosite 2, whereas competition with UFH suggested that 24 and 21 do not target the equivalent exosite on the catalytic domain of FXa. Instead, we predict that inhibitor 24 binds to a site on the A3 domain of full-length FXa. If future structural biology studies confirm this prediction, inhibitor 24 would be the first molecule that disrupts catalytic function through A3 recognition.

Despite variance in the putative binding sites, the respective activities of these inhibitors against the two related serine proteases, thrombin and FXa, are essentially equivalent. This is an interesting coincidence because the binding sites are significantly different at an atomic level. Although monosulfated benzofurans may appear to be relatively nonselective between thrombin and FXa, further structural modification of the scaffold/groups can be expected to yield selective agents. Thus, although the affinity of 24 is the best observed so far against FXa, these agents cannot be deemed as promising clinically relevant candidates at the present time.

Yet, the monosulfated benzofurans are interesting because of their allosteric mechanism of action. Targeting an allosteric site can potentially provide two advantages: selectivity of recognition and ability to fine-tune inhibition potential. As compared to orthosteric sites, especially of coagulation enzymes, allosteric sites are more structurally diverse, which enhances the probability of higher selectivity. In addition, allosterism relies on energetic coupling between the active site and site of inhibitor binding, which arises in the form of conformational changes that can theoretically be modulated by appropriate inhibitor design. Orthosteric or competitive inhibition, however, blocks all activity. Thus, the observation that molecule 24 is a true allosteric inhibitors bodes well for further structure-based design of molecules that may exhibit better selectivity and tunability of inhibition.

Allosterism in the 24–FXa interaction was deduced from fluorescence quenching experiments, which showed a significant change in conformation in or near the active site. Inhibitor 24 induced less susceptibility of the active site fluorophore to quenching by sodium iodide. This is a classic experiment used in the literature to decipher significant conformational changes. The results suggest that monosulfated benzofurans probably bring about a physical closing of the active site so as to restrict access to species as small as iodide. The physical closing of the active site is not expected to involve changes in the α-helix/β-sheet content of the enzyme, which implies that other techniques such as CD and FTIR are less likely to report on the allosteric nature of interactions being studied here.

How is this physical closing of the active site brought about? The A3 domain has been known to be a key regulator of factor IX activation by FXIa. It has been suggested that Arg184 within the A3 domain undergoes a dramatic movement upon activation of zymogen FXI to FXa. More specifically, Arg184 moves from a hindered position in its cavity in FXI, where it is bound to Ser268, Asp488, and Asn566, to an exposed orientation in FXIa so as to enable its interaction with factor IX. Our proposed binding site of 24 is in a pocket adjacent to Arg184 (Figure 7C). It is likely that inhibitor 24 restricts the movement of the catalytic domain through its interactions with the loop that hosts Arg184 and its interacting partners including Asn566. Another way to explain the same point is that inhibitor 24 possibly transforms FXIa into its zymogen-like conformation in which Arg184 movement is hindered resulting in steric restriction on access to the active site present on the catalytic domain. This results in inhibition. This allosteric effect theory is of much interest and will be the subject of further work on structure-based design of advanced molecules.
Overall, this fundamental work has realized promising monosulfated benzo[uran dimers and trimers as potent inhibitors of human FXa. Our work shows that the molecules display an allosteric mechanism of inhibition that induces conformational changes in the enzyme’s active site. Molecular modeling studies have predicted a specific recognition site for inhibitor 24 (and possibly other analogs), which is a unique site that is recognized to play a major role in FXa biochemistry. The work highlights the idea that the A3 binding domain on full-length FXa may be targeted for the design of advanced allosteric FXa regulators.

**Experimental Procedures**

**Materials.** Human factor XIa (FXa and active-site labeled fluorescein-EGF-DXa (fEGF-DXa)) was purchased from Haematologic Technologies (Essex Junction, VT). Recombinant FXa containing only the catalytic domain (DXa-CD) was a gift from Dr. Alireza Rezaie (St. Louis University, MO). Chromogenic substrate S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline) was purchased from Diapharma (West Chester, OH). Stock solutions of FXa were prepared in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.1% PEG8000. The buffer used in inhibition studies was 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.1% PEG8000, and 0.02% Tween80, while that used for all other studies was devoid of 0.02% Tween80. All of the other chemicals were of biochemical grade and purchased either from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA). The sulfated small molecules used in this study were prepared earlier, as described in a series of articles, and were more than 95% pure. In these studies, purity was assessed by a combination of techniques including HPLC/UPLC, HR-MS, and/or elemental analysis.

**Screening of a Library of Factor Xa Inhibition.** A library of 65 sulfated small molecules were screened against FXa using a chromogenic substrate hydrolysis assay adopted to 96-well microplate format (FlexStation III, Molecular Devices, Sunnyvale, CA) following previous reports on traditional screening. Briefly, potential inhibitors (or control solvent) and FXa were added to pH 7.4 buffer at 37°C so as to provide final concentrations of 300 μM and 0.765 nM, respectively, incubated for 10 min, and S-2366 added to each well to give 330 μM concentration. The initial rate of S-2366 hydrolysis (<10% substrate conversion) was then measured from the increase in absorbance at 405 nm as a function of time. The residual FXa activity (%), was calculated from the ratio of the initial rates in the presence and absence of a potential inhibitor. At least two independent experiments were performed, and the data averaged to calculate the mean and standard deviation.

**Quantification of the Inhibition of Wild-Type Factor Xa or Factor XIa-Catalytic Domain.** The inhibition profile of molecules displaying more than 40% relative inhibition in the initial screen was quantified using the 96-well microplate assay, which was modified from our previous reports. Briefly, 5 μL of FXa (wild-type or catalytic domain) was added to 85 μL of pH 7.4 buffer followed by the addition of 5 μL of inhibitor solutions, which were prepared as serial dilutions of the stock in 2/3rd (or 5/6th) decrements. The enzyme’s final concentration was 0.765 nM. The mixture was then incubated for 10 min at 37°C followed by the addition of 5 μL of S-2366 (final concentration = 330 μM) to each well, and the initial rate of increase in A_{405} was recorded to calculate the residual activity (Y, in %), which was plotted as a function of the log of inhibitor concentration (log[I]₀) and fitted using eq 1 (below) to derive the concentration of the inhibitor that results in 50% inhibition of enzyme activity (IC₅₀) and the Hill slope (HS). In this equation, Yₐ and Y₀ are the maximum and minimum values of the residual activity (Y), respectively.

\[
Y = Y₀ + \frac{Yₐ - Y₀}{1 + 10^{(log[I]₀ - log(IC₅₀)}/HS}
\]

**Fluorescence Spectroscopic Studies of Sulfated Inhibitors Binding to FXa.** The fluorescence emission spectrum of fluoresceinylated FXa (fEGF-DXa) in the presence and absence of 21 and 24 was measured by exciting at 480 nm in pH 7.4 buffer at 37°C using a QM4 spectrophuorometer (Photon Technology International, Birmingham, NJ). The excitation and emission slit widths were set at 1 nm. A semimicrocuvette having a 2 mm and 10 mm path length on the excitation and emission sides, respectively, containing 250 μL total volume and inhibitors 21 and 24 at 120 μM and 8 μM, respectively, and fEGF-DXa at 74 nM was used. The wavelength of maximal fluorescence emission was found to be 522 nm for both 21 and 24.

The affinity of both inhibitors was measured with a similar setup by recording the fluorescence intensity at λ_{522} nm as a function of the concentration of the ligands. The relative change in fluorescence (ΔF/F₀) as a function of the inhibitor concentration could be fitted using quadratic binding eq 2 to yield the dissociation constant of interaction. In this equation, ΔF_{MAX} represents the maximal change in fluorescence observed when the enzyme is saturated with the inhibitor.

\[
\Delta F = \Delta F_{MAX} \left[ (\frac{[\text{I}]}{K_{D}}) + \left( \frac{[\text{I}] + K_{D}}{K_{M}} \right) \right] \frac{K_{M} [\text{I}]}{2 [\text{I}]}
\]

Collisional quenching studies were performed with sodium iodide in pH 7.4 buffer at 37°C. The quenching of active site fluorescein fluorescence of fEGF-DXa (74 nM) by NaI (0–0.175 M) was measured as reported in the literature in the presence (40 μM) and absence of inhibitor 24.

**Michaelis–Menten Kinetics in the Presence of Inhibitors.** The initial rate of S-2366 hydrolysis by FXa was measured, as described earlier, in pH 7.4 buffer at 37°C. The concentration of substrate (0.01–1.6 mM) was varied, and the concentrations of inhibitor (0–16 μM) and enzyme (0.765 nM) were held constant. The initial rate of hydrolysis was calculated from the linear increase in A_{405} at substrate concentration. The hyperbolic profile of the initial rate versus S-2366 concentration was fitted using the standard Michaelis–Menten equation to obtain the K_{M} and V_{MAX} values, where V_{MAX} is the maximum velocity of the enzyme reaction, and K_{M} is the Michaelis–Menten constant.

**Competitive Inhibition Studies Using Unfractionated Heparin as a Competitor.** Inhibition of FXa by inhibitors 21 and 24 was measured in the presence of fixed concentrations of UFH (0–16 μM) in pH 7.4 buffer at 37°C on FlexStation III (Molecular Devices, Sunnyvale, CA). Serial dilutions of 21 and 24 were made in such a manner that each dilution was 5/6th of the previous. Briefly, 5 μL of FXa (final concentration = 1.5 nM) was added to 85 μL of pH 7.4 buffer followed by the addition of 5 μL of inhibitor solutions. The mixture was then incubated for 10 min followed by the addition of 5 μL of S-2366 (345 μM). The initial rate of S-2366 hydrolysis was measured from A_{405} increase. The apparent IC₅₀ was obtained using eq 1.

**Molecular Modeling Studies.** A model for the full-length active FXa was generated by replacing the inactive catalytic domain of thezymogen form (PDB ID: 2B83) with the activated catalytic domain (PDB ID: 1zom) crystal structure using Pymol, version 1.5.0.4 (Schrodinger, LLC). Further modeling was performed using the protein preparation tool of Tropos Sybyl-X, version 2.1 (www.tripos.com/sybyl). Hydrogens were added to the chimeric structure and minimized keeping all heavy atoms as aggregates. Inhibitor 24 was modeled in Sybyl and docked into the structure of the chimera using GOLD at eight probable sites of binding without any constraints.
These sites were defined as 24 Å around residues Lys8, Arg136, Gln153, Lys252, Lys325, Lys357, Asn566, and Arg584 (shown in Figure 7A). For each site, a 1000 genetic algorithm run was employed in which the early termination option was disabled. Automatic cavity detection was permitted. Each docked pose was scored using GOLDSCORE, and the top two poses were retained. Triplicate docking runs were employed to ensure the docked poses were reproducible, giving us 6 docked poses per site. Average RMSD across the docked poses was ascertained using an in-house code utilizing the OEChem toolkit, version 1.7.7 (OpenEye Scientific Software, Inc., Santa Fe, NM, USA).

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**Notes**
The authors declare no competing financial interest.

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**REFERENCES**
(1) Cove, C., L.; Hylek, E. M. An updated review of target-specific oral anticoagulants used in stroke prevention in atrial fibrillation, venous thromboembolic disease, and acute coronary syndromes. *J. Am. Heart Assoc.* 2013, 2, e000136.
(2) Henry, B., L.; Desai, U. R. Anticoagulants. In *Burger’s Medicinal Chemistry, Drug Discovery and Development*, 7th ed.; Rotella, D., Abraham, D. J., Eds.; John Wiley and Sons: New York, 2010; pp 365–408.
(3) Liew, A.; Eikelboom, J. W.; O’Donnell, M.; Hart, R. G. Assessment of anticoagulation intensity and management of bleeding with old and new oral anticoagulants. *Can. J. Cardiol.* 2013, 29, S34–44.
(4) Simonson, C. Z.; Steiner, T.; Tietze, A.; Damgaard, D. Dagabatran-related intracerebral hemorrhage resulting in hematomata expansion. *J. Stroke Cerebrovasc. Dis.* 2014, 23, e133–134.
(5) Pfeilschifter, W.; Luger, S.; Brunrhorst, R.; Lindhoff-Last, E.; Foerch, C. The gap between trial data and clinical practice - An analysis of case reports on bleeding complications occurring under dabigatran and rivaroxaban anticoagulation. *Cerebrovasc. Dis.* 2013, 36, 115–119.
(6) Villa, L. A.; Malone, D. C.; Ross, D. Evaluating the efficacy and safety of apixaban, a new oral anticoagulant, using Bayesian meta-analysis. *Int. J. Hematol.* 2013, 98, 390–397.
(7) Cossette, B.; Pelletier, M. E.; Carrier, N.; Turgeon, M.; Lecclair, C.; Charron, P.; Eichenberg, D.; Fayad, T.; Farand, P. Evaluation of bleeding risk in patients exposed to therapeutic fractionated or low-molecular-weight heparin: a cohort study in the context of a quality improvement initiative. *Ann. Pharmacother.* 2010, 44, 994–1002.
(8) Schumacher, W. A.; Uettgen, J. M.; Quan, M. L.; Seifert, D. A. Inhibition of factor XIa as a new approach to anticoagulation. *Arterioscler., Thromb., Vasc. Biol.* 2010, 30, 388–392.
(9) Wu, W.; Sinha, D.; Shikov, S.; Yip, C. K.; Wala, T.; Billings, P. C.; Lear, J. D.; Walsh, P. N. Factor XI homodimer structure is essential for normal proteolytic activation by factor XIIa, thrombin, and factor Xa. *J. Biol. Chem.* 2008, 283, 18655–18664.
(10) Emsley, J.; McEwan, P. A.; Gailani, D. Structure and function of factor XI. *Blood* 2010, 115, 2569–2577.
(11) Beng, Y.; Verhamme, I. M.; Smith, S. B.; Sun, M. F.; Matafonov, A.; Cheng, Q.; Smith, S. A.; Morrissey, J. H.; Gailani, D. The dimeric structure of factor X and zymogen activation. *Blood* 2013, 121, 3962–3969.
(12) Baglia, F. A.; Walsh, P. N. Thrombin-mediated feedback activation of factor XI on the activated platelet surface is preferred over contact activation by factor XIIa or factor Xa. *J. Biol. Chem.* 2000, 275, 20514–20519.
(13) Von dem Borne, P. A.; Bajzar, L.; Meijers, J. C.; Nesheim, M. E.; Bouma, B. N. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. *J. Clin. Invest.* 1997, 99, 2323–2327.
(14) Renne, T.; Oschatz, C.; Seifert, S.; Muller, F.; Antovic, J.; Karlman, M.; Benz, P. M. Factor XI deficiency in animal models. *J. Thromb. Haemost.* 2009, 7 (Suppl 1), 79–83.
(15) Gailani, D.; Lasky, N. M.; Broze, G. J., Jr. A murine model of factor XI deficiency. *Blood Coagul Fibrinolys.* 1997, 8, 134–144.
(16) Yamashita, A.; Nishihira, K.; Kitazawa, T.; Yoshikashi, K.; Soeda, T.; Esaki, K.; Imamura, T.; Hattori, K.; Asada, Y. Factor XI contributes to thrombus propagation on injured neointima of the rabbit iliac artery. *J. Thromb. Haemost.* 2006, 4, 1496–1501.
(17) Seligsohn, U. Factor XI deficiency in humans. *J. Thromb. Haemost.* 2009, 7 (Suppl 1), 84–87.
(18) Gomez, K.; Bolton-Maggs, P. Factor XI deficiency. *Haemophilia* 2008, 14, 1183–1189.
(19) Asakai, R.; Chung, D. W.; Davie, E. W.; Seligsohn, U. Factor XI deficiency in Ashkenazi Jews in Israel. *N. Engl. J. Med.* 1991, 325, 153–158.
(20) Duga, S.; Salomon, O. Factor XI deficiency. *Semin. Thromb. Hemost.* 2009, 35, 416–425.
(21) Al-Horani, R. A.; Ponnusamy, P.; Mehta, A. Y.; Gailani, D.; Desai, U. R. Sulfated pentagalloypglucoside is a potent, allosteric, and selective inhibitor of factor XIa. *J. Med. Chem.* 2013, 56, 867–878.
(22) Karuturi, R.; Al-Horani, R. A.; Mehta, S. C.; Gailani, D.; Desai, U. R. Discovery of allosteric modulators of factor XIa by targeting hydrophobic domains adjacent to its heparin-binding site. *J. Med. Chem.* 2013, 56, 2415–2428.
(23) Sinha, D.; Badellino, K. O.; Marcinkiewicz, M.; Walsh, P. N. Allosteric modification of factor XIa functional activity upon binding to polyanions. *Biochemistry* 2004, 43, 7593–7600.
(24) Gunnarsson, G. T.; Desai, U. R. Designing small, nonsugar activators of antithrombin using hydropathic interaction analyses. *J. Med. Chem.* 2002, 45, 1233–1243.
(25) Gunnarsson, G. T.; Desai, U. R. Exploring new nonsugar sulfated molecules as activators of antithrombin. *Bioorg. Med. Chem. Lett.* 2007, 13, 679–683.
(26) Raghuraman, A.; Rajz, M.; Hindle, M.; Desai, U. R. Rapid and efficient microwave-assisted synthesis of highly sulfated organic scaffolds. *Tetrahedron Lett.* 2007, 48, 6754–6758.
(27) Raman, K.; Karuturi, R.; Swarup, V. P.; Desai, U. R.; Kubera, B. Discovery of novel sulfonated small molecules that inhibit vascular tube formation. *Bioorg. Med. Chem. Lett.* 2012, 22, 4467–4470.
(28) Raghuraman, A.; Liang, A.; Krishnasamy, C.; Lauck, T.; Gunnarsson, G. T.; Desai, U. R. On designing non-saccharide, allosteric activators of antithrombin. *Eur. J. Med. Chem.* 2009, 44, 2626–2631.
(29) Al-Horani, R. A.; Liang, A.; Desai, U. R. Designing nonsaccharide, allosteric activators of antithrombin for accelerated inhibition of factor Xa. *J. Med. Chem.* 2011, 54, 6125–6138.
(30) Verghese, P.; Liang, A.; Sidhu, P. P.; Hindle, M.; Zhou, Q.; Desai, U. R. First steps in the direction of synthetic, allosteric, direct inhibitors of thrombin and factor Xa. *Bioorg. Med. Chem. Lett.* 2009, 19, 4126–4129.
(31) Sidhu, P. S.; Liang, A.; Mehta, A. Y.; Abdel Aziz, M. H.; Zhou, Q.; Desai, U. R. Rational design of potent, small, synthetic allosteric inhibitors of thrombin. *J. Med. Chem.* 2011, 54, 5522–5531.
(32) Sidhu, P. S.; Abdel Aziz, M. H.; Sarkar, A.; Mehta, A. Y.; Zhou, Q.; Desai, U. R. Designing allosteric regulators of thrombin. Exosite 2 features multiple subsites that can be targeted by sulfated small molecules for inducing inhibition. J. Med. Chem. 2013, 56, 5059–5070.

(33) Sidhu, P. S.; Mosier, P. D.; Zhou, Q.; Desai, U. R. On scaffold hopping: challenges in the discovery of sulfated small molecules as mimetics of glycosaminoglycans. Bioorg. Med. Chem. Lett. 2013, 23, 355–359.

(34) Whelihan, M. F.; Orleo, T.; Gissel, M. T.; Mann, K. G. Coagulation procofactor activation by factor Xa. J. Thromb. Hemost. 2010, 8, 1532–1539.

(35) Lakowicz, J. R. Quenching of Fluorescence. In Principles of Fluorescence Spectroscopy, 3rd ed.; Springer: New York, 2006, pp 278–330.

(36) Yang, L.; Sun, M. F.; Gailani, D.; Rezaie, A. R. Characterization of a heparin-binding site on the catalytic domain of factor Xa: mechanism of heparin acceleration of factor Xa inhibition by the serpins antithrombin and Cl-inhibitor. Biochemistry 2009, 48, 1517–1524.

(37) Zhao, M.; Abdel-Razek, T.; Sun, M. F.; Gailani, D. Characterization of a heparin binding site on the heavy chain of factor XI. J. Biol. Chem. 1998, 273, 31153–31159.

(38) Ho, D. H.; Badellino, K.; Baglia, F. A.; Walsh, P. N. A binding site for heparin in the apple 3 domain of factor XI. J. Biol. Chem. 1998, 273, 16382–16390.

(39) Badellino, K. O.; Walsh, P. N. Localization of a heparin binding site in the catalytic domain of factor Xa. Biochemistry 2001, 40, 7569–7580.

(40) Abdel Aziz, M. H.; Sidhu, P. S.; Liang, A.; Kim, J. Y.; Mosier, P. D.; Zhou, Q.; Farrell, D. H.; Desai, U. R. Designing allosteric regulators of thrombin. Monosulfated benzofuran dimers selectively interact with Arg173 of exosite 2 to induce inhibition. J. Med. Chem. 2012, 55, 6888–6897.

(41) Papagrignioriou, E.; McEwan, P. A.; Walsh, P. N.; Emsley, J. Crystal structure of the factor XI zymogen reveals a pathway for transactivation. Nat. Struct. Mol. Biol. 2006, 13, 557–558.

(42) Lin, J.; Deng, H.; Jin, L.; Pandey, P.; Quinn, J.; Cantin, S.; Rynkiewicz, M. J.; Gorga, J. C.; Bibbins, F.; Celatka, C. A.; Nagafuji, P.; Bannister, T. D.; Meyers, H. V.; Babine, R. E.; Hayward, N. J.; Weaver, D.; Benjamin, H.; Stassen, F.; Abdel-Meguid, S. S.; Strickler, J. E. Design, synthesis, and biological evaluation of peptidomimetic inhibitors of factor Xa as novel anticoagulants. J. Med. Chem. 2006, 49, 7781–7791.

(43) Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. 1997, 267, 727–748.

(44) Raghuraman, A.; Mosier, P. D.; Desai, U. R. Finding a needle in a haystack. Development of a combinatorial virtual screening approach for identifying high specificity heparin/heparan sulfate sequence(s). J. Med. Chem. 2006, 49, 3553–3562.

(45) Hu, X.; Balaz, S.; Shelver, W. H. A practical approach to docking of zinc metalloproteinase inhibitors. J. Mol. Graphics Modelling 2004, 22, 293–307.

(46) Gohlke, H.; Hendlich, M.; Klebe, G. Knowledge-based scoring function to predict protein-ligand interactions. J. Mol. Biol. 2000, 295, 337–356.

(47) Henry, B. L.; Monien, B. H.; Bock, P. E.; Desai, U. R. A novel allosteric pathway of thrombin inhibition. Exosite II mediated potent inhibition of thrombin by chemo-enzymatic, sulfated dehydropolymers of 4-hydroxycinnamic acids. J. Biol. Chem. 2007, 282, 31891–31899.

(48) Svilagyi, A.; Nussinov, R.; Csermely, P. Allo-network drugs: extension of the allosteric drug concept to protein–protein interaction and signaling networks. Curr. Top. Med. Chem. 2013, 13, 64–77.

(49) Thompson, A. D.; Dugan, A.; Gestwicki, J. E.; Mapp, A. K. Fine-tuning multiprotein complexes using small molecules. ACS Chem. Biol. 2012, 7, 1311–1320.

(50) Hedstrom, L. Serine protease mechanism and specificity. Chem. Rev. 2002, 102, 4501–4524.