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First steps in the direction of synthetic, allosteric, direct inhibitors of thrombin and factor Xa

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ABSTRACT

Designing non-saccharide functional mimics of heparin is a major challenge. In this work, a library of small, aromatic molecules based on the sulfated DHP scaffold was synthesized and screened against thrombin and factor Xa. The results reveal that (i) selected monomeric benzofuran derivatives inhibit the two enzymes, *albeit* weakly; (ii) the two enzymes recognize different structural features in the benzofurans studied suggesting significant selectivity of recognition; and (iii) the mechanism of inhibition is allosteric. The molecules represent the first allosteric small molecule inhibitors of the two enzymes.

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Thrombin and factor Xa are pivotal enzymes of the coagulation cascade. Both enzymes are the prime target of the current heparin-based anticoagulant therapy, which has been in use for more than seven decades. Despite its usefulness, heparin is associated with several adverse effects including an enhanced risk for bleeding.[1,2] In addition, the animal origin of the drug is a cause for concern with respect to viral or other potentially infectious agent contamination. Likewise, recent incidences of oversulfated chondroitin sulfate contaminating unfractionated heparin (UFH) preparations also highlight the need for new heparin-like anticoagulants.[3–5]

Molecules that functionally mimic heparin without its adverse effects are highly desirable. Heparin is a polysaccharide that is decorated with numerous sulfate and carboxylate groups. Designing heparin mimics, especially not based on a saccharide scaffold, to specifically recognize its prime targets such as antithrombin, thrombin and factor Xa, is a major challenge. Non-sugar scaffolds that can bear multiple sulfate and carboxylate groups and are as large as a typical active sequence in heparin are difficult to synthesize. Additionally, designing such scaffolds is fraught with the problem of poor specificity arising from surface exposed heparin binding sites on proteins.[6,7] Finally, robust computational tools available to reliably predict the interactions of highly anionic molecules with heparin-binding proteins are not yet available.

Our effort to design large non-saccharide scaffolds that functionally mimic heparin resulted in the chemo-enzymatic synthesis of sulfated DHPs. Sulfated DHPs are synthetic variants of the naturally available lignin and a specific sulfated DHP, CDSO3 (Fig. 1), was found to potently inhibit thrombin and factor Xa with an IC_{50} of 18–34 nM under physiological conditions.[8] More importantly, CDSO3 also inhibited blood clotting under ex vivo conditions with potency comparable to the low molecular weight heparins.[9]

CDSO3 represents a library of structures arising from multiple inter-monomeric linkages (e.g., β-5, β-*O*-4, β-β and 5-5), variable sulfation and carboxylation of oligomeric chains, the presence of numerous chiral centers, and variable chain length (Fig. 1).[10] These four major variables introduce phenomenal structural diversity in a typically preparation of sulfated DHP. For example, a simple calculation reveals that 262,144 distinct decamer structures are possible for CDSO3.

Mechanistically, CDSO3 binds to exosite II of thrombin to allosterically disrupt the catalytic apparatus resulting in inhibition.[8]

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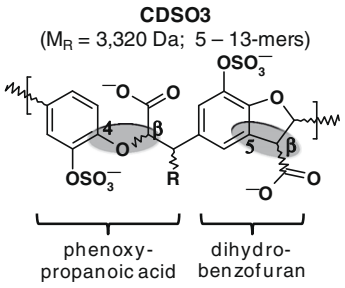

Figure 1. Oligomeric structure of a chemo-enzymatically synthesized CDSO3 consisting of β-5 and β-*O*-4 inter-monomeric linkages. Substituent R can be either –H, –OH, or –OSO_3^-. Chiral centers are identified using curvy bonds. CDSO3 can be thought of as being made from dihydro-benzofuran and phenoxy-propanoic acid monomeric units joined either in an alternative or successive manner. The average chain length of CDSO3 is 5–13-monomer units.

This is the first example of an exclusive exosite II-mediated inhibition mechanism and represents an exciting opportunity for designing new anticoagulants.

In this work, a small library of 17 β-5-like monomeric benzofuran derivatives was synthesized and screened against thrombin and factor Xa. The results reveal that (i) monomeric CDSO3-based structures are inhibitory, *albeit* the potency is weak; (ii) thrombin and factor Xa appear to recognize different structural features suggesting significant selectivity of recognition; and (iii) the inhibition mechanism is allosteric.

Results and discussion. *Rationale for the design of the benzofuran library:* *β*-5 and β-*O*-4-linked chemo-enzymatically prepared CDSO3 can be thought of as being made from dihydro-benzofuran and phenoxy-propanoic acid monomeric units. These monomeric units may be alternatively, successively or randomly linked (see Fig. 1). Each unit may or may not bear sulfate group(s) to give the heterogeneous CDSO3. Assessing the role of both these structural units requires the availability of a large library of sulfated and carboxylated, aromatic molecules containing multiple stereocenters. As a first step, we focused on synthesizing a small, achiral benzofuran library to assess whether the smallest structural unit of CDSO3, that is, a β-5-like monomer, possesses thrombin and factor Xa inhibitory property.

Synthesis and description of the benzofuran library: The synthesis of the β-5-like benzofuran monomer library is described in detail in the Supplementary data. Briefly, laccase-mediate oxidative coupling of catechol and ethylacetoacetate was used to prepare the parent 5,6-dihydroxy-benzofuran-3-carboxylic acid ethyl ester monomer **1E** (Fig. 1), as reported in literature.[11] Monomer **1E** served as a starting point for differentially introducing the multiple sulfate and carboxylate groups on the scaffold. Most synthetic steps used in the construction of the library involved simple functional group transformations (see Supplementary data). Yet, the synthesis of library members containing both *O*-sulfate and carboxylate groups, especially **4AS**, **5AS**, **6AS** and **7AS**, was not trivial. The synthesis of polysulfated small, aromatic molecules is known to be challenging.[12] Common chromatographic techniques used to purify organic molecules fail to work well with these highly water soluble molecules. Additionally, the stability of these highly anionic molecules is suspect. In fact, molecule **1XS** was found to be fairly unstable in aqueous solution, the reason for which is unclear at the present time. We utilized a microwave-based sulfating protocol developed in our laboratory[13] followed by size exclusion and cation exchange chromatography to synthesize the targeted sulfated benzofuran ethyl esters in good to high yields (see Schemes I–III in Supplementary data).

Conversion of the ester to the acid functionality worked only with potassium *t*-butoxide in anhydrous DMSO containing one equivalent of H_2O as the traditional hydrolytic conditions (NaOH/EtOH and HCl/EtOH) resulted in the breakdown of the aromatic *O*-sulfate group. The library of 17 small molecules so synthesized contained members devoid of anionic groups, for example, **1E**, to those bearing two sulfate and one carboxylate groups, for example, **6AS**.

Thrombin and factor Xa inhibition properties: Inhibition of thrombin and factor Xa by β-5-like benzofuran derivatives was followed by spectrophotometric determination of the initial rate of hydrolysis of appropriate chromogenic substrate, as previously described in our work.[8] Spectrozyme TH and S-2772 were used as substrates of thrombin and factor Xa, respectively. Appropriate controls to correct for changes introduced by small volumes of organic solvents, such as DMSO used for dissolving highly hydrophobic molecules, were performed. The fractional decrease in the rate of initial hydrolysis of Spectrozyme TH (thrombin) or S-2772 (factor Xa) in the presence of 0.4–4.3 mM benzofuran derivative as compared to that in its absence corresponded to the inhibition potential of the molecule (see Supplementary data for details).

Of the 17 molecules screened, only **1A**, **2A**, **4AS**, **5AS**, **6AS**, **7A** and **7AS** were found to exhibit inhibitory properties (Fig. 3). This indicates that only selected β-5-like monomeric units possess the capability to induce direct inhibition of thrombin and factor Xa. The minimal concentration necessary to display inhibition was found to be approximately 400 μM, which corresponds to a moderate level of inhibition potential. Yet, interesting structure–activity relationships are evident. All ester containing molecules were found to exhibit no inhibition suggesting a key role for the –COO^- group. Whereas no inhibitor displays more than 40% inhibition of thrombin at 2.9 mM, at least four out of seven (**4AS**, **5AS**, **6AS**, and **7A**) inhibit factor Xa better (>40%) at 2.6 mM (Fig. 3). This suggests a preference of the benzofuran scaffold for targeting factor Xa. More importantly, inhibitors **5AS** and **6AS** display 86% and 75% inhibition of factor Xa, which is much better than all other active inhibitors, suggesting significant selectivity of recognition by the enzyme. The common 'pharmacophore' present in these two molecules is the 3-COO^- and 6-$OSO3^-$ unit, which is absent in all other structures. Interestingly, factor Xa inhibition by **4AS**, which is a regioisomer of **5AS**, was much weaker (42%) further supporting preferential recognition hypothesis. Inhibitors **5AS** and **6AS** more closely resemble the native CDSO3 monomeric unit than inhibitors **7A** or **7AS**, which possess a non-native, extended COO^- bearing linker (see Fig. 2).

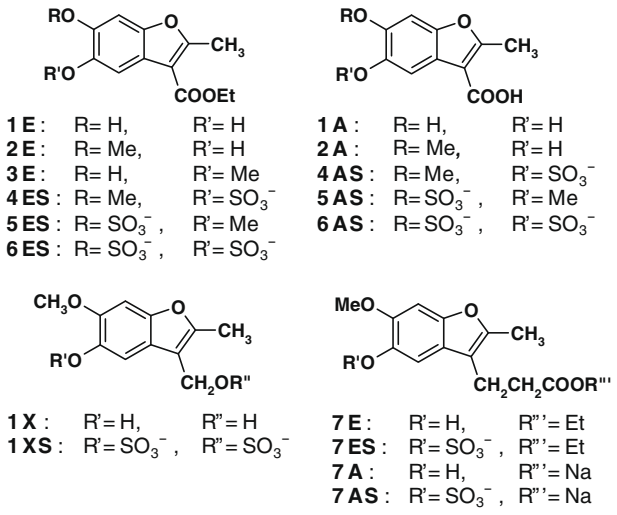

Figure 2. Structures of 17 benzofuran derivatives present in the small synthetic library screened against factor Xa and thrombin.

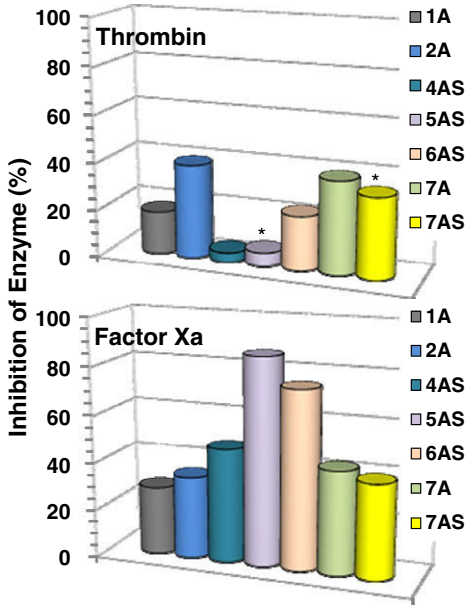

Figure 3. Inhibition of thrombin (upper plot) and factor Xa (lower plot) by benzofuran derivatives **1A**, **2A**, **4AS**, **5AS**, **6AS**, **7A**, and **7AS**. The monomers were present at 2.9 mM (thrombin) or 2.6 mM (factor Xa) concentration, except for the asterisk (∗) labeled cases for which the concentration was 4.3 mM. All other derivatives in the library were found to be essentially inactive below 4.3 mM. The standard error in these experiments was less than 10%.

Mechanism of factor Xa inhibition: To understand the molecular basis for the inhibitory potential of the benzofuran derivatives, the kinetics of S-2772 hydrolysis by factor Xa at pH 7.4 and 25 °C in the presence and absence of **5AS** was determined. S-2772 is a chromogenic substrate of factor Xa. The initial rate of hydrolysis varied in a hyperbolic manner with the concentration of the substrate, as expected (Fig. 4), from which the Michaelis constant (K_M) and maximal velocity of the reaction (V_{MAX}) were derived (see Supplementary data). The K_M and V_{MAX} for S-2772 in the absence of **5AS** was found to be 441 ± 86 μM and 197 ± 22 mAU $min^{-1}μM^{-1}$, respectively. In the presence of **5AS**, these values changed to 61 ± 13 μM and 54 ± 4 mAU $min^{-1}μM^{-1}$, respectively. This suggests that the presence of **5AS** significantly affects the binding of the chromogenic substrate to the active site of the enzyme. More specifically, the interaction of **5AS** with factor Xa results in a sevenfold increase in affinity of the substrate for the enzyme and a 3.6-fold decrease in the catalytic rate of hydrolysis. Thus, the presence of **5AS** brings about structural changes in the active site of thrombin, which significantly alters the formation of the factor Xa–S-2772 Michaelis complex as well as induces dysfunction of the catalytic apparatus of the enzyme. Considering that the parent inhibitor, that is, CDSO3, is known to bind exosite II of thrombin,[8] these results support the hypothesis that the benzofuran-based synthetic small molecules also inhibit factor Xa and thrombin through an exosite II-mediated allosteric dysfunction of the enzymatic catalytic triad.

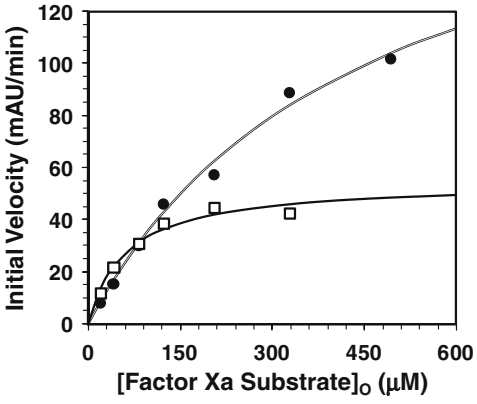

Figure 4. Hydrolysis of substrate S-2772 by factor Xa in the presence (□) and absence (●) of 2.6 mM **5AS**. The solid lines represent the fits to Michaelis–Menten equation to derive K_M and V_{MAX} values. See text for details.

Significance: A large number of inhibitors have been designed in the past decade to inhibit thrombin and factor Xa. These include peptide-based biomolecules, peptidomimetics, natural products and small hydrophobic structures.[14–16] Nearly all of these inhibitors, especially the small organic molecules, target the active site of the enzymes. Small molecules that do not function as competitive inhibitors of thrombin and factor Xa are unknown and the molecules studied here are the first example of allosteric inhibitors.

The benzofuran-based inhibitors studied here are not potent inhibitors of the two procoagulant enzymes. This is not too unexpected because (a) these first generation inhibitors are fairly small molecules containing sub-optimal binding features, (b) only one type of monomeric structural unit (benzofuran) is present in these molecules as compared to the two structural units present in CDSO3 (see Fig. 1), and (c) the CDSO3 binding site on thrombin and factor Xa is likely to be large, possibly involving multiple benzofuran and phenoxy propanoic acid structural units to generate nanomolar affinity. It is to be expected that coupling the most potent structures found here, that is, **5AS** and **6AS**, with phenoxypropanoic acid-based monomeric units will result in better inhibition profile. In fact, the average chain of CDSO3 is 5–13-mer in length,[8,10] which implies that this process may have to be optimized through several iterations. Thus, the screening of the small benzofuran-based library represents a first step in the design of allosteric inhibitors of thrombin and factor Xa.

The design of allosteric inhibitors is typically challenging because of inadequate information on the nature of the binding site and the mode of interaction. This inadequacy is amplified with heparin mimics because of the highly surface exposed interaction involving primarily electrostatic phenomenon. Yet, the novel allosteric mechanism of these small synthetic molecules affords an opportunity to discover radically different molecules from the known ligands with possibly different pharmacological and toxicological profile. Thus, the synthetic molecules designed on the basis of the CDSO3 scaffold may results in potent functional mimics of heparin.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.013.

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