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A small group of sulfated benzofurans induces steady-state submaximal inhibition of thrombin

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ABSTRACT

Despite the development of promising direct oral anticoagulants, which are all orthosteric inhibitors, a sizable number of patients suffer from bleeding complications. We have hypothesized that allosterism based on the heparin-binding exosites presents a major opportunity to induce sub-maximal inhibition of coagulation proteases, thereby avoiding/reducing bleeding risk. We present the design of a group of sulfated benzofuran dimers that display heparin-binding site-dependent partial allosteric inhibition of thrombin against fibrinogen ($\Delta Y = 55–75\%$), the first time that a small molecule (MW < 800) has been found to thwart macromolecular cleavage by a monomeric protease in a controlled manner. The work leads to the promising concept that it should be possible to develop allosteric inhibitors that reduce clotting, but do not completely eliminate it, thereby avoiding major bleeding complications that beset anticoagulants today.

It is estimated that nearly 10% of the adult population will be treated with anticoagulants at least once in their lifetime. Orally bioavailable agents constitute frequently used anticoagulants these days but their risk of bleeding remains substantial. A number of patients on oral anticoagulants suffer from bleeding consequences,[1] which raises considerable harm.

All current direct oral anticoagulants (DOACs) target the active site of a coagulation enzyme, e.g., thrombin or factor Xa.[2] In this orthosteric inhibition mechanism, the only parameter available for regulation of clotting activity is the inhibitory potency (either IC_{50} or K_I). In contrast, allosteric inhibition mechanism offers two parameters for regulating clotting activity, i.e., potency and efficacy (ΔY). Such dual parameter regulation is not possible for orthosteric inhibitors because of their all ($\Delta Y = 100\%$) or none ($\Delta Y = 0\%$) property.

Allosteric inhibition also promises to offer enhanced level of selectivity because allosteric sites typically tend to be less homologous.[3,4] This has certainly been found to be true for a group of allosteric inhibitors of thrombin that we have been studying for the past few years. We had reasoned that appropriate sulfated non-saccharide glycosaminoglycan mimetics (NSGMs) could be designed to target exosite 2 of thrombin and induce allosteric inhibition (Fig. 1). To realize this, we first developed sulfated low molecular weight lignins[5] as polymeric mimetics of the highly sulfated polysaccharide, heparin, which binds to an exosite of certain serine proteases with 10–20 μM affinity.[6] However, the sulfated lignins inhibited several coagulation proteases.[7] To enhance selectivity, we designed sulfated benzofuran monomers (SBMs), which preferentially inhibited thrombin and factor Xa,[8] *albeit* displaying a high IC_{50}. Further improvement in design led to sulfated benzofuran dimers (SBDs, Fig. 1) that selectively inhibited thrombin by interacting with Arg173 on the periphery of exosite 2.[9,10] This was a major advance. Yet, the allosteric SBDs displayed efficacies ≥90%, which does not truly afford an opportunity for efficacy-based regulation (i.e., based on ΔY).

Allosteric agents that display efficacies, or alternatively responses, in the intermediate range (e.g., 30–70%) have been well-known for receptors and oligomeric proteins. Such agents are referred to as partial antagonists. However, partial allosterism has been extremely challenging to discover for monomeric proteins or proteases. We recently discussed the first example of an allosteric partial inhibitor of a monomeric protease, a sulfated coumarin analog, which displayed sub-maximal efficacy.[11] The analog

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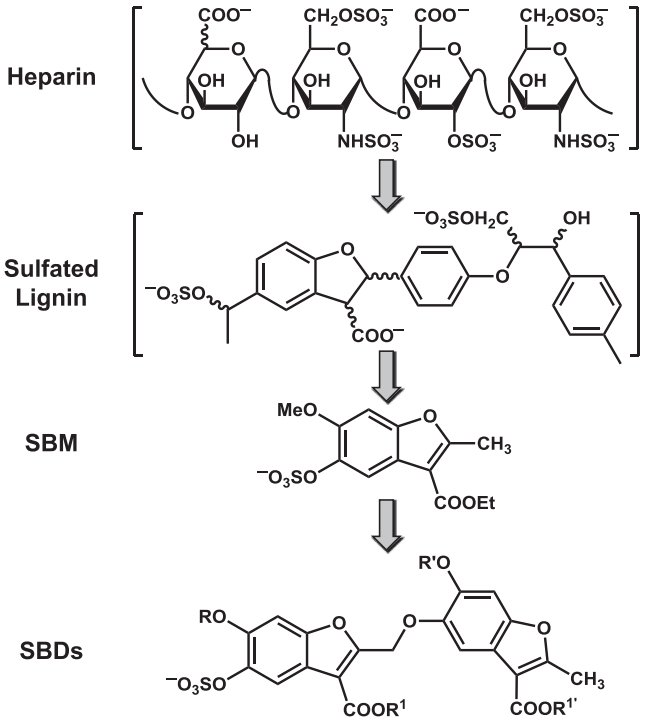

Fig. 1. The development of allosteric partial inhibitors of thrombin that target exosite 2 of thrombin. SBMs = sulfated benzofuran monomers; SBDs = sulfated benzofuran dimers. R, R′, R^1 and $R^{1\prime}$ groups represent alkyl or aryl substituents (see Table 1).

reduced thrombin's hydrolysis of a small chromogenic substrate with a maximal efficacy of only about 50%. However, this partial allosterism was lost for fibrinogen, thrombin's natural macromolecular substrate. We reasoned that alternative small, allosteric partial inhibitors of thrombin that function even against the macromolecular substrate should be possible to design/discover considering that exosite 2 displays multiple hydrophobic sub-sites that are coupled with its catalytic triad.[12]

To realize such agents, we focused on a prototypic SBD, **1** (Fig. 1), which had been designed earlier but displayed ΔY of 75%.[9] We posited that modifications in aromatic and ester substituents of **1** may lead to a more favorable partial inhibition characteristics (ΔY ~30–70%). We synthesized a library of 16 SBDs with variations in R, R′, R^1 and $R^{1\prime}$ substituents (Fig. 1). We decided that whereas predecessor **1** carries methyls or ethyls at these positions, the analogs would carry larger lipophilic substituents, which could possibly engage the hydrophobic sub-domains present in exosite 2 better.[13] The synthesis of these SBDs was achieved in 7–14 steps involving protection–deprotection, nucleophilic substitution, free radical bromination and sulfation reactions (see Supplementary Material and Schemes S1–S7). We also synthesized 12 new SBMs (see Supplementary Table S1), which were also studied.

Thrombin inhibition was studied using chromogenic substrate hydrolysis assay at pH 7.4 and 25 °C in the presence of 2.5 mM $CaCl_2$, as described elsewhere.[9–12] Of the 13 SBDs studied, 11 were found to be 2–3-fold more potent than **1** identified earlier (Table 1).[10] Of these, **2c** carrying a $PhCH_2CH_2$ group at the R′ position, instead of a CH_3 present in prototype **1**, displayed the best potency (IC_{50} = 1.8 μM, Fig. 2). In contrast, **2o** and **2p** carrying sulfate groups at the R′ position failed to inhibit thrombin at concentrations lower than 300 μM suggesting a sensitive structure–activity relationship (SAR).

Although the discovery of higher potency was interesting, the most exciting finding was the property of partial inhibition for this series of agents. Of the 11 active inhibitors, nine displayed ΔY in the range of 55 to 75% (Table 1, Fig. 2a). Inhibitor **2c** inhibited thrombin with an efficacy of 58% at saturation, a characteristic of possibly major consequences with regard to regulation of enzyme function. Such partial inhibition profile is not possible for orthosteric agents. Interestingly, two agents, i.e., **2h** and **2i**, inhibited

Table 1
Inhibition of human thrombin by sulfated benzofuran dimers (SBDs).

	R	R′	R^1	$R^{1\prime}$	IC_{50} (μM)[a]	ΔY (%)[a]
1[b]	Me	Me	Et	Et	6.2 ± 2.7[c]	75 ± 1[c]
2a	Me	$C_6H_{11}CH_2$	Et	Et	2.6 ± 0.1	63 ± 1
2b	Me	Bn[d]	Et	Et	2.0 ± 0.1	57 ± 1
2c	Me	$BnCH_2$	Et	Et	1.8 ± 0.1	58 ± 1
2d	Me	pOMOM-Bn	Et	Et	3.3 ± 0.2	61 ± 2
2e	Me	*m*OMOM-Bn	Et	Et	4.0 ± 0.5	59 ± 5
2f	Me	*p*O-iPr-Bn	Et	Et	2.3 ± 0.1	74 ± 3
2g	Me	*m*O-iPr-Bn	Et	Et	2.6 ± 0.1	57 ± 1
2h	$C_6H_{11}CH_2$	$C_6H_{11}CH_2$	Et	Et	8.7 ± 0.8	93 ± 4
2i	$C_6H_{11}CH_2$	Bn	Et	Et	3.6 ± 0.1	90 ± 2
2j	$C_6H_{11}CH_2$	Me	Et	Et	3.8 ± 0.3	77 ± 3
2k	Me	Me	Et	Bn	4.9 ± 0.2	68 ± 2
2l	Me	Me	Et	$BnCH_2$	3.2 ± 0.2	58 ± 3
2m	Me	Me	Et	*p*OMOM-Bn	2.6 ± 0.1	66 ± 2
2n	Me	Bn	X[e]	Et	14 ± 0.5	74 ± 2
2o	Me	Y[f]	Et	Et	>300 [g]	–[h]
2p	Me	SO_3Na	Et	Et	>300 [g]	–
Hirudin peptide (54–75)					1.3[i]	
Fibrinogen peptide (410–427)					130[i]	

[a] IC_{50} and ΔY were measured spectrophotometrically using a chromogenic substrate hydrolysis assay in 20 mM Tris-HCl buffer, pH 7.4, containing 25 mM $CaCl_2$, 100 mM NaCl and 0.1% PEG 8000 at 25 °C.
[b] IC_{50} and ΔY taken from Ref. 10.
[c] Errors represent ± 1 S.E.
[d] Bn = benzyl.
[e] X = $-CH_2CH_2OSO_3Na$.
[f] Y = Bn-*m*OSO_3Na.
[g] No inhibition was observed at 300 μM.
[h] Not applicable.
[i] Values taken from Ref. 18.