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Generation of potent coagulation protease inhibitors utilizing zinc-mediated chelation

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Abstract—Inhibition of coagulation proteases such as thrombin, fXa, and fVIIa has been a focus of ongoing research to produce safe and effective antithrombotic agents. Herein, we describe a unique zinc-mediated chelation strategy to streamline the discovery of potent inhibitors of fIIa, fXa, and fVIIa. SAR studies that led to the development of selective inhibitors of fXa will also be detailed.

Direct inhibition of activated proteases involved in the coagulation cascade has been a common strategy employed by the pharmaceutical industry in an effort to generate new and safer antithrombotics.[1–3] While initial efforts focused mainly on inhibition of thrombin (fIIa),[1] more recent efforts have involved the generation of selective inhibitors of factor Xa (fXa)[2,3] and factor VIIa (fVIIa).[3] These proteases mediate key steps along the coagulation cascade and accordingly, the pharmacological effect of inhibition of these targets may be of therapeutic value. Currently, there are a variety of small molecule fXa and fIIa inhibitors under clinical evaluation for the treatment of thromboembolic disease while only protein agents are in trials for factor VIIa. As the data from these trials become available, the therapeutic value of fXa and/or fVIIa inhibition will perhaps become more clear.

At Axys Pharmaceuticals, we exploited a unique zinc-mediated chelation strategy to streamline the discovery of potent inhibitors of fIIa, fXa, and fVIIa.[4] Herein, we describe the development of small molecule inhibitors that demonstrate nanomolar inhibition for fXa, fIIa, and fVIIa. SAR studies that led to the development of selective inhibitors of fXa will also be described.

We have previously reported on a novel mechanism for inhibiting serine proteases in which Zn^{2+} potentiates the inhibition of active site-directed, small molecule inhibitors via formation of a highly stabilized ternary complex. A crystal structure of bis(5-amidino-2-benzimidazolyl) methane (BABIM) in trypsin has previously been reported by Katz et al.,[5] and illustrates this novel binding mode (Fig. 1) in which Zn^{2+} is simultaneously coordinated to electron pairs on the O-α of Ser195 and N-ε2 of His57 (trypsin numbering system) as well as to

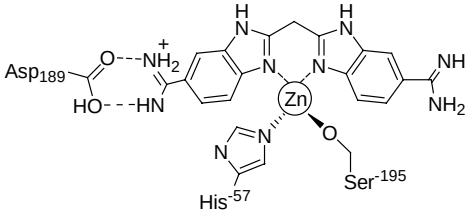

Figure 1. Zinc-mediated tetrahedral binding motif.

Keywords: Factor Xa; Factor VIIa; Thrombin; Coagulation; Protease inhibitor; Zinc chelation.
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the benzimidazole nitrogen atoms. A tetrahedral complex is formed between the bidentate ligand, zinc, and protease. The amidine group of the 5-amidinobenzimidazole provides an anchor to the protein through a strong salt bridge with Asp189. This kinetically driven interaction provides a template for the tetrahedral complex (ligand, Zn, and protease) to form. The 'bridging' of inhibitor to the active site mediated by Zn^{2+} routinely affords 1000-fold binding enhancement. This mode of inhibition relies on the spatial orientation of electron pairs presented by residues within the catalytic triad. From structural studies, this presentation is known to be highly conserved amongst trypsin-like serine proteases. Reports from Katz and Luong[6] describe X-ray crystallographic work of analogous zinc–inhibitor complexes in several trypsin-like serine proteases. Additionally, a report from Janc et al.[7] outlines the kinetic components of this unique binding mode.

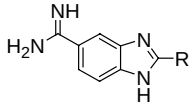
Recognizing that human plasma contains 15 μM zinc[8] and that this novel approach to serine protease inhibition could be applied to the coagulation proteases, we focused on identifying inhibitors of fXa, fIIa, and fVIIa. We initiated our research program by generating a range of bidentate scaffolds and analyzing the inhibitory effects of these agents upon selected proteases in the presence and absence of Zn^{2+}. The compounds were assayed with the addition of 150 μM $ZnCl_2$ or 1 M EDTA to the assay buffer.[9] As the on-rates and zinc K_d for each scaffold can vary slightly, the two assay conditions allowed for the discrete measurement of the inhibitory potency of (zinc/ligand) complex and the apo-ligand, respectively, within a 1 h timeframe.[7]

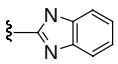
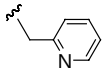
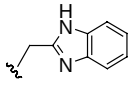
Initially, we chose to generate scaffolds having a 5-amidinobenzimidazole as the P1 element and we varied the distal chelating moiety using nitrogen-containing heterocycles. Over 30 scaffolds were generated to explore binding properties. Five selected scaffolds are presented in Table 1. These scaffolds possessed good starting points for further modification as all compounds pass Lipinksi and Veber rules, and have molecular weights <290 and polar surface areas <107 $Å^2$. Scaffolds **1**–**5** all demonstrated enhanced binding in the presence of Zn^{2+} for fXa, fVIIa, and fIIa, with fXa demonstrating the largest shifts in binding potency. Interestingly, in the presence of Zn^{2+}, scaffold **5** showed the greatest inhibition to fXa, fIIa, and fVIIa. Scaffolds **1**–**4** showed very good inhibition versus fXa, although they exhibited noticeably less activity against fIIa and fVIIa. In order to analyze these results, it is important to consider that analogs **1**–**3** form a five-membered chelate when coordinated to Zn^{2+}, while analogs **4** and **5** form a six-membered chelate. As the Zn^{2+} K_d determinations of the five- and six-membered chelates are comparable (roughly 10–30 μM),[7] it is reasonable to assume that the K_i of each scaffold is more dependent upon the flexibility of each protease to accommodate particular spatial arrangements.

In order to develop a plan to gain greater selectivity for fXa versus thrombin, we chose to investigate small changes on the distal ring of scaffold **5** as it demonstrated the greatest inhibition in the presence and absence of zinc for all proteases, fXa, fIIa, and fVIIa. Additionally, general SAR information gained from a study on this scaffold might be transferable to one of the more selective scaffolds. To assist this effort, we obtained a crystal structure of **5** bound to thrombin (depicted in Fig. 2).

As expected, the amidine forms a salt bridge with Asp189 and a tetrahedral complex is formed between the benzimidazole nitrogens, zinc, and catalytic residues, Ser190 and His57. Notably, Lys60F, of the thrombin

Table 1. Inhibition of selected scaffolds on factor Xa, thrombin, and fVIIa in the presence and absence of Zn^{2+}



Compound	R	+Zn^{2+}/−Zn^{2+} K_i (μM)		
		fXa	Thrombin	fVIIa
1		0.03/38 Δ = 1270	1.1/—	0.90/370
2		0.027/45.6 Δ = 1700	0.72/60	1.45/—
3		0.046/72 Δ = 1565	0.32/113	0.46/440
4		0.06/>150 Δ = 2500	10.5/>150	39/—
5		<0.001/2.7 Δ = >2700	0.001/70	0.074/26

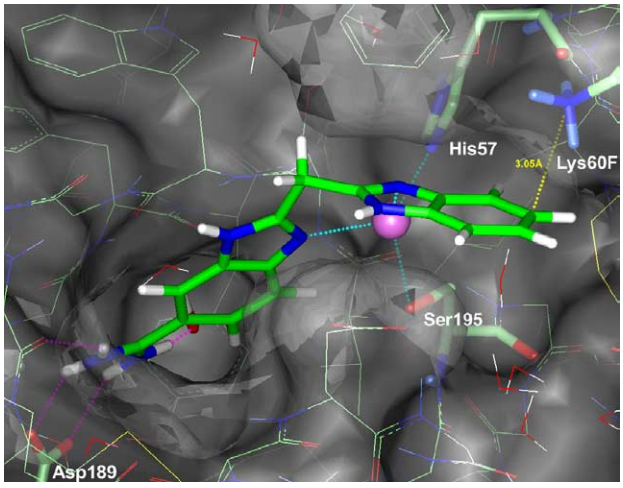


Figure 2. Crystal structures of **5** and Zn^{2+} bound to fIIa.[10]

'60s loop', is 3.05 Å from the distal benzimidazole ring and may be involved in a π–hydrogen bond.[11] Other proteases, such as fXa and fVIIa, do not in general possess an extended 60s loop as seen in thrombin and are not likely to have a lysine at this site. In an attempt to take advantage of this difference, we designed analogs that would be less prone to participate in π–hydrogen bond interactions with the thrombin Lys60F. We hypothesized that by disrupting the electron density of the π-system of the distal aryl ring with electron-deficient substituents, we could decrease this interaction. To this end, we generated a variety of analogs of **5** with varying moieties on the distal ring as depicted in Table 2. It was anticipated that the binding affinity of these analogs to proteases that lack the ability to π–hydrogen bond (such as fXa and fVIIa) would be unaffected. On the other hand, the binding to fIIa was expected to decrease with electron-deficient arenes creating an overall increase in selectivity for fXa and fVIIa.

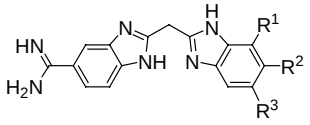
Both mono- and di-substitution patterns of the distal ring were evaluated. It is important to note that both tautomers of the benzimidazole ring system are capable of presenting the requisite sp^2 nitrogen lone pair for chelation to the zinc, therefore two distinct binding modes can be adopted if the ring is unsymmetrically substituted. Mono-substituted analogs **6**–**8** and bis-substituted analogs **13**–**15** may all adopt two distinct binding modes, while symmetrically substituted analogs **9**–**12** can only adopt one.

Comparison of **5** to substituted analogs **6**–**8** initially led us to believe that our hypothesis to modulate π–hydrogen bonding to improve selectivity for fXa versus thrombin was an appropriate strategy. The incorporation of electron-withdrawing groups such as in fluorine analog **6** and chlorine-containing derivative **7** caused a significant decrease in binding to fIIa, while causing basically no change in inhibition for fXa. Interestingly, the incorporation of an electron-donating methoxy group (as in analog **8**) caused only a slight decrease in binding to both fIIa and fVIIa, although it decreased the binding to fXa to a greater extent. It is possible that steric effects play a more dominant role with this particular substitution than any electronic effects.

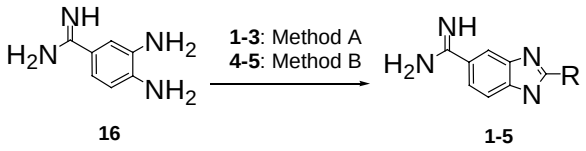
Compounds **9**–**15** were generated to explore bis-substitution SAR patterns. Upon inspection, it is evident that the only analogs that showed a decrease in binding to fXa were **10** and **11**, while the others maintained good activity. Clearly, larger groups such as chlorine and methoxy in the bis-2,3 di-substitution pattern offer inferior fXa binding to the bis-1,3 arrangement. As the dimethyl analog **12** showed equivalent potency to **5**, sterics probably play a role.

At this time, it is difficult to draw rigorous conclusions regarding the substitution patterns upon the distal benzimidazole and the binding capability of each analog. The steric, electronic, and H-bonding interactions all

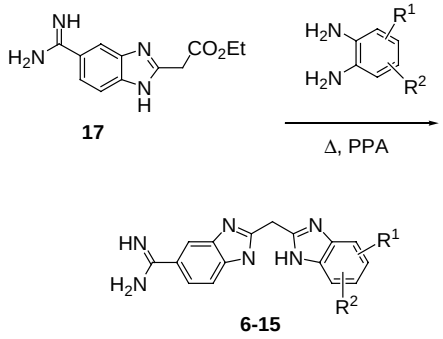
Table 2. SAR on scaffold **5**



Compound	R^1	R^2	R^3	fXa + Zn^{2+} K_i (nM)	Zn^{2+} selectivity for Xa versus	
					fIIa	fVIIa
5	H	H	H	1	1	74
6	H	F	H	1	190	540
7	H	CI	H	1	1600	760
8	H	OMe	H	40	1	8
9	H	F	F	6	600	80
10	H	CI	CI	140	210	170
11	H	OMe	OMe	80	40	200
12	H	Me	Me	2	1300	185
13	F	H	F	6	170	16
14	CI	H	CI	3	16,000	633
15	OMe	H	F	3	1150	14,300



Scheme 1. Reagents and conditions: Method A: RCHO, benzoquinone, ethanol, Δ; Method B: RCO_2Et, neat PPA, Δ.



Scheme 2. General synthesis for the generation of **6**–**15**.

contribute to the complexity of each example. What is clear though is that small and subtle changes upon the distal benzimidazole of **5** can cause dramatic affects upon binding to fIIa and fVIIa while, for the most part, minimal change is noted upon fXa binding. As a consequence, some very potent and selective fXa inhibitors have now been identified. Analogs **6**, **7**, **12**, **14**, and **15** all have fXa inhibition constants less than 10 nM and demonstrate a greater than 100-fold selectivity versus fIIa and fVIIa.

Finally, the in vitro anticoagulant effects (2xPT and 2xaPTTs) of all analogs described in this communication are >10 μM. These relatively poor in vitro coagulation results may be thought to be surprising at first, given the excellent in vitro potency versus the purified enzyme. It is the driving forces (kinetics versus thermodynamics) of each assay that must be understood to comprehend this 'mismatched' effect. First, it is well known that it can take up to 1 h to achieve maximum binding inhibition when the zinc-mediated inhibition mechanism is operating.[7] This is a thermodynamic effect based on the slow on rate of the tetrahedral complex. Second, and in contrast, the in vitro coagulation assay monitors blood coagulation within 30 s, which does not allow time for the tetrahedral complex to form and thus the potency of full zinc-mediated inhibition cannot be monitored by such a rapid coagulation assay.

All analogs described were prepared in a straightforward manner. Scaffolds **1**–**5** were prepared by the action of 5-amidino-3,4-diaminobenzamidine (**16**)[12] on the appropriate aldehyde and/or ester component as illustrated in Scheme 1. Analogs **6**–**15** were prepared from commercially available diamines and 5-carbamimidoyl-1*H*-benzoimidazol-2-yl-acetic acid ethyl ester (**17**) as previously reported[4] and depicted in Scheme 2.

A range of low molecular weight scaffolds have been shown to be potent inhibitors of the coagulation proteases fIIa, fVIIa, and fXa in the presence of $ZnCl_2$. Systematic incorporation of substituents on the distal benzimidazole moiety of scaffold **5** has resulted in the generation of potent and selective inhibitors of fXa.

Acknowledgments

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9. The assay conditions used were identical to those described in Ref. 7 with the following additional details. Factor VIIa (Enzyme Research) was incubated at 7 nM together with recombinant soluble human tissue factor (11 nM) with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, CH_3SO_2-D-CHA-But-Arg-pNA (Centerchem), supplied at the K_m (500 μM). Thrombin (Calbiochem) was incubated at 12.7 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, Tosyl-Gly-Pro-Lys-pNA (Centerchem), supplied at the K_m (25 μM). Factor Xa (Haematologic Technologies) was incubated at 2 nM with variable concentrations of inhibitor in 50 mM Tris (pH 7.4), 150 mM NaCl, 5.0 mM $CaCl_2$, 0.05% Tween 20, and 10% DMSO. The reaction was initiated with the addition of substrate, CH_3OCO-D-Cha-Gly-Arg-pNA (Centerchem), supplied at the K_m (1.0 mM).
10. PDB deposition number for this X-ray structure is 1c1u.
11. For more information on π–hydrogen bonding effects, see: Allen, F. H.; Davies, J. E.; Galloy, J. J.; Johnson, O.; Kennard, O.; Macrae, C. F.; Mitchell, E. M.; Mitchell, G. F.; Smith, J. M.; Watson, D. G. *J. Chem. Inf. Comput. Sci.* **1991**, *31*, 187.
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