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Neutral Inhibitors of the Serine Protease Factor Xa

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Abstract—A neutral inhibitor of the serine protease factor Xa was identified via a high-throughput screen of a commercial library. The initial lead **1** demonstrated reversible and competitive inhibition kinetics for factor Xa and possessed a high degree of selectivity versus other related serine proteases. Initial modeling efforts and the generation of a series of analogues of **1** are described.

Factor Xa, a member of the trypsin-like serine protease class of enzymes, is a pivotal amplification protease in the blood coagulation cascade. It is the proteolytic component of the prothrombinase complex that converts prothrombin to thrombin. Thrombin, in turn, converts fibrinogen to fibrin which polymerizes to form insoluble fibrin, a major component of blood clots. In the coagulation cascade, the prothrombinase complex is the convergent point of the surface-activated intrinsic pathway and the tissue factor-activated extrinsic pathway. Therefore, potent and selective inhibitors of factor Xa are potentially valuable therapeutic agents for the treatment of thromboembolic disorders.

A high-throughput screen of a commercial library identified compound **1**[1] as an inhibitor of factor Xa. Upon resynthesis, purification and characterization of **1**, we verified that the activity as observed in the high-throughput library screen was indeed accurate and due to the reported structure of compound **1**. Enzymatic studies showed that this compound operated by a competitive mechanism and had good to excellent selectivity versus related serine proteases. Interestingly, the factor Xa potency ranged from 0.042 to 2.1 μM depending upon the assay conditions employed (see Table 1).[2,3] For instance, when the assay was performed in a pH 8.2 buffer the K_i' was 2.1 μM. Lowering the pH of the buffer to 7.4 caused a 50-fold enhancement in potency while the addition of HSA (human serum albumin) at physiological pH produced only a 4-fold increase in factor Xa inhibition. It appears that the HSA binds the compound in solution and reduces inhibition.

Compound **1** is comprised of a 2-amino-5-methyl-benzoic acid core with a *p*-fluoroaniline amide occupying one terminus and a 3-chloro-benzo[*b*]thiophene-2-carboxamide at the other. The structure of **1** has many characteristics that differentiate it from the majority of factor Xa inhibitors in the literature,[4–6] although there have been other reports of anthranilic acids being utilized as a core scaffold.[5,6] Principally, **1** is neutral and does not have the highly basic amidine moiety that defines the majority of factor Xa inhibitors. An amidine moiety on typical small molecule drug candidates is often associated with low oral bioavailability, short half-life and rapid clearance. For this reason, we found structure **1** a promising lead. Our initial efforts focused on identifying its binding mode and probing its SAR.

Molecular Modeling

To aid the SAR studies, a binding mode hypothesis was developed. A Monte Carlo conformational search was performed on **1** employing both the MM2 and Merck Molecular force fields in the gas phase and with a water solvation model.[7–11] These results suggested that **1** can adopt the characteristic L-shape conformation that has been observed for many factor Xa inhibitors that bind from the S_1 to S_4 pockets of serine proteases.[12] The optimized solvent conformation of **1** is consistent with either terminal ring serving as the P_1 element (both the

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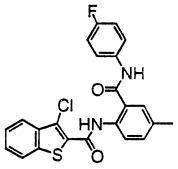
p-fluoroaniline ring or the 3-chloro-benzo[*b*]thiophene ring can be docked into the S_1 pocket). At the time this work was done two facts led to the 3-chloro-benzo[*b*]thiophene ring being selected as the P_1 element. Primarily, it was realized that the chlorine could occupy a similar position to that observed for the iodine atom of 4-iodobenzo[*b*]thiophene-2-carboxamidine in u-PA crystal structures.[13,14] Also, Lilly Research Laboratories reported X-ray studies of a 2,3-substituted benzothiophene analogue in which the benzothiophene moiety fits nicely into the S_1 pocket of the closely related protease, thrombin.[15]

Thus, a model of a possible binding mode of **1** to factor Xa was built using the solvated conformers from the Monte Carlo search which were docked with the 3-chloro-benzo[*b*]thiophene ring in the S_1 pocket. The recently reported high resolution (1.95 Å) apo factor Xa crystal structure was used as the model for the enzyme.[13] Two of the S_1 pocket water molecules (one that bridges between Asp189 and Gly218 C=O and one above Tyr228) were retained since **1** has no polar functionality itself to interact with the enzyme at these sites. A modified version of the flexible docking program Hammerhead developed at Axys Pharmaceuticals[16] was employed to first dock the starting conformers rigidly into the enzyme and then optimize the polar and steric interactions by allowing the ligand to flex. The resulting bound structure closely resembles the low energy conformations of **1** in solution.

Table 1. K_i' (μM) of **1** screened against selected serine proteases



1

Protease	K_i' (μM)
Factor Xa[a]	2.1
Factor Xa[b]	0.57
Factor Xa[c]	0.042
Thrombin[a]	125
Factor VIIa[a]	>900
Plasmin[a]	>150
Trypsin[a]	>150
uPa[a]	>150
tPa[a]	>150
aPC[a]	>150

[a]pH 8.2.
[b]pH 7.4 + HAS.
[c]pH 7.4.

In our model (Fig. 1), the sulfur of the benzothiophene ring is directed toward Ser195 of the catalytic triad (3.17 Å), while the remainder of the ring makes van der Waals contact with the S_1 residues and water molecules. The chlorine atom does indeed adopt a position above Gly218 similar to the iodine in 4-iodobenzo[*b*]thiophene-2-carboxamidine, a u-PA inhibitor.[13,14] Both amide bonds of the core 2-amino-5-methyl-benzamide are twisted out of plane to allow the turn necessary for accessing the S_4 pocket of factor Xa. The non-planarity of the two amide dihedral angles (both ca. −35°) between the benzothiophene and core rings results in a total torsional angle of roughly −72°. This twist is sufficient to direct the *p*-fluoroaniline into the S_4 pocket. The oxygen of this proximal amide is directed away from Gly214 towards solvent. Meanwhile, the NH is directed towards the Gly214 carbonyl, although the distance is too long for an H-bond (4.94 Å). The torsional angles of the remaining distal amide bond are of opposite sign (ca. 25–30°) to those on the proximal amide with the carbonyl directed towards Trp215 NH (5.01 Å). The resulting geometry allows the distal *p*-fluoroaniline ring to bind in the 'hydrophobic box' created by Trp215, Tyr99, and Phe235 of factor Xa and directs the fluorine atom toward the back of the S_4 pocket (5.26 Å from the Ile175 carbonyl).

Chemistry

In order to probe the SAR of **1**, we produced an initial set of closely related analogues. The design of this first set of derivatives was entirely based upon compounds that could be generated from commercially available

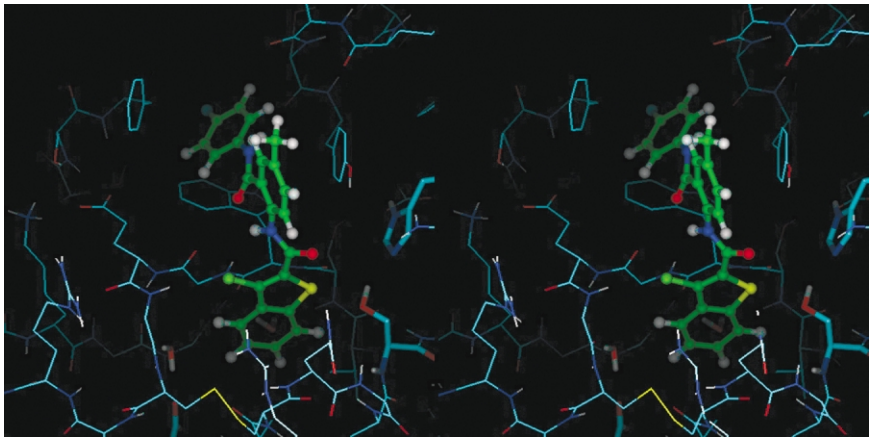
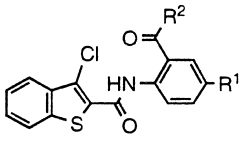
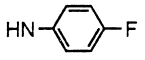
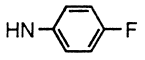
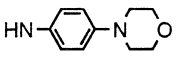
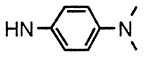
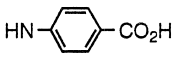
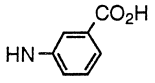
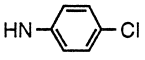
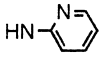
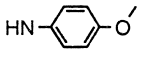
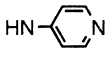
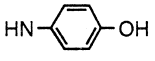
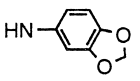
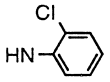
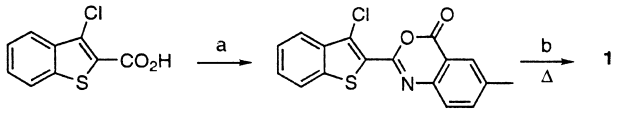


Figure 1. Stereoview of the docked conformation of **1** (ball-and-stick) into the S_1 and S_4 sites of factor Xa. The catalytic residues His57 and Ser195 as well as Asp189 and the S_1 water molecules retained in the docking studies are shown as polytube representations.

Table 2. Distal site analogues



Compound	R^1	R^2	Factor Xa K_i' (μM) pH 7.4 + HSA
1	CH_3		0.57
2	H		3.1
3	CH_3		436
4	CH_3		> 1200
5	H		> 150
6	H		> 150
7	H		> 150
8	CH_3		> 1200
9	CH_3		0.21
10	H		7.8
11	CH_3		2.1
12	CH_3		22.1
13	CH_3		97
14	CH_3		17.7
15	CH_3		> 1200



Scheme 1. Reagents: (a) 2-amino-5-methylbenzoic acid, CDI, DMF; (b) *p*-fluoroaniline.

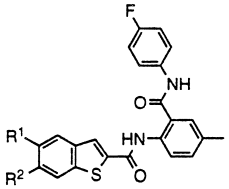
precursors. These analogues, as illustrated in Table 2, were all prepared by a straight-forward two-step procedure that progressed via a nucleophilic ring opening of intermediate benzoxazines. For example, **1** was synthesized as shown in Scheme 1.[17]

Commercially available 3-chloro-benzo[*b*]thiophene-2-carboxylic acid was treated with 2-amino-5-methyl-benzoic acid in the presence of carbonyl diimidazole in DMF. The resulting 2-benzo[*b*]thiophen-2-yl-6-methyl-benzo[*d*][1,3]oxazi-4-one was then treated with *p*-fluoroaniline to afford **1** in 58% overall yield. Analogues **2**–**15** were generated in an analogous manner utilizing the requisite benzothiophenes and anthranilic acid precursors.

Based on the modeling indications that the distal *p*-fluoroaniline ring was binding in the S_4 pocket, we initiated our search by replacing this group with a range of functionality. In short, the replacement of the *p*-fluoroaniline with larger and more hydrophilic groups, that is, 4-morpholino-4-yl-phenylamine (**3**), *N*,*N*-dimethyl-benzene-1,4-diamine (**4**), 4-aminobenzoic acid (**5**), and 5-aminobenzoic acid (**6**) all decreased factor Xa affinity dramatically. Additionally, more flexible hydrophilic groups such as a direct fused morpholine (**7**) and piperizine (**8**) also decreased factor Xa potency. These findings supported our initial binding hypothesis as the S_4 pocket is lipophilic and would discourage generic hydrophilic groups which do not make distinct interactions with the protein. As suspected, a slightly larger, more hydrophobic group, such as the chlorine analogue **9**, enhanced factor Xa potency roughly 3-fold. Less dramatic modifications to the *p*-fluoroaniline group, as in analogues **10**–**14**, were well-tolerated by the enzyme, although **15** had very poor activity. The most reasonable explanation for this would be that the *ortho* chloro group forces this ring to twist and adopt a less favorable configuration. Not unexpectedly, removal of the methyl group on the central ring of **1** (as indicated by **2**) decreased factor Xa affinity since this deletion decreases the overall lipophilicity of the molecule.

In an effort to improve the P_1 binding element, two regioisomeric hydroxy analogues of **1** were generated as depicted in Table 3. Both analogues saw a 5- to 10-fold decrease in fXa potency. The slight loss in potency would indicate that although the hydroxy groups did

Table 3. P_1 modifications



Compd	R^1	R^2	Factor Xa K_i' (μM) pH 7.4 + HSA
17	OH	H	3.5
18	H	OH	10

not make productive interactions with the protein, there remains room in the S_1 pocket to accommodate moieties on the benzothiophene which could in fact improve binding. A more thorough investigation at this location may prove quite fruitful.

It was realized that incorporation of an amidine group on the P_1 side could have a positive effect upon binding through the likely interaction with Asp189. Rationally, we chose not to make this modification as this change was likely to alter the binding mode and thereby confuse the SAR picture. It has been our experience that many benzamidine-based compounds bind in the S1 pocket of factor Xa and an inhibitor will adopt an appropriate binding mode to accommodate such a strong salt bridge interaction. Additionally, incorporation of an amidine would have derailed us from the original attraction of this neutral lead series.

Subsequent to this work, reports on the development of anthranilic acid analogues as factor Xa inhibitors surfaced.[18,19] In particular, a report by Eli Lilly indicates that the alternative binding mode (in which the *p*-fluoroaniline is in the S1 pocket) is also highly likely for **1** and should also be considered for the above described analogues.

Conclusion

The identification of **1**, a neutral, uncharged factor Xa inhibitor, exemplifies the ability of identifying desirable lead molecules via high-throughput screening of suitably selected libraries. The SAR of the initial series of analogues generated is consistent with our initial binding hypotheses.

References and Notes

1. Compound **1** was purchased from Maybridge Chemicals.
2. Kinetic measurements were performed in 96-well U-bottom microtiter plates (Falcon) using a SpectraMax spectrophotometric plate reader (Molecular Devices). Factor Xa (7 nM) was combined with inhibitor at varying concentrations in 50 mM Tris (pH 7.4 or 8.2 as indicated), 110 mM NaCl, 0.6 mM $MgCl_2$, 5 mM $CaCl_2$, 0.001% Antifoam A (Sigma) and 10% DMSO for 1 h at room temperature. Measurements in human serum albumin (HSA) assay conditions were performed with the addition of 0.45 mM HSA. Reactions were initiated by the addition of substrate (0.5 mM MeOC-Nle-Gly-Arg-pNA, Boehringer Mannheim) and the rate of substrate hydrolysis was measured by monitoring the change in absorbance (A_{405}) over 5 min. K_i apparent (K_i') calculations were performed by a nonlinear regression fit to the Morrison equation as described.
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17. **Synthesis of 1:** In a 100 mL round-bottom flask under a nitrogen atmosphere, 3-chloro-benzo[*b*]thiophene-2-carboxylic acid (0.030 g, 1.4 mmol), anhydrous DMF (30 mL) and 1,1-carbonyldiimidazole (0.23 g, 1.4 mmol) were stirred at room temperature for 20 min. 2-Amino-5-methyl-benzoic acid (0.18 g, 1.2 mmol) was added and the mixture was stirred for another 12 h. The precipitate was filtered and dried over P_2O_5 in a vacuum oven to give 295 mg of an off-white solid. This solid was placed in a 50 mL round-bottom flask and *p*-fluoroaniline was added (0.67 g, 6 mmol). The mixture was heated to 180 °C for 30 min, the crude residue was taken up in CH_2Cl_2, and purified by column chromatogrpahy on SiO_2 with 20% EtOAc/CH_2Cl_2 as the eluent to give **1** as an off-white solid: 0.31 g, 0.7 mmol, 58%. MS (ESI) calcd for $C_{23}H_{16}ClFN_2O_2S$: 438.1, found: MH^+ 439.0. ^1H NMR (300 MHz, DMSO) δ 11.42 (s, 1H), 10.62 (s, 1H), 8.29 (dd, 1 H, $J=8.4$, 1.2 Hz), 8.16–8.11 (m, 1H), 7.97–7.92 (m, 1H), 7.77–7.72 (m, 3H), 7.64–7.60 (m, 2H), 7.45 (dd, 1H, $J=8.5$, 1.6 Hz), 7.26–7.17 (m, 2H), 2.40 (s, 3H).
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