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Structure–Activity Relationships of Substituted Benzothiophene-anthranilamide Factor Xa Inhibitors†

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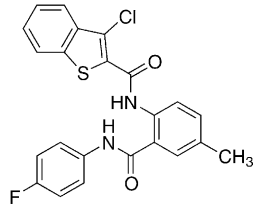
Abstract—Compound **1** was identified by high throughput screening as a novel, potent, non-amidine factor Xa inhibitor with good selectivity against thrombin and trypsin. A series of modifications of the three aromatic groups of **1** was investigated. Substitution of chlorine or bromine for fluorine on the aniline ring led to the discovery of subnanomolar factor Xa inhibitors. Positions on the anthranilic acid ring that can accommodate further substitution were also identified.

The serine protease factor Xa (f Xa) plays a central role in the coagulation cascade, located at the convergence point of the intrinsic and extrinsic pathways. As a component of the prothrombinase complex, f Xa catalyzes the conversion of prothrombin to thrombin.[2] Thrombin induces platelet activation and aggregation, and catalyzes the formation of polymerizable fibrin. Because inhibition of f Xa prevents thrombin formation but does not affect pre-existing thrombin, f Xa inhibitors may cause less impairment of hemostasis, and hence may be more effective and safer anticoagulants than direct thrombin inhibitors.[3] The effort to identify small molecule f Xa inhibitors has thus become a major focus in anticoagulant research.[4]

Our high throughput screening efforts led to the identification of compound **1** as a potent inhibitor of human f Xa ($K_{i,app} = 11$ nM), with good selectivity against other related serine proteases ($K_{i,app} > 5000$ nM for thrombin and trypsin).[5–7] Compound **1** was a unique lead, as it does not contain the benzamidine group characteristic of the majority of f Xa inhibitors, nor any other basic or charged groups thought to be important for binding to the S1 pocket of f Xa.[8] Because benzamidine-containing compounds often display unfavorable pharmacokinetic properties, compound **1** was a good starting point for optimization to an orally available f Xa inhibitor. In this paper, we describe our initial optimization efforts on this template.



1 fXa $K_{i,app}$ = 11 nM

Target compounds were constructed by coupling of the substituted aniline to the central ring, followed by attachment of the benzothiophene, as illustrated for the synthesis of compound **31** (Scheme 1). Commercially available 3-methoxy-2-nitrobenzoic acid was converted to the acid chloride, and coupled with 4-chloroaniline.

†Some of these data have been previously reported. See ref 1.

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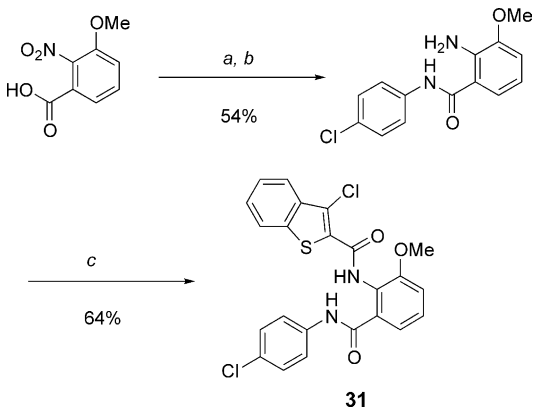


Scheme 1. Reagents and conditions: (a) (i) $SOCl_2$, DMF, CH_2Cl_2, rt; (ii) 4-chloroaniline, NEt_3, CH_2Cl_2, rt; (b) $SnCl_2{\cdot}2H_2O$, EtOAc, reflux (c) 3-chlorobenzothiophene-2-carbonyl chloride, pyridine, 0 °C to rt.

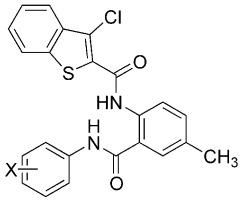
The nitro group was then reduced with tin chloride to afford the aniline intermediate. Alternatively this intermediate could be prepared by addition of an aniline to an isatoic anhydride derivative. Reaction of the aniline intermediate with commercially available 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride completed the synthesis of **31**.

We began our investigation with the fluorobenzene ring of **1**. The data in Table 1 show that this portion of the template was highly sensitive to changes in substituent type and position. Removal of the fluorine (**2**) had little effect on fXa activity. Replacement with bromine (**3**) or chlorine (**4**) increased potency by more than 20-fold to afford subnanomolar inhibitors. However, moving the chlorine to the 3-position (**5**) caused a large loss in potency ($K_{i,app} = 260$ nM), and moving the chlorine to the 2-position (**6**) gave an inactive compound. Methyl and methoxy substituents at C-4 (**7**, **8**) afforded similar potency to the lead compound, while increasing the size of the 4-substituent to ethoxy (**9**) caused a drastic loss of potency ($K_{i,app} > 5000$ nM). Electron-withdrawing substituents at C-4 were detrimental to fXa activity, as shown by comparing the 4-trifluoromethyl compound (**13**, $K_{i,app} > 5000$ nM) with the 4-methyl compound (**7**, $K_{i,app} = 7.1$ nM). Similarly, substitution with a carboxylic acid (**10**), ester (**11**) or nitrile (**12**) decreased fXa potency. Small electron or hydrogen-bond donating substituents such as 4-amino (**14**) and 4-aminomethyl (**15**) also caused a decrease in potency. By analogy to crystallographic data for related analogues,[9] as well as molecular modeling studies reported for a similar template,[8] this template is presumed to bind to fXa in a U-shaped conformation with the aniline ring in the S1 pocket and the benzothiophene in the S4 pocket. The sensitivity of the aniline ring to introduction of large and/or polar substituents is consistent with this binding mode, considering the limited size and hydrophobic nature of the S1 pocket.

We also investigated the replacement of the phenyl ring with heterocycles (Table 2). For pyridine analogues, the template was sensitive to the position of the pyridine nitrogen. The 2-pyridyl compound (**17**) had similar potency to the unsubstituted phenyl compound, while 3-pyridyl (**16**) unexpectedly caused a 10-fold loss of activity. Isosteric replacement of 2-pyridyl with 2-thiazole (**19**) afforded an inactive compound. Consistent with the phenyl series, the addition of a chlorine to the 2-pyridyl compound improved potency, although to a lesser extent (**18** vs **17**, **4** vs **2**).

After determining that a 4-chloro substituent was optimal for the terminal benzene ring, we investigated substitution on the central anthranilamide ring (Table 3). The C-5

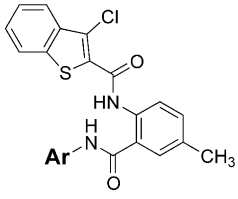
Table 1. Effect of aniline ring substituents on fXa inhibition



Compd	X	fXa $K_{i,app}$ (nM)[a,b,c]
1	4-F	11
2	H	19
3	4-Br	0.46
4	4-Cl	0.32
5	3-Cl	260
6	2-Cl	> 5000
7	4-Me	7.1
8	4-OMe	16
9	4-OEt	> 5000
10	4-CO_2H	4700
11	4-CO_2Me	> 5000
12	4-CN	150
13	4-CF_3	> 5000
14	4-NH_2	280
15	4-CH_2NH_2	1400

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. See ref 7 for assay conditions.
[b] $K_{i,app}$ values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are < 30% of the mean.
[c] All compounds had $K_{i,app}$ values for human thrombin and bovine trypsin of > 5000 nM.

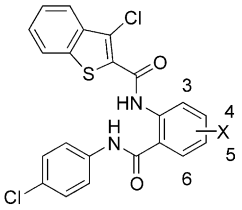
Table 2. Replacement of aniline with heteroaromatic rings



Compd	Ar	fXa $K_{i,app}$ (nM)[a,b,c]
2	Benzene	19
16	Pyridin-3-yl	210
17	Pyridin-2-yl	30
18	5-Cl-pyridin-2-yl	2.3
19	Thiazol-2-yl	> 5000

[a] $K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. See ref 7 for assay conditions.
[b] $K_{i,app}$ values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are < 30% of the mean.
[c] All compounds had $K_{i,app}$ values for human thrombin and bovine trypsin of > 5000 nM.

Table 3. Effect of anthranilic acid ring substituents on fXa inhibition



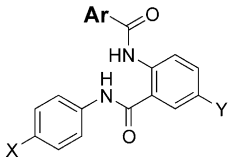
Compd	X	fXa $K_{i,app}$ (nM)[a,b,c]
4	5-CH_3	0.32
20	5-Cl	0.60
21	H	7.0
22	5-F	5.0
23	5-OH	73
24	5-NO_2	>500
25	5-NH_2	16
26	5-(4-Me-piperazin-1-yl)	470
27	5-(Pyrrolidin-1-yl)	>5000
28	5-OCH_2(4-MeOPh)	>5000
29	3-Cl	24
30	3-OH	18
31	3-OMe	22
32	3-Me	58
33	3-OCH_2CO_2Et	22
34	4-Me	7.2
35	4-F	15
36	4-Cl	26
37	4-CF_3	100
38	6-F	26
39	6-Cl	>5000
40	6-Me	>5000
41	3-Me,5-Cl	1.8
42	3-OMe,5-Cl	2.2
43	3-OH,5-Cl	3.7
44	4-F,5-Cl	7.0
45	4-Me,5-Cl	11

[a]$K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. See ref 7 for assay conditions.
[b]$K_{i,app}$ values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean.
[c]All compounds had $K_{i,app}$ values for human thrombin and bovine trypsin of >5000 nM.

position of this ring was found to be quite sensitive to substitution. Isosteric replacement of the methyl substituent with chlorine (**20**) retained subnanomolar fXa potency. However, removal of the C-5 substituent (**21**), or substitution with fluorine (**22**) decreased activity by 15- to 20-fold. Small polar protic groups such as hydroxy (**23**) and amino (**25**) also caused a decrease of activity (50- to 200-fold). Larger substituents, either electron-withdrawing (**24**) or electron-donating (**26–28**), drastically reduced inhibitory activity. Substitution at other positions of the anthranilamide ring affected potency to varying degrees. At C-3, chloro (**29**), hydroxy (**30**) and methoxy (**31**) substituents all afforded compounds with similar potency to the unsubstituted compound (**21**), while substitution with methyl (**32**) gave 8-fold lower activity ($K_{i,app} = 58$ nM). Extension of the 3-methoxy substituent, for example with an ethyl carboxylate (**33**), caused no reduction in activity, suggesting a potential site for further exploration.

At C-4, the methyl (**34**), fluoro (**35**) and chloro (**36**) compounds had similar potency to the unsubstituted compound (**21**). Increasing the electron-withdrawing nature of the substituent caused a decrease in potency (**37** vs **34**). At the 6-position, any substituent larger than hydrogen or fluorine abolished activity (**38–40**). Crystallographic data for related analogues suggests that the C-5 substituent extends into a small pocket and can make contact with the disulfide bridge between Cys191 and Cys220.[9] Chlorine and methyl appear to be the best fit for this interaction. The C-6 position is also in close proximity to the disulfide bridge, leaving insufficient space available for substitution. The C-3 and C-4 positions are exposed to solvent, consistent with the lack of effect of substitution on potency.

Table 4. Effect of substituting or replacing benzothiophene ring on fXa inhibition



Compd	X	Y	Ar	fXa $K_{i,app}$ (nM)[a,b,c]
1	F	Me	3-Cl-benzothiophen-2-yl	11
46	F	Me	Benzothiophen-2-yl	590
47	F	Me	3-Me-benzothiophen-2-yl	25
48	F	Me	3-Cl-thiophen-2-yl	390
4	Cl	Me	3-Cl-benzothiophen-2-yl	0.32
49	Cl	Me	3-MeO-benzothiophen-2-yl	5.9
50	Cl	Me	3-HO-benzothiophen-2-yl	4400
51	Cl	Me	3-Cl-thiophen-2-yl	130
52	Cl	Me	3-Cl-4-($MeSO_2$)-thiophen-2-yl	14
53	Cl	Me	3-MeO-thiophen-2-yl	160
54	Cl	Me	3-MeO-4-Br-thiophen-2-yl	54
55	Cl	Me	2-Br-phenyl	960
56	Cl	Me	Naphthalen-2-yl	78
57	Cl	Me	2-MeS-5-Cl-pyrimidin-4-yl	>5000
58	Cl	Me	2,4-diMe-thiazol-5-yl	210
59	Cl	Me	2-Me-pyridin-3-yl	>5000

[a]$K_{i,app}$ is the apparent K_i value, defined as $IC_{50}/2$ when the IC_{50} value is determined at a substrate concentration equal to the K_m. See ref 7 for assay conditions.
[b]$K_{i,app}$ values are averaged from multiple determinations ($n \geq 2$), and the standard deviations are <30% of the mean.
[c]All compounds had $K_{i,app}$ values for human thrombin and bovine trypsin of >5000 nM.

We were also interested in exploring disubstitution of the central ring. Because of the high potency of the 5-Cl analogue and the low tolerance for substitution at C-6, we concentrated our effort on 5-Cl analogues with a second substituent at C-3 or C-4. The 3,5-disubstituted compounds (**41–43**) all showed improved potency over the corresponding 3-monosubstituted analogues, and were nearly as potent as the 5-chloro analogue (**20**). In contrast, the 4,5-disubstituted analogues (**44** and **45**) showed no change in potency relative to the corresponding 4-monosubstituted compounds, gaining no benefit from the introduction of the 5-chloro group. Potencies for the 3,5-disubstituted compounds are consistent with the C-3 position being solvent exposed. The primary binding interaction on the central ring for these compounds is made by the 5-chloro substituent. It is less clear why the 4,5-disubstituted compounds are not more potent than the 4-monosubstituted compounds.

Concurrent with our studies of the aniline and anthranilic acid rings, we investigated substitution or replacement of the benzothiophene ring (Table 4). Substitution at the 3-position of the benzothiophene was required for potency, with chloro or methyl substituents giving the best result (**1** and **47** vs **46**). The larger 3-methoxy substituent reduced potency by ca. 20-fold (**49** vs **4**), while the small polar 3-hydroxy substituent caused a potency loss of more than 10,000-fold (**50**). The loss in activity of the C-3 unsubstituted analogue (**46**) and the 3-hydroxy analogue (**50**) could both be due to conformational effects. The chloro and methyl substituents should cause the benzothiophene to rotate out of the plane of the adjacent amide bond. For compounds **46** and **50**, the benzothiophene would be expected to be coplanar with the amide bond, due to lack of steric hindrance (**46**) or hydrogen bonding between the hydroxy group and the adjacent carbonyl group (**50**). Changing from benzothiophene to thiophene caused a 30- to 400-fold loss of potency (**1** vs **48** and **4** vs **51**). Some potency was regained when a methyl sulfone or bromo substituent was added at the 4-position of the thiophene (**52** vs **51** and **54** vs **53**). These results suggest that benzothiophene gives a better fit in the S4 pocket than does thiophene. However, a C-4 substituent on the thiophene may improve S4 binding. Replacement of the benzothiophene with other ring systems such as benzene, naphthalene, pyrimidine, thiazole and pyridine (**55–59**), all caused loss of fXa activity to varying degrees, although in most cases consideration was not given to optimal substitution of these ring systems. The most potency was retained by the isosteric 2-naphthyl analogue (**56**, $K_{i,app} = 78$ nM).

Compounds **1**, **4**, and **20** were tested for anticoagulant activity using the in vitro prothrombin time (PT) assay. Despite their fXa inhibitory activity, none of the compounds prolonged PT 2-fold at concentrations up to 500 μM. This result was attributed to the poor solubility of these compounds, along with their high lipophilicity, which likely results in high plasma protein binding. Related results have been reported for a similar series of lipophilic fXa inhibitors and for a series of lipophilic thrombin inhibitors.[8c,10] In these reports, potency of compounds in PT (fXa inhibitors) or in vitro activated partial thromboplastin time (APTT, thrombin inhibitors) assays did not correlate solely with activity against fXa or thrombin, but was additionally a function of lipophilicity.

We have explored structure–activity relationships around the novel non-amidine fXa inhibitor **1** by systematic modifications of each of the aryl rings. Small hydrophobic substituents were found to be optimal at C-3 on the benzothiophene ring. On the central ring, halogen or methyl substitution at C-5 is critical for high fXa potency, and a second substituent may be introduced at the 3-position. The only significant increase in potency was obtained by adding a chloro or bromo substituent to the 4-position of the aniline ring. These substitutions resulted in subnanomolar non-amidine fXa inhibitors. Further optimization of this template will be the subject of future publications.

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5. Compound **1** was originally purchased from Maybridge Chemical Company. A recent publication from Axys Pharmaceuticals has appeared detailing their initial SAR investigations of this compound: Shrader, W. D.; Young, W. B.; Sprengeler, P. A.; Sangalang, J. C.; Elrod, K.; Carr, G. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1801. Our independent results described herein are consistent with the findings of the Axys group.
6. A detailed description of the identification and characterization of compound **1** is the subject of another publication: Liang, A. M.; Light, D. R.; Kochanny, M.; Rumennik, G.; Trinh, L.; Lentz, D.; Post, J.; Morser, J.; Snider, M. *Biochem. Pharmacol.* In press.
7. **Enzyme assay procedures**.[11] The activities of human fXa, human thrombin and bovine trypsin were determined kinetically as the initial rate of cleavage of a peptide *p*-nitroanilide by the enzyme. The assay was performed at room temperature in flat-bottom microtiter plates in a final volume of 200 μL. The reaction mixture consisted of 50 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 7.5, with enzyme and substrate at the following concentrations: (1) fXa assay: 0.04–1 nM fXa and 164 μM S-2222; (2) thrombin assay: 16 nM thrombin and 300 μM S-2302; and (3) trypsin assay: 16 nM bovine trypsin and 127 μM S-2266. Standard techniques with at least four substrate dilutions were used to determine the K_m for a given enzyme and substrate. The substrate concentration listed is equal to the K_m. Controls without the test inhibitors or with a reference compound were also run in each assay plate. Enzyme was incubated with test compounds for 10 min; the reaction was then started by the addition of the substrate. Reaction rates were determined by measuring the rate of the absorbance change at 405 nm in a ThermoMax microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Data analysis methods. IC_{50} values for inhibitors were determined from the dose response curve by fitting the data to the Hill equation with an automated analysis method using a computer spreadsheet. $K_{i,app}$ values were calculated as the $IC_{50}/2$, with the IC_{50} value determined at a substrate concentration equal to K_m [$K_{i,app} = IC_{50}/(1 + [S]/K_m) = IC_{50}/2$]. For inhibitors with $K_{i,app}$ values less than 3 nM, IC_{50} values were determined by fitting data to a modification of the Morrison equation to correct for the proportion of inhibitor bound to the enzyme relative to the free inhibitor.[12] $K_{i,app}$ values are the mean of multiple determinations ($n \geq 2$). Standard deviations are $< 30\%$ of the mean.

8. (a) A similar series of neutral anthranilamide and diaminobenzene-derived fXa inhibitors has also been disclosed recently by researchers from Eli Lilly & Co.: Herron, D. K.; Goodson, T., Jr.; Wiley, M. R.; Weir, L. C.; Kyle, J. A.; Yee, Y. K.; Tebbe, A. L.; Tinsley, J. M.; Mendel, D.; Masters, J. J.; Franciskovich, J. B.; Sawyer, J. S.; Beight, D. W.; Ratz, A. M.; Milot, G.; Hall, S. E.; Klimkowski, V. J.; Wikel, J. H.; Eastwood, B. J.; Towner, R. D.; Gifford-Moore, D. S.; Craft, T. J.; Smith, G. F. *J. Med. Chem.* **2000**, *43*, 859. (b) Yee, Y. K.; Tebbe, A. L.; Linebarger, J. H.; Beight, D. W.; Craft, T. J.; Gifford-Moore, D.; Goodson, T., Jr.; Herron, D. K.; Klimkowski, V. J.; Kyle, J. A.; Sawyer, J. S.; Smith, G. F.; Tinsley, J. M.; Towner, R. D.; Weir, L.; Wiley, M. R. *J. Med. Chem.* **2000**, *43*, 873. (c) Masters, J. J.; Franciskovich, J. B.; Tinsley, J. M.; Campbell, C.; Craft, T. J.; Froelich, L. L.; Gifford-Moore, D. S.; Hay, L. A.; Herron, D. K.; Klimkowski, V. J.; Kurz, K. D.; Metz, J. T.; Ratz, A. M.; Shuman, R. T.; Smith, G. F.; Smith, T.; Towner, R. D.; Wiley, M. R.; Wilson, A.; Yee, Y. K. *J. Med. Chem.* **2000**, *43*, 2087.

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