Non-Amidine-Containing 1,2-Dibenzamidobenzene Inhibitors of Human Factor Xa with Potent Anticoagulant and Antithrombotic Activity

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Introduction. Human factor Xa (fXa) is a trypsin-like serine protease which serves a critical role in blood coagulation events.[1] Competitive inhibitors of fXa have demonstrated potent anticoagulant activity in vitro and antithrombotic efficacy in preclinical models in vivo.[2] Within the past decade, research efforts to identify small molecule inhibitors of this coagulation enzyme as novel therapies for thromboembolic disorders have increased.[3]

We recently disclosed a series of 1,2-dibenzamidobenzene derivatives that are selective, sub-micromolar inhibitors of fXa (**1**, Figure 1).[4] In contrast to most inhibitors of fXa reported which contain amidine functionality,[5] these compounds use selected hydrophobic groups for occupying both the S1 and S4 binding domains of the enzyme.[6] In the case of **1**, the 4-methoxyphenyl and 4-*tert*-butylphenyl groups reside in the S1-site and "aryl binding" S4-site of fXa, respectively.[7] Incorporation of an S1 binding amidine into these constructs yielded **2**.[8] Amidine **2** was significantly more potent than **1** in both binding affinity for fXa and prothrombin time (PT) activity (Table 1).[9] Moreover, the potency observed with **2** in vitro translated into potent antithrombotic efficacy in vivo. There is precedent that amidine-containing molecules, in general, display undesired pharmacokinetic properties after oral administration.[10] We describe here our efforts to identify fXa inhibitors that have potency similar to that of amidine **2** but lack this undesirable S1 binding functionality altogether.

Previous reported structure−activity relationship (SAR) studies of **1** revealed that 4-arylbenzamides are acceptable S4 binding elements.[4a] Derivative **3** was of particular note since it had a binding affinity for fXa similar to that of **1**; however, **3** was at least 5-fold more potent in PT assays (Table 1). This suggested the increased hydrophilicity of **3** significantly reduced interactions with plasma proteins, therefore allowing the fXa binding affinity to more effectively translate into potent anticoagulant activity.[11] This was further extended to the 1-(4-pyridyl)piperidine-containing derivative **4**.[12] Since the 1-(4-pyridyl)piperidine compound **4** (pK_a ∼ 9) is significantly more basic than the 4-phenylpyridine-containing **3** (pK_a ∼ 5), compound **4** should carry a positive charge at plasma pH, further reducing undesirable interactions with plasma proteins. Consistent with this hypothesis, both **3** and **4** had similar PT activities even though **3** had ∼4-fold higher binding affinity for fXa. Derivatives of **4** with enhanced binding affinity for fXa would be expected to be even more potent anticoagulants.

In this communication, we describe the SAR of altering the linkage of the 1-(4-pyridyl)piperidine to the central ring of **4** in order to optimize the placement of this group in the S4-site of fXa. Toward this end, we prepared various urethane and urea derivatives and measured their fXa binding affinity. Most of these compounds were found to have significantly higher binding affinity for fXa than **4**. Further, these data provided opportunity to examine the translation of fXa binding affinity into anticoagulant potency in this structurally related series. The most potent anticoagu-

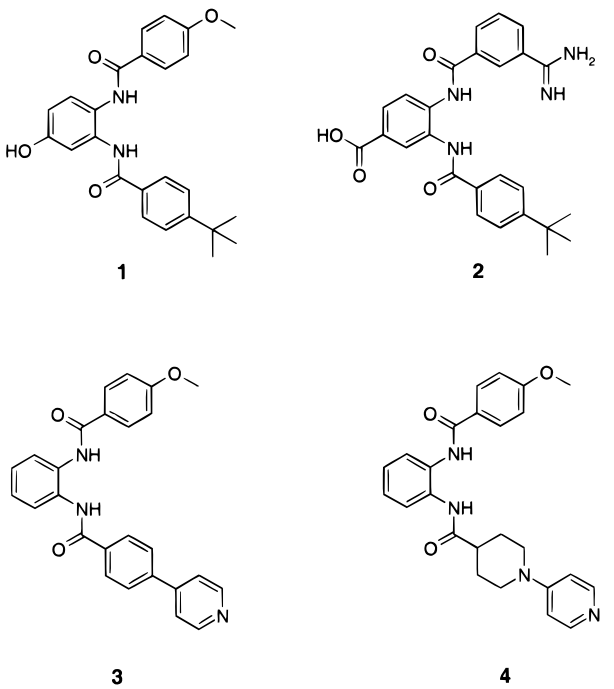

Figure 1. 1,2-Benzamidobenzene inhibitors of human factor Xa **1**−**4**.

Table 1. Human fXa Binding and Prothrombin Time Activity of 1,2-Benzamidobenzene Inhibitors **1**−**4**

compd	K_{ass}[a] (× 10^6 L/mol)	PT[b] (μM)
1	6.6	>50
2	470	0.83
3	9.2	9.5
4	2.3	9.7

[a] K_{ass} represents the apparent association constant as measured by the methods of Smith, G.F.; et al.[8] [b] PT is defined as the concentration of compound (μM) required to double the time to clot formation in the prothrombin time assay as described in ref 7.

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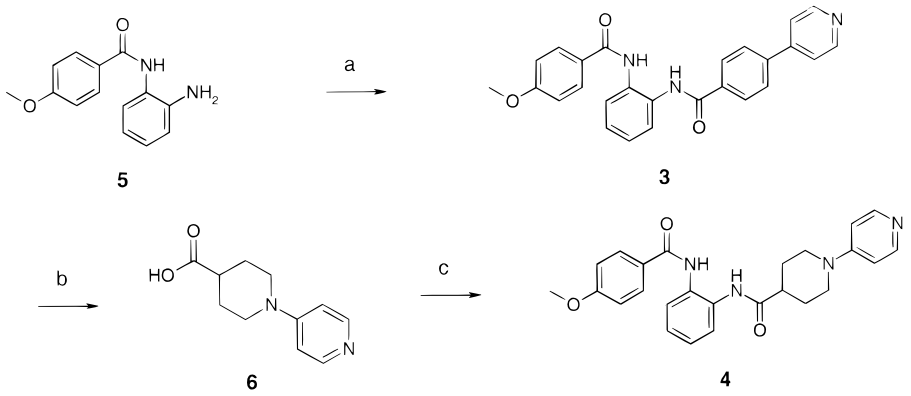
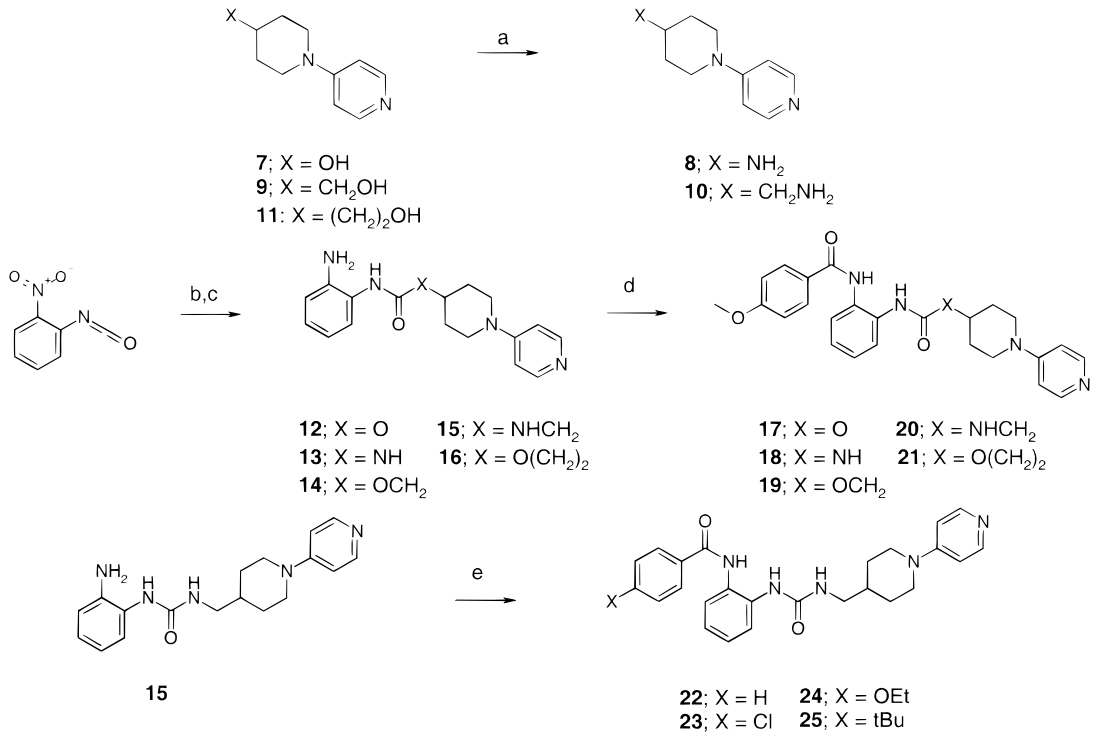
Scheme 1. Preparation of **3** and **4**[a]



[a] Reagents: (a) 4-(4-pyridyl)benzoic acid, thionyl chloride; then, **5**, pyridine, $CHCl_3$; (b) 4-chloropyridine hydrochloride, triethylamine, EtOH, 120 °C; (c) oxalyl chloride; then, **5**, pyridine, $CHCl_3$.

Scheme 2. Preparation of **17**−**25**[a]



[a] Reagents: (a) phthalimide, triphenylphosphine, diethylazodicarboxylate, THF; then, hydrazine monohydrate, EtOH, 70 °C; (b) **7**−**11**, $CHCl_3$/THF; (c) H_2, 10% Pd/C, EtOH; (d) *p*-anisoyl chloride, pyridine, $CHCl_3$; (e) aryl acid chloride, pyridine, $CHCl_3$.

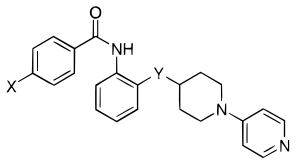
lant identified in these studies was examined in a rabbit arterio-venous shunt model of thrombosis in vivo, and these results are compared to amidine **2** of the related 1,2-dibenzamidobenzene series. Finally, with representative compounds, selectivity for inhibition of fXa versus other proteases is also discussed.

Chemistry. The synthesis of **3** and **4** is outlined in Scheme 1.[13] Treatment of N^1-(4-methoxybenzoyl)-1,2-benzenediamine (**5**)[4a] with 4-(4-pyridyl)benzoyl chloride[14] afforded **3**. The amide lead **4** was obtained from **5** and the acid chloride of **6**.[12]

Synthesis of the urethane and urea derivatives **17**−**25** is described in Scheme 2.[13] Preparation of **17**−**21** required the 1-(4-pyridyl)piperidine-containing intermediates **7**−**11**. Using a procedure similar to that described for the synthesis of acid **6**, 4-hydroxypiperidine and 4-chloropyridine afforded **7**. Conversion of this alcohol to amine **8** was accomplished using a two-step sequence involving a Mitsunobu reaction with phthalimide followed by a hydrazine deprotection sequence. Primary carbinol **9** was obtained by lithium aluminum hydride mediated reduction of the corresponding ethyl ester of acid **6**. Amine **10** was prepared using the same procedure described for **8**. Finally, alcohol **11** was obtained using the same protocol developed for constructs **6** and **7**. Reaction of **7**−**11** with 2-nitrophenyl isocyanate followed by reduction of the nitro functionality afforded the anilines **12**−**16**. The final compounds **17**−**21** were completed by treatment of **12**−**16** with 4-anisoyl chloride, respectively. The ureas **22**−**25** were prepared by reaction of aniline **15** with the corresponding aryl acid chlorides.

Results and Discussion. Illustrated in Table 2 are the results obtained with **17**−**25** in fXa binding affinity and PT assays in vitro. The constructs **17**−**21** have two characteristics worth mentioning. First, they maintain

Table 2. Human fXa Binding and Prothrombin Time Activity of **21**−**29**



compd	X =	Y =	K_{ass}[a] (× 10^6 L/mol)	PT[b] (μM)
17	OMe	NH(CO)O	17	2.6
18	OMe	NH(CO)NH	12	2.4
19	OMe	NH(CO)OCH_2	20	1.9
20	OMe	NH(CO)$NHCH_2$	62	0.96
21	OMe	NH(CO)$O(CH_2)_2$	2.5	>15
22	H	NH(CO)$NHCH_2$	5.9	6.2
23	Cl	NH(CO)$NHCH_2$	100	0.58
24	OEt	NH(CO)$NHCH_2$	15	2.6
25	*t*Bu	NH(CO)$NHCH_2$	0.04	

[a] K_{ass} represents the apparent association constant as measured by the methods of Smith, G.F.; et al.[8] [b] PT is defined as the concentration of compound (μM) required to double the time to clot formation in the prothrombin time assay as described in ref 7. K_{ass} and PT values are the average of at least three separate experiments with a standard deviation of less than 20%.

the 1,2-diamide-like functionality of lead compounds **1**−**4**. Second, these derivatives sequentially extend the 1-(4-pyridyl)piperidine group from the central core of **4**, allowing for alternate placement of this group in the S4-site of fXa. Published SAR studies in the 1,2-dibenzamidobenzene series revealed the benefits of the central amides to high fXa binding affinity.[4] Furthermore, molecular modeling studies of this series consistently indicated H-bonding interactions between the carbonyl of the amide linking the S4 binding group and Gly218 of fXa.[7] Modeling studies with **4** also displayed these particular interactions (Figure 2).[15] These studies with **4** further revealed that the 1-(4-pyridyl)piperidinyl functionality could reside "deeper" in the S4 binding site of fXa, which could further enhance binding affinity to the enzyme.

All of the derivatives **17**−**21** had higher binding affinity for fXa relative to lead compound **4**. Extension of the amide linkage of **4** by one atom, as in urethane **17** and urea **18**, afforded ∼7-fold and ∼5-fold more potent analogues, respectively. Even more active compounds resulted when the amide linker was extended by two atoms. In this case, the urea **20** was ∼27-fold more potent than **4** and had >3-fold greater binding affinity for fXa than carbamate **19**. Extension of the amide linkage of **4** by three atoms, as in **21**, provided comparable fXa binding activity. Overall, these data suggest that various positioning of the 4-(1-piperidinyl)-pyridine group within the S4-site of fXa is tolerated and high binding affinity for fXa can result for each.[16]

Having identified several linkers that provided enhanced fXa binding affinity, we focus on SAR of the 4-methoxyphenyl group. Assuming that this substituent serves as the S1 binding element, SAR trends consistent with those observed earlier in the 1,2-dibenzamidobenzene series were expected.[4a,8] The phenyl analogue **22** indicates the contribution of the 4-methoxy substituent to fXa binding affinity. The ∼10-fold decrease in activity of **22** compared to **20** is consistent with previously reported results. Similarly, 4-chlorophenyl also serves as a suitable S1 binding functionality as exemplified by compound **23**. Compounds **24** and **25** confirm the sensitivity of the S1-site to the size of the 4-substituent of the phenyl ring. Overall, these results strongly suggest that the 4-substituted benzamide in this series resides in the S1-site of fXa.

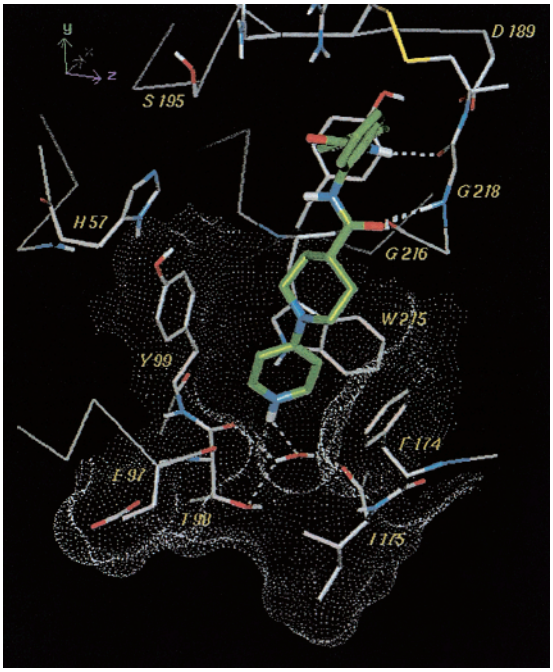


Figure 2. Proposed energy-minimized binding model of the compound **4** complexed with the active site of factor Xa. The orientation presented is looking directly into the S1 and S4 binding pockets. Intermolecular hydrogen bonds are shown as dashed lines, and the molecular surface for the S4−S4′ region is highlighted by white dots.

The range of the fXa binding affinity of compounds **17**−**25** enables an assessment of how this activity translates into anticoagulant potency for this series of fXa inhibitors. Among all these structurally related compounds, a clear relationship between higher binding affinity for fXa and an increase in anticoagulant potency was observed. Even though this correlation exists within this series of inhibitors, it does not hold when directly comparing these with other inhibitors of fXa that display different physiochemical properties.[17] For example, compound **23** was ∼2-fold more potent than amidine **2** as an anticoagulant, even though it was 4.5-fold less active in fXa binding assays. Additionally, compound **20** was ∼7-fold less potent in fXa binding assays, but had similar activity in the PT assay. These data illustrate the importance of considering both the fXa binding affinity and anticoagulant activity in vitro when attempting to compare the relative antithrombotic potential of fXa inhibitors that arise from distinct structural classes.

The antithrombotic activity for **23**, a non-amidine inhibitor of fXa with comparable activity to potent amidines in vitro, was evaluated in vivo. To choose an appropriate animal model, we measured the PT activity of **23** in plasma from various animal species.[18] The similarity between the rabbit and human plasma dose−response curves prompted us to evaluate **23** in a rabbit arterio-venous shunt model. As illustrated in Figure 3, **23** displayed dose-dependent antithrombotic efficacy after intravenous administration. An ED_{50} was obtained at a dose of 1.8 mg/kg/h. Initial evaluation of these data

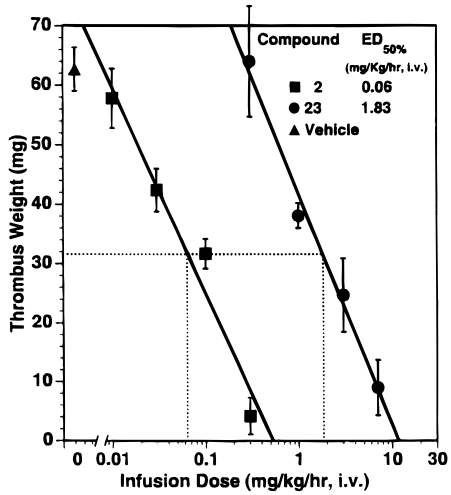


Figure 3. Antithrombotic dose response for compounds **2** and **23** in an AV shunt model of thrombosis in the rabbit during continuous infusion of compound for 30 min. Thrombosis was induced during the final 15 min of the infusion period.

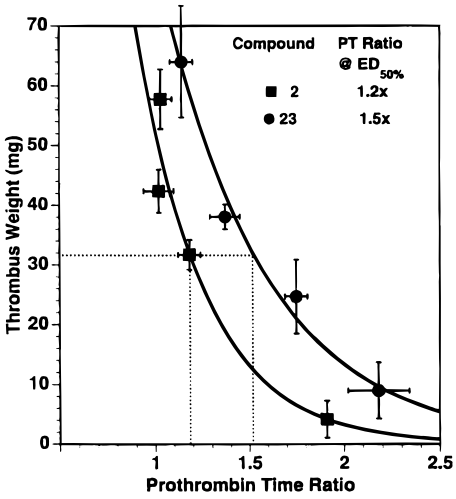


Figure 4. Relationship between prothrombin time ex vivo and antithrombotic response in the AV shunt model of thrombosis in the rabbit. Extension of prothrombin time reflects the average of samples collected from each animal before and after the period of thrombosis.

suggests amidine **2** was significantly more antithrombotic in this model, requiring only a dose of 0.06 mg/kg/mL to achieve an ED_{50} response. In contrast, inspection of the relationship between PT ex vivo and clot size suggests they have similar antithrombotic activity in vivo (Figure 4). Since **2** and **23** have similar dose–response curves in rabbit PT measurements, similar antithrombotic effects result at comparable plasma concentrations of these two inhibitors.[18] Individual pharmacokinetic properties of **2** and **23** likely explain potency differences observed in dose.[17]

Since many trypsin-like proteases have essential physiological functions,[19] an important development consideration for fXa inhibitors is the ability of the molecule to bind selectively. Toward this end, both **20** and **23** were evaluated in several enzyme binding assays (Table 3). Both compounds displayed >500-fold selectivity for fXa versus several coagulation (IIa, fXIa, fXIIa, and activated protein C) and fibrinolytic enzymes (plasmin, t-PA, urokinase). Further, these compounds had no significant binding to bovine trypsin.

Table 3. Binding Affinity of Compounds **20** and **23** for Various Enzymes

enzyme	K_{ass} for **20**[a] ($\times 10^6$ L/mol)	K_{ass} for **23**[a] ($\times 10^6$ L/mol)
fXa	62	100
IIa	<0.01	0.06
fXIa	<0.01	<0.01
fXIIa	<0.01	<0.01
activated protein C	<0.01	<0.01
plasmin	<0.01	0.09
t-PA	<0.01	0.14
urokinase	<0.01	<0.01
trypsin (bovine)	<0.01	<0.01

[a] K_{ass} represents the apparent association constant as measured by the methods of Smith, G.F.; et al.[8]

Conclusions

Variation of the linkage which connects the central ring of **4** to the S4 binding 1-(4-pyridyl)piperidine group produced several potent nonamidine inhibitors of human fXa. Similar to amidine-containing inhibitors of fXa, this series shows efficient translation of enzyme binding affinity into anticoagulant potency. The most potent compound, **23**, was 43-fold more active than lead molecule **4** in fXa binding assays and ~10-fold more potent in prothrombin clotting time assays. Dose-dependent antithrombotic efficacy was observed with **23** when evaluated intravenously in a rabbit arterio-venous shunt model in vivo. In this thrombosis model, similar PT ex vivo and antithrombotic effects in vivo were noted with **23** and the potent amidine **2** of the 1,2-dibenzamidobenzene series. Compound **23** was highly selective for binding to fXa. These potent and selective nonamidine containing inhibitors of fXa not only represent novel anticoagulants, they are also useful tools to further our preclinical understanding of competitive fXa inhibition.

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Supporting Information Available: Mean and standard deviations of measurements in vitro, elemental analyses, and ^{1}H NMR and MS spectral data for **3**, **4**, and **17**–**25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The pK_a values for compounds **2**, **4**, and **23** were 11.0 ± 0.2 (3.9 ± 0.1-acid), 8.7 ± 0.02, and 8.9 ± 0.1, respectively. The LogD values measured at pH = 7.4 for compounds **2**, **4**, and **23** were −1.0, 1.1, and 1.9, respectively.
(18) The importance of using species anticoagulant selectivity profiles to guide the selection of the appropriate animal model was revealed in our earlier reported studies.[8] The dose response of prothrombin clotting times in various species plasma for **2** and **23** was determined as described in ref 8. The PT concentrations of **2** and **23** in rabbit plasma were 0.84 ± 0.05 μM and 0.78 ± 0.03 μM, respectively. The PT for **23** was >20 μM in both rat and dog plasma.
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