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Discovery and characterization of a potent and selective non-amidine inhibitor of human factor Xa

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Abstract

Benzothiophene-anthranilamide **1** (3-chloro-*N*-[2-[[(4-fluorophenyl)amino]carbonyl]-4-methylphenyl]benzo[*b*]thiophene-2-carboxamide) was discovered by high throughput screening to be a highly potent and selective non-amidine inhibitor of human factor Xa with a K_i of 15 ± 4 nM. Compound **1** is a selective inhibitor of human factor Xa as suggested by the $K_{i(app)}$ determined for nine other human serine proteases and bovine trypsin. The activity of reconstituted human prothrombinase complex was inhibited by compound **1** when assayed in physiological concentrations of the substrate prothrombin. However, 27-fold higher inhibitor concentrations were needed to achieve the same level of inhibition than were required for the inhibition of free factor Xa, due in part to non-specific binding of the inhibitor to phospholipid under the assay conditions. Failure to demonstrate enzymatic cleavage of compound **1** suggests that compound **1** is solely an inhibitor rather than a substrate for factor Xa. The inhibition of factor Xa by compound **1** was reversible upon dilution of the enzyme/inhibitor mixture. Analyses of the inhibition mechanism with Dixon, Cornish–Bowden, and Lineweaver–Burk plots showed that compound **1** is a linear mixed-type inhibitor with 5-fold higher affinity for free factor Xa than the factor Xa/substrate complex. The linear mixed-type inhibition suggests that compound **1** binds to the active site region of factor Xa, but its binding cannot be fully displaced by the substrate S2222 (1:1 mixture of *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide and *N*-benzoyl-Ile-Glu(γ-OMe)-Gly-Arg-*p*-nitroanilide hydrochloride). Thus, the inhibition mechanism for compound **1** is novel compared to most serine protease inhibitors including amidine-containing factor Xa inhibitors, which rely on binding to the S1 pocket of the enzyme active site. Compound **1** represents an attractive, novel structural template for further development of efficacious, safe, and potentially orally active human factor Xa inhibitors.

Keywords: Human factor Xa; Anticoagulant; Benzothiophene-anthranilamide

1. Introduction

Human factor Xa, a serine protease located at the convergence of the intrinsic and extrinsic blood coagulation pathways, catalyzes the activation of prothrombin to thrombin. Due to its focal function and upstream location from thrombin in the coagulation cascade, selective inhibition of factor Xa rather than thrombin may be a more effective and safer treatment for thrombotic disorders. Indeed, such effective and safe anticoagulant properties have been demonstrated with the naturally occurring small protein factor Xa inhibitors antistasin and tick anticoagulant peptide [1]. Considerable efforts have been made in recent years to discover small molecule factor Xa inhibitors [2]. Potent and selective small molecule factor Xa inhibitors have been demonstrated to be effective and safe anticoagulants in animal thrombosis models [3–8]. Until recently, most small molecule factor Xa inhibitors utilized a basic amidine moiety as an arginine side chain mimetic to achieve sufficient potency [2,9–16]. This highly basic functionality, however, has been implicated in the observed

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Abbreviations: SucAAPF-pNA, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; MeOSucAAPV-pNA, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; S2222, 1:1 mixture of *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide and *N*-benzoyl-Ile-Glu(γ-OMe)-Gly-Arg-*p*-nitroanilide hydrochloride; S2302, *H*-D-Pro-Phe-Arg-*p*-nitroanilide dihydrochloride; S2266, *H*-D-Val-Leu-Arg-*p*-nitroanilide dihydrochloride; S2288, *H*-D-Ile-Pro-Arg-*p*-nitroanilide dihydrochloride; Spectrozyme TH, *H*-D-HHT-Ala-Arg-*p*-nitroanilide 2AcOH; PCPS, phospholipid vesicles containing 80% phosphatidylcholine and 20% phospatidylserine; PPACK-thrombin, D-Phe-Pro-Arg-chloromethylketone-inhibited human α-thrombin; PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time; TFA, trifluoracetic acid; GLA-domain, γ-carboxyglutamic acid-containing domain.

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suboptimal pharmacokinetic properties including low oral bioavailability [16–18]. Efforts to improve the pharmacokinetic and pharmacodynamic properties of the amidine-containing factor Xa inhibitors have focused largely on replacing the highly basic amidine group with less basic or neutral groups to bind to the S1 site [19–21]. In this paper, we report the discovery by high throughput screening and characterization of benzothiophene-anthranilamide **1**, a novel, highly potent and selective non-amidine inhibitor of human factor Xa.

2. Materials and methods

2.1. Materials

Human factor Xa, human α-thrombin, human prothrombin, human plasmin, rat factor Xa, rabbit factor Xa, and dog factor Xa were purchased from Enzyme Research Laboratories, Inc. Human factor Va, Gla-domainless human factor Xa, human factor VIIa, and human factor XIa were obtained from Haematologic Technologies, Inc. Human tissue plasminogen activator (2-chain), human urokinase, SucAAPF-pNA, MeOSucAAPV-pNA, and bovine serum albumin (fraction V, RIA grade) were purchased from Sigma Chemical Co. Bovine trypsin was purchased from Boehringer Mannheim. S2222, S2302, S2266, S2288, S2366, and S2444 were purchased from Kabi Pharmacia Hepar, Inc. Spectrozyme TH was obtained from American Diagnostica, Inc. Phosphatidylserine and phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. The benzothiophene-anthranilamide **1** and 3-chlorobenzo[*b*]thiophene-2-carboxylic acid were initially obtained from Maybridge through Ryan Scientific, Inc. 4-Fluoroaniline and 2-amino-5-methylbenzoic acid were purchased from the Aldrich Chemical Co. The synthesis of compound **6a** has been described [22]. Compound **1** [23], 2-[[(3-chlorobenzo[*b*]thien-2-yl)carbonyl]amino]-5-methylbenzoic acid, and 2-amino-*N*-(4-fluorophenyl)-5-methyl benzamide were synthesized at Berlex.

2.2. Human factor Xa and other serine protease inhibition assays

All assays measured the inhibitory effect of compounds on the initial cleavage rate of the peptide-*p*-nitroanilide substrates by serine proteases. Detailed assay conditions on the assay buffers and the concentrations of enzymes, substrates, and cofactors used in factor Xa and other serine protease assays are described in Table 1. Buffer A contained 50 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol 6000, pH 7.5. Buffer B was similar to Buffer A except that it contained 0.02% NaN_3 and 5 mM instead of 2.5 mM $CaCl_2$. Assays were performed in a final volume of 200 μL in flat-bottom 96-well microtiter plates at room temperature. One hundred microliters of serine protease (final assay concentration as specified in Table 1) was incubated with 50 μL of compounds (4× final assay concentrations) for 10 min. At the end of the incubation, reaction was initiated by adding 50 μL of peptide-*p*-nitroanilide substrate (final assay concentration as specified in Table 1). The increase in absorbance at 405 nm was measured kinetically for 2–5 min in a ThermoMax plate reader (Molecular Devices). Control experiments without compound or with a reference inhibitory compound were run in parallel in the same plate. The substrate concentration used was equal to the K_m. Standard techniques with at least four substrate dilutions were used to determine the K_m for a given enzyme and substrate. The final DMSO concentration in the assays was 0.1% or less. At least six serially diluted compound concentrations were used in the assays. The $K_{i(app)}$ values were determined from the concentration–response curve by linear fit of the data to the Hill equation with an automated analysis method using a Microsoft Excel spreadsheet. In this report, $K_{i(app)}$ is defined as the concentration of inhibitor resulting in 50% inhibition of the protease activity under the following conditions: (a) for the free protease assays, $K_{i(app)}$ was determined under conditions where the chromogenic peptide substrate was equal in concentration to the K_m of the substrate, and (b) for the prothrombinase assay, $K_{i(app)}$ was determined under conditions where the prothrombinase complex was assayed with a physiological concentration of the substrate, prothrombin (1.2 μM). At least two independent determinations were made for each assay.

2.3. Human prothrombinase inhibition and in vitro coagulation assays

The activation of human prothrombin to thrombin by human prothrombinase was measured in a two-step assay in Buffer A + 0.05% bovine serum albumin in a 96-well microtiter plate at room temperature. Thirty microliters of pre-assembled human prothrombinase (consisting of final concentrations of 1 pM factor Xa, 5 nM factor Va, and 20 μM PCPS) was incubated with 10 μL of the compound (5× final concentration) prior to the addition of 10 μL of human prothrombin (final concentration, 1.2 μM). Incubation was carried out for 6 min before the addition of 50 μL of 20 mM EDTA in the assay buffer to stop the prothrombinase reaction. This was followed by 100 μL of 0.2 mM Spectrozyme TH (in the assay buffer containing 10 mM EDTA). The activity of the thrombin product was then measured by monitoring the rate of cleavage of Spectrozyme TH by thrombin at 405 nm. The amount of thrombin generated was determined from a thrombin standard curve. Controls without compound or with an inhibitory compound were run in the same plate. Six serially diluted compound concentrations were used in the assays. The $K_{i(app)}$ values were determined as described above. The *in vitro* coagulation assays (PT, aPTT, and TT) were

Table 1
Serine protease assay protocols

Assay	Enzyme (concentration) Source (supplier)	Cofactor (concentration) Source (supplier)	Substrate (concentration) Supplier (buffer)
(1) Human factor XIa	Factor XIa (0.5 nM) Plasma (HTI)[a]	None	S2366 (250 μM) Kabi[b] (Buffer B)
(2) Human tissue plasminogen activator	2-Chain tPA (10 nM) Cell line (Sigma)	None	S2288 (371 μM) Kabi (Buffer A)
(3) Human urokinase	Urokinase (2 nM) Cell line (Sigma)	None	S2444 (32 μM) Kabi (Buffer A)
(4) Human plasmin	Plasmin (20 nM) Plasma (ERL)[c]	None	S2302 (146 μM) Kabi (Buffer A)
(5) Bovine trypsin	Trypsin (16 nM) Pancreas (BMC)[d]	None	S2266 (127 μM) Kabi (Buffer A)
(6) Human chymotrypsin	Chymotrypsin (4 nM) Pancreas (ART)[e]	None	SucAAPF-pNA (80 μM) Sigma (Buffer A)
(7) Human cathepsin G	Cathepsin G (43 nM) Blood (ART)	None	SucAAPF-pNA (1900 μM) Sigma (Buffer A)
(8) Human neutrophil elastase	Elastase (6 nM) Blood (ART)	None	MeOSucAAPV-pNA (60 μM) Sigma (Buffer A)
(9) Human factor Xa	Factor Xa (0.04–1 nM) Plasma (ERL)	None	S2222 (164 μM) Kabi (Buffer A)
(10) Rat factor Xa	Factor Xa (1 nM) Plasma (ERL)	None	S2222 (360 μM) Kabi (Buffer A)
(11) Rabbit factor Xa	Factor Xa (1 nM) Plasma (ERL)	None	S2222 (164 μM) Kabi (Buffer A)
(12) Dog factor Xa	Factor Xa (1 nM) Plasma (ERL)	None	S2222 (263 μM) Kabi (Buffer A)
(13) Human prothrombinase complex	Factor Xa (1 pM) Plasma (ERL)	(a) Factor Va (5 nM) Plasma (HTI) (b) Phospholipid (20 μM) Synthetic (APL)[f]	Prothrombin (1200 μM) ERL (Buffer A + 0.05% BSA) [thrombin quantified with Spectrozyme TH (ADI)][g]
(14) Human thrombin	Thrombin (20 nM) Plasma (ERL)	None	S2302 (300 μM) Kabi (Buffer A)
(15) Human factor VIIa/tissue factor (TF) complex	Factor VIIa (10 nM) Plasma (ERL)	Soluble TF (100 nM) Recombinant (BBS)[h]	S2266 (800 μM) Kabi (Buffer B)
(16) Bovine factor Xa	Factor Xa (1 nM) Plasma (ERL)	None	S2222 (164 μM) Kabi (Buffer A)

[a] Haematological Technologies, Inc.
[b] Kabi Pharmacia Hepar, Inc.
[c] Enzyme Research Laboratories, Inc.
[d] Boehringer Mannheim Corp.
[e] Athens Research and Technology, Inc.
[f] Avanti Polar Lipids, Inc.
[g] American Diagnostica, Inc.
[h] Berlex Biosciences.

performed in human plasma (N = 3) as previously described [24].

2.4. High throughput screening of compound libraries against human factor Xa

High throughput screening of compound libraries was performed using an orthogonal matrix-based method [25]. This method was based on screening pools of ten compounds with each compound being assayed twice in the presence of two sets of nine other compounds. The active compound was predicted by determining which compound was present in an active pool both times that a pool containing that compound was tested. Compounds were assayed against human factor Xa at a final 1 μM concentration per compound. Assay conditions were the same as those described in the section above except that 3 nM human factor Xa was used. Compounds with

inhibitory activity equal to or greater than 50% at 1 μM were re-assayed individually. $K_{i(app)}$ values of the confirmed hits were then determined as described above.

2.5. Determination of the susceptibility of compound 1 to cleavage by human factor Xa

Compound **1** (final concentration, 50 μM) was incubated with human factor Xa (final concentration, 10 μM) in Buffer A in a total volume of 600 μL for 21 hr at room temperature. A compound **1** control without factor Xa, and a factor Xa control without compound **1** were also run in parallel. At the end of the incubation, aliquots of samples (70 μL) were removed and frozen immediately in a dry ice alcohol bath. Samples (10 μL) were then analyzed by HPLC on a Dynamax C-18 column using a 70–95% gradient of 0.1% TFA/CH_3CN vs. 0.1% TFA/H_2O with a flow rate of 1.0 mL/min. Eluted compounds were detected by absorbance at 254 nm and quantified by comparison to the HPLC results of standard compounds of known concentration run under identical conditions.

2.6. Determination of the reversibility of the inhibition of human factor Xa by compound 1

Human factor Xa (final concentration, 1 μM) was incubated with or without compound **1** (final concentration, 1.2 μM) in Buffer A in a total volume of 100 μL for up to 22 hr at room temperature. At the end of various incubation times, 5 μL of the incubation mixture was diluted 100-fold in Buffer A. This was immediately followed by a further 10-fold dilution of 20-μL diluted samples in 182 μM S2222 in Buffer A in a 96-well microtiter plate. The final mixture contained 164 μM S2222 and 1 nM factor Xa in 200 μL volume. The cleavage rate of S2222 by factor Xa was measured immediately at 405 nm for 5 min in a ThermoMax plate reader. The recovery of the factor Xa activity upon dilution of the factor Xa/compound **1** mixtures taken at different time points was calculated as the percentage of the factor Xa activity of the corresponding factor Xa control without compound **1**.

2.7. Measurement of the cleavage of N-benzoyl-arginine-p-nitroanilide by human factor Xa

Human factor Xa (final concentration, 200 nM) was incubated with or without inhibitor (final concentration, 10 μM) in 150 μL of Buffer A for 20 min at room temperature in a 96-well microtiter plate. Reactions were started by the addition of 50 μL of *N*-benzoyl-arginine-*p*-nitroanilide to a final concentration of 156 μM in a 200-μL assay volume. Cleavage of *N*-benzoyl-arginine-*p*-nitroanilide by the enzyme was measured by monitoring the increase in absorbance at 405 nm at 30-min intervals for up to 8 hr at room temperature in a ThermoMax plate reader. The rate of cleavage of *N*-benzoyl-arginine-*p*-nitroanilide by human factor Xa was found to be linear for the first 4 hr of the assay and used to calculate the inhibitory activity of the test compounds. Inhibitory activities of the test compounds were expressed as percent inhibition using the following equation: $\%\,\text{Inhibition} = [[(\text{Rate without compound}) - (\text{Rate with compound})]/(\text{Rate without compound})] \times 100\%$.

3. Results

3.1. Discovery of benzothiophene-anthranilamide 1, a non-amidine factor Xa inhibitor, by high throughput screening

The benzothiophene-anthranilamide, compound **1** (3-chloro-*N*-[2-[[(4-fluorophenyl)amino]carbonyl]-4-methylphenyl]benzo[*b*]thiophene-2-carboxamide) purchased from Maybridge (Fig. 1), was discovered by high throughput screening to be a potent inhibitor of human factor Xa. It displayed a $K_{i(app)}$ value of 17 ± 9 nM for human factor Xa when the small peptide-*p*-nitroanilide, S2222, was used as the substrate (Table 2). The inhibitory activity and the structure of compound **1** were confirmed by re-synthesis of compound **1**, by assay and analysis of the re-synthesized compound **1** with NMR, mass spectrometry, and HPLC. Unlike many factor Xa inhibitors described thus far [2,9–16], compound **1** does not contain the strongly basic amidine functionality.

3.2. Serine protease and factor Xa species selectivity of benzothiophene-anthranilamide 1

Selectivity of compound **1** was determined for six trypsin-like serine proteases involved in blood coagulation or fibrinolysis, three chymotrypsin-like serine proteases, bovine trypsin, and factor Xa from four non-human species. Results in Table 2 show that compound **1** was highly selective for human factor Xa in solution vs. other soluble serine proteases. As compared to the $K_{i(app)}$ of 17 ± 9 nM for factor Xa, the $K_{i(app)}$ values of compound **1** for the ten other serine proteases tested were in the range of 10–200 μM. The effect of compound **1** on the prothrombinase complex was measured by monitoring the amount of thrombin generated from a physiological concentration

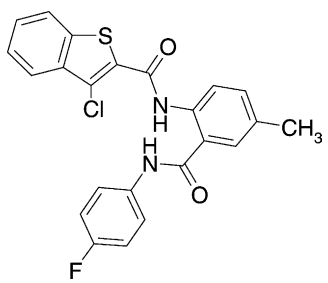


Fig. 1. Structure of compound **1**.

Table 2
Potency and selectivity of compound **1**

Serine protease	$K_{i(app)}$ (nM)
Human proteases	
Trypsin-like	
Human factor Xa	17 ± 9
Human prothrombinase	454 ± 13
Human thrombin	>10,000
Human factor VIIa/TF	>10,000
Human factor XIa	>200,000
Human tPA	>10,000
Human plasmin	>50,000
Human urokinase	>50,000
Bovine trypsin	>10,000
Chymotrypsin-like	
Human chymotrypsin	>50,000
Human cathepsin G	>50,000
Human neutrophil elastase	>50,000
Non-human factor Xa	
Rat factor Xa	53 ± 3
Rabbit factor Xa	34 ± 0
Dog factor Xa	89 ± 23
Bovine factor Xa	38 ± 4

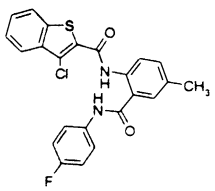
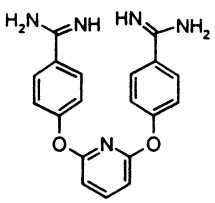
Assay conditions were as described in Section 2. Values are means ± SD, N = 3–29. Abbreviations: TF, tissue factor; and tPA, tissue plasminogen activator.

of prothrombin by the enzyme complex. Thrombin was quantified by activity measurement after the prothrombinase reaction was quenched with 10 mM EDTA that resulted in a complete inhibition of the activity of prothrombinase under the present assay conditions (data not shown). The activity of the reconstituted human prothrombinase complex was inhibited by compound **1**, but 27-fold higher inhibitor concentrations were required to achieve similar levels of inhibition than those required for inhibition of free factor Xa. When tested on non-human factor Xa, the $K_{i(app)}$ values of compound **1** were 2- to 5-fold higher than that for human factor Xa.

3.3. Binding of benzothiophene-anthranilamide *1* to phospholipid

The observation that the $K_{i(app)}$ of compound **1** for the prothrombinase was higher than that for factor Xa led us to examine possible reasons for this difference in apparent affinity. Because compound **1** is hydrophobic, we hypothesized that compound **1** could bind to phospholipid vesicles required in the prothrombinase assay. When tested in the factor Xa assay with a small chromogenic peptide substrate, in the presence and absence of phospholipid, the potency of compound **1** for factor Xa decreased by 19-fold when 20 μM phospholipid was present in the assay (Table 3). In contrast, the potency of a less hydrophobic inhibitor, compound **6a**, a simple arylamidine factor Xa inhibitor, was not affected by the phospholipid. Factor Xa binds to phospholipid through the N-terminal γ-carboxyglutamic acid (Gla)-containing domain in the presence of calcium ion [26], and this interaction may mask the interaction site of compound **1** on factor Xa to reduce the apparent potency of the compound. To investigate such a possibility, the potency of compound **1** for Gla-domainless factor Xa was determined and compared with that for regular factor Xa. Results in Table 4 showed that in the absence of phospholipid the potency of compound **1** for Gla-domainless factor Xa was similar to that for factor Xa. In the presence of 20 μM phospholipid, however, the potency of compound **1** for the Gla-domainless factor Xa was decreased by an extent (28-fold) similar to that observed with factor Xa (19-fold). Since the Gla-domainless factor Xa is unable to bind to phospholipid, these results suggested that compound **1** interacted with

Table 3
Effect of phospholipid on the potency of compounds **1** and **6a**

Inhibitor	Structure	$K_{i(app)}$ (nM) (no PCPS)	$K_{i(app)}$ (nM) (20 μM PCPS)	Ratio ± PCPS
Compound **1**		17 ± 9	318 ± 111	19.0
Compound **6a**		592 ± 46	649 ± 26	1.1

Human factor Xa (1 nM) was incubated with inhibitor in the presence and absence of 20 μM PCPS prior to the addition of substrate S2222. Assay conditions were as described in Section 2. Values are means ± SD, N = 4–29.

Table 4
Effect of phospholipid on the potency of compound **1** against factor Xa or Gla-domainless factor Xa

Enzyme	$K_{i(app)}$ (nM) (no PCPS)	$K_{i(app)}$ (nM) (20 μM PCPS)	Ratio ± PCPS
Factor Xa	17 ± 9	318 ± 111	19
Gla-domainless factor Xa	16 ± 7	464 ± 17	28

Human factor Xa (1 nM) or Gla-domainless human factor Xa (1 nM) was incubated with compound **1** in the presence or absence of 20 μM PCPS prior to the addition of substrate S2222. Assay conditions were as described in Section 2. Values are means ± SD, N = 6–29.

phospholipid, thereby masking its ability to inhibit factor Xa. Direct binding of compound **1** to phospholipid and subsequent separation of the compound/phospholipid complex by gel filtration column chromatography was attempted, but analysis was complicated by compound aggregation under the conditions used. Nevertheless, when a preincubated mixture of phospholipid and compound **1** was eluted on a Sephadex G25 column, the concentration of compound **1** in the combined flow-through fractions that contained the phospholipid vesicles was 2- to 3-fold higher than that in the corresponding fractions from the parallel control experiment with compound **1** alone (data not shown). These results are consistent with the hypothesis that the loss of potency of compound **1** in the factor Xa assays in the presence of phospholipid is due to the binding of compound **1** to phospholipid. The phospholipid-binding property of compound **1** is consistent with the hydrophobic nature of the compound and contributes to the higher $K_{i(app)}$ obtained in the prothrombinase assays.

3.4. Susceptibility of benzothiophene-anthranilamide *1* to cleavage by human factor Xa

Since compound **1** contained two peptide-like amide bonds, we evaluated whether compound **1** could be cleaved by factor Xa, hence functioning as a substrate. A second substrate molecule would compete with the chromogenic peptide substrate S2222 at the substrate-binding site, thus showing apparent inhibition of peptidase activity as measured by this assay. This possibility was investigated by incubating compound **1** in the presence and absence of excess human factor Xa (10 μM) for up to 21 hr at room temperature. Samples taken at different time points were analyzed by HPLC for potential cleavage products. Standards corresponding to five possible cleavage products of compound **1** were either purchased or synthesized: (a) 4-fluoroaniline, (b) 3-chlorobenzo[*b*]thiophene-2-carboxylic acid, (c) 2-amino-5-methylbenzoic acid, (d) 2-[[(3-chlorobenzo[*b*]thien-2-yl)carbonyl]amino]-5-methylbenzoic acid, and (e) 2-amino-*N*-(4-fluorophenyl)-5-methyl benzamide. The HPLC elution profiles of the standards were determined beforehand (data not shown). The results in Fig. 2 demonstrated that compound **1** was stable over the experimental period in the absence of factor Xa. After 21 hr of incubation with excess factor Xa (10 μM), compound **1** remained essentially intact with a peak area 90% of the control, and no material was detectable that co-migrated with the potential cleavage products. Compound **1** thus acted as an inhibitor rather than a substrate for human factor Xa.

3.5. Reversibility of inhibition of human factor Xa by benzothiophene-anthranilamide *1*

Reversibility of the inhibition of human factor Xa by compound **1** was examined by determining the recovery of factor Xa activity upon dilution of a preincubated enzyme/inhibitor mixture taken at different time points over about a 5-hr incubation period. As shown in Fig. 3, >95% of factor Xa activity was recovered after preincubating the enzyme with equimolar compound **1** over about 5 hr compared with control factor Xa incubated in the absence of inhibitor. Even after overnight incubation with the inhibitor, >90% of factor Xa activity still remained (results not shown). These results indicate that the inhibition of human factor Xa by compound **1** is freely reversible.

3.6. Mechanism of inhibition of human factor Xa by benzothiophene-anthranilamide *1*

The mechanism of inhibition of human factor Xa by compound **1** was determined by measuring factor Xa activity in the presence of increasing concentrations of the inhibitor and the chromogenic peptide substrate S2222. Kinetics data were analyzed graphically by three types of plots (Fig. 4). As shown in the Dixon plot (Fig. 4A), all four lines were linear and intersected at a common point above the *x*-axis ([inhibitor]) and to the left of the *y*-axis (1/*V*), suggesting either competitive or linear mixed-type inhibition with $\alpha > 1$ [27]. Since it was not possible to distinguish between these two types of inhibition from a Dixon plot, kinetics data were analyzed further graphically by both the Cornish–Bowden plot (Fig. 4B) and the Lineweaver–Burk plot (Fig. 4C) [27,28]. Although the four linear lines in the Cornish–Bowden plot did not intersect at a common point, they did intersect below the *x*-axis ([inhibitor]) and to the left of the *y*-axis (*S*/*V*) (Fig. 4B). Taken together, the results of the Dixon and the Cornish–Bowden plots indicated a linear mixed-type inhibition mechanism for compound **1** [28] with $K_i < K_i'$ or $\alpha > 1$ where K_i' ($=\alpha K_i$) is the equilibrium dissociation constant of the enzyme–inhibitor–substrate (EIS) complex [27]. The linear mixed-type inhibition mechanism was further supported by the Lineweaver–Burk plot analysis of the kinetics data (Fig. 4C). The Lineweaver–Burk plot shows that all four lines with different compound concentrations intersect above the *x*-axis (1/[S]), indicating a linear mixed-type inhibition with $\alpha > 1$ and $\beta = 0$ [27]. Table 5 summarizes the kinetics constants and inhibition constants of compound **1** determined by Lineweaver–Burk analysis of

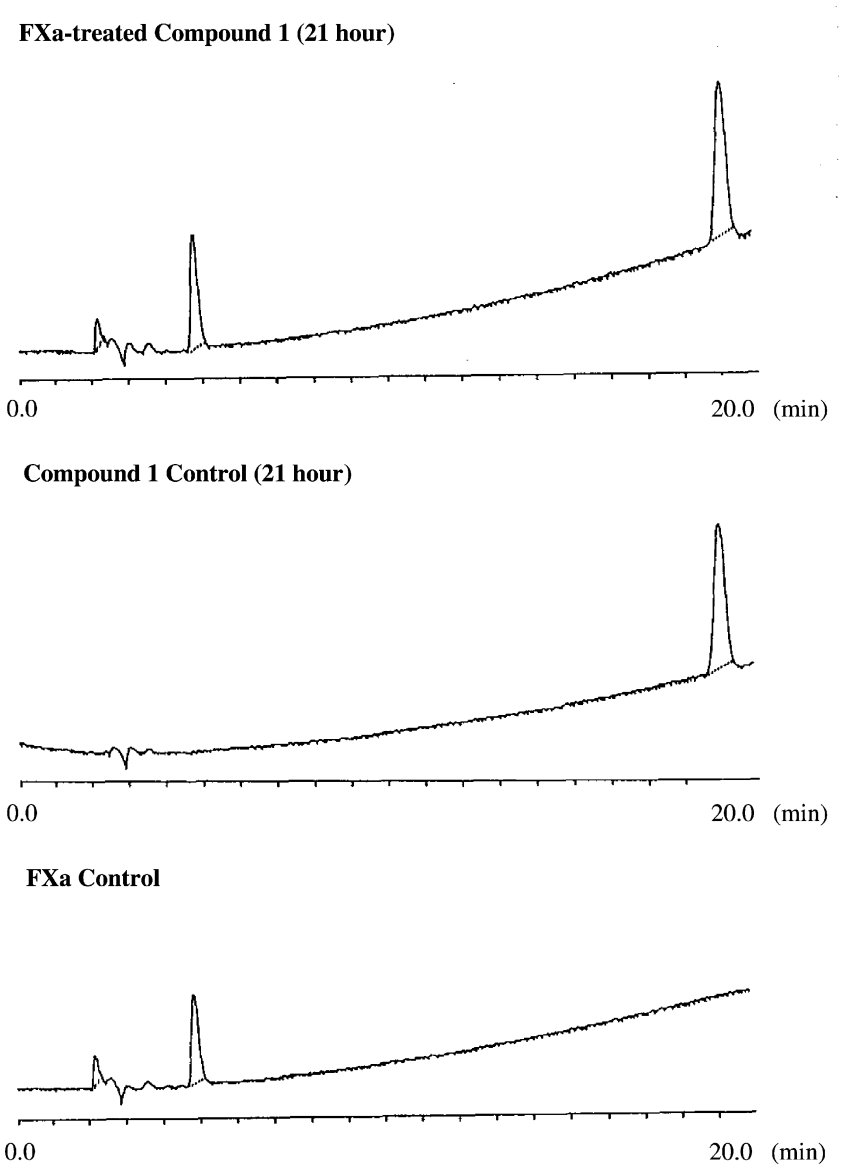


Fig. 2. HPLC tracings of compound **1** after incubation with or without human factor Xa for 21 hr. Compound **1** (final concentration, 50 μM) was incubated with or without human factor Xa (final concentration, 10 μM) at room temperature for 21 hr. At the end of the incubation, aliquots of samples were removed and frozen immediately in a dry ice alcohol bath. Then samples (10 μL) were analyzed by HPLC on a Dynamax C-18 column using a 70–95% gradient of 0.1% TFA/CH_3CN vs. 0.1% TFA/H_2O with a flow rate of 1.0 mL/min. Eluted compounds were detected by absorbance at 254 nm and quantified by comparison to the HPLC results of standard compounds of known concentration run under identical conditions. A sample of pure factor Xa (10 μM) was shown to elute as a minor peak early in the HPLC run.

the kinetics data using the Enzyme Kinetics Pro software version 2.31 (ChemSW Inc.). The 5-fold higher K_i' (82 nM) as compared to the K_i(15 nM) of compound **1** suggests that the affinity of compound **1** for free factor Xa is about 5-fold greater than that for the factor Xa–substrate complex. The linear mixed-type inhibition for compound **1** is in contrast to the competitive inhibition documented for the amidine-containing factor Xa inhibitors [10,14,29–31].

Table 5
Summary of kinetics constants of human factor Xa

	K_m (μM)	V_{max} (pmol/min)	K_i (nM)	K_i' (nM)
S2222	133 ± 1	623 ± 16		
Compound **1**			15 ± 4	82 ± 3

Values are means ± SD, N = 4.

*3.7. Effect of benzothiophene-anthranilamide **1** on the cleavage of N-benzoyl-arginine-p-nitroanilide by factor Xa*

N-Benzoyl-arginine-*p*-nitroanilide, a substrate with only P1 and P1′ components, was used to further characterize the binding site of compound **1** on factor Xa. This more limited interaction with the factor Xa active site is in contrast to the binding interaction of S2222 whose binding

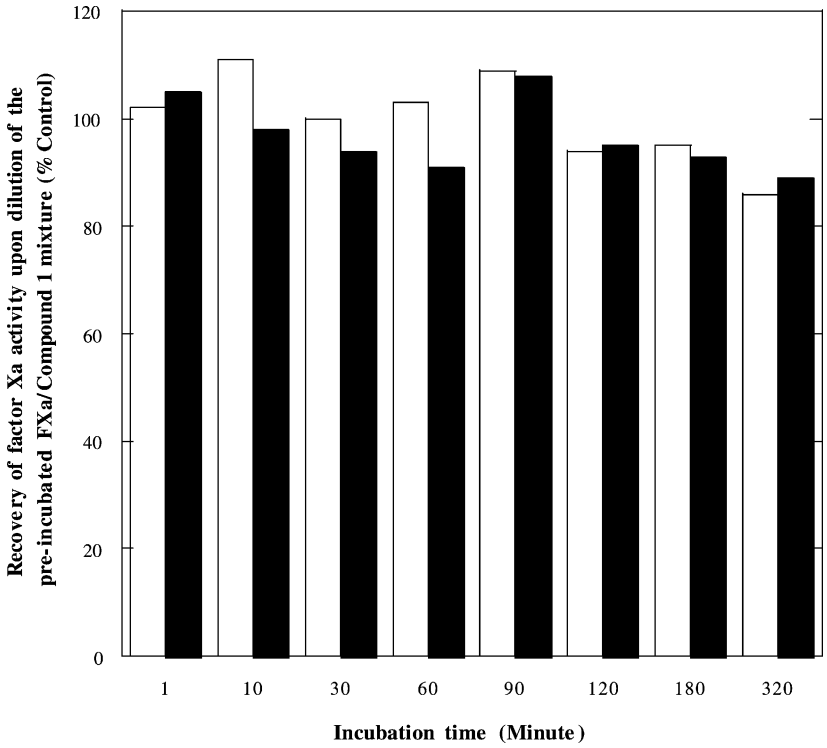


Fig. 3. Reversibility of inhibition of human factor Xa by compound **1** upon dilution of the preincubated FXa/inhibitor mixture into substrate solution. Human factor Xa (final concentration, 1 μM) was incubated in the presence or absence of compound **1** (final concentration, 1.2 μM) at room temperature. Details of the experimental procedures were as described in Section 2. Data represented by solid bars and open bars were the results of two independent experiments. Factor Xa activity of 381 ± 21 pmol S2222 cleaved/min was obtained in the absence of inhibitor at zero incubation time. The average percent recovery of factor Xa activity upon dilution of the preincubated factor Xa/compound **1** mixture at different time points over the 320-min incubation period was 98 ± 7% (mean ± SD, N = 16).

interactions extend from the S4 to the S1′ subsites. The amidine inhibitor ZK-807834 (CI-1031) was included as a positive inhibitory control. ZK-807834 is a potent competitive inhibitor with a K_i of 0.11 nM for human factor Xa [29]. A crystal structure of the ZK-807834/factor Xa complex showed that the amidine moiety of the inhibitor formed a salt bridge with Asp^{189} in the S1 pocket of the enzyme [31]. Because the binding interactions for *N*-benzoyl-arginine-*p*-nitroanilide occur only in the S1 and S1′ sites, it is a very poor substrate for factor Xa. The rate of cleavage is measurable only with high concentrations of enzyme (200 nM) over an extended reaction time (4 hr). An average rate of 217 pmol of *N*-benzoyl-arginine-*p*-nitroanilide cleaved/hr by factor Xa was obtained under the present experimental conditions (N = 2). The cleavage of *N*-benzoyl-arginine-*p*-nitroanilide by factor Xa was inhibited 90% by 10 μM compound **1** and 100% by 10 μM ZK-807834, respectively (data not shown). These results suggest that compound **1** can exclude substrate binding in the vicinity of the S1 and/or S1′ site of the enzyme.

4. Discussion

A majority of potent factor Xa inhibitors identified to date contain a highly basic amidine P1 moiety that mimics an arginine side chain to achieve high potency [2,9–16]. Amidine-containing factor Xa inhibitors, in general, display poor oral bioavailability and suboptimal pharmacokinetic properties such as high clearance rate and short plasma half-life [16–18]. It has become a major effort of the pharmaceutical industry to identify non-amidine factor Xa inhibitors with improved oral bioavailability and pharmacokinetic properties while retaining high potency and selectivity. Efforts in this regard have focused on replacing the highly basic amidine group with a less basic or neutral S1 binding functionality. A neutral P1 moiety in thrombin inhibitors has been shown to increase metabolic stability and oral absorption [32]. Efforts in the factor Xa field have resulted in recent success in design and discovery of potent and selective non-amidine factor Xa inhibitors. A series of non-amidine factor Xa inhibitors containing piperidinyl-pyridine [19,33], piperazinyl-pyridine [34], or phenyl-pyridine [35] as the S1 binding group have been described. Factor Xa inhibitors containing piperidinyl-pyridine have been shown to possess low nanomolar potency for factor Xa (IC_{50} = 3 nM) and high selectivity over thrombin (10,000-fold) and to compare favorably with the amidine inhibitor DX-9065a [19]. Structurally related piperazine-sulfonamide containing non-amidine factor Xa inhibitors with similar potencies have also been discussed as promising factor Xa inhibitors [36]. Potent and selective

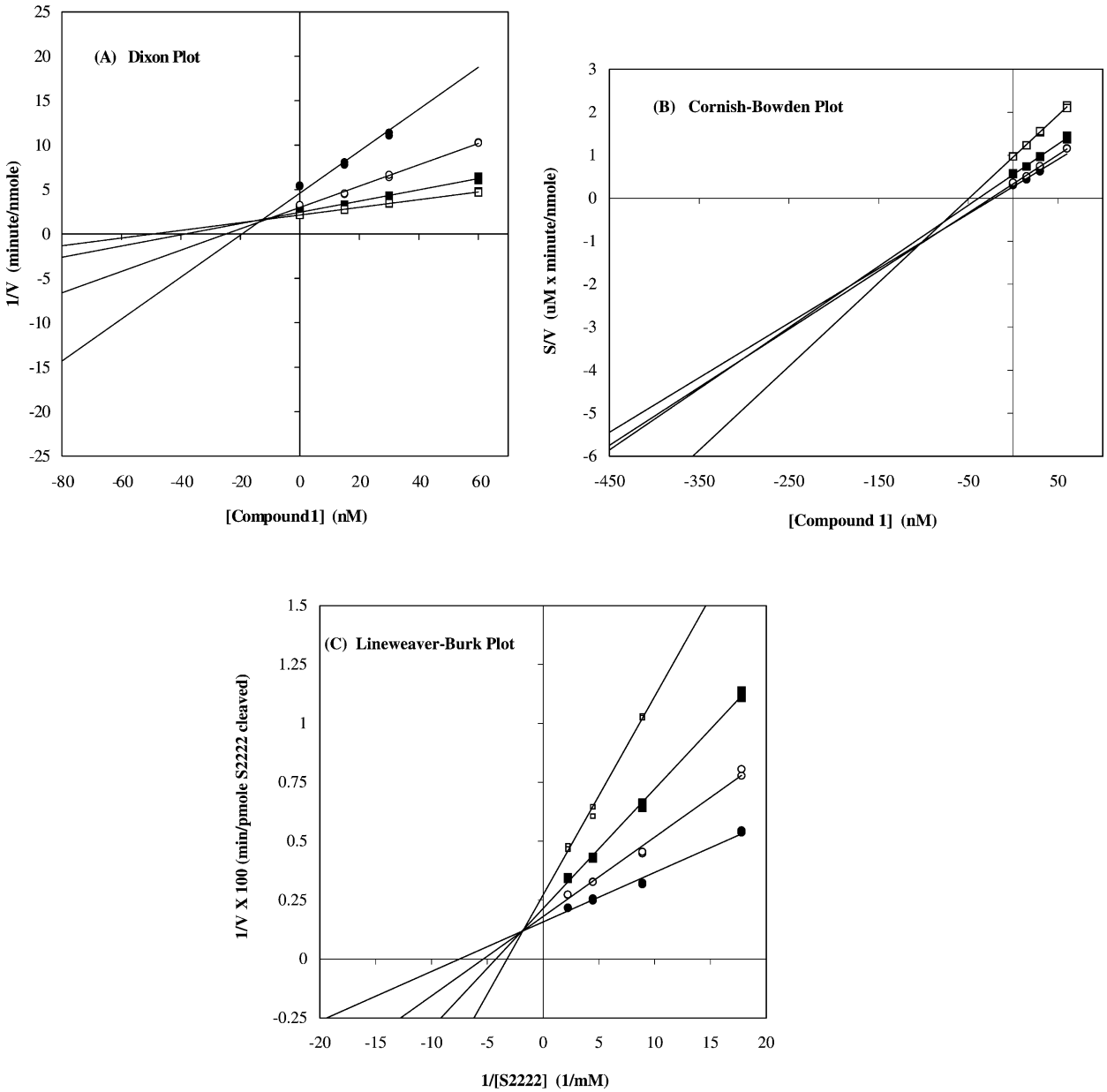


Fig. 4. Inhibition of human factor Xa catalyzed cleavage of S2222 by compound **1**. In panels (A) and (B), human factor Xa (1 nM) was preincubated for 10 min with increasing concentrations of compound **1** (0, 15, 30, and 60 nM). Reaction was started by the addition of substrate S2222 at: 56.25 μM (●), 112.5 μM (○), 225 μM (■), and 450 μM (□). In panel (C), human factor Xa (1 nM) was preincubated for 10 min with a final concentration of compound **1** of: 0 nM (●), 15 nM (○), 30 nM (■), and 60 nM (□). Reaction was started by the addition of increasing concentrations of substrate S2222 (56.25, 112.5, 225, and 450 μM). The initial rate of substrate cleavage was determined by measuring the kinetics of the absorbance increase at 405 nm at room temperature. Data were plotted as (A) a Dixon plot, (B) a Cornish–Bowden plot, and (C) a Lineweaver–Burk plot.

non-amidine factor Xa inhibitors based upon a 1,2-dibenzamidobenzene structure [15,21], on an N^2-aroylanthranilamide template [37], or on a pyrazole-based template [38] have all been reported.

The benzothiophene-anthranilamide compound **1** was discovered as a potent factor Xa inhibitor (K_i = 15 ± 4 nM) from high throughput screening of compound libraries. Failure of the enzymatic cleavage of its amide bonds by factor Xa confirms that compound **1** is not a substrate for this protease and is consistent with the hypothesis that it exerts its inhibitory effect by interfering with the enzyme activity rather than by competing with the substrate S2222. The nanomolar potency and the non-amidine nature make compound **1** a very attractive template for the potential development of orally available and potent factor Xa inhibitors. To qualify compound **1** as a lead template, its selectivity, reversibility, and mechanism of inhibition were further characterized in the present studies. During preparation of this manuscript, an independent report [39] verified the potency and selectivity we show here for compound **1**, but without information to support the mechanism of inhibition.

The results presented here demonstrate that compound **1** is a highly selective, reversible, mixed-type inhibitor for human factor Xa. Its selectivity for nine other human serine proteases and bovine trypsin is remarkable for a "hit" from high capacity screening. In contrast to the amidine factor Xa inhibitor ZK-807834, the potencies ($K_{i(app)}$) of compound **1** for rat, rabbit, dog, and bovine factor Xa were similar and within 2- to 5-fold of that for human factor Xa. When tested in the prothrombinase assay with a physiological concentration of the substrate, prothrombin, the $K_{i(app)}$ of compound **1** was higher than the $K_{i(app)}$ determined for free factor Xa. The phospholipid-binding properties of compound **1** may account, in part, for the higher $K_{i(app)}$ of compound **1** in the prothrombinase assay. The phospholipid-binding properties of compound **1** suggest that it is lipophilic, which is consistent with the poor aqueous solubility of compound **1**. These properties of compound **1** may also contribute to its low potency in the *in vitro* human plasma-based coagulation assays with PT, aPTT, and TT values greater than 100 μM. An increase in lipophilicity has been associated with reduced *in vitro* anticoagulation activity (determined in the prothrombin time assay) for a series of *N*-[(7-amidino-2-naphthyl)-methyl]aniline-derived factor Xa inhibitors [40]. Optimization of the structure of compound **1** to improve its aqueous solubility and potency in plasma-based coagulation assays is therefore essential for further development of lead compounds based on this template.

The mixed-type inhibition demonstrated in the present studies indicates that compound **1** binds to the active site region of factor Xa but cannot be fully displaced by the substrate S2222. The chromogenic peptide substrate, S2222, is a 1:1 mixture of *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide and *N*-benzoyl-Ile-Glu(γ-OMe)-Gly-Arg-*p*-nitroanilide hydrochloride. Based on its structure and the crystal structure of structurally related chloromethylketone-type inhibitors in serine proteases, for example PPACK-thrombin [41], it is likely that S2222 has extensive interactions with factor Xa between the S1′ and S4 subsites. The inability of S2222 to fully displace compound **1** from factor Xa as manifested by the mixed-type inhibition mechanism suggested the existence of a non-overlapping binding site(s) for compound **1** and S2222 on factor Xa. The inhibition of the cleavage of *N*-benzoyl-arginine-*p*-nitroanilide by compound **1** suggests that the binding site or part of the binding site of compound **1** is located in the vicinity of S1 and/or S1′ sites on factor Xa. Further X-ray crystal structure analysis of the factor Xa/compound **1** complex would provide more definite information for the binding site of compound **1** on factor Xa. The finding of a K_i' value (82 ± 3 nM) about 5-fold higher than that of K_i (15 ± 4 nM) for compound **1** implies that the inhibitor binds to free factor Xa more tightly than to the ES complex. In a study of the inhibition of aminopeptidase P by pyrrolidines and thiazolidines, a linear mixed-type inhibition was also found. In this case, an $\alpha < 1$ was found for simple structures, implying that the inhibitor has higher affinity for the ES complex than for the free enzyme [42]. However, an $\alpha > 1$ was found for more sterically hindered compounds, implying higher affinity binding to the free enzyme as is reported here for compound **1** and factor Xa.

The mixed-type inhibition mechanism for compound **1** is distinct from the competitive inhibition for the amidine inhibitors including ZK-807834, an amidine factor Xa inhibitor in clinical development [10,14,29–31] and some recent non-amidine factor Xa inhibitors [37,43,44]. Non-amidine factor Xa inhibitors based on both 1,2-dibenzamidobenzene and N^2-aroylanthranilamide templates have been shown kinetically to behave as competitive inhibitors of human factor Xa [37,43]. A recent report on the crystal structure of human factor Xa complexed with non-amidine inhibitors showed that the surrogates of the benzamidine group interact with the enzyme S1 pocket via hydrogen bonding and hydrophobic contact rather than through direct interaction with Asp^{189} [44].

Results of the present studies show that compound **1** is a highly potent, selective non-amidine factor Xa inhibitor with a mixed-type inhibition mechanism distinguishing it from the competitive inhibition displayed by the amidine inhibitors. Compound **1**, therefore, may provide a novel and viable template to develop efficacious, safe, and potentially orally active factor Xa inhibitors. The structural optimization of compound **1** has been presented at the 221st American Chemical Society Meeting[1] and is the subject of a separate publication [23].

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References

[1] Vlasuk GP, Ramjit D, Fujita T, Dunwiddie CT, Nutt EM, Smith DE, Shebuski RJ. Comparison of the *in vivo* anticoagulant properties of

[1] Kochanny M, Adler M, Cheeseman S, Chou YL, Davey DD, Eagen KA, Ewing J, Fitch R, Griedel BD, Karanjawala R, Lee W, Lentz D, Liang A, Morrissey MM, Phillips G, Post J, Sacchi KL, Sakata ST, Shaw KJ, Snider M, Subramanyam B, Trinh L, Vergona R, Walters J, Wang YX, White KA, Whitlow M, Wu SC, Ye B, Zhao Z. Development of highly potent, selective, and orally available non-amidine factor Xa inhibitors. In: Abstracts, 221st ACS National Meeting, San Diego, CA, 1–5 April 2001. MEDI-16.

standard heparin and the highly selective factor Xa inhibitors antistasin and tick anticoagulant peptide (TAP) in a rabbit model of venous thrombosis. Thromb Haemost 1991;65:257–62.

[2] Zhu BY, Scarborough RM. Recent advances in inhibitors of factor Xa in the prothrombinase complex. Curr Opin Cardiovasc Pulmon Renal Invest Drugs 1999;1:63–87.

[3] Hara T, Yokoyama A, Tanabe K, Ishihara H, Iwamoto M. DX-9065a, an orally active, specific inhibitor of factor Xa, inhibits thrombosis without affecting bleeding time in rats. Thromb Haemost 1995;74: 635–9.

[4] Kawasaki T, Sato K, Sakai Y, Hirayama F, Koshio H, Taniuchi Y, Matsumoto Y. Comparative studies of an orally-active factor Xa inhibitor, YM-60828, with other antithrombotic agents in a rat model of arterial thrombosis. Thromb Haemost 1998;79:410–6.

[5] Sinha U, Ku P, Malinowski J, Zhu BY, Scarborough RM, Marlowe CK, Wong PW, Lin PH, Hollenbach SJ. Antithrombotic and hemostatic capacity of factor Xa versus thrombin inhibitors in models of venous and arteriovenous thrombosis. Eur J Pharmacol 2000;395:51–9.

[6] Abendschein DR, Baum PK, Martin DJ, Vergona R, Post J, Rumennik G, Sullivan ME, Eisenberg PR, Light DR. Effect of ZK-807834, a novel inhibitor of factor Xa, on arterial and venous thrombosis in rabbits. J Cardiovasc Pharmacol 2000;35:796–805.

[7] Heran C, Morgan S, Kasiewski C, Bostwick J, Bentley R, Klein S, Chu V, Brown K, Colussi D, Czekaj M, Perrone M, Leadley Jr R. Antithrombotic efficacy of RPR208566, a novel factor Xa inhibitor, in a rat model of carotid artery thrombosis. Eur J Pharmacol 2000;389:201–7.

[8] Abendschein DR, Baum PK, Verhallen PR, Eisenberg PR, Sullivan ME, Light DR. A novel synthetic inhibitor of factor Xa decreases early reocclusion and improves 24-hr patency after coronary fibrinolysis in dogs. J Pharmacol Exp Ther 2001;296:567–72.

[9] Nagahara T, Yokoyama Y, Inamura K, Katakura S, Komoriya S, Yamaguchi H, Hara T, Iwamoto M. Dibasic (amidinoaryl) propanoic acid derivatives as novel blood coagulation factor Xa inhibitors. J Med Chem 1994;37:1200–7.

[10] Taniuchi Y, Sakai Y, Hisamichi N, Kayama M, Mano Y, Sato K, Hirayama F, Koshio H, Matsumoto Y, Kawasaki T. Biochemical and pharmacological characterization of YM-60828, a newly synthesized and orally active inhibitor of human factor Xa. Thromb Haemost 1998;79:543–8.

[11] Phillips GB, Buckman BO, Davey DD, Eagen KA, Guilford WJ, Hinchman J, Ho E, Koovakkat S, Liang A, Light DR, Mohan R, Ng HP, Post JM, Shaw KJ, Smith D, Subramanyam B, Sullivan ME, Trinh L, Vergona R, Walters J, White K, Whitlow M, Wu S, Xu W, Morrissey MM. Discovery of *N*-[2-[5-[amino(imino)methyl]-2-hydroxyphenoxy]-3,5-difluoro-6-[3-(4,5-dihydro-1-methyl-1*H*-imidazol-2-yl)-phenoxy]pyridin-4-yl]-*N*-methylglycine (ZK-807834): a potent, selective, and orally active inhibitor of the blood coagulation enzyme factor Xa. J Med Chem 1998;41:3557–62.

[12] Quan ML, Ellis CD, Liauw AY, Alexander RS, Knabb RM, Lam G, Wright MR, Wong PC, Wexler RR. Design and synthesis of isoxazoline derivatives as factor Xa inhibitors. J Med Chem 1999;42:2760–73.

[13] Han Q, Dominguez C, Stouten PFW, Park JM, Duffy DE, Galemmo Jr RA, Rossi KA, Alexander RS, Smallwood AM, Wong PC, Wright MM, Luettgen JM, Knabb RM, Wexler RR. Design, synthesis, and biological evaluation of potent and selective amidino bicyclic factor Xa inhibitors. J Med Chem 2000;43:4398–415.

[14] Chu V, Brown K, Colussi D, Choi Y-M, Green D, Pauls HW, Spada AP, Perrone MH, Leadley Jr RL, Dunwiddie CT. *In vitro* characterization of a novel factor Xa inhibitor, RPR 130737. Thromb Res 2000;99: 71–82.

[15] Wiley MR, Weir LC, Briggs S, Bryan NA, Buben J, Campbell C, Chirgadze NY, Conrad RC, Craft TJ, Ficorilli JV, Franciskovich JB, Froelich LL, Gifford-Moore DS, Goodson Jr T, Herron DK, Klimkowski VJ, Kurz KD, Kyle JA, Masters JJ, Ratz AM, Milot G, Shuman RT, Smith T, Smith GF, Tebbe AL, Tinsley JM, Towner RD, Wilson A, Yee YK. Structure-based design of potent, amidine-derived inhibitors of factor Xa: evaluation of selectivity, anticoagulant activity, and antithrombotic activity. J Med Chem 2000;43:883–99.

[16] Dudley DA, Bunker AM, Chi L, Cody WL, Holland DR, Ignasiak DP, Janiczek-Dolphin N, McClanahan TB, Mertz TE, Narasimhan LS, Rapundalo ST, Trautschold JA, Van Huis CA, Edmunds JJ. Rational design, synthesis, and biological activity of benzoxazinones as novel factor Xa inhibitors. J Med Chem 2000;43:4063–70.

[17] Fevig JM, Cacciola J, Alexander RS, Knabb RM, Lam GN, Wong PC, Wexler RR. Preparation of meta-amidino-*N*,*N*-disubstituted anilines as potent inhibitors of coagulation factor Xa. Bioorg Med Chem Lett 1998;8:3143–8.

[18] Fevig JM, Buriak Jr J, Stouten PFW, Knabb RM, Lam GN, Wong PC, Wexler RR. Preparation of pyrrolidine and isoxazolidine benzamidines as potent inhibitors of coagulation factor Xa. Bioorg Med Chem Lett 1999;9:1195–200.

[19] Faull AW, Mayo CM, Preston J, Stocker A. Novel aminoheterocyclic derivatives as inhibitors of factor Xa. Exp Opin Ther Patents 1996;6: 795–9.

[20] Choi-Sledeski YM, Becker MR, Green DM, Davis R, Ewing WR, Mason HJ, Ly C, Spada A, Liang G, Cheney D, Barton J, Chu V, Brown K, Colussi D, Bentley R, Leadley R, Dunwiddie C, Pauls HW. Aminoisoquinolines: design and synthesis of an orally active benzamidine isostere for the inhibition of factor Xa. Bioorg Med Chem Lett 1999;9:2539–44.

[21] Masters JJ, Franciskovich JB, Tinsley JM, Campbell C, Campbell JB, Craf TJ, Froelich LL, Gifford-Moore DS, Hay LA, Herron DK, Klimkowski VJ, Kurz KD, Metz JT, Ratz AM, Shuman RT, Smith GF, Smith Y, Towner RD, Wiley MR, Wilson A, Yee YK. Non-amidine-containing 1,2-dibenzamidobenzene inhibitors of human factor Xa with potent anticoagulant and antithrombotic activity. J Med Chem 2000;43:2087–92.

[22] Phillips G, Davey DD, Eagen KA, Koovakkat SK, Liang A, Ng HP, Pinkerton M, Trinh L, Whitlow M, Beatty AM, Morrissey MM. Design, synthesis and activity of 2,6-diphenoxypyridine-derived factor Xa inhibitors. J Med Chem 1999;42:1749–56.

[23] Chou YL, Davey DD, Eagen KA, Griedel BD, Karanjawala R, Phillips GB, Sacchi KL, Shaw KJ, Wu SC, Lentz D, Liang A, Trinh L, Morrissey MM, Kochanny MJ. Structural-activity relationship of substituted benzothiophene-anthranilamide factor Xa inhibitors. Bioorg Med Chem Lett 2003;13:507–11.

[24] Post JM, Sullivan ME, Abendschein D, Ewing J, Hinchman JW, Light DR. Human *in vitro* pharmacodynamic profile of the selective factor Xa inhibitor ZK-807834 (CI-1031). Thromb Res 2002;105: 347–52.

[25] Devlin JJ, Liang A, Trinh L, Polokoff MA, Senator D, Zheng W, Kondracki J, Kretschmer PJ, Morser J, Lipson SE, Spann R, Loughlin JA, Dunn KV, Morrissey MM. High capacity screening of pooled compounds: identification of the active compound without re-assay of pool members. Drug Dev Res 1996;37:80–5.

[26] Stenflo J. Contributions of Gla and EGF-like domains to the function of vitamin K dependent coagulation factors. Crit Rev Eukaryot Gene Expr 1999;9:59–88.

[27] Segel IH. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. New York: Wiley; 1975.

[28] Cornish-Bowden A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem J 1974;137:143–4.

[29] Light D, Liang A, Rumennik G, Clarke J, McLean K, Post J, Trinh L, Phillips G, Davey D, Guilford W, Morser J, Morrissey M, Sullivan M. *In vitro* inhibition of factor Xa (Xa) and prothrombinase (PTase) by BX-807834 (BX), a potent, selective active site inhibitor. FASEB J 1998;12:A719.

[30] Hara T, Yokoyama A, Ishihara H, Yokoyama Y, Nagahara T, Iwamoto M. DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. Thromb Haemost 1994;71:314–9.

[31] Adler M, Davey DD, Phillips GB, Kim SU, Jancarik J, Rumennik G, Light DR, Whitlow M. Preparation, characterization, and the crystal structure of the inhibitor ZK-807834 (CI-1031) complexed with factor Xa. Biochemistry 2000;39:12534–42.

[32] Feng DM, Gardell SJ, Lewis SD, Bock MG, Chen Z, Freidinger RM, Naylor-Olsen AM, Ramjit HG, Woltman R, Baskin EP, Lynch JJ, Lucas R, Shafer JA, Dancheck KB, Chen IW, Mao SS, Krueger JA, Hare TR, Mulichak AM, Vacca JPJ. Discovery of a novel, selective, and orally bioavailable class of thrombin inhibitors incorporating aminopyridyl moieties at the P1 position. J Med Chem 1997;40: 3726–33.

[33] Faull AW. Aminoheterocyclic compounds with antithrombotic/ anticoagulant effect. Patent Corporation Treaty Publication No. WO-09729104; 1997.

[34] Smithers MJ, Preston J, Stocker A. Aminoheterocyclic derivatives as antithrombotic or anticoagulant agents. Patent Corporation Treaty Publication No. WO-09728129; 1997.

[35] Preston J, Stocker A, Tumer P, Smithers MJ, Rayner JW. Heterocycle derivatives that inhibit factor Xa. Exp Opin Ther Patents 1998;8:1361–7.

[36] Tawada H, Ho F, Moriya N, Terashita Z. Sulfonaimde derivatives, their production and use. Patent Corporation Treaty Publication No. WO-09854164; 1998.

[37] Yee YK, Tebbe AL, Linebarger JH, Beight DW, Craft TJ, Gifford-Morre D, Goodson Jr T, Herron DK, Klimkowski VJ, Kyle JA, Sawyer JS, Smith GF, Tinsley JM, Towner RD, Weir L, Wiley MR. N^2-Aroylanthranilamide inhibitors of human factor Xa. J Med Chem 2000;43:873–82.

[38] Pinto DJP, Orwat MJ, Wang S, Fevig JM, Quan ML, Amparo E, Cacciola J, Rossi KA, Alexander RS, Smallwood AM, Luettgen JM, Liang L, Aungst BJ, Wright MR, Knabb RM, Wong PC, Wexler RR, Lam PYS. Discovery of 1-[3-(aminomethyl)phenyl]-*N*-[3-fluoro-2′(methylsulfony)-[1,1′-biphenyl]-4-yl]-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamide (DPC423), a highly potent, selective, and orally bioavailable inhibitor of blood coagulation factor Xa. J Med Chem 2001;44:566–78.

[39] Shrader WD, Young WB, Sprengeler PA, Sangalang JC, Elrod K, Carr G. Neutral inhibitors of the serine protease factor Xa. Bioorg Med Chem Lett 2001;11:1801–4.

[40] Hirayama F, Koshio H, Katayama N, Kurihara H, Taniuchi Y, Sato K, Hisamichi N, Sakai-Moritani Y, Kawasaki T, Matsumoto Y, Yanagisawa I. The discovery of YM-60828: a potent, selective and orally-bioavailable factor Xa inhibitor. Bioorg Med Chem 2002;10:1509–23.

[41] Bode W, Turk D, Karshikov A. The refined 1.9-Å X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human α-thrombin: structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure–function relationships. Protein Sci 1992;1:426–71.

[42] Stockel-Maschek A, Mrestani-Klaus C, Stiebitz B, Demuth H, Neubert K. Thioxo amino acid pyrrolidides and thiazolidides: new inhibitors of proline specific peptidases. Biochim Biophys Acta 2000;1479:15–31.

[43] Herron DK, Goodson Jr T, Wiley MR, Weir LC, Kyle JA, Yee YK, Tebbe AL, Tinsley JM, Mendel D, Masters JJ, Franciskovich JB, Sawyer JS, Beight DW, Ratz AM, Milot G, Hall SE, Klimkowski VJ, Wikel JH, Eastwood BJ, Towner RD, Gifford-Moore DS, Craft TJ, Smith GF. 1,2-Dibenzamidobenzene inhibitors of human factor Xa. J Med Chem 2000;43:859–72.

[44] Maignan S, Guilloteau JP, Pouzieux S, Choi-Sledeski YM, Becker MR, Klein SI, Ewing WR, Pauls HW, Spada AP, Mikol V. Crystal structures of human factor Xa complexed with potent inhibitors. J Med Chem 2000;43:3226–32.