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Original article

Design of novel aminopyrrolidine factor Xa inhibitors from a screening hit

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ABSTRACT

Starting from a hit identified by focused screening, 3-aminopyrrolidine factor Xa inhibitors were designed. The binding mode as determined by X-ray structural analysis as well as the pharmacokinetic behaviour of selected compounds is discussed.

1. Introduction

Prevention of coagulation is required in a wide range of patients presenting with conditions such as venous thromboembolism, atrial fibrillation and acute coronary syndromes. Anticoagulants currently in clinical use include the parenterally administered unfractionated heparin and low molecular weight heparins (LMWHs) as well as the orally administered coumarins. Heparin and the LMWHs are indirect inhibitors of thrombin and factor Xa. The coumarins act by inhibiting γ-carboxylation in the biosynthesis of coagulation factors prothrombin, VII, IX and X. This mechanism leads to a slow on-set of action. Additionally, the coumarins suffer from substantial food and drug interaction and high protein binding [1], rendering it very difficult to maintain a balanced plasma exposure. Careful and regular monitoring of the patient is therefore required [2].

During the last decades substantial efforts have been devoted to the search for an orally bioavailable replacement of the coumarins. Out of countless research programs, the first compound to have reached the market was Ximelagatran [3] from AstraZeneca, which is a double prodrug of the direct thrombin inhibitor Melagatran. Ximelagatran was launched in France in 2004 under the trade name Exanta, but has been withdrawn from the market due to concerns about long-term liver damage and possible risk of heart attacks [4]. In the meantime Pradaxa® [5], (developed by Boehringer Ingelheim), like Ximelagatran a double prodrug of a direct thrombin inhibitor, has been approved by the European Medicines Agency in spring 2008 and has since been launched in Germany and the UK [6]. Johnson & Johnson/Bayer have submitted an application for US new drug approval (NDA) of its oral F.Xa inhibitor Xarelto® [7], and just recently the drug was approved in Canada [8]. Bristol-Myers Squibb's oral F.Xa inhibitor Apixaban [9] is currently in phase III clinical studies [10]. Oral anticoagulants which have been studied in phase III clinical trials have been discussed in recent review articles [11]. Several oral factor Xa inhibitors are being investigated in phase II clinical trials. The results of these studies have been reviewed extensively [12].

The serine protease factor Xa has a unique role in the coagulation cascade. It is located at the convergence point of the intrinsic and extrinsic pathways and therefore represents an interesting target for the development of a novel anticoagulant. During the past few years enormous efforts have been devoted to the search

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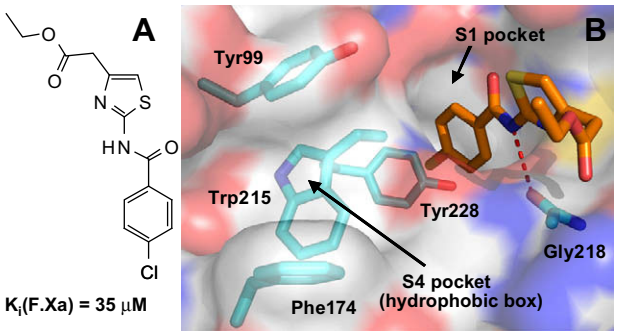


Fig. 1. (A) Hit from biased screen. (B) Model of screening hit bound to the active site of F.Xa.

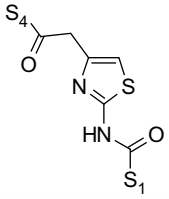
for an orally active and selective factor Xa inhibitor resulting in a great number of disclosed structures [13].

Here we report the identification of novel 3-aminopyrrolidine factor Xa inhibitors starting from a hit originated from a biased screen. Recently a similar series of aminopyrrolidine factor Xa inhibitors has been published by a research group from Bristol-Myers Squibb [14].

2. Results and discussion

Like other proteases of the coagulation cascade, factor Xa possesses an S_1 specificity pocket recognizing basic motifs by means of the Asp189 side chain at its bottom. Inhibitors with typical basic S_1 binding elements such as amidines suffer often from poor bioavailability. At the outset of this project, ample precedence in the literature indicated that chlorinated phenyl or heteroaromatic rings would be good neutral motifs for binding to the S_1 pocket [15]. A focused screen of the Roche compound collection was performed under the following procedure: (i) selecting all chlorinated planar cycles without substituents in either *ortho* position, (ii) docking these into the coordinates of a published protein crystal structure (PDB code 1nfy) with the constraint of holding the C–Cl bond in a position allowing a chlorine–aromatic interaction with Tyr228 and (iii) removing typical GPCR ligands and highly flexible compounds from the solution set. This resulted in about 8000 compounds, for which single point

Table 1
Binding affinity, functional activity and selectivity of aminothiazoles **1**–**6**.



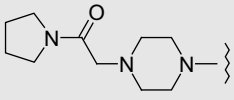
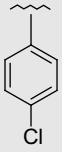
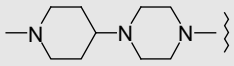
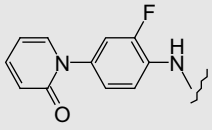
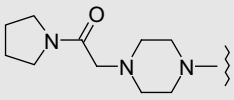
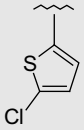
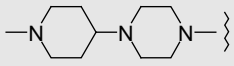
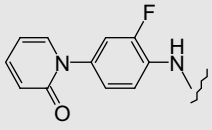
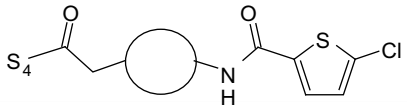
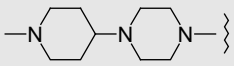
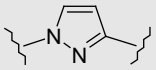
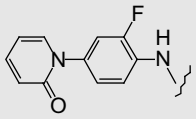
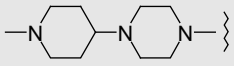
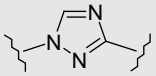
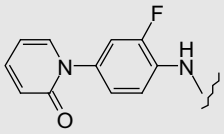
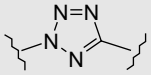
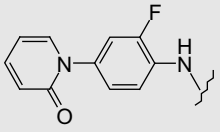
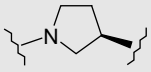
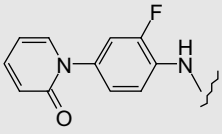
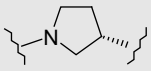
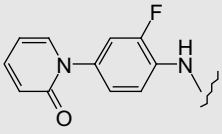
Compound	S1	S4	K_i (μM)		Thrombin F.VIIa	2 × PT (μM)	2 × aPTT (μM)	log *D*	Solubility (μg/mL)
			F.Xa	Thrombin					
1			0.442	2.8	6.3	17.8	32.0	2.2	>580
2			0.162	8.6	53	8.9	20.9	0.6	>620
3			0.112	>35	>310	45.6	70.1	>3.0	<1.0
4			0.099	5.9	60	12.2	33.8	2.1	>640
5			0.032	15.3	480	4.4	10.4	1.0	>620
6			0.018	>35	>1950	43.8	73.3	>3.0	<1.0

Table 2
Variation of central heterocycle (N-linked derivatives): influence of binding affinity and functional activity of compounds **7**–**13**.



Compound	Central heterocycle	S4	F.Xa	2 × PT (μM)	2 × aPTT (μM)	log *D*	Solubility (μg/mL)
7			0.074	4.2	5.6	0.1	>601
8			0.006	6.4	2.7	>3	<1
9			0.175	11.4	22.6	−0.5	>600
10			0.006	4.7	2.0	2.4	1.0
11			0.011	13.3	19.5	2.4	<1.0
12			0.008	1.5	1.8	2.3	197
13			1.272	n.d.	n.d.	2.3	166

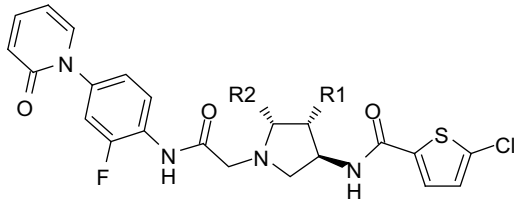
measurements at 20 μM concentration were run in a standard chromogenic enzyme assay. A total of 60 compounds gave signals below 70% control at this concentration.

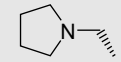
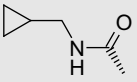
The aminothiazole hit shown in Fig. 1 is a weak inhibitor of factor Xa. The modeled binding mode of this compound indicated that it essentially only binds into the S_1 pocket and does not extend into the lipophilic S_4 pocket formed by the aromatic side chains of Tyr99, Phe174 and Trp215 ('hydrophobic box'). Typical factor Xa inhibitors gain a significant amount of their affinity by binding into this pocket. Based on this model, it was hypothesized that inhibitory activity of the screening hit could be improved when replacing the ester functionality by an amide group with the potential to extend into the S_4 binding pocket. To that end, a great variety of amide analogues of the screening hit were prepared, using structurally diverse commercial amines as well as amines which are described in the literature as good S_4 pocket binding elements.

Compared to the screening hit, amide derivatives **1**–**3** (Table 1) show an >100-fold increase in binding affinity. While the 3-fluorophenyl-pyridone [16] moiety proved to be most suitable for enzyme affinity, the methyl–piperidinyl–piperazine [17] group is by far superior with respect to activity in the *ex vivo* clotting assays PT and aPTT. This can be explained by the substantially lower lipophilicity of compound **2** which is also reflected in a reduced log *D* value and an increased aqueous solubility. Enzyme affinity and *ex vivo* activity could be further improved by replacing the 4-chlorophenyl S_1 moiety with a 2-chlorothiophene substituent [18] (entries 4–6, Table 1). Again, the methyl–piperidinyl–piperazine derivative **5** is superior with respect to functional activity in plasma. As in the chlorophenyl series, the 3-fluoro-phenyl-pyridone S_4 moiety results in good enzyme affinity, but is – due to its lipophilicity – poorly active in the clotting assays.

In an attempt to further improve activity in the clotting assays, the central thiazole core was replaced by various nitrogen heterocycles (Table 2). Results for the pyrazole derivative **7** and the triazole derivative **9** show that this core replacement leads to a substantial loss of binding affinity in the methyl–piperidinyl–piperazine

Table 3
Variation of substituents on central pyrrolidine moiety influence of binding affinity, functional activity, microsomal stability and CYP inhibition profile of compounds **12**–**26**.



Compound	R1	R2	F.Xa (nM)	2 × PT (μM)	2 × aPTT (μM)	Microsomal clearance human (μl/min/mg microsomal protein)	Microsomal clearance rat (μl/min/mg microsomal protein)
12	H	H	8	1.5	1.8	15.2	79.2
14	OH	H	10	1.8	2.0	0.0	15.8
15	OMe	H	3	1.7	0.5	19.6	42.3
16	OEt	H	10	3.6	2.0	74.7	68.3
17	–O–CH_2–CHF_2	H	20	4.3	3.3	9.0	35.0
18	–O–CH_2–CH_2–OH	H	22	2.4	1.3	8.4	12.6
19	–CH_2–OH	H	27	2.9	2.6	21.7	35.4
20	Me (*rac*)	H	14	5.5	3.0	76.3	165.8
21	Cyclopropyl (*rac*)	H	43	12.9	9.2	267.2	109.3
22	F	H	31	8.2	6.2	16.1	65.9
23	H	–CH_2–OH	95	4.9	10.9	7.0	17.6
24	H	–CH_2–OMe	94	7.2	8.3	34.5	102.9
25	H		132	5.6	10.9	61.4	115.5
26	H		93	5.4	10.4	7.5	19.5

subclass. However, the 3-fluorophenyl-pyridone derivatives **8**, **10** and **11** are all characterized by excellent potency. For the triazole compound **10** an optimal balance between binding affinity and lipophilicity was achieved leading to a favourable plasma activity. Unfortunately, this compound is characterized by a low bioavailability in the rat (Table 4).

Replacing the aromatic by an saturated central core resulted in the aminopyrrolidine derivative **12** with *R*-configuration, displaying excellent functional activity in plasma. The corresponding *S*-enantiomer **13** has 160-fold lower enzyme affinity. The pyrrolidine moiety allows the introduction of additional substituents. This set of compounds is shown in Table 3. The methoxy derivative **15** is the most potent compound of this series.

X-ray structural analysis confirmed its absolute configuration and shows that the ligand binding mode (Fig. 2) is as expected from the literature precedence [19]. The peripheral aromatic moieties are found in their usual positions. The chlorothiophene ring is located in the S1 pocket, with the chlorine atom displacing a structural water molecule and being engaged in a hydrophobic interaction with Tyr228 (not visible in figure). 3-Fluorophenyl-pyridone is situated in the S4 pocket ('hydrophobic box'). The aminopyrrolidine linker is in a strain-free conformation. The amide group adjacent to the S4 substituent lies with its plane normal to that of the fluorophenyl moiety and the fluorine atom is found exclusively on the 'right-hand' side of the hydrophobic box as depicted. The interaction with the enzyme is mostly governed by van der Waals and π–π interactions with few polar contacts, namely two rather long hydrogen bonds between the carbonyl of Gly216 and the amide NH adjacent to the S_4 moiety of the inhibitor (d ∼ 2.9 Å) and between the carbonyl of Gly218 and the amide NH adjacent to the S_1 moiety (d ∼ 3.3 Å) as well as a charge dipole interaction between the protonated pyrrolidine N and the carbonyl of Gly216 (d ∼ 3.5 Å). The methoxy group packs well over the disulphide bridge 191:220 and against the side chain of Gln192 which is somewhat disordered.

Compared to the methoxy derivative **15**, the hydroxy compound **14**, the ethoxy compound **16** and the methyl compound **20** show slightly reduced binding affinity which is also reflected in a lower functional activity. For compounds bearing larger substituents such as the difluoro-ethoxy moiety, the hydroxyethoxy moiety, the hydroxymethyl moiety and the cyclopropyl group (compounds **17**–**19** and **21**) the reduction in binding and functional activity is more pronounced indicating that these groups do not fit well in the shallow cavity formed by disulphide bridge 191:220 and the side

Table 4
Physicochemical properties and pharmacokinetic profile of compounds **10**, **14** and **18**.

Compound no.	Solubility (mg/mL)	Permeability (10^{-6} cm/s)	log *D*	Human microsomal clearance[a] (mL/min/kg)	Rat microsomal clearance[a] (mL/min/kg)	SDPK rat *i.v.*				SDPK rat p.o.			
						Dose (mg)	Clearance (mL/min//kg)	$T_{1/2}$ (h)	V_{ss} (l/kg)	Dose (mg)	$T_{1/2}$ (h)	T_{max} (h)	*F* (%)
10	1.0	0.9	2.4	0.4	6.0	0.5	25.0	0.3	0.3	1.0	0.2	1.1	10
14	516	1.5	n.m.	0.0	15.8	0.5	74.0	0.25	1.1	1.0	0.9	0.25	38
18	>713	0.4	1.6	6.8	12.6	1.0	57.1	0.35	1.1	1.0	2.7	0.25	16

[a] *In vitro* CL was extrapolated into *in vivo* CL by physiological scaling [33].

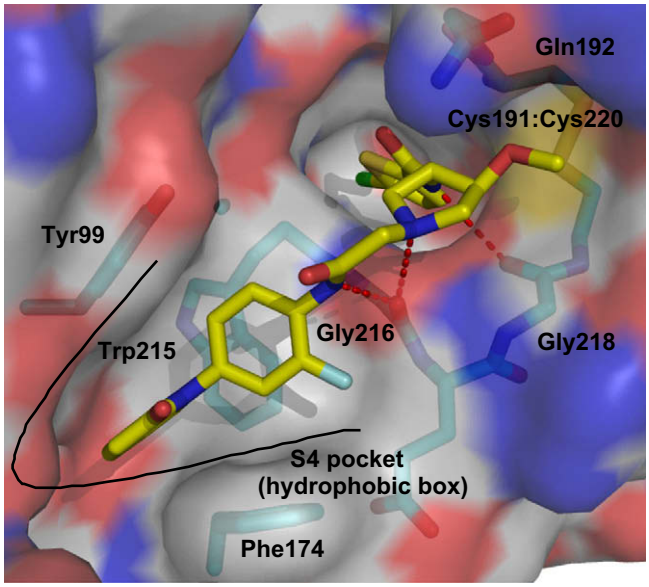


Fig. 2. Crystal structure of compound **15** bound to the active site of F.Xa. Methods are described in Chapter 4.2.

chain of Gln192. The reduced binding affinity of fluorine compound **22** might be explained by a diminished interaction between the dipole of the carbonyl of Gly216 and the fluorinated pyrrolidine which can be attributed to a reduced basicity caused by the electron-withdrawing substituent. Introduction of a substituent next to the ring nitrogen of the pyrrolidine core leads to a substantial loss in activity (compounds **23**–**26**).

Unfortunately, the most active aminopyrrolidine derivative – compound **15** – is rapidly cleared by human and rat microsomes (Table 3). The hydroxy derivative **14** and the hydroxyethoxy derivative **18** are somewhat less potent in the clotting assays but have improved microsomal stability.

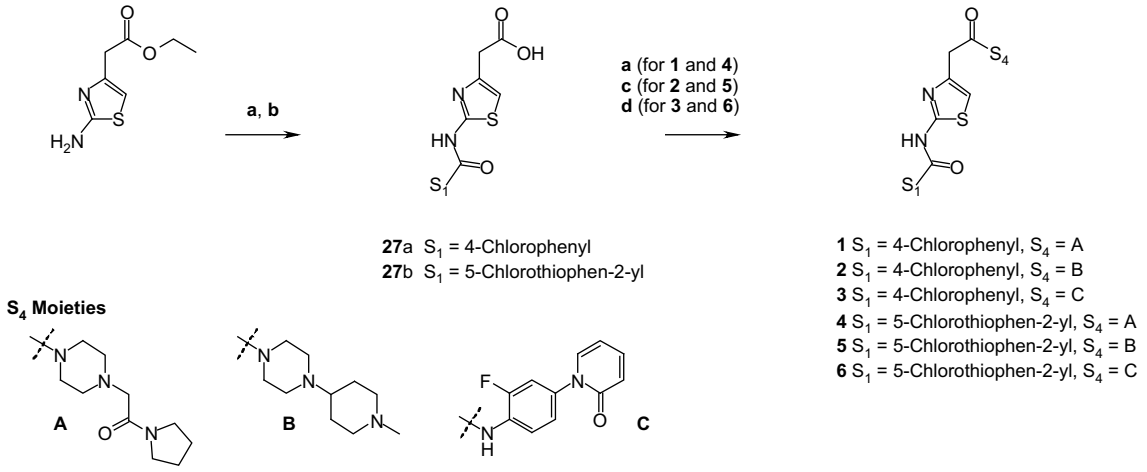
Pharmacokinetic parameters of compounds **14** and **18** are given in Table 4. Derivative **14** has a moderate oral bioavailability, while only poor plasma exposure is reached after oral administration of derivative **18**. This can be explained by its reduced membrane permeability. Low microsomal clearance of compounds **14** and **18** did unfortunately not transfer to low *in vivo* clearance (Table 4). Both derivatives have high plasma clearance in the rat. It was shown that for both compounds renal clearance contributes for at least 50% of drug elimination. Active secretion is assumed. Also other non-P450 elimination pathways like hydrolytic cleavage might be involved. Detailed investigation of elimination processes has however not been performed.

3. Chemistry

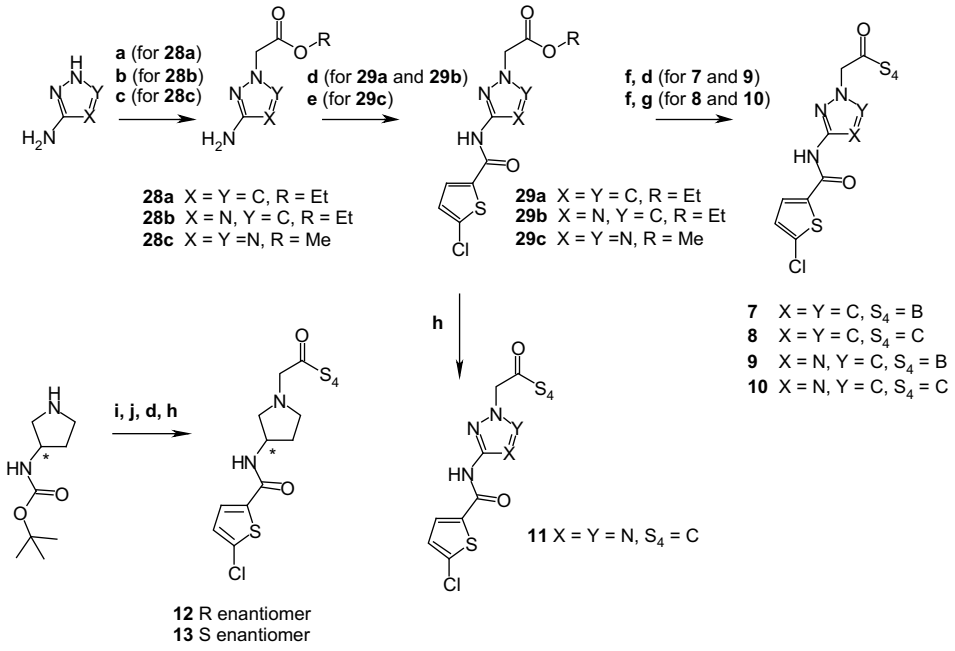
The synthesis of the aminothiazole derivatives **1**–**6** (Scheme 1) started by coupling (2-amino-thiazol-4-yl)-acetic acid ethyl ester with either 4-chlorobenzoic acid (entries 1–3) or 5-chloro-thiophene-2-carboxylic acid (entries 4–6) using BOP as coupling reagent. Subsequently the esters were hydrolyzed to the corresponding acids **27a** and **27b** which were then coupled with the appropriate amines in the presence of optimized coupling reagents (2-piperazin-1-yl-1-pyrrolidin-1-yl-ethanone and BOP for introduction of S_4 moiety A, 1-(1-methyl-piperidin-4-yl)-piperazine and TBTU for moiety B, 1-(4-amino-3-fluoro-phenyl)-1*H*-pyridin-2-one [20] and BOP-Cl for moiety C).

The preparation of derivatives **7**–**11** (Scheme 2) started with the alkylation of the respective aminoheterocycles under optimized or literature conditions. 3-Aminopyrazole was alkylated with ethyl bromoacetate in the presence of potassium *tert*-butylate. 3-Amino-1,2,4-triazole was alkylated with ethyl iodoacetate using sodium hydride as base. 5-Amino-1*H*-tetrazole was reacted with methyl chloroacetate with KOH in refluxing MeOH [21]. All three alkylations provided the desired regioisomers in modest yield. Structures of **28a**, **28b** and **28c** were assigned with the help of NOE experiments. The aminoheterocycles were coupled with 5-chloro-thiophene-2-carboxylic acid under optimized conditions (BOP for **28a** and **28b**, EDCI/DMAP for **28c**) to give **29a**, **29b** and **29c**. Esters **29a** and **29b** were hydrolyzed and coupled with the appropriate amines in the presence of optimized coupling reagents (1-(1-methyl-piperidin-4-yl)-piperazine and BOP for the introduction of S_4 moiety B, 1-(4-amino-3-fluoro-phenyl)-1*H*-pyridin-2-one and BOP-Cl for moiety C) to give the final products **7**–**10**. The tetrazole derivative **29c** was directly reacted with the amine leading to S_4 moiety C in the presence of $AlMe_3$ to give final product **11**.

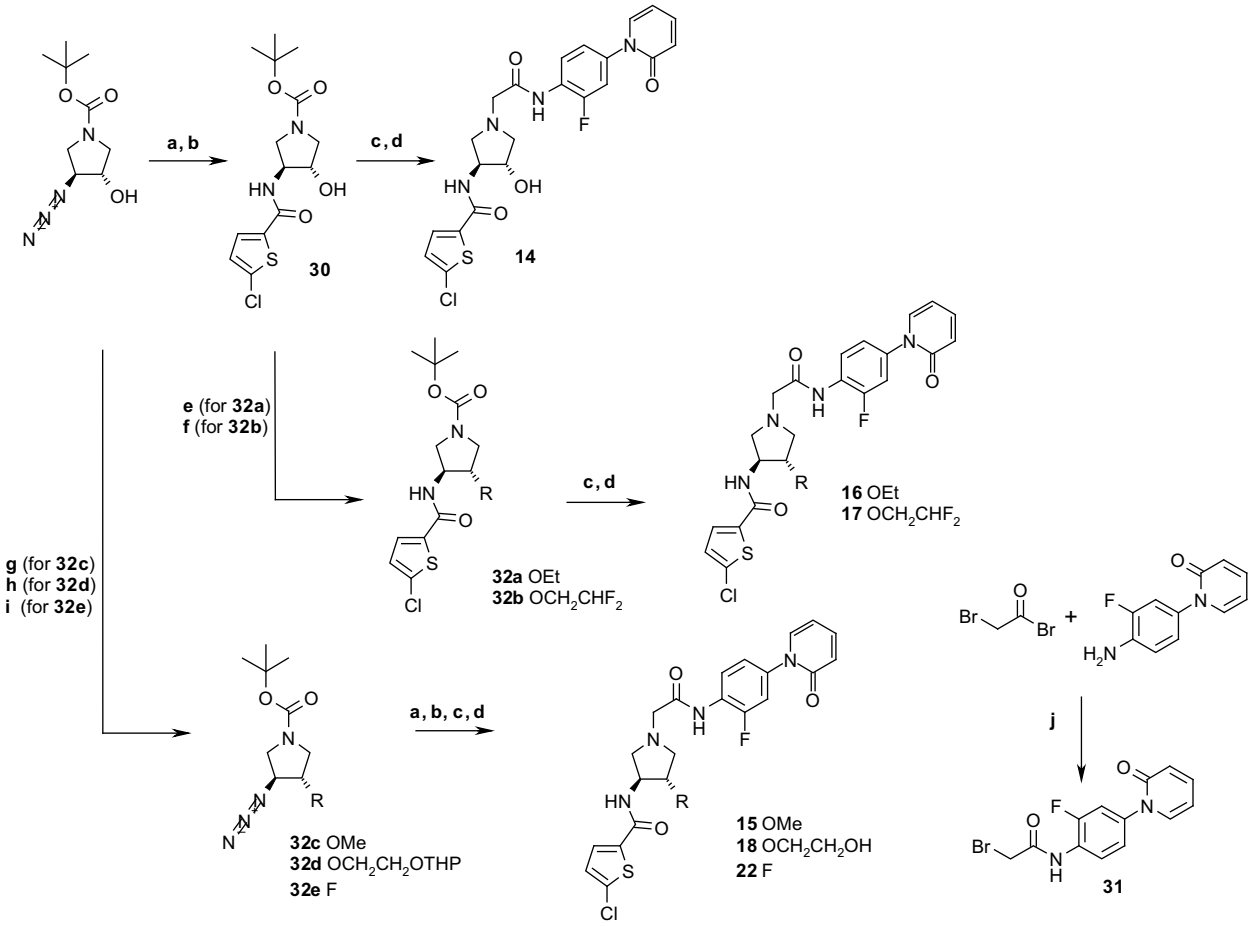
The aminopyrrolidine derivatives **12** and **13** (Scheme 2) were prepared starting with the alkylation of *R*- or *S*-3-*Boc*-amino-pyrrolidine with ethyl bromoacetate. Cleavage of the Boc protecting group, coupling with 5-chloro-thiophene-2-carboxylic acid using BOP as coupling reagent and $AlMe_3$-mediated reaction with 1-(4-



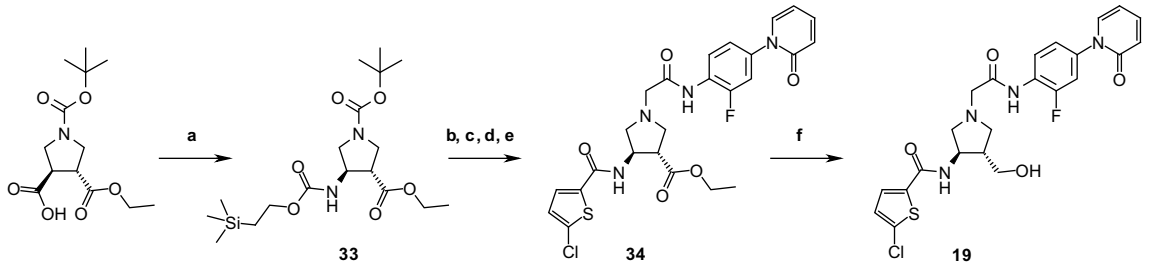
Scheme 1. Preparation of 2-aminothiazole derivatives **1**–**6**. Conditions: (a) BOP, DIPEA, DMF, r.t.; (b) NaOH, MeOH/H_2O, 0 °C; (c) TBTU, TEA, CH_2Cl_2, r.t.; (d) BOP-Cl, DIPEA, MeCN, DMF, r.t.



Scheme 2. Preparation of derivatives **7**–**13**. Conditions: (a) ethyl bromoacetate, KOtBu, DMF, 0 °C; (b) ethyl iodoacetate, NaH, DMF, 0 °C; (c) methyl chloroacetate, KOH, MeOH, reflux; (d) BOP, DIPEA, THF or DMF, r.t.; (e) EDCI, TEA, DMAP, CH_2Cl_2, r.t.; (f) NaOH, EtOH/H_2O, r.t.; (g) BOP-Cl, DIPEA, MeCN, r.t.; (h) $AlMe_3$, toluene, 90 °C; (i) ethyl bromoacetate, TEA, THF, r.t. to 50 °C; (j) 4 N HCl in dioxane, r.t.



Scheme 3. Preparation of aminopyrrolidine derivatives **14**–**18** and **22**. Conditions: (a) H_2, PtO_2, MeOH, r.t.; (b) BOP, DIPEA, DMF, r.t.; (c) 4 N HCl in dioxane, r.t.; (d) 31, TEA, DMF, r.t.; (e) EtI, Ag_2O, MeCN/THF 3:1, r.t.; (f) 2,2-difluoroethyl trifluoromethanesulfonate, NaH, DMF, 0 °C; (g) MeI, Ag_2O, MeCN/THF 5:1, r.t.; (h) 2-(2-bromoethyl)tetrahydro-2*H*-pyran, NaH, DMF, r.t.; (i) DAST, CH_2Cl_2, −78 °C; (j) TEA, THF, r.t.



Scheme 4. Preparation of aminopyrrolidine derivative **19**: (a) diphenylphosphorylazide, 2-(trimethylsilyl)ethanol, TEA, toluene, 80 °C; (b) TBAF, acetonitrile, 80 °C; (c) isobutyl chloroformate, *N*-methyl morpholine, acetonitrile, r.t.; (d) 4 N HCl in dioxane; (e) 31, K_2CO_3, MeCN; (f) $NaBH_4$, EtOH, r.t.

amino-3-fluoro-phenyl)-1*H*-pyridin-2-one leading to S_4 moiety C provided final products **12** and **13**.

The preparation of the hydroxy-substituted aminopyrrolidine derivative **14** (Scheme 3) involved the hydrogenation of (3*S*,4*S*)-3-azido-4-hydroxy-pyrrolidine-1-carboxylic acid *tert*-butyl ester [22] as a first step. The resulting aminoalcohol was then coupled with 5-chloro-thiophene-2-carboxylic acid using BOP as coupling reagent to give intermediate **30**. Deprotection of the pyrrolidine moiety and subsequent alkylation with bromide **31** yielded the final compound. Bromide **31** which was also used in many of the syntheses described below was obtained by reaction of 1-(4-amino-3-fluoro-phenyl)-1*H*-pyridin-2-one [20] with bromoacetyl bromide in the presence of TEA.

The first step in the preparation of the substituted aminopyrrolidine derivatives **16** and **17** was the alkylation of the hydroxy intermediate **30** with ethyl iodide or 2,2-difluoroethyl trifluoromethanesulfonate respectively. The pyrrolidine ring of **32a** and **32b** was deprotected and then alkylated with bromide **31** to lead to the desired final products.

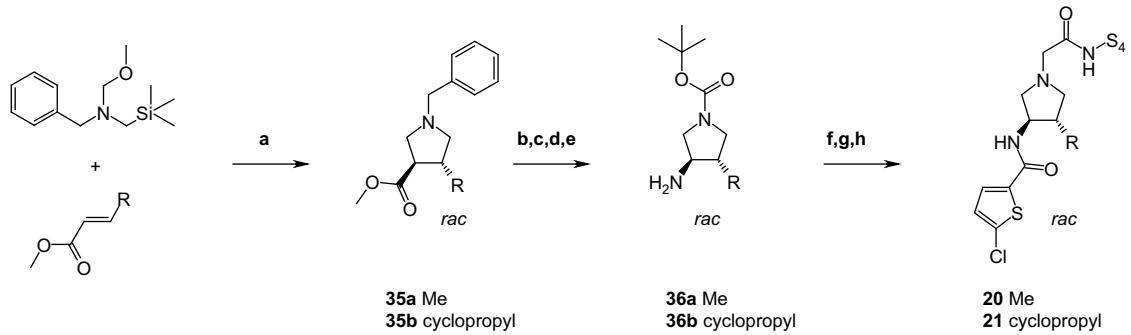
Derivatives **15** and **18** were obtained starting by alkylation of (3*S*,4*S*)-3-azido-4-hydroxy-pyrrolidine-1-carboxylic acid *tert*-butyl ester with methyl iodide and Ag_2O or 2-(2-bromoethyl)tetrahydro-2*H*-pyran and NaH respectively. Intermediates **32c** and **32d** were hydrogenated and subsequently coupled with 5-chloro-thiophene-2-carboxylic acid. Then the pyrrolidine ring was deprotected and alkylated with bromide **31**.

The fluoro compound **22** was obtained by treating (3*S*,4*S*)-3-azido-4-hydroxy-pyrrolidine-1-carboxylic acid *tert*-butyl ester with DAST which according to the literature [23] leads to the trans isomer. The resulting intermediate **32e** was converted to the final product as described for compounds **15** and **18**.

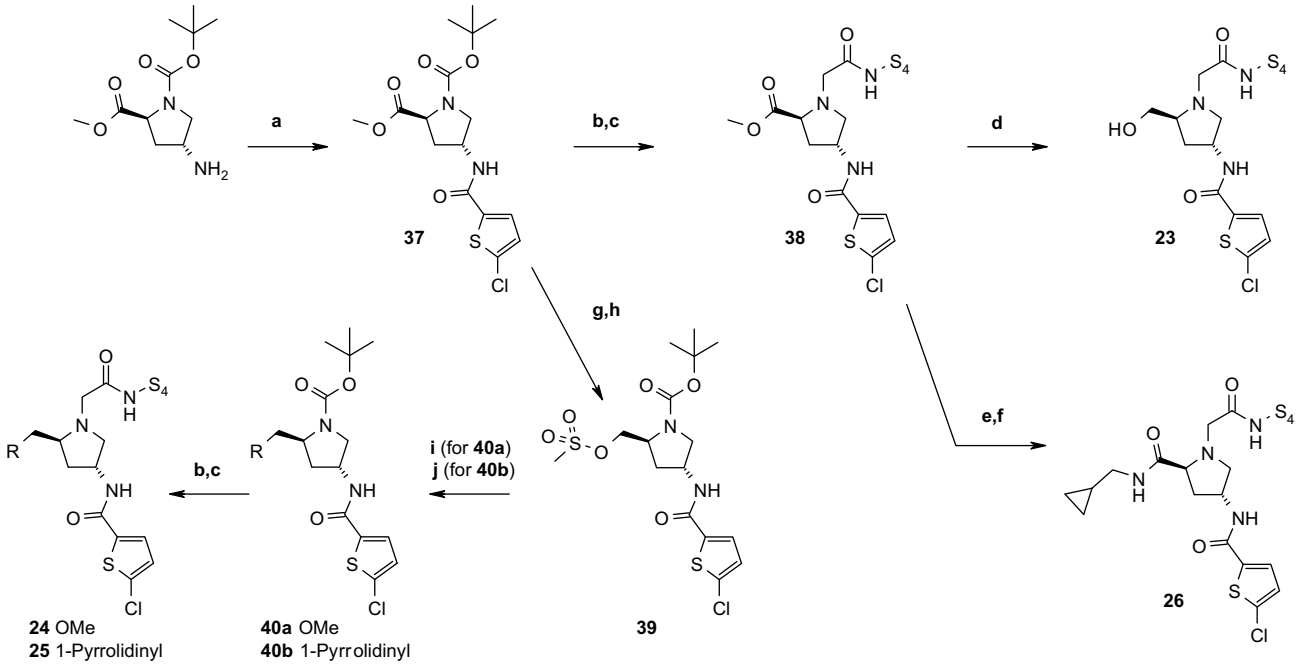
The synthesis of the hydroxymethyl compound **19** (Scheme 4) started with a Curtius rearrangement of pyrrolidine-1,3,4-tricarboxylic acid 1-*tert*-butyl ester 3-ethyl ester in the presence of 2-(trimethylsilyl)ethanol. Cleavage of the Teoc group with TBAF, coupling with 5-chloro-thiophene-2-carboxylic acid with isobutyl chloroformate as coupling reagent, deprotection of the pyrrolidine moiety and alkylation with bromide **31** provided the ester intermediate **34** which was reduced with sodium borohydride to provide the desired final product **19**.

The synthesis of alkylsubstituted aminopyrrolidine derivatives **20** and **21** (Scheme 5) was accomplished starting with a 1,3-dipolar cycloaddition reaction between the appropriate α,β-unsaturated esters and the azomethine ylide generated by TFA treatment of *N*-(methoxy-methyl)-*N*-(trimethylsilylmethyl)benzyl-amine [24]. The resulting pyrrolidines **35a** and **35b** were debenzylated by hydrogenation and Boc-protected. After ester hydrolysis, a Curtius rearrangement provided the aminopyrrolidines **36a** and **36b**. Coupling with 5-chloro-thiophene-2-carboxylic acid, cleavage of the Boc protecting group and alkylation of the pyrrolidine with bromide **31** gave the targeted final products.

The proline-derived compounds **23**–**26** (Scheme 6) were prepared starting with a coupling reaction between *N-Boc-trans*-4-amino-L-proline methyl ester and 5-chloro-thiophene-2-carboxylic acid. The resulting intermediate **37** was deprotected and alkylated with bromide **31** to give ester **38** which was subsequently either reduced with sodium borohydride to give the hydroxymethyl derivative **23** or hydrolyzed and coupled with cyclopropylmethylamine to give amide **26**. Intermediate **37** could alternatively be reduced to the corresponding hydroxymethyl compound using $NaBH_4$ in the presence of lithium chloride which was then transformed into the mesylate **39** and subsequently reacted with methanolate to give **24** or with pyrrolidine to give **25**. Intermediate 37 could alternatively be reduced to the corresponding hydroxymethyl compound using NaBH4 in the presence of lithium chloride which was then transformed into the mesylate 39 and subsequently reacted with methanolate to give 40a or with pyrrolidine to give 40b. The final products 24 and 25 were obtained by cleavage of the Boc protecting group and alkylation with bromide 31.



Scheme 5. Preparation of aminopyridine derivatives **20** and **21**. Conditions: (a) TFA, CH_2Cl_2, 0 °C; (b) H_2, Pd/C 10%, MeOH, r.t.; c) Boc_2O, DMAP, CH_2Cl_2, r.t.; (d) 1 N NaOH THF, 0 °C; (e) DPPA, DIPEA, toluene or dioxane, 90 °C; (f) BOP, DIPEA, DMF, r.t.; (g) 4 N HCl, dioxane, r.t.; (h) 31, TEA, THF, r.t.



Scheme 6. Preparation of proline-derived aminopyridine derivatives **23**–**26**. Conditions: (a) BOP, DIPEA, DMF, r.t.; (b) 4 N HCl in dioxane, r.t.; (c) 31, TEA, DMF, r.t.; (d) $NaBH_4$, MeOH, 0 °C; (e) 1 N NaOH, THF, r.t.; (f) cyclopropylmethylamine, BOP, DIPEA, DMF (g) $NaBH_4$, LiCl, EtOH, THF, r.t.; (h) MsCl, DIPEA, CH_2Cl_2, 0 °C; (i) NaOMe, MeOH/THF, reflux; (j) pyrrolidine, THF, r.t.

4. X-ray structural analysis: experimental

4.1. Clotting assays

Prothrombin time PT: 2 × PT prolongation is a measure for the ability of an inhibitor to prevent clotting *via* the extrinsic pathway of the coagulation cascade. Experimental procedure: human citrated plasma is spiked with at least 6 concentrations of inhibitor. Clotting is initiated by addition of exogenous tissue factor (Innovin). Clotting time is determined by a turbidity measurement. The concentration of inhibitor necessary to double control clotting time is determined by fitting the data to an exponential regression.

Activated partial thromboplastin time aPTT: 2 × prolongation of aPTT serves as a measure for the ability of the inhibitor to interfere with the coagulation cascade *via* the intrinsic pathway. Experimental procedure: human citrated plasma is spiked with at least 6 concentrations of inhibitor. Clotting *via* the intrinsic pathway is initiated by addition of Actin® FS (ellagic acid in soy phosphatides). Clotting time is determined by a turbidity measurement. The concentration of inhibitor necessary to double control clotting time is determined by fitting the data to an exponential regression.

See Ref. [25] for further experimental details.

4.2. X-ray structural analysis

Short form Factor Xa was produced as previously described [26] as the Arg150Glu mutant. Compound **15** was pre-incubated with the protein and crystals grown by addition of 0.1 M bis–tris pH 6.5, 25% PEG3350 in microbatch mode. Crystals appeared after a week and grew to full size within two weeks. Before data collection, crystals were transferred to crystallization buffer supplemented with 20% glycerol and flash-frozen in liquid N_2. Diffraction data were measured on a Bruker FR591 X-ray generator, with 0.2 mm focus, run at 50 kV/60 mA and equipped with an Osmic focussing monochromator and an Oxford Cryostream cooler run at 100 K. The Marresearch345 (dtb) image plate detector was placed 150 mm from the crystal and scanned with 0.15 mm pixel size. Exposure times were 600 s for 0.5° frames. Data from 360 frames were processed to 1.95 Å resolution using XDS [27]. The space group is $P2_1$ with unit cell dimensions $a = 48.96$, $b = 77.19$, $c = 74.76$ Å, $\beta = 92.12°$. For 133,552 observations of 36,457 reflections, the merging R factor on intensities was 11.9% (38.5% in the outermost shell, 1.95–2.01 Å), with completeness 90.3% (95.9%) and I/σ 7.8 (3.0). Data reduction used the CCP4 package [28]. The structure was solved by molecular replacement using 2bok.pdb as model. Model building with Moloc [29,30] and Coot [31] and refinement with Refmac5 [32] gave final overall crystallographic R factors of 20.3% (working) and 27.5% (free), with values in the outer shell (1.95–2.0 Å) of 24.1% and 34.4%, respectively, for 5088 atoms, including one Ca^{++} ion, two Na^{+} ions, and 576 water molecules. The observed monoclinic form is similar in packing to the commonly found orthorhombic form (e.g. 2bok.pdb) and may arise due to partial degradation of the calcium loop. In one unit cell two independent, but very similar protein molecules are observed. The inhibitor density in both molecules is clear and unambiguous. Coordinates have been deposited at the Protein Data Bank with the PDB code 2vvc.

X-ray structures of further complexes between factor Xa and selected inhibitors from Tables 2 and 3 are deposited in the Protein Data Bank as well under the following PDB codes: 2vvv (compound **10**), 2vvu (compound **12**), 2vwn (compound **14**), 2vwo (compound **22**), 2vwl (compound **23**), 2vwm (compound **26**).

5. Conclusion

A weakly active aminothiazole factor Xa inhibitor was identified from a biased screen. Guided by molecular modeling, the binding affinity of the initial lead was improved. Aminothiazole derivatives with nanomolar affinity were identified. Replacement of the central aminothiazole core by various nitrogen-containing aromatic heterocycles resulted in inhibitors with even better binding affinity and good functional activity in plasma clotting assays. Further improvement of functional activity was achieved by the

introduction of a pyrrolidine moiety as central core. The presence of the pyrrolidine ring allowed the modulation of binding affinity, functional activity and *in vitro* pharmacokinetic parameters by introduction of a large variety of ring substituents. Potent inhibitors with good microsomal stability were identified.

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