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Nonbenzamidine Tetrazole Derivatives as Factor Xa Inhibitors

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Abstract—Factor Xa (fXa) is an important serine protease that holds the central position linking the intrinsic and extrinsic activation mechanisms in the blood coagulation cascade. Therefore, inhibition of fXa has potential therapeutic applications in the treatments of both arterial and venous thrombosis. Herein we describe a series of tetrazole fXa inhibitors containing benzamidine mimics as the P_1 substrate, of which the aminobenzisoxazole moiety was found to be the most potent benzamidine mimic. SR374 (**12**) inhibits fXa with a K_i value of 0.35 nM and is very selective for fXa over thrombin and trypsin.

Introduction

Factor Xa (fXa) is the site of convergence of the intrinsic and the extrinsic coagulation pathways. Factor Xa in combination with factor (Va) and calcium forms the prothrombinase complex that converts prothrombin to thrombin via proteolysis. This activation is a highly amplified process.[1] The concentration of fXa is lower than the concentration of thrombin at the thrombotic site, and hence a smaller dose of a fXa inhibitor may prevent thrombosis effectively. Furthermore, since fXa inhibitors affect coagulation specifically, this mechanism may decrease the risk of abnormal bleeding. Preclinical studies has indicated less bleeding risk with fXa inhibitors than thrombin inhibitors.[2] Therefore, orally active fXa inhibitors should represent an important advance in the management of both chronic arterial and venous thrombosis.

A large number of small molecule fXa inhibitors have been disclosed in recent years.[3] We have reported on a series of benzamidine fXa inhibitors containing a vicinally-substituted heterocyclic core.[4] Pyrazole, triazole, and tetrazole compounds with a *m*-benzamidine P_1 moiety linked to a nitrogen in the 5-membered heterocycle have been shown to be potent fXa inhibitors.[4] Optimization of the pyrazole series has led to a series of extremely potent fXa inhibitors which culminated in clinical development candidate DPC 423.[5,6] Herein we would like to report on a series of tetrazole fXa inhibitors in which the benzamidine moiety has been replaced with a less basic P_1 residue.

Results and Discussion

We have reported on a variety of benzamidine mimics which we employed in an isoxazoline and a pyrazole series that afforded potent and selective fXa inhibitors with improved pharmacokinetic profile.[5–8] We now wish to report on a series of tetrazole based fXa inhibitors which employ the same set of benzamidine mimics that we have previous described (Table 1). In this case, aminobenzisoxazole **2** was found to be the most potent inhibitor, which was only 26-fold less active than the corresponding benzamidine **1**. Chloroaniline **3** was approximately 150-fold less potent, while *p*-methoxy **4**, *m*-benzylamine **5**, and methoxyaniline **6** were >200-fold less active. As we have previously shown,[5–8] these benzamidine mimics have excellent selectivity over thrombin and trypsin, with the possible exception of benzylamine **5**, which while 1000-fold selective over thrombin, is only 5-fold selective over trypsin with a K_i value of 65 nM.

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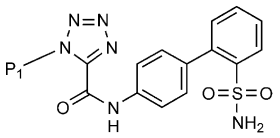
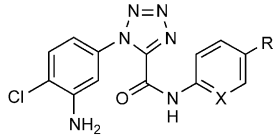
Table 1. Benzamidine mimics: comparison of fXa potency and selectivity

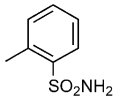
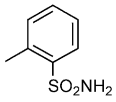
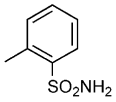
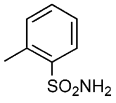
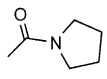


Compd	P_1	fXa K_i (nM)	Thrombin K_i (nM)	Trypsin K_i (nM)
1		0.037	300	6.4
2		0.98	1500	>1600
3		5.4	1600	>1600
4		8.0	10,000	>1600
5		13	>21,000	69
6		19	4000	>1600

fXa, thrombin, and trypsin K_i values were obtained using human purified enzymes. See ref 16 for assay conditions.

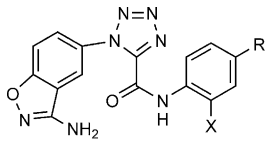
Table 2. Optimization employing chloroaniline as the P_1 substrate

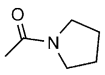
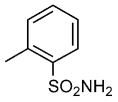
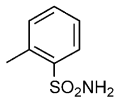
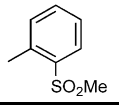


Compd	R	X	FXa K_i (nM)	Thrombin K_i (nM)	Trypsin K_i (nM)
7		N	8.0	7900	>1600
3		CH	5.4	1600	>1600
8		CCl	3.3	1600	>1600
9		CF	1.2		
SW712 (**10**)		CF	3.0	1200	>1600

fXa, thrombin, and trypsin K_i values were obtained using human purified enzymes. See ref 16 for assay conditions.

Table 3. Optimization employing aminobenzisoxazole as the P_1 substrate



Compd	X	R	FXa K_i (nM)	Thrombin K_i (nM)	Trypsin K_i (nM)
11	F		2.9	>21,000	>1600
2	H		0.98	1500	>1600
SR374 (**12**)	F		0.35	600	>5200
13	F		0.39	400	>1600

fXa, thrombin, and trypsin Ki values were obtained using human purified enzymes. See ref 16 for assay conditions.

Attempts to further optimize the chloroaniline and aminobenzisoxazole series are shown in Tables 2 and 3. *Ortho*-halogen substitution on the inner phenyl ring afforded slight improvement in fXa affinity (1.5–4.5 fold). Replacement of the terminal phenylsulfonamide with a pyrrolidinylamide group decreased the fXa affinity by 2.5-fold. The chloroanilines showed good selectivity for fXa over thrombin and trypsin as shown in Table 2. The modest decrease in potency with the pyrolidinylamide modification was also observed when aminobenzisoxazole was employed as the P_1 moiety (**11** vs **2**). On the other hand, *ortho*-fluoro substitution increased fXa affinity by 3-fold (**12**). As we have previously reported for both the isoxazoline and the pyrazole series, replacement of the sulfonamide group with a methysulfone moiety did not effect fXa affinity (**13**). The fluoro-substituted analogues **12** and **13** are only 10-fold less potent than benzamidine SN357 and they both show excellent selectivity for fXa relative to thrombin and trypsin.

In order to elucidate the binding mode of this series of compounds, a crystal structure of SW712 (**10**) complexed to human thrombin was obtained (Fig. 1).[9] In the thrombin crystal structure, a 64-degree dihedral angle was observed between the chloroaniline ring and the tetrazole ring, leading to a 3.1 Angstrom contact between the backbone nitrogen of Glu^{192} and the N2 of the tetrazole ring. A similar interaction between the pyrazole and the backbone nitrogen of residue Glu^{192} was observed in the crystal structure of the DPC 423 fXa complex.[12] The presence of a hydrogen bond acceptor in this position appears to be important for activity. The oxygen of the amide of the linker group and the backbone nitrogen of Gly^{218} are within hydrogen bonding range with a distance of 3.0 Angstroms.

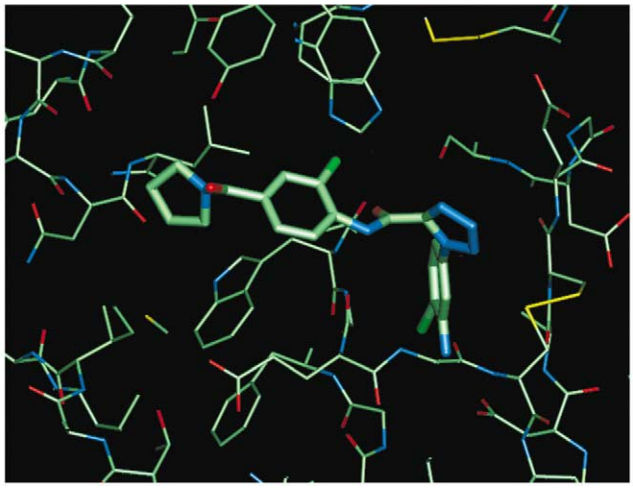


Figure 1. Structure of SW712 complexed to human thrombin.

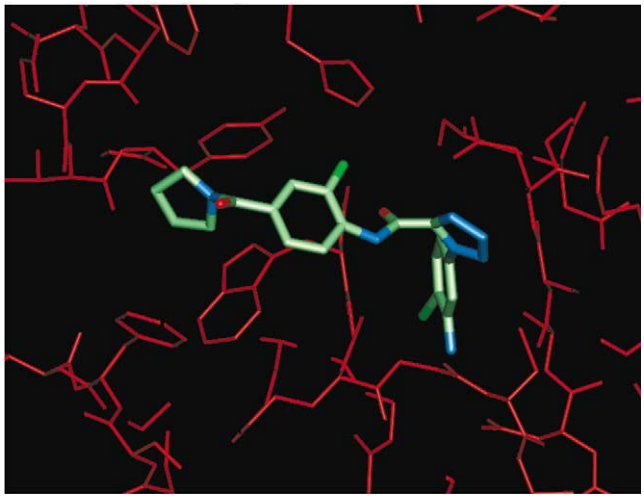


Figure 2. Structure of SW712 (atomtype) from the human thrombin-SW712 complex superimposed on the coordinates of fXa (red).

The pyrolidine group is found in the P_4 pocket with a 4.2 Angstrom distance to Trp^{215}. The nitrogen of the chloroaniline interacts with side-chain oxygens of Asp^{189} with contacts of 2.9 and 3.0 Angstroms. The chlorine of the chloroaniline interacts with Ala^{190} with a contact of 3.8 Angstroms. The increased steric bulk associated with the substitution of Ser^{190A} in trypsin (Ala is found in fXa) would explain the selectivity of this series of compounds over trypsin. Specifically, the oxygen of the serine side-chain would sterically clash with the chlorine of the inhibitor. Given that sequence identity between fXa and thrombin, we superimposed the coordinates of SW712 from the thrombin complex on the structure of fXa.[13] Analogous interactions could be found in the model of SW712 superimposed into the fXa active site as were observed in the thrombin crystal structure (Fig. 2).

Table 4. Dog PK profile and antithrombotic efficacy of SR374 compared to **1**

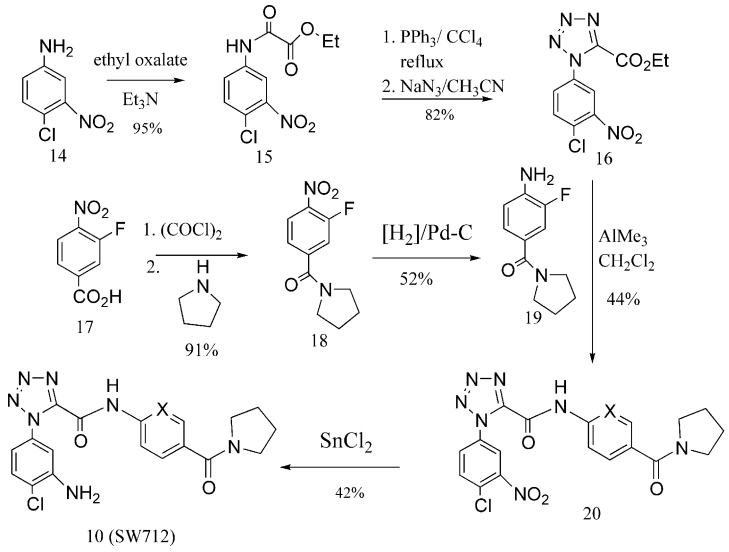
Compd	fXa K_i (nM)	Dose (mg/kg)	Clearance (L/h/kg)	$t_{1/2}$ (h)	ID_{50} (μmol/kg/h)
1	0.037	1.0 (iv)	0.62	0.65	0.13
SR374 (**12**)	0.36	0.5 (iv); 0.2 (po)	0.30	3.1	5.8

PK Data were obtained dog N-in-1 studies in dogs. SR374 has a bioavailability of 74%. ID_{50}'s were obtained in the rabbit A-V shunt model.[14]

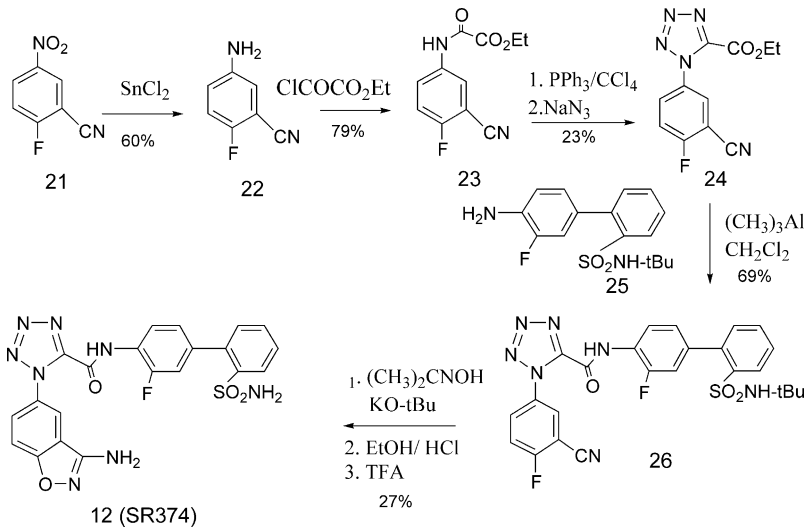
The pharmacokinetic profile of SR374 (**12**) was studied in dogs. Compared with benzamidine **1**, the clearance was reduced by two-fold and the half-life was increased by five-fold (Table 4). More significantly, SR374 showed 74% bioavailability in dog. The antithrombotic efficacy of SR374 was studied in the rabbit A-V shunt model.[14] Following intravenous administration, the ID_{50} was found to be 5.8 μmol/kg.

Synthesis

The preparation of the chloroaniline derivatives is exemplified by the synthesis of SW712 (**10**) shown in



Scheme 1. Synthesis of SW712.



Scheme 2. Synthesis of SR374.

Scheme 1. 4-Chloro-3-nitroaniline was reacted with ethyl oxalate and Et_3N in CH_2Cl_2 to give compound **15**, which was converted to tetrazole **16** by heating to reflux with PPh_3 in CCl_4, followed by reaction with NaN_3. 3-Fluoro-4-nitrobenzoic acid was converted to the corresponding acyl chloride and then coupled with pyrrolidine to give compound **18**. Reduction of **18** afforded aniline **19**, which was reacted with tetrazole **16** under Weinreb conditions to produce amide **20**. Reduction of the nitro group of **20** with $SnCl_2$ gave the chloroaniline SW712 (**10**), which was purified by HPLC to afford the final compound as the TFA salt.

The preparation of the aminobenzisoxazole derivatives is exemplified by the synthesis of SR374 (**12**) as shown in Scheme 2. 2-Fluoro-5-nitrobenzonitrile was reduced to aniline **22** with $SnCl_2$. The tetrazole **24** was formed using the same methods as described above for the chloroaniline series employing aniline **22** as starting material. Weinreb coupling of tetrazole **12** and biaryl derivative **25**[15] provided amide **26**. The aminobenisoxazole ring was then formed by reaction of **26** with acetone oxime and potassium *t*-butoxide, followed by heating to reflux with HCl and ethanol. Removal of the *t*-butyl group with TFA gave the final product as the TFA salt.

Conclusions

We have designed and synthesized a series of tetrazole compounds vicinally-substituted with P_1 and P_4 residues. The benzamidine group in the picomolar inhibitor **1** was replaced with a variety of less basic and nonbasic moieties. Chloroaniline and aminobenzisoxazole were found to be the most potent benzamidine replacements with low nanomolar to subnanomolar fXa affinity. SR374 (**12**) has a fXa K_i of 0.35 nM and was found to be more selective compared to benzamidine **1**. The pharmacokinetic profile of aminobenzisoxazole SR374 was improved relative to benzamidine **1** with a half-life of 3.1 h and oral bioavailability of 74%. SR374 also showed antithrombotic efficacy with an ID_{50} of 5.8 μmol/kg/h.

Acknowledgements

We wish to thank Earl Crain, Angela Smallwood, and Carol Watson for technical assistance.

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9. Crystals of human thrombin–hirugen complex were prepared by the method described by Skrzypczak-Jankun et al.[10]

Soaks of the enzyme-inhibitor complex were prepared by the method described in Galemmo et al.[11] Data were collected on an R Axis II image plate mounted on a Rigaku RU200 rotating anode; crystals diffracted to 2.5 Å. Data were refined using CNX (Molecular Structure Inc.) with a final R-value of 0.209. Unambigous electron density was observed for the inhibitor.

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16. **Enzyme affinity assays**. FXa, thrombin, and trypsin K_i values were obtained from human purified enzymes. All assays are run in micro-titer plates using a total volume of 250 μL in 0.1 M Sodium Phosphate Buffer containing 0.2 M NaCl and 0.5% polyethylene glycol 6000 at pH 7.0. The compounds are run at 10 μM, 3.16 μM, 1.0 μM, 0.316 μM, 0.1, 0.0316, 0.01, and 0.00316 μM. Plates are read for 30 min at 405 nm. Rates are determined for the controls (no inhibitor) and for the inhibitors. %Enzyme activity is determined from these rates and used in the following formula to determine K_i: $K_i = 1000 * \text{Inhibitor Concentration}/(((K_m + S) - S * \text{ACT})/(\text{ACT} * K_m) - 1)$; where $S =$ Substrate Concentration; ACT = % Enzyme Activity for inhibitor. All compounds were tested in duplicate studies and were compared with the same internal standards. These assays are described in detail in refs 17 and 18.

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