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Thrombin inhibitors built on an azaphenylalanine scaffold

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Abstract—A series of azaphenylalanine derivatives were investigated as novel thrombin inhibitors based on the prodrug principle. By systematic structural modifications we have identified optimal groups for this series that led us to potent inhibitors of thrombin incorporating the benzamidine fragment at the P1 position, and their potentially orally active benzamidoxime prodrugs. The binding modes in the thrombin active site of two representative compounds were identified by X-ray crystallographic analysis.

Thromboembolic disorders are the major cause of morbidity and mortality in Western societies. At present, clinical regulation of thrombosis still involves the administration of heparin and its derivatives, or oral anticoagulants of the dicumarol type, which all indirectly inhibit thrombin, a trypsin-like serine protease that plays a key role in blood coagulation processes. These drugs have several limitations in both their effectiveness and bleeding risk, leading to the need for extensive monitoring. An injectable form of the small-molecule direct thrombin inhibitor, argatroban, has been approved by the FDA, but only for the relatively rare condition of heparin-induced thrombocytopenia.[1] Orally active thrombin inhibitors have been reported recently, for example, the most advanced oral thrombin inhibitor ximelagatran.[2] However, an ideal, clinically useful direct thrombin inhibitor is still a high priority in medicinal chemistry research.[3]

In earlier studies we identified a novel series of non-covalent inhibitors built on the conformationally restricted azaphenylalanine scaffold.[4,5] In this type of compound, the α-carbon of the original peptidomimetic structure[6] was replaced by nitrogen and the stereogenic centre of the central amino acid was omitted, with the result that the overall conformation was changed.[7,8]

Most direct thrombin inhibitors have a strong base that fits in the S1 specificity pocket of thrombin. The strong base, often a guanidine or amidine, is protonated at the pH of the intestinal tract, thus being a poor candidate for intestinal absorption. Development of new inhibitors has focused on introducing less basic substituents to improve intestinal absorption.[3f,g] The *N*-hydroxylated derivatives, amidoximes are less basic because of the introduced the oxygen atom. They are not protonated under physiological conditions and should lead to sufficient oral absorption and therefore to improved bioavailability.

Because of these properties, and taking advantage of the well-investigated reduction of benzamidoximes to benzamidines,[9] we have designed compounds with benzamidoxime moieties instead of benzamidine.

In this report we describe the synthesis, SAR (structure–activity relationship) and a binding mode of thrombin inhibitors with an azaphenylalanine scaffold, which are based on the prodrug principle.

All target compounds in Tables 1 and 2 were prepared employing essentially the same strategy as described in Scheme 1. Commercially available 4-cyanobenzaldehyde and 3-cyanobenzaldehyde **1** were transformed into the corresponding Boc-protected hydrazones **2** using *tert*-butylcarbazate followed by catalytic hydrogenation on Pd/carbon to give intermediates **3**. Coupling of these Boc-protected hydrazines **3** with cyclic amines gave the

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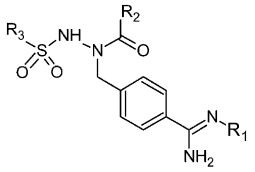
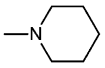
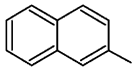
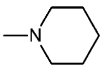
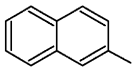
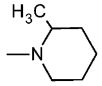
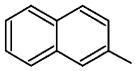
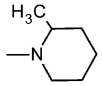
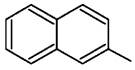
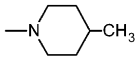
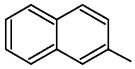
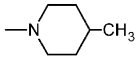
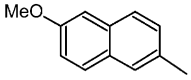
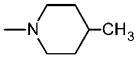
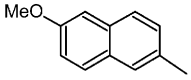
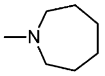
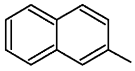
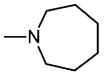
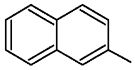
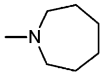
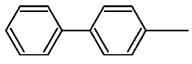
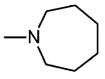
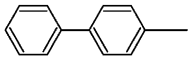
Table 1. Inhibitory activities of *para* substituted benzamidines and benzamidoximes[12]



Compd	R_1	R_2	R_3	K_i (μM)		
				Thrombin	Trypsin	Factor Xa
6a	OH			1.9	>68	>75
7a	H			0.095	13	66
6b	OH			0.43	45	>75
7b	H			0.085	26	53
6c	OH			0.77	>68	>75
6d	OH			0.38	>68	>75
7d	H			0.045	18	>75
6e	OH			0.21	13	>75
7e	H			0.032	10	69
6f	OH			1.6	>400	ND
7f	H			0.15	23	ND

ND not determined.

required Boc-protected carbazamides **4** in a one-pot synthesis, utilizing commercially available triphosgene. Deprotecting the Boc group in compounds **4** by treatment with gaseous HCl in AcOH and subsequent reaction with aryl sulfonyl chlorides led to compounds **5**. Methoxy-substituted naphthalenesulfonyl chloride was prepared from 6-hydroxy-2-naphthalenesulfonic acid sodium salt and thionyl chloride according to literature procedures.[10,11] Finally, hydroxylamine was used to convert the nitriles **5** to the target benzamidoximes **6**, and cyano compounds were treated with ethanolic HCl and reacted with ammonium acetate to give the corresponding amidines **7**.

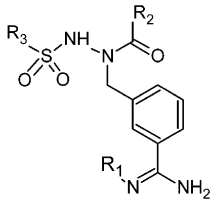
Although we propose in vivo reduction of benzamidoxime to the benzamidine, we have also investigated the SAR of benzamidoximes.

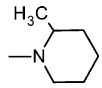
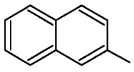
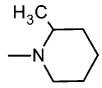
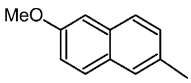
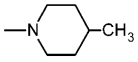
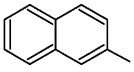
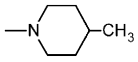
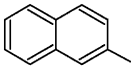
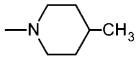
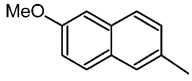
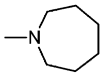
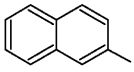
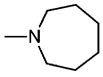
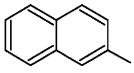
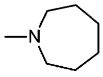
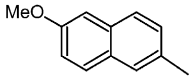
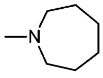
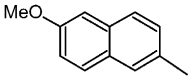
The enzyme inhibition constants of the target benzamidines (**7**) and their benzamidoxime analogues (**6**) toward thrombin and the structurally related serine proteases FXa and trypsin are listed in Tables 1 and 2.

Comparing the in vitro inhibitory potencies of the *para* substituted (Table 1) and *meta* substituted (Table 2) moieties on the aromatic ring in the P1 part of the molecule, it is evident that, of the benzamidines, *meta* isomers are preferred for binding in the S1 specificity pocket. In contrast, the most potent of the benzamidoximes is the *para* substituted compound **6e**, with a K_i of 210 nM.

In evaluation for trypsin-selectivity, *para* substituted analogues exhibited better selectivity ratio than *meta* analogues, particularly among benzamidines (e.g., the

Table 2. Inhibitory activities of *meta* substituted benzamidines and benzamidoximes[12]



Compd	R_1	R_2	R_3	K_i (μM)		
				Thrombin	Trypsin	Factor Xa
6g	OH			12	67	5.4
6h	OH			4.2	47	111
6i	OH			0.47	>68	>75
7i	H			0.004	0.11	5.3
7j	H			0.011	ND	ND
6k	OH			0.59	32	>75
7k	H			0.005	0.14	3.9
6l	OH			0.61	17	103
7l	H			0.009	0.053	3.0

ND not determined.

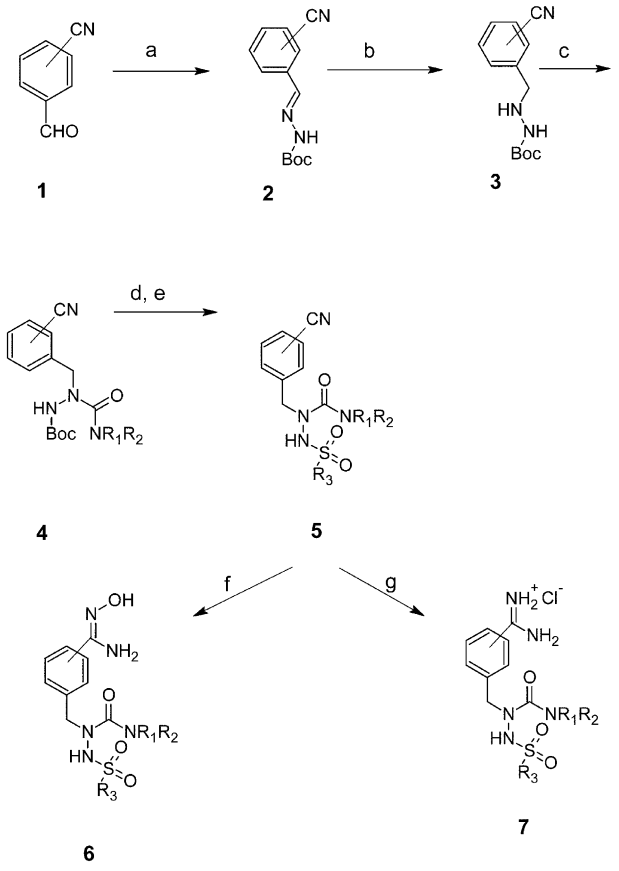
para isomer **7e** (312-fold) versus the *meta* isomer **7k** (28 fold)).

Methylation and enlargement of the piperidine ring resulted in increased hydrophobicity at the P2 part of the molecule, with increased potency of the benzamidines and benzamidoximes. In the series of *para* substituted benzamidoximes, introduction of a 2- or 4-methyl group on the piperidine ring led to 4,5 fold (**6b**, $K_i = 427$ nM) and 2.5-fold (**6c**, $K_i = 768$ nM) increase in activity, respectively. Incorporation of an azepine ring in this part of the molecule yielded the most potent benzamidoxime compound of the series, **6e**, with a K_i of 210 nM. The favourable effect of this modification was not so evident in the series of *para* benzamidines where the maximum increase in potency was 3-fold (**7a**, $K_i = 950$ nM versus **7e**, $K_i = 320$ nM). Introduction of a 2-methyl group on the piperidine in the series of *meta* substituted benzamidoximes resulted in compounds with low activity (**6g**, $K_i = 12$ μM; **6h**, $K_i = 4$ μM). In contrast, a methyl group on the *p*-position on the piperidine ring, or introduction of the azepine ring, led to compounds with moderate thrombin inhibitory potency (**6i**, $K_i = 469$ nM; **6k**, $K_i = 594$ nM; **6l**, $K_i = 610$ nM).

Thus, among the benzamidines, *meta* isomers are preferred for binding in the S1 specificity pocket and substitution in the P2 part of the molecule led us to potent inhibitors of thrombin with K_i values lowered to 4 nM (**7i**).

Variation of the sulfonamide aryl moiety, which fits into the S3 binding pocket, led to improvements in potency in the series of *para* substituted benzamidoximes. Replacement of the naphthyl group with the 6-methoxy-2-naphthyl group led to compound **6d** which showed enhanced potency over **6c**. 4-Substituted biaryl replacement (compounds **6f** and **7f**) resulted in significant losses of potency. Presumably, the biaryl groups of this series reach deeper in the S3 pocket of the thrombin active site, and probably bump into side wall of the pocket, causing less favourable binding affinity.

In order to understand the in vitro structure–activity relation, the binding conformations in the active site of human α-thrombin of the *meta*-substituted benzamidine, compound **7k**, and the *para*-substituted benzamidoxime, compound **6d**, were determined by X-ray crystallography. A simple superposition of the structures of **6d** and **7k** in the active site of thrombin is shown in Figure 1.



Scheme 1. (a) Boc-$NHNH_2$, EtOH, reflux; (b) H_2, Pd/C, MeOH; (c) (1) $(Cl_3CO)_2CO$, CH_2Cl_2; (2) cyclic amine, DIEA; (d) HCl (g), AcOH; (e) aryl sulfonyl chloride, CH_2Cl_2, Et_3N; (f) NH_2OH, EtOH, reflux; (g) HCl/EtOH, NH_4OAc.

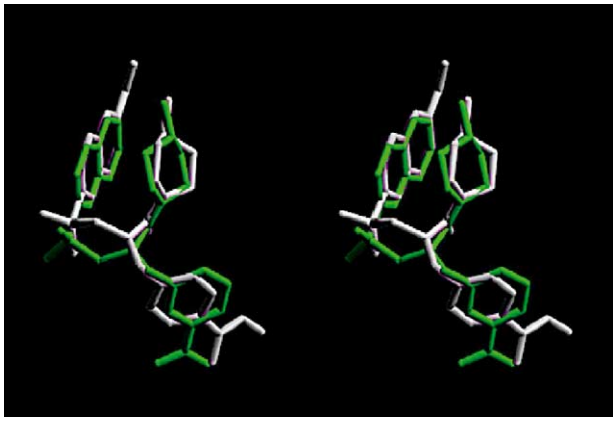


Figure 1. Binding conformation of **6d** (gray) superimposed onto **7i** (green) as bound to the active site of thrombin.

The complexes, refined at a resolution 1.73 Å, showed that **6d** and **7k** bind in a compact form similar to that of other molecules of the Argatroban/NAPAP family.[13] Benzamidine or benzamidoxime occupies the S1 selectivity pocket, the 4-methylpiperidine ring nestles under the YPPW loop of the lipophilic S2 binding pocket and the naphthalene group enters the aryl binding site.

The *meta*-substituted benzamidine of compound **7k** goes straight to the bottom of the S1 pocket (Fig. 2) in the position that facilitates optimal twined contacts with Asp 189 of thrombin, while the *para*-substituted benzamidoxime in **6d** is directed to the side wall of the S1 pocket. The benzamidoxime oxygen points towards the plane of the benzyl group of Tyr 228. The distance between them is 3.5 Å, suggesting an interaction between hydrogen of the benzamidoxime hydroxyl group and the π-electron system of the ring.[14] Only one nitrogen of benzamidoxime forms a hydrogen bond with Asp 189 and two weak bonds with the carbonyl oxygens of Trp 215 and Ala 190. In **7k** there is a total of seven hydrogen bonds at the bottom of the S1 pocket. Two of them are with Asp 189, three with other residues and two with water molecules. **6d** additionally lacks a hydrogen bond with the backbone carbonyl oxygen of Gly 216. This is probably a consequence of the scaffold position of the azaphenylalanine functionality in the active site pocket and *para* substitution of the benzamidoxime ring. The third reason for the observed reduced affinity of **6d** versus **7k** is probably the absence of any hydrogen bond with water molecules that are in contact with protein. **7k** on the other hand forms two such contacts and is thus indirectly bonded to six surrounding residues in the active site through a dense hydrogen bond network of water molecules. Furthermore, the difference in pK_a values of the benzamidine (pKa = 11.6) and benzamidoxime (pK_a = 4.5 or 7.0 depending on the tautomeric form) plays a role in binding of the inhibitor to the aspartate Asp 189 of the S1 binding pocket.[15]

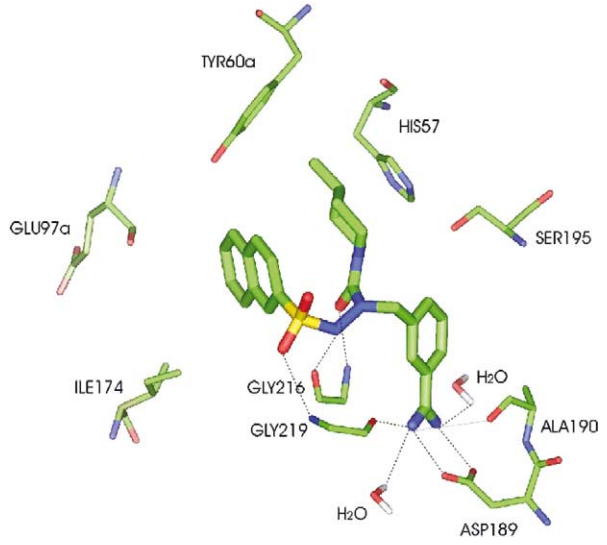


Figure 2. Schematic representation of inhibitor **7i** bound in the active site of thrombin. Dashed lines indicate hydrogen bonds.

In summary, we have prepared and evaluated a series of thrombin inhibitors with an azaphenylalanine scaffold and reduced stereogenicity that incorporate a benzamidine or weakly basic benzamidoxime element at the P1 position. Several benzamidine based thrombin inhibitors with low nanomolar potency and their benzamidoxime analogues, for which we propose in vivo reduction, were identified.

Further SAR development and studies directed to demonstrate antithrombotic activity in animal models following oral administration are in progress; the results will be published in due course.

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