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THE DESIGN OF POTENT AND SELECTIVE INHIBITORS OF THROMBIN UTILIZING A PIPERAZINEDIONE TEMPLATE: PART 2

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Abstract: Potent and selective thrombin inhibitors have been prepared with a piperazinedione template and L-amino acids. Likewise, incorporation of D-amino acids led to potent inhibitors with a novel mode of binding. Herein, the structure activity relationships and structural aspects of these compounds will be described.

Introduction:

It has been well established that the inhibition of thrombin is a critical therapeutic target for the treatment of venous and arterial thrombosis, pulmonary embolism and restenosis following angioplasty.[1-3] Current therapies such as coumadin, heparin and hirudin or hirulog are effective, but suffer from several side effects. Although a number of low molecular weight thrombin inhibitors have been reported, these inhibitors suffer from several problems including: 1) lack of a high selectivity for thrombin over other trypsin-like serine proteases; 2) low oral bioavailability; and 3) short duration of action.[1-3]

In the previous publication, we have shown that potent and selective inhibitors of thrombin can be developed utilizing a piperazinedione template (compound **1**, Figure 1).[4] This template was designed from a series of highly potent bicylic compounds[5-7] with the goal of decreasing molecular weight and increasing the potential for oral activity. In addition, the chemistry to prepare these piperazinediones was significantly less challenging. These monocyclic inhibitors maintained good affinity for thrombin and selectivity versus trypsin. Selectivity was imparted by the arginine mimic, which fits well in the P1 pocket of thrombin, but was too large for the P1 pocket of trypsin. Unfortunately, even though the most potent analogue in this series (compound **1**) showed antithrombotic efficacy in an *in vivo* rat model of acute arterial thrombosis (3.2-fold shift in mean occulsion time vs. control), however it lacked oral bioavailability a dog model.

The compounds of the previous publication were prepared from L-amino acids, which led to S-stereochemistry at the chiral carbon of the piperazidione template.[4] Herein, we report on the structure activity relationships (SAR) of the monocyclic analogues prepared with D-amino acids.[8] These inhibitors display a unique SAR and most interestingly, bind in a novel conformation to thrombin.

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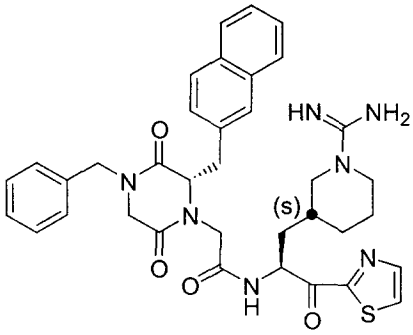
Figure 1:



Compound **1**
Trypsin IC_{50} =11000 nM
Thrombin IC_{50} = 170 nM
Thrombin K_i = 13.5 nM

Experimental:

Chemistry: The piperazinedione inhibitors were prepared and purified as previously described except that a D-amino acid was substituted for the L-amino acid in the reaction sequence.[4]

Pharmacology: The inhibitors were evaluated for binding to thrombin and trypsin as previously described.[4,9] Promising compounds were further evaluated in an *in vivo* model of antithrombotic efficacy in a rat model of acute arterial thrombosis[10] and finally, oral bioavailability was evaluated in the dog.

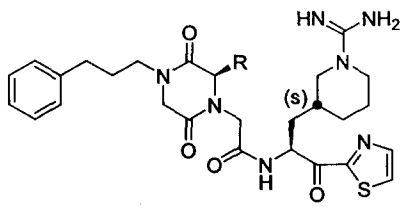
Results and Discussion:

Preparation of the prototypical D-enantiomer[4] yielded compound **3** that possessed good affinity for thrombin (IC_{50} = 170 nM, K_i = 55 nM) and was also 50-fold selective for thrombin over trypsin (Figure 2). X-ray crystallographic analysis of **3** bound to thrombin revealed that this analogue bound significantly differently from that of the L-stereochemistry series (Figure 3). Specifically, the guanidine still formed strong hydrogen bonds to Asp^{189} in the P1 pocket. However, the catalytic Ser^{195} did not interact with the carbonyl of the ketothiazole. In fact, the ketothiazole points into solvent, with its carbonyl forming a hydrogen bond to the amide of Gly^{219}. The benzyl group occupies the (d)S3 pocket, while the phenylpropyl group binds in the S2 pocket.

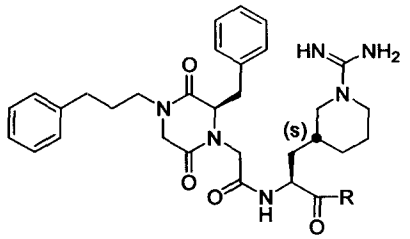
This unusual binding mode for the D-monocyclic piperazinedione (**3**) prompted a further evaluation of the structure-activity relationships. Interestingly, this binding mode should potentially allow the incorporation of novel heterocycles in the (d)S3 and S2 pockets. Also, since Ser^{195} is not covalently bound to the ketothiazole carbonyl, it may then be possible to replace this electrophilic carbonyl with simple amides, esters or an acid. Also, the highly basic P1 group could be potentially replaced with groups more amenable to oral biological activity.

The X-ray crystal structure suggested that we could occupy the (d)S3 with a variety of aromatic residues. The beta-naphthyl group was well-tolerated (**4**). However, a diphenyl group (**5**) was not tolerated leading to a loss of affinity for thrombin and a loss of selectivity. Interestingly, incorporation of the 3,4-dichlorobenzyl group lead to a 2-fold increase in affinity for thrombin versus **3** (**6**, IC_{50} = 80 nM, K_i = 8 nM)

Figure 2:



Compound No.	R	Trypsin IC_{50} (nM)	Thrombin IC_{50} (nM)	Thrombin K_i (nM)
2	$-CH_3$	340	880	n.d.
3	$-CH_2Ph$	9100	170	55
4	$-CH_2$(beta-Naphthyl)	18200	160	55
5	$-CH(Ph)_2$	8500	350	n.d.
6	$-CH_2(3,4\text{-}Cl_2\text{-}Ph)$	7800	80	8
7	$-CH_2$(4-Cl-Ph)	17000	280	n.d.
8	$-CH_2$(3-Cl-Ph)	4800	390	20
9	$-CH_2$(2-Cl-Ph)	9700	18	1.2



10	**-2-benzthiazole**	**3700**	**150**	**80**
11	**-piperidinyl amide**	**52% @ 100uM**	**6600**	**825**
12	**-pyrrolidinyl amide**	**87000**	**660**	**130**
13	**-methyl amide**	**23000**	**440**	**55**

while maintaining 100-fold selectivity. Preparation of the mono-chloro compounds (**7** – **9**) led to the most potent analogue in this series (**9**). Specifically, the incorporation of the 2-chlorobenzyl group resulted in an analogue with significant affinity for thrombin (**9**, IC_{50} = 18 nM, K_i = 1.2 nM) and greater than 500-fold selectivity. This compound was active in the *in vivo* rat arterial thrombosis model showing a 3.2-fold shift in mean occlusion time (MOT) versus control. Unfortunately, compound **9** lacked sufficient oral bioavailability in the dog. The lack of oral bioavailability may be a result of the basic guanidine and/or the molecular weight

(MW = 792.28).

Further SAR evaluation revealed that the thiazole group could be replaced with a benzothiazole (**10**) or methyl amide (**13**) while maintaining reasonable affinity and selectivity. The activity of the methyl amide is consistent with the X-ray structure, in that the thiazole did not appear to be making significant interactions. However, larger amides including pyrrolidinyl and piperidinyl were not well tolerated (**11** and **12**).

Finally, the phenylpropyl group was also optimized. Shorter chains, including phenyl ethyl (**14**) and benzyl (**15**) resulted in significant losses of affinity for thrombin. Interestingly, the aromatic ring can be substituted with several groups (**16 - 22**) with little effect on potency and selectivity with respect to the parent compound (**3**). It was also shown that the phenyl group could simply be replaced with a methyl group (**23**).

Surprisingly, incorporation of arginine in the P1 position was not tolerated and only resulting in a moderately potent trypsin inhibitor (**24**). In fact, **24** possessed twenty-fold less affinity for thrombin than **3**.

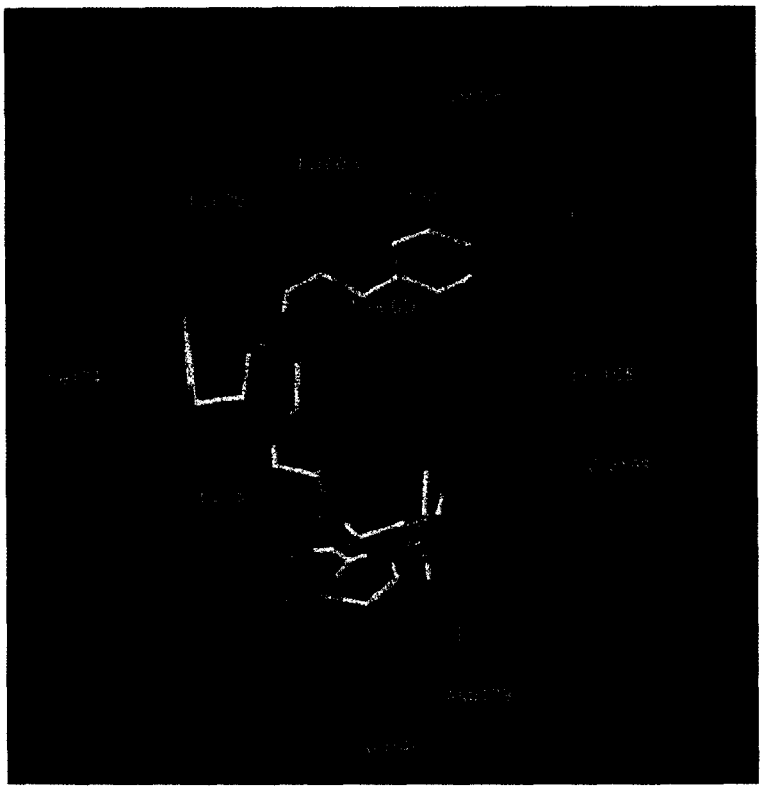
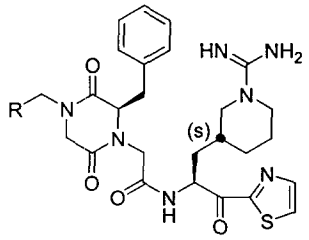
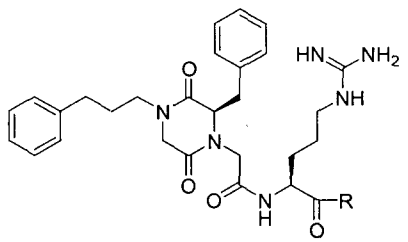


Figure 3. The above figure is a graphical representation of the molecular interactions between compound **3** and thrombin as seen in the X-ray structure of the binary complex. Substrate-binding subsites S1'-S3 of thrombin only are shown. The inhibitor is shown in thick lines with the following atom coloring: carbon (white), oxygen (red), nitrogen (blue), and sulfur (yellow). Hydrogen bonds are shown as yellow dashed lines and significant hydrophobic contacts are highlighted with dashed magenta arcs. The S1', S1, S2, S3 sites are highlighted with labeled cyan boxes.

Figure 4:



Compound No.	R	Trypsin IC_{50} (nM)	Thrombin IC_{50} (nM)	Thrombin K_i (nM)
14	-CH_2Ph	45000	990	n.d.
15	-Ph	21000	1230	n.d.
16	-$(CH_2)_2$-4-MeO-Ph	14000	160	n.d.
17	-$(CH_2)_2$-4-Me-Ph	4100	210	n.d.
18	-$(CH_2)_2$-4-Cl-Ph	4800	110	n.d.
19	-$(CH_2)_2$-4-NO_2-Ph	9100	210	n.d.
20	-$(CH_2)_2$-4-iPr-Ph	7000	370	n.d.
21	-$(CH_2)_2$-4-OH-Ph	25000	240	n.d.
22	-$(CH_2)_2$-3,4-Cl_2-Ph	7200	250	n.d.
23	-$(CH_2)_2$-CH_3	15000	340	32



24	-thiazole	96	3400	n.d.
25	-OH	20% @ 100uM	20% @ 100uM	n.d.
26	-NH_2	15% @ 100uM	35% @ 100uM	n.d.

Replacement of the thiazole of **24** with an –OH or –NH_2 yielded inactive analogues (**25** and **26**). A crystal structure of **24** bound to thrombin illustrated that **24** binds in a substrate-like mode, with the arginine ketothiazole binding in S1-S1' and Ser^{195} hydroxyl forms a covalent bond with the inhibitor. The phenylpropyl group binds in the dS3 pocket, the template carbonyl is not positioned to make a hydrogen bond

to $Gly^{216}NH$, and the benzyl substituent is pointing into solvent. Lack of favorable interactions with the enzyme by the piperazinedione ring and the benzyl group helps to explain the poor inhibition. Modeling of an arginine containing compound (e.g. **24**) based on the conformation of thrombin-bound **3** showed that for this compound to bind similar to **3**, the chi2 (χ_2) angle of the arginine side chain would need to be -27°, where an angle of 180° is preferred. Also, some hydrophobic interactions between the enzyme and the piperidine ring are not available for **24**. Thus, this may help explain the lack of activity for compounds **24 - 26**.

In conclusion, incorporation of D-amino acids into the piperazinedione template revealed a unique binding mode for these molecules to thrombin, and highlights the advantage of performing routine X-ray crystallography in the drug discovery process to delineate structure-activity relationships and binding modes. These X-ray experiments were performed by soaking the inhibitor into pregrown thrombin-hirugen crystals. This approach is simpler than performing co-crystallization experiments with thrombin and each individual inhibitor. Thus, this is an attractive approach for drug discovery programs.

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