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THE DESIGN OF POTENT AND SELECTIVE INHIBITORS OF THROMBIN UTILIZING A PIPERAZINEDIONE TEMPLATE: PART 1

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Abstract: Utilizing X-ray crystallography and molecular modeling, highly potent and selective peptidomimetic thrombin inhibitors have been designed containing a rigid piperazinedione template. The synthesis and biological activity of these compounds will be described.

Introduction:

Thrombin is a trypsin-like serine protease that plays a critical role in blood coagulation. Specifically, thrombin is responsible for the conversion of the plasma soluble protein fibrinogen to insoluble fibrin, which is the matrix of clots. Similar to other serine proteases, thrombin utilizes a catalytic triad of amino acids (Ser^{195}, His^{57}, and Asp^{102}) to initiate amide bond cleavage C-terminal to a basic residue, in particular arginine. Specific inhibitors of thrombin have therapeutic utility in the treatment of venous and arterial thrombosis, pulmonary embolism, and restenosis following angioplasty. Also, thrombin has been implicated as a contributor to atherosclerosis, inflammation, and neurodegenerative diseases. Current antithrombotics such as coumadin, heparin, and thrombin inhibitors including hirudin and hirulog have been evaluated clinically. However, these compounds have been shown to cause excessive bleeding and resulted in the need for continual blood monitoring. Therefore, the development of potent, selective, and orally active small molecule inhibitors of thrombin is of significant commercial and therapeutic interest.[1-3]

Previously, we reported on the development of a series of potent and selective thrombin inhibitors based upon a novel bicyclic template (Figure 1).[4-6] Unfortunately, the preparation of the template was long and cumbersome, and even though several of the resulting inhibitors possessed subnanomolar affinity for thrombin, oral activity was not achieved. However, in this series it was discovered that selectivity for thrombin over other serine proteases was obtained by incorporating 3-amidino-piperidinyl alanine to bind in the P1 pocket of thrombin.[5] Therefore, we incorporated this selective P1 pharmacophore into a different template that was synthetically less challenging and of lower molecular weight in order to increase the potential for oral activity. Thus, we chose to examine a piperazinedione template.

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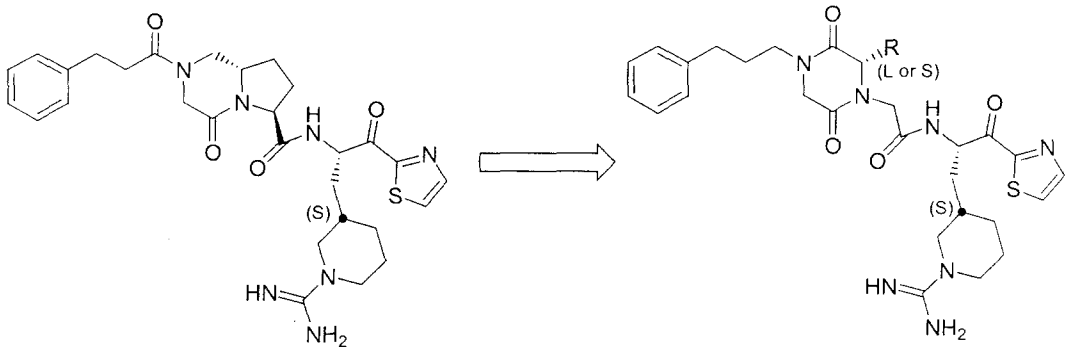
Thrombin is unique among trypsin-like serine proteases, in that it contains an insertion loop formed by residues Tyr60A - Trp60D. Molecular modelling suggested that this piperazinedione template could be further exploited to access additional binding interactions in the insertion loop of thrombin by varying the R group (See Figure 1), thereby enhancing potency and selectivity. Herein, we will report the structure activity relationships (SAR) for some piperazinedione analogues wherein the R group is derived from L-amino acids.[8]

Figure 1:

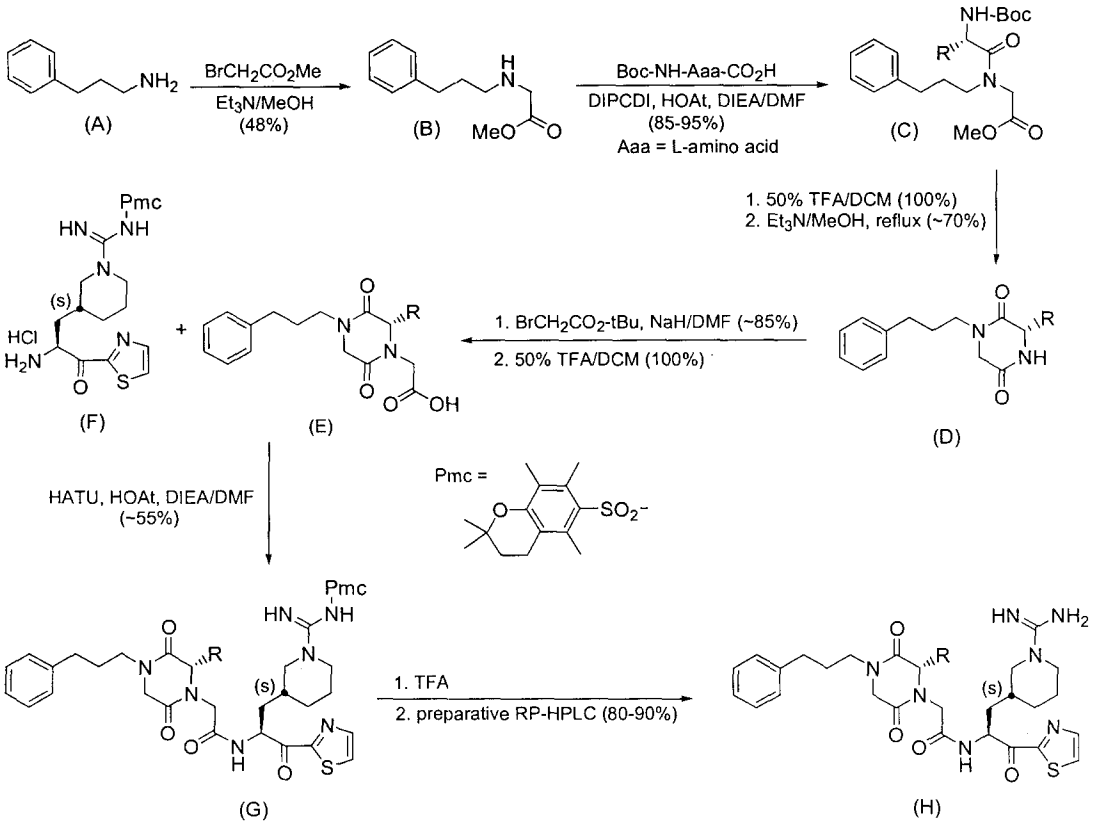


Experimental:

Chemistry: The piperazinedione based inhibitors were prepared by a convergent synthetic strategy in 10-15% overall yield, as shown in Scheme 1. Specifically, 3-phenyl-1-propylamine (**A**) was condensed with bromomethyl acetate under basic conditions to provide **B** in 48% yield. This amine was coupled to the desired L-amino acid in the presence of *N,N'*-diisopropylcarbodiimide (DIPCDI), 1-hydroxyazabenzotriazole (HOAt) and *N,N*-diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF) to provide **C**. 1-Hydroxybenzotriazole was not effective for this hindered coupling and the use of HOAt was required.[7] Removal of the *tert*-butyloxycarbonyl (*t*-Boc) protecting group with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM), followed by suspension in refluxing methanol with an excess of triethylamine yielded the piperazinedione template (**D**). Alkylation of the amide nitrogen with *tert*-butyl bromoacetate in the presence of sodium hydride (NaH) in DMF, followed by deprotection with 50% TFA in DCM yielded (**E**). Compound **E** was then coupled to the protected arginine mimetic (e.g., **F**) utilizing *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) with HOAt in the presence of DIEA to provide the protected target (**G**). Since the guanidino group of **F** was protected with the 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) group[9], it was readily deprotected with TFA and the crude product (**H**) was purified by preparative reversed phase HPLC.

Pharmacology: Compounds were evaluated for their ability to inhibit the serine proteases, thrombin and trypsin. Kinetic assays were conducted at 37°C using a kinetic spectrophotometric plate reader. A typical assay consisted of an optimized concentration of human enzyme in buffer (0.5 nM final concentration for thrombin and trypsin) combined with 2 μl of inhibitor dilutions (creating a final concentration range of 1 nM -

Scheme 1:



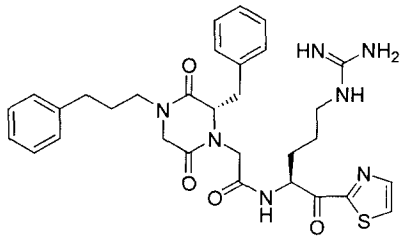
100 μM), and preincubated for 1 h at room temperature. The assay was initiated by the addition of an appropriate synthetic substrate (Chromozym TH for thrombin, and S-2222 for trypsin), respectively at predetermined 2 X K_m. Absorbance at 405 nM was determined over 10 minutes and percent inhibition was calculated from the slope of the progress curves during the linear part of the time course at each concentration. The IC_{50} was defined as that concentration of test substance that inhibits 50% of the respective protease activity. Non-linear regression analysis was used to estimate a K_i by use of Michaelis-Menten and reciprical Dixon plot fits.

Results and Discussion:

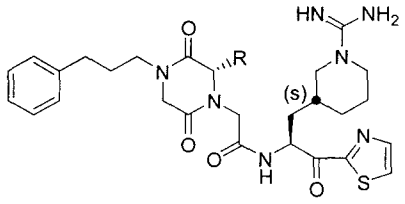
The initial prototypic compound (**1**) in which an arginine type side chain was incorporated in the P1 position and R was a benzyl group with L-stereochemistry at the chiral carbon of the piperazinedione template showed effective inhibition of thrombin, but possessed greater affinity for trypsin (Figure 2).

X-ray crystallographic analysis of **1** bound to thrombin provided insights into the mode of binding. Specifically, the arginine side chain was binding in the P1 pocket forming strong hydrogen bonds to Asp^{189}, and the catalytic Ser^{195} forms a covalent bond to the carbonyl of the ketothiazole. The benzyl group occupies

Figure 2:



Compound **1**
Trypsin IC_{50} = 90 nM
Thrombin IC_{50} = 130 nM



Compound No.	R	Trypsin IC_{50} (nM)	Thrombin IC_{50} (nM)	Thrombin K_i (nM)
2	$-CH_2Ph$	80% @ 100uM	240	24
3	-H	91200	4700	495
4	$-CH_3$	24000	760	65
5	$-(CH_2)_3CH_3$	42700	1600	98
6	$-CH(CH_3)_2$	74800	1800	n.d.
7	$-CH_2CH(CH_3)_2$	69% @ 100uM	4300	165
8	$-CH(CH_3)CH_2CH_3$	67% @ 100uM	3400	200
9	$-CH_2(C_6H_{11})$	18500	430	75
10	$-CH_2$(4-MeO-Ph)	3800	350	43
11	$-CH_2$(4-Cl-Ph)	33400	170	40
12	$-CH_2$(3-Pyr)	40% @ 100uM	1000	n.d.
13	$-CH(Ph)_2$	9700	750	n.d.
14	$-CH_2CH_2Ph$	29000	590	105
15	$-CH_2$(beta-Naphthyl)	27000	45	30

the (d)S3 pocket while the phenylpropyl group extends into solvent without making significant interactions with the enzyme (Figure 3). In addition, a carbonyl from the piperazinedione template was shown to form a hydrogen bond to the Gly^{216} amide. Upon incorporation of the selective P1 moiety (**F**) a significant enhancement in selectivity was obtained as seen in compound **2**. Therefore, we decided to further examine the

SAR of this series by varying R in order to take advantage of potential interactions in the insertion loop.

In general, replacement of the benzyl group with aliphatic moieties (**3** - **9**, Figure 2) led to compounds with reasonable selectivity for thrombin over trypsin (~20-50 fold). However, all of these compounds were relatively weak inhibitors, with the most potent compound being **9** (IC_{50} = 430 nM, K_i = 75 nM).

Incorporation of aromatic residues at this position provided more promising results. Both the para-methoxy and para-chloro derivatives (**10** and **11**) possessed good affinity for thrombin and were selective versus trypsin. Interestingly, **11** was approximately 200-fold selective for thrombin over trypsin. Basic and bulky groups were not well tolerated (e.g. 3-pyridyl and diphenyl, **12** and **13**). In addition, extension of the phenyl group further into the pocket (**14**) led to a loss of activity versus **2**. The incorporation of a beta-naphthyl group in this position resulted in an analogue (**15**) with the best affinity for thrombin (IC_{50} = 45 nM, K_i = 30 nM), along with the best selectivity versus trypsin (~200-fold).

Since the X-ray crystal structure of compound **1** bound to thrombin suggested that the phenylpropyl

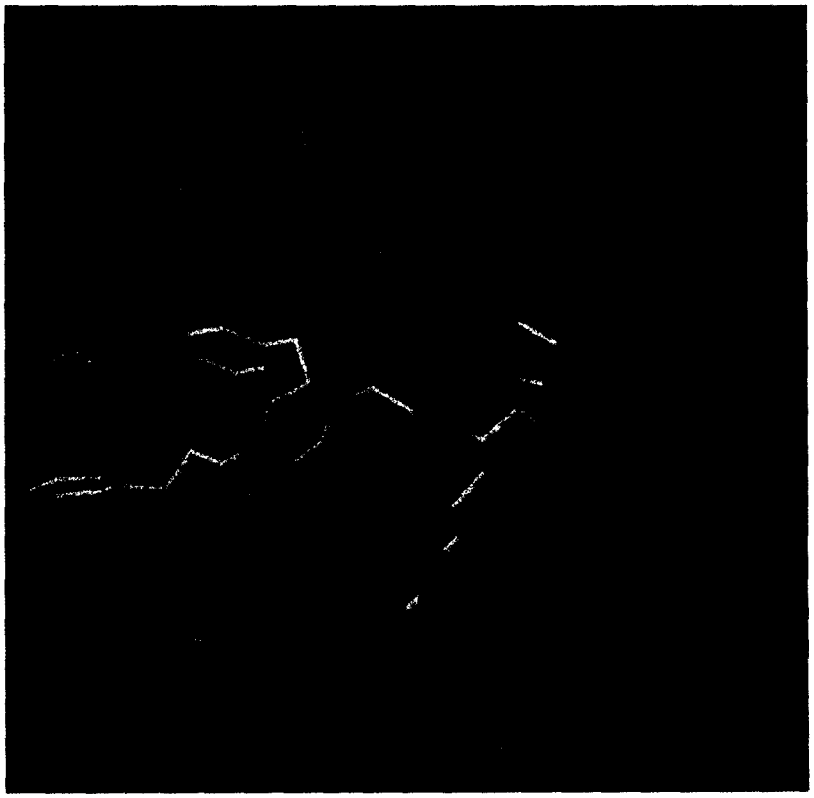
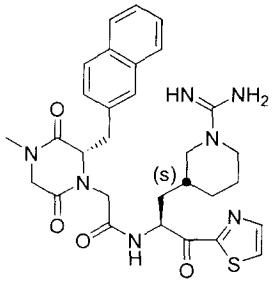
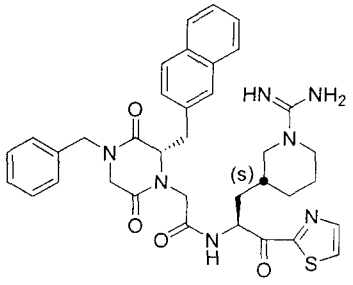


Figure 3. The above figure is a graphical representation of the molecular interactions between compound **1** and thrombin as seen in the X-ray structure of the binary complex. Substrate-binding subsites S1'-S3 of thrombin only are shown. The inhibitor is shown in thick lines with the following atom coloring scheme: 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.

group was solvent exposed and not contributing to the overall binding energy, this group was replaced in compound **15** with a methyl (**16**) and a benzyl (**17**) group. These substitutions were predicted to enhance binding affinity and result in analogues of lower molecular weight with increased potential for oral activity.



Compound **16**



Compound **17**

Unfortunately, compound **16** had significantly less affinity for thrombin (IC_{50} = 1270 nM, K_i = 140 nM) than compound **15**, but maintained good selectivity versus trypsin (IC_{50} = 26600 nM). Compound **17** was more potent than compound **16** comparing favorably with **15** (IC_{50} = 170 nM, K_i = 13.5 nM) while maintaining selectivity versus trypsin (IC_{50} = 11100 nM). Thus, compound **17** was evaluated for antithrombotic efficacy in an *in vivo* rat model of acute aterial thrombosis[10] and possessed a 3.2 fold shift in the mean occlusion time versus control (0.75 mg/kg bolus followed by 50 ug/kg/min continuous infusion). Unfortunately, **17** lacked sufficient oral bioavailability in rats to be considered for further development. However, this series may provide an interesting starting point for developing compounds with improved pharmacokinetic properties.

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