Communications to the Editor

Retro-Binding Tripeptide Thrombin Active-Site Inhibitors: Discovery, Synthesis, and Molecular Modeling

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Thrombin, a serine protease product of the blood coagulation cascade, plays a central role in hemostasis and thrombosis.[1] Thrombin cleaves fibrinogen to form fibrin and activates Factor XIII, which cross-links and stabilizes the formed clot. By limited proteolysis, thrombin activates Factors V, VIII, and XI, which promote further thrombin production. Thrombin is a very potent stimulator of platelet shape change, aggregation, and secretion. In addition, thrombin is mitogenic for vascular smooth muscle and can activate endothelial cells, promoting cell adhesion. Because of these actions, the proteolytic activity of thrombin plays a major role in arterial as well as venous thrombosis; therefore, thrombin inhibition continues to be an important target for the development of new therapeutic agents.

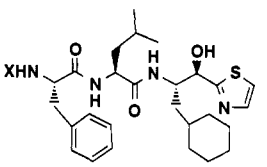
A large number of synthetic inhibitors of thrombin have been patterned originally after substrates or naturally occurring inhibitors, most of which are derivatives of arginine or benzamidine. Four thrombin active-site inhibitors which typify current chemotypes are D-Phe-Pro-Arg-H (RGH 2958) and various analogs,[2] NAPAP,[3] MD 805,[4] and cyclotheonamide A.[5] Their modes of binding have been determined from crystallographic studies.[6] They share a common feature: each inhibitor forms antiparallel β hydrogen bonds to Gly216.[7] Thus, the amino acid residues in the active site are in an orientation similar to those of the natural substrates of thrombin.

We report here a new series of potent thrombin active-site inhibitors. We believe from structure–activity relationships and the modeled bound conformation for this new series that the mode of binding is in a "retro" fashion: the tripeptide N-terminus is proximal to the active-site serine hydroxyl (Ser195), and the peptide backbone forms a parallel β hydrogen bond to Gly216. We describe briefly below the various synthetic analogs and the biological studies that have led to the elucidation of this binding conformation.

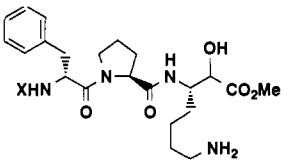
Results and Discussion. Screening of in-house compounds had identified **1** as an active-site inhibitor of thrombin. Although similar in structure, **2** displays no *in vitro* activity, showing the critical nature of the 6-aminohexanoyl moiety for potency. We have shown previously that **4**, an analog of RGH 2958, is an active-site inhibitor of thrombin that binds in a fashion similar to PPACK.[8] After examining the modes of binding for the various known thrombin inhibitors from their solid-state structures (*vide ante*), we proposed that **1** could bind in one of two fashions. In one mode of binding, the peptide backbone of **1** is oriented in the same direction as **4**; the hydroxythioimidazole of **1** functions as an isostere of the hydroxy ester of **4**, and the aromatic residue interacts with the distal pocket of thrombin.[9] Unlike **1** and **2**, **3** and **4** display comparable potency, presumably because of a vastly different binding orientation for **1** and not solely because of the difference in absolute stereochemistry of the phenylalanine residues.

The other binding mode would align the amide backbone in a reverse fashion (compared to natural substrates). We modeled this new binding orientation of **1** *via* docking studies.[12] In the modeled bound conformation, the 6-aminohexanoyl moiety binds at the specificity pocket, and the aromatic and the cyclohexyl residues bind at the

Table 1. *In Vitro* Inhibition of Thrombin Catalytic Activity for 1 and Related Compounds



1,2

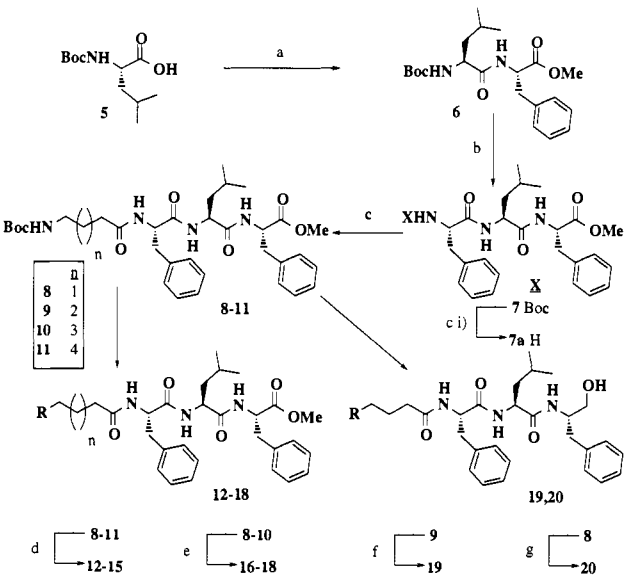


3,4

compd	X	IC_{50} (μm)[a]
1	$H_2N(CH_2)_5C(=O)$—	7.5
2	H	>530
3	$H_2N(CH_2)_5C(=O)$—	17.6
4	H	20–30

[a] *In vitro* inhibition of thrombin catalytic activity on 10 μM substrate s-2238 (D-Phe-Pip-Arg-pNA).[10]

Scheme 1[a]



[a] (a) (i) EDAC·HCl, HOBt, DMF, L-Phe(OMe); (b) (i) TFA, 0 °C; (ii) EDAC·HCl, HOBt, DMF, NMM, Boc-L-Phe; (c) (i) TFA, 0 °C; (ii) EDAC·HCl, HOBt, NMM, $BocNHCH_2(CH_2)_nCH_2CO_2H$ (n = 1–4); (d) (i) TFA, 0 °C; (e) (i) TFA, 0 °C; (ii) $H_2NC(SO_3H)NH$, Et_3N, EtOH; (f) (i) $LiBH_4$, MeOH, THF; (ii) TFA, 0 °C; (g) (i) $LiBH_4$, MeOH, THF; (ii) TFA, 0 °C; (iii) $H_2NC(SO_3H)NH$, Et_3N, EtOH.

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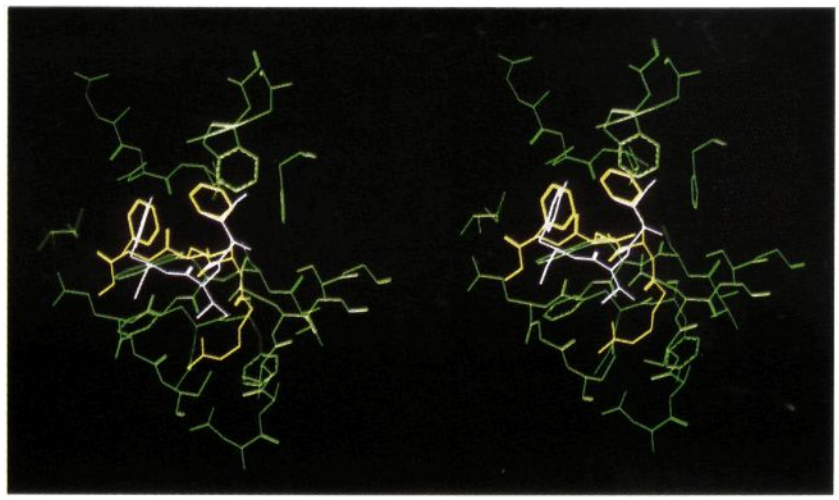
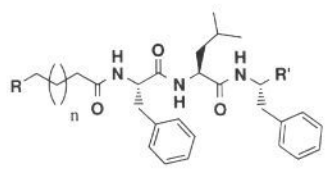


Figure 1. The minimized conformation of **16**, superimposed upon the hirudin(rHV2)/α-thrombin complex. Compound **16** is shown in yellow, hirudin Ile1-Tyr3 is in white, and the various residues defining the active-site region of thrombin are in green.

Table 2. *In Vitro* Inhibition of Thrombin Catalytic Activity: Side Chain Optimization (Chain Length and N-Terminal Functionality)



compd	n	R	R′	IC_{50} (μM)
12	1	H_2N	CO_2CH_3	>300
13	2	H_2N	CO_2CH_3	57.1
14	3	H_2N	CO_2CH_3	80
15	4	H_2N	CO_2CH_3	330
16	1	$H_2N(HN)CHN$	CO_2CH_3	1.41
17	2	$H_2N(HN)CHN$	CO_2CH_3	154
18	3	$H_2N(HN)CHN$	CO_2CH_3	>200
19	2	H_2N	CH_2OH	100
20	1	$H_2N(HN)CHN$	CH_2OH	2.5
7a[a]	–	–	CO_2CH_3	>333

[a] The TFA salt of **7**, shown in Scheme 1.

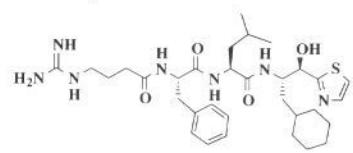
proximal and the distal pockets, respectively. The hydroxythioimidazole is exposed to solvent.

Consistant with this model, the cyclohexyl and the (hydroxymethyl)thioimidazole moieties in **1** (residues binding in or near the distal pocket) could be replaced by a phenyl and a methyl ester residue, respectively. This gave compound **14**. This simplified retro-binding peptide was found to be 10-fold less potent than **1** and suitable as a template for further study (see Scheme 1).

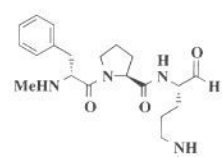
Analogs with various aliphatic side chain lengths and amino or guanidino termini were prepared, and their biological activities were determined (see Table 2). Analog **16** having a 4-guanidinobutanoyl moiety was the most potent active-site inhibitor of thrombin. Thrombin is a highly selective hydrolytic serine protease with the typical site of cleavage occurring after an arginine residue in the various natural substrates.[11] The sensitivity in potency to the side chain length and the preference for a guanidine residue over a primary amine in this series of inhibitors is consistent with the N-terminal side chain binding in the specificity pocket. The primary alcohols of **13** and **16**, **19** and **20**, respectively, were also prepared, and both were found to be modestly less active than the corresponding methyl esters.

After the optimal guanidine-terminated side chain was determined for the simplified analogs, this residue was affixed to **2** to give **21** (the optimized analog of **1**). Compound **21** displays classic competitive kinetics for the inhibition of thrombin with a K_i of 22 nM. In comparison to GYKI-14,766, **21** displays better selectivity for thrombin *vs* trypsin and plasmin but less than Factor Xa. Compound **21** (BMS 182,627) represents a promising new thrombin active-site inhibitor series.

Table 3. A Comparison of *in Vitro* Activity for **21** and GYKI-14,766 *vs* Several Serine Proteases



21 (BMS 182,627)



GYKI-14,766

	IC_{50} (μM)			
compd	thrombin	trypsin	plasmin	Factor Xa
21	0.12 [K_i = 22 nM] (1)	1.5 (12.5)[a]	143 (1200)	6.0 (50)
GYKI-14,766	0.008 (1)	0.008 (1)	0.230 (29)	4.0 (500)

[a] Selectivity ratio: IC_{50}/IC_{50} (thrombin).

Modeling. We have modeled the binding of **16** and several other analogs in the active site of thrombin using a combination of distance geometry and energy minimizations.[12] Compounds in this series, as exemplified by **16**, bind to the active site of thrombin in a fashion similar to the first three amino acid residues of hirudin (Ile1, Thr2, and Tyr3).[15-17] The key structural features include the formation of a β-parallel hydrogen bond with Gly216 by Phe1-O and Phe3-N, the extension of the 4-guanidinobutanoyl moiety into the specificity pocket, and the orientation of the amino acid side chains in regions similar to the corresponding three residues of hirudin. Hence, the phenyl moiety of the Phe1 side chain of **16** occupies the proximal pocket, a region defined by His57, Tyr60A, Trp60D, and Leu99, and the phenyl residue of the Phe3 side chain occupies the distal pocket.[18] Likewise, the Leu2 side chain of **16** is in a similar position to Thr2 in the hirudin/thrombin complex. A stereoscopic view of the modeled conformation of **16** superimposed upon the hirudin–thrombin complex is shown in Figure 1.

Studies have shown that the hydrogen bond or salt bridge between the α-amino group of Ile1 (hirudin) and thrombin makes a significant contribution to the binding energy.[19] In addition, various modifications to the N-

terminus of hirudin have led to a loss of binding energy. Acetylation of this terminal amine eliminates the positive charge and decreases the binding energy by 22.7 kJ/mol. However, in this new series of inhibitors, we find the amine- or the guanidine-terminated side chain is an important pharmacophore that is required for thrombin active-site inhibition as shown through a comparison of **21** and **16**, **2** and **7a**, respectively. The protonated guanidine or protonated amine terminus interacts with Asp189 in the specificity pocket to significantly enhance binding. This new interaction compensates for the loss of electrostatic interactions at the N-terminus upon acylation. Also, unlike Ile1-Tyr3 of hirudin, the lack of a bulky C-terminal attachment makes it easier for these inhibitors to avoid unfavorable steric interactions with Ser195 and other residues in its vicinity *via* a slight shift in its backbone (Figure 1). Our studies are continuing in this area with a focus on structural changes that will enhance potency and selectivity but reduce molecular weight.

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Supplementary Material Available: The coordinates for the modeled bound conformations of compound **16** are given relative to the coordinates of the PPACK-α-thrombin structure 1DWE from the Protein Data Bank (3 pages). Ordering information is given on any current masthead page.

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(10) Chromogenic substrate assay: The inhibitors were incubated in an assay buffer (145 mM NaCl, 5 mM KCl, 1 mg/mL poly(ethylene glycol) (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, pH 7.4) with human α-thrombin (0.1 unit/mL) at room temperature for 3 min. The enzymatic reaction was started with 10 μM s-2238 substrate and continued at room temperature. The change in optical density was measured at 405 nm. A kinetic microplate reader (Molecular Devices Corporation V_{max}) was used to measure the change in optical density over time. The K_m for s-2238 under these conditions is 2.5 μM. Each value represents the average of at least triplicate determinations.

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(12) The coordinates of α-thrombin were taken from the structure of PPACK-α-thrombin (ref 7). Molecular models of the inhibitors were constructed using SYBYL and minimized with BatchMin prior to DGEOM (ref 13) docking. Compound **16** was docked using DGEOM with three hydrogen bond distance constraints: (1) the guanidino terminus of the 4-guanidinobutanoyl moiety hydrogen bonds to thrombin Asp189, (2) the Phe1 O hydrogen bonds to thrombin Gly216 N, and (3) the Phe3 N hydrogen bonds to thrombin Gly216 O. Fifty trial DGEOM structures were generated for the inhibitor. These were minimized in the active site of thrombin with the MacroModel/BatchMin (ref 14) software package employing the AMBER (ref 20) united atom force field. The protein atoms were constrained with a harmonic constraint of 100 kJ/Å. The inhibitor was allowed to minimize without any constraints. The minimization employed a distance-dependent dielectric constant of 2*r*, the electrostatic cutoff was 12 Å, the van der Waals cutoff was 7 Å, and the hydrogen bonding cutoff was 4.0 Å. The most energetically favorable conformer was chosen as the model for the bound structure. Compound **1** was docked using a similar DGEOM and minimization protocol. A set of distance constraints similar to that for compound **16** was used for DGEOM docking of **1**. The results were visualized using SYBYL (ref 21) running on a Silicon Graphics 420VGX.

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