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Retro-Binding Thrombin Active Site Inhibitors: Identification of an Orally Active Inhibitor of Thrombin Catalytic Activity

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Abstract—A series of retro-binding inhibitors of human α-thrombin was prepared to elucidate structure–activity relationships (SAR) and optimize in vivo performance. Compounds **9** and **11**, orally active inhibitors of thrombin catalytic activity, were identified to be efficacious in a thrombin-induced lethality model in mice.

Thrombin, a serine protease product in the blood coagulation cascade, plays a central role in hemaostasis and thrombosis.[1] Thrombin cleaves fibrinogen to form fibrin and activates Factor XIII, which crosslinks and stabilizes the formed clot. By limited proteolysis, thrombin activates Factors V, VIII, and XI, which promote further thrombin production. Through the activation of protease-activated receptors (PARs), thrombin induces platelet shape change, aggregation, and secretion.[2] In addition, thrombin is mitogenic for vascular smooth muscle and can activate endothelial cells, thereby promoting cell adhesion. Through proteolytic activity, thrombin plays a major role in arterial and venous thrombosis; therefore, inhibition of thrombin catalytic activity continues to be a target for developing new therapeutic agents.[3]

Early efforts directed toward preparing inhibitors of thrombin catalytic activity were patterned after substrates and naturally occurring inhibitors. Notably, potent small molecule inhibitors that bound in a similar fashion to the N1–N3 residues of hirudin were unknown until disclosure by our group.[4,5] Molecular modeling studies supported the binding hypothesis that was later proven by solving the crystallographic structure of the ternary complex among human α-thrombin, hirugen, and BMS-183,507.[6–8]

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In this report, we describe studies that led to the identification of **11**, a retro-binding orally active inhibitor of thrombin catalytic activity.

The synthesis of compound **1** is representative of the syntheses carried out to prepare compounds **2**–**10** (Scheme 1).

The studies reported herein focus on the enhancement of the metabolic stability for the retro-binding series of inhibitors. The C-terminal methyl ester was viewed as a significant metabolic liability and was the focus of our primary efforts. Compound **1**, with optimal residues for N1 and N2, was one of the most potent analogues prepared in this series.[5,9] In early studies with less potent analogues, replacement of the N3 phenyl moiety with cyclohexyl yielded a 10-fold enhancement in potency. In combination with *p*-nitrophenylanaline (N1) and *allo*-threonine (N2), replacement of the N3 phenyl with cyclohexyl gave analogues that had similar binding affinity for thrombin (Table 1). In addition, a variety of functionalities at the C-terminus were tolerated calling into question the need for functionality at this site.

Removal of the N3 residue, affording compound **6**, led to a four order of magnitude loss in potency, (Table 2). Analogues prepared from both optical antipodes of α-methyl benzyl amine and α-methyl phenethylamine were examined for thrombin inhibition. Compounds **7**

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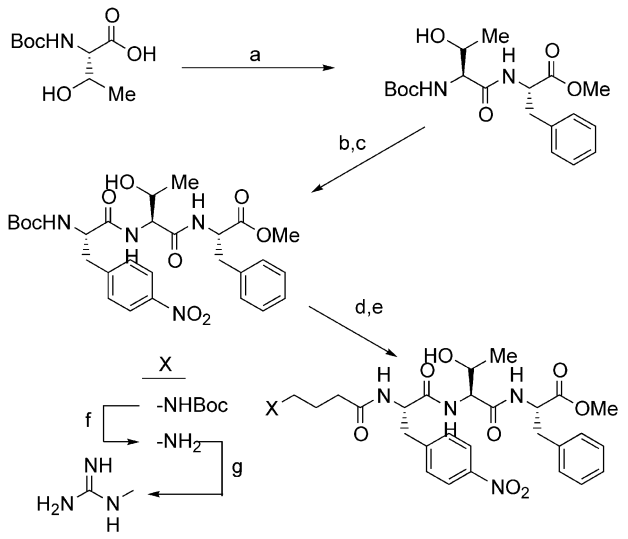


Scheme 1. Synthesis of compound **1**: (a) EDAC, HOBt, L-4-Nitro-Phe(OMe), DMF, 0 °C, 82%; (b) TFA, CH_2Cl_2, 0 °C, 95%; (c) EDAC, HOBt, L-4-Nitro-Phe(OMe), DMF, 0 °C, 89%; (d) TFA, CH_2Cl_2, 0 °C, 95%; (e) EDAC, HOBt, $BocNH(CH_2)_3C(O)OH$, DMF, 0 °C, 88%; (f) TFA, CH_2Cl_2, 0 °C, quant; (g) Et_3N, $H_2NC(SO_3H)NH$, DMF, 0–20 °C, 79%.

and **9** having the same relative stereochemistry as L-phenylalanine were found to be the more potent diasteromers. However, neither analogue was as potent as the preferred analogues described in Table 1. Metabolic stability studies utilizing human and mouse liver S-9 fractions identified cleavage of the amide bond between the N1 and N2 residues as the predominate mode of degradation for compound **7**.

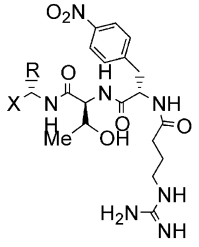
The crystallographic structure of the ternary complex among human α-thrombin, hirugen and BMS-183,507, showed the NH of the amide bond linking N1 and N2 is directed toward solvent. Potentially the amide linkage may be alkylated in an attempt to reduce the rate of enzymatic cleavage. The impact of this change, upon the bound conformation of the inhibitor, must be considered. Molecular modeling studies of BMS-183,507 had shown that the bound rotomeric configuration of the *allo*-threonine side chain to be the lowest energy of the available conformations. Subsequent modeling studies concluded that the *N*-methylation of the amide nitrogen of the Phe residue would be expected to render as lowest energy the bound rotomeric conformation of the *allo*-thronine side chain while introducing two energetically similar rotomeric populations about the amide bond (Fig. 1).

Compound **11** was prepared as shown in Scheme 1 utilizing **13** and was shown to be is a potent inhibitor of thrombin catalytic activity displaying classic competitive kinetics with a K_i of 7.0 nM.[11] In addition, this analogue displays excellent selectivity for the thrombolytic enzymes plasmin and t-PA (Table 3).[12] Data for GYKI-14,766 is given for comparison. In addition, a modified thrombin time (TT) was used to determine the direct inhibition of thrombin activity in a protein rich environment.[13] In this assay, **11** doubled clotting time (in vitro) at 0.085 μM (GYKI-14,776: 0.40 μM) (Table 4).[14] Compound **11** was chosen for further evaluation in vivo.

GYKI-14,776 and **11** were examined in a thrombin-induced lethality model in anesthetized mice.[13] Two modes of administration were examined, iv and oral. Compound **11** is a very effective agent at 10 min and 1 h when given by iv administration. This analogue is also effective when dosed orally with an ED_{50} and 44 mg/kg.

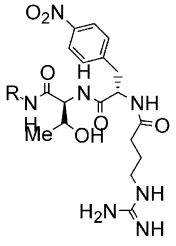
In conclusion, combinations of the preferred N1–N3 residues, previously identified, led to the most potent analogues in this retro-binding series. The terminal methyl ester may be removed with a modest loss in potency and the major metabolic liability, cleavage of the amide bond between N1 and N2, was effectively

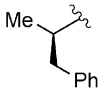
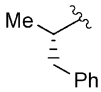
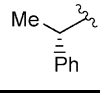
Table 1. N3 SAR



Compd	R	X	Thrombin inhibition[10] IC_{50} (nM)
1	-Ph	-C(O)OMe	8.3±2.0
2	-Ph	$-CH_2OH$	19±2.0
3	-Chx	-C(O)OMe	16±3.0
4	-Chx	$-CH_2OH$	19±2.0
5	-Chx	$-CO_2H$	55±22

Table 2. SAR from modification of the N3 position



Compd	R	Thrombin inhibition IC_{50} (nM)
6	-H	230,000
7		43±6.0
8		260±33
9		72±6.0
10		310±31

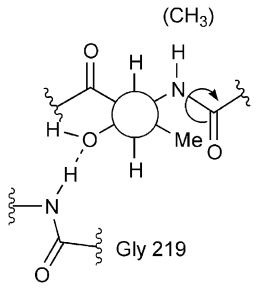
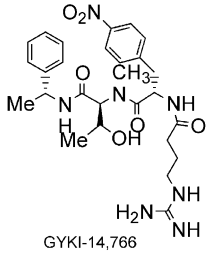
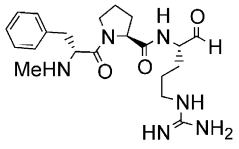


Figure 1. Newman projection of the β-hydroxy side chain of the N2 residue as determined from the inhibitor/protein crystallographic studies. The curve arrow signifies the two energetically similar rotomeric populations about the amide bond when the amide nitrogen is alkylated with a methyl residue.

Table 3. Inhibition of key serine proteases by 11 and GYKI-14,766 [IC_{50} (μM)]


GYKI-14,766


11

Compd	Thrombin	Factor Xa	Plasmin	t-PA	Trypsin
11	0.030 ± 5 (K_i = 7.0 nM)	16	25	34	0.045
GYKI-14,766	0.008[b]	4.6	0.23	0.70	0.008[a]

[a]GYKI-14,766 was found to exhibit slow tight binding kinetics for the inhibition of thrombin and trypsin.[15]

Table 4. Efficacy (ED_{50}) in the mouse thrombin challenge model for BMS-190,623 and GYKI-14,766. Dosages are given in mg/kg

Compd	iv 10 min	iv 1 h	oral 1 h
11	0.04	3.0	44
GYKI-14,766	0.21	2.2	23

addressed through methylation of the amide nitrogen. Compound **11** is a potent inhibitor of thrombin catalytic activity with a K_i of 7.0 nM. Notably this analogue is highly selective over the thrombolytic enzymes plasmin and t-PA. In vivo studies in a mouse model utilizing human α-thrombin showed that **11** was effective when administered iv or orally. We are continuing to explore the implications of the retro-binding motif to the design of new inhibitors. In the future, we will report on extensions of this work to other serine proteases.

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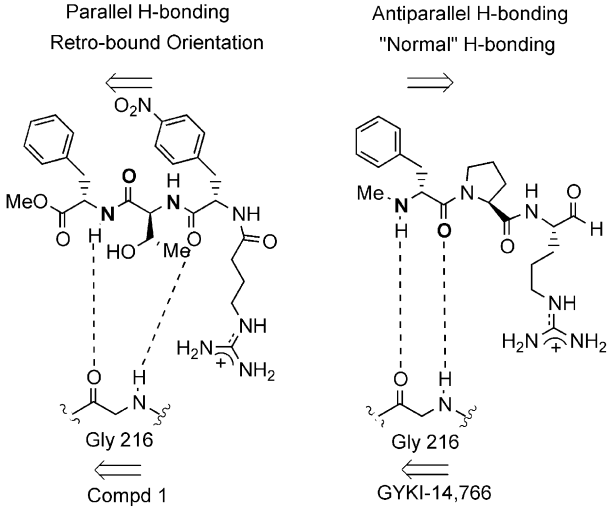
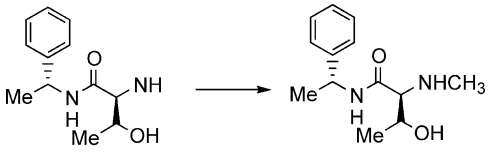
8. The majority of thrombin inhibitors and substrates bind in a manner that allows the formation of a pair of hydrogen-bonds to Gly 216 in a substrate-like or anti-parallel orientation. D-Phe-Pro-Arg-derived inhibitors such as GYKI-14,766, bind to thrombin forming this antiparallel arrangement with Gly 216. In contrast, **1** forms a parallel hydrogen-bond arrangement, a key feature of the retro mode of binding.



9. N1-3 terminology is used to signify the three amino acids bound in the active site of thrombin where the N terminus of N1 is proximal to Ser 195.
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 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.
11. *N*-Methyl allo-threonine containing intermediate **13** was prepared as given below:


12 **13**

A solution of **12** (4.04 g, 12.0 mmol, 1.0 equiv), formaldehyde (0.024 M of 37% an aqueous solution in methanol, 500 mL, 1.0 equiv), diisopropylethylamine (2.4 mL, 13.8 mmol, 1.2 equiv) and palladium hydroxide on carbon (1.24 g, 31% by weight) was stirred inder an atmosphere of hydrogen for 3.5 h. After flushing with nitrogen and filtering though a pad of

Celite, the solution was concentrated in vacuo, dissolved in chloroform and washed twice with saturated aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and concentrated in vacuo to give a white solid (2.68 g, 95% yield as a 9:1 mixture of the mono- and di-methylated amines. This mixture was utilized without further purification.

12. Inhibition of bovine pancreatic trypsin was measured in an assay containing 2 mM $CaCl_2$, 50 mM Tris pH 8.0 and 30 μM carbobenzyloxy-Val-Gly-Arg-pNA as substrate. The inhibitor was combined with trypsin assay buffer and incubated for 3 min, after which the substrate was added and absorption was measured using a microplate reader (Molecular devices V_{max}) at 405 nM. Inhibition of human plasmin was measured in an assay containing 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4 and 100 μM S-2251 (D-Val-Leu-Lys-pNA). The inhibitor was incubated with the enzyme for 3 min, after which the substrate was added and the rate of hydrolysis was measured as for thrombin and trypsin. Inhibition of tissue plasminogen activator (t-PA) was measured in an assay containing 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4 and 100 μM methanesulfonyl-D-cyclohexyltyrosyl-Gly-Arg-pNA as substrate. The inhibitor was incubated with the enzyme for 3 min, after which the substrate was added and the rate of hydrolysis was measured as for thrombin. Inhibition of Factor Xa was measured in an assay containing 145 mM NaCl, 5 mM KCl, 1 mg/mL polyethylene glycol (PEG-8000), 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4 and 100 μM S-2222. The inhibitor was incubated with the enzyme for 3 min, after which the substrate S-2222 was added and the rate of hydrolysis was measured as for thrombin. The IC_{50} values are reported with corresponding standard deviations.

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