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IN VITRO EVALUATION AND CRYSTALLOGRAPHIC ANALYSIS OF A NEW CLASS OF SELECTIVE, NON-AMIDE-BASED THROMBIN INHIBITORS

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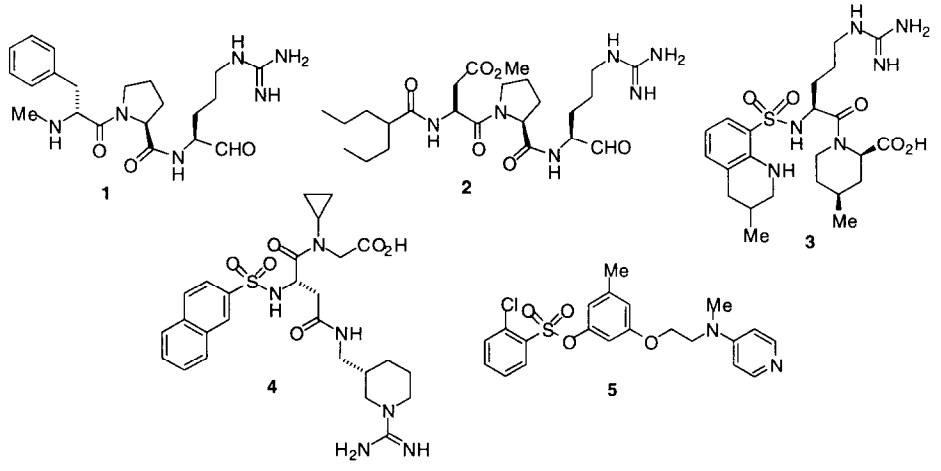
Abstract: We describe the in vitro evaluation and crystallographic analysis of a new class of potent and selective, non-aminoacid-based, small-molecule thrombin inhibitors, exemplified by **14**. This class of achiral inhibitors lacks an amide-based backbone, exhibits nM inhibition of thrombin, and is selective for thrombin. Compound **14** does not interact with the active-site catalytic apparatus and is anchored to the enzyme via a single network of hydrogen bonds to Asp189 of the S1 pocket.

Orally bioavailable antithrombotic agents based on inhibition of the trypsin-like serine protease thrombin are of intense interest due to a number of limitations associated with heparin, low-molecular-weight heparin (LMWH), and warfarin.[1] For long-term anticoagulation, the insufficiencies of currently available agents include (a) their indirect mechanisms of thrombin inactivation, (b) the need for constant monitoring to assure effective drug plasma levels and avoidance of bleeding complications, and/or (c) lack of oral bioavailability.

In connection with our goal of developing orally bioavailable thrombin inhibitors, we were intrigued by the disclosure of a nonpeptide-based series exemplifed by compound **5**.[2] This series presented a radical departure from aminoacid-based templates, such as efegatran (**1**), CVS 1123 (**2**), argatroban (Novastan, **3**), and napsagatran (Ro-46-6240, **4**) for a number of reasons: (1) compound **5** possessed impressive potency despite its structural simplicity and/or the absence of electrophilic groupings in comparison to compounds such as **1**–**4**;[3] (2) the guanidino group was replaced with a guanidino mimic;[4] and (3) the aminoacid framework, which we also regarded as one potential structural liability towards overall in vivo performance,[5] was eliminated.



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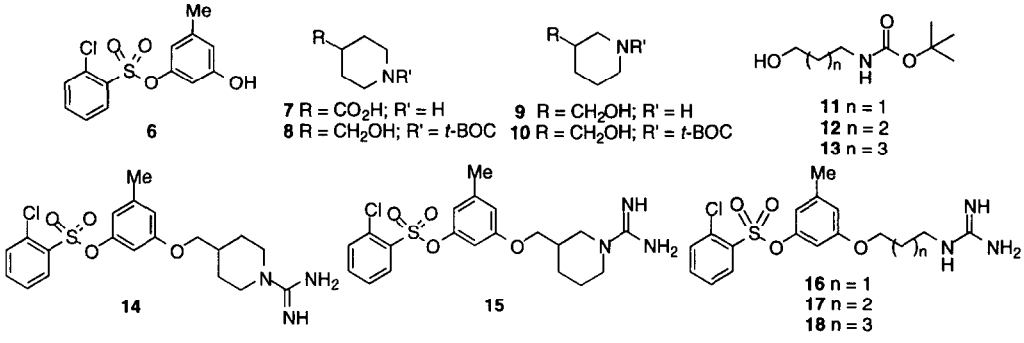
Crystallographic analysis of **5** bound to thrombin revealed the presence of a single, intermolecular hydrogen bond (pyridine NH to Asp189).[10] Modeling suggested that substitution of the pyridine with groups possessing additional H-bonding capacity may potentially enhance in vitro potency. In this communication, we report the in vitro potency and selectivity of a guanidino-based series of analogs of **5** and compare the crystal structures of thrombin complexed with **5**, **14**, and **17**. The results of this study, coupled with our earlier observations concerning guanidino[6] and backbone replacements,[10] provide additional structural insights as we advance towards obtaining oral bioavailabilty.

The syntheses of **14–18** were accomplished in a straightforward manner[7] from **6**. Isonipecotic acid (**7**) was converted to **8** ((a) di-*t*-butyl dicarbonate (1 equiv), $NaHCO_3$ (2 equiv), dioxane/H_2O (1:1), 24 h (91%); (b) BH_3•THF (1 equiv), THF, 0 °C to 25 °C (84%)). Alcohol **8** was mesylated (MsCl (1 equiv), NEt_3, CH_2Cl_2 (93%)), coupled to the sodium salt of **6** (generated with NaH (1.1 equiv), DMF) at 50 °C for 3 h (69%), deprotected (4 N HCl in dioxane, 2 h (79%)), and treated with aminoiminomethanesulfonic acid (2 equiv, NEt_3 DMF, 12 h (49%)) to provide **14**. Alcohol **10** was prepared from **9** (di-*t*-butyl dicarbonate (1 equiv), NEt_3, dioxane, 2 h (91%) and converted to **15** analogously to **14** (15% yield from **10**; 4 steps). Compounds **16–18** were prepared from **6** in 3 steps by (a) Mitsunobu coupling[8] of alcohols **11–13**, respectively, (PPh_3 (1.5 equiv), DEAD (1.5 equiv), alcohol **11**, **12**, or **13** (1.5 equiv), THF, 0 °C to 25 °C), (b) *t*-BOC removal with HCl in dioxane, and (c) guanidinylation with 2 equiv of aminoiminomethanesulfonic acid (DIEA; DMF) for **16** and **17**, or 1.5 equiv 1*H*-pyrazole-1-carboxamidine•HCl in DMF for **18**. The yields from this 3-step sequence were 70%, 68%, and 60% for **16–18**, respectively.



Compounds **14–18** were evaluated for inhibition of thrombin, FXa, plasmin, trypsin, chymotrypsin, urokinase, and elastase using standard chromogenic assays (Table 1).[9] Compound **14** exhibited remarkable specificity for thrombin. At the highest concentration tested (200 μM), the compound showed no detectable inhibition of plasmin, chymotrypsin, urokinase, or elastase and marginal inhibition of FXa and trypsin. Other members of this guanidino series also exhibited specificity towards thrombin, but not to the same extent. None of the members of the series showed any inhibition of plasmin, urokinase, or elastase at the highest screening dose.

Table 1. In Vitro Inhibition of Serine Proteases (K_i (μM))

Compound	Thrombin	Factor Xa	Plasmin	Urokinase	Trypsin	Chymotrypsin	Elastase
5	0.011 ± 0.001	160 ± 43	>200	>200	11 ± 0.84	49 ± 10	>200
14	0.0046 ± 0.001	80 ± 14	>200	>200	25 ± 1.9	>200	>200
15	0.32 ± 0.021	29 ± 7.4	>200	>200	74 ± 7	72 ± 8.9	>200
16	0.033 ± 0.0019	>200	>200	>200	23 ± 1.8	>200	>200
17	0.013 ± 0.0016	110 ± 53	>200	>200	46 ± 5.4	49 ± 15	>200
18	0.26 ± 0.032	120 ± 29	>200	>200	30 ± 2.3	80 ± 8.4	>200

Compounds **5**, **14**, and **17** bound to thrombin were examined crystallographically with the overlay in Figure 1 showing this comparison.[15] Compounds **5**, **14**, and **17** do not interact with the catalytic triad, nor do they form direct H-bonds to residues 214–216, conserved residues involved in substrate main-chain recognition. The central orcinol template and the arylsulfonate group interact with the S_2 and distal aryl-binding pockets as described for **5** in detail elsewhere.[10]

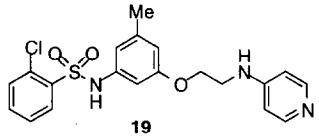
The major differences in the structures of **14** and **17** vs. **5** occur in the vicinity of the S_1 pocket. Whereas **5** forms a single H-bond with Asp189 (shown in lower right corner of Figure 1), compounds **14** and **17** form two H-bonds with Asp189, one H-bond with the carbonyl of Gly218, and one H-bond to the S_1-pocket water molecule (shown as a red ball in Figure 1), which is observed in both unliganded thrombin and nearly all thrombin inhibitor complexes. In addition, the P_1 sidechain of **17** follows the back edge of the ring systems of both **5** and **14**, although there are clear differences in the presentation of the guanidino groups of **17** and constrained **14** to Asp189.

Figure 1. Overlap of Crystal Structures of Compounds **5** (Orange), **14** (Magenta), and **17** (Gray) Bound to Thrombin

The results of the present study are noteworthy. In light of the empirical estimates of the contribution of H-bonding to intermolecular interactions, the introduction of three additional H-bonds in the S_1 pocket surprisingly did not improve potency by more than a factor of 2.[14] Apparently, there are other structural and physicochemical attributes of the S_1-binding substituents which contribute overall binding energetics. Nonetheless, the potency of compound **14** is impressive since the compound possesses a hydrophobic backbone and lacks amide functionalities capable of hydrogen bonding with the enzyme.

Interestingly, compound **14** fills a different region of thrombin's S_1 specificity pocket in comparison to **5** in that the bulky hydrophobic piperidine appendage of **14** sits much higher in thrombin's S_1 pocket than the pyridine fragment of **5**. In addition, the potency of this series is dependent upon chain length and an extended substitution pattern about the piperidine ring system is most preferred (**14** vs. **15**). This bulkiness, hydrophobicity, and position may also account in part for its enhanced potency and selectivity relative to **17**. Compounds **14–18** also compare favorably in their thrombin inhibition potency to other recently reported analogs of **5** such as **19** (BM 14.1248; K_i = 23 nM),[11] and **20** (K_i = 70 nM).[12]

In summary, we have described a novel series of non-aminoacid, small-molecule thombin inhibitors, which exhibit high potency and impressive selectivity (>10^3 ×) towards thrombin. These achiral inhibitors lack any functionality that modify the active-site catalytic residues and do not form H-bonds with conserved residues responsible for recognition of the peptide substrate backbone. Inhibitors **14** and **17** derive their remarkable selectivity solely through interaction with the specificity pockets of thrombin. Despite increased H-bonding in the S_1 pocket, these compounds do not exhibit vastly improved potency relative to **5**. The insights presented in this paper, in concert with library approaches for physicochemical diversification,[13] provide useful information as the focus begins to shift from in vitro optimization to in vivo performance.



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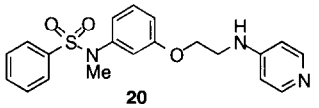

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9. Dissociation constants were determined at 37 °C in a 96-well format using a Molecular Devices plate reader. Varying concentrations of inhibitors in 10 μL dimethylsulfoxide were added to wells with 280 μL of assay buffer (pH 7.5) which contained 50 mM HEPES, 0.2 M NaCl, 1% dimethylsulfoxide, 0.05% β-octyl-glucoside and substrate and incubated for 30 min at 37 °C. Reactions were initiated by the addition of 10 μL of enzyme in assay buffer without dimethylsulfoxide and substrate and the change in absorbance at 405 nm was monitored for 30 min. Apparent dissociation constants ($K_i$app) were obtained as the inverse slope from plots of the ratio of initial velocity in the presence of inhibitor to initial velocity in the absence of inhibitor as a function of inhibitor concentration. The dissociation constant (K_i) was calculated using the equation $K_i = K_i\text{app}/(1 + S/K_m)$ where S is the substrate concentration. K_m values for each enzyme-substrate pair were determined from double reciprocal plots using the same final buffer conditions as K_i determinations. Substrates, substrate concentrations and K_m values for each enzyme were: succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide, 100 μM and 320 μM for human α-thrombin; benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, 100 μM and 2300 μM for human factor Xa; carbobenzyloxy-Phe-Val-Arg-*p*-nitroanilide, 170 μM and 190 μM for human urokinase; tosyl-Gly-Pro-Lys-*p*-nitroanilide, 100 μM and 690 μM for human plasmin; benzoyl-Phe-Val-Arg-*p*-nitroanilide, 100 μM and 160 μM for bovine trypsin; suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, 100 μM and 60 μM for bovine chymotrypsin; suc-Ala-Ala-Pro-Val-*p*-nitroanilide, 100 μM and 2600 μM for human elastase.

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15. Crystallographic data for **5**: R = 0.18 at 2.2 Å resolution; **17** R = 0.20 at 1.9 Å resoluton; **14** R = 0.205 at 2.0 Å resolution.