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RATIONAL DESIGN OF SELECTIVE THROMBIN INHIBITORS

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Abstract. Thrombin inhibitors with functionalized benzamidines as surrogates for arginine were designed, synthesized, and characterized. Amino acid sequence difference in the position 190 between thrombin and trypsin was exploited in the design to enhance selectivity over trypsin. A representative compound **6** showed high potency (Ki of 45.5 nM) and extremely high specificity over trypsin (over 10,000 fold). © 1997 Elsevier Science Ltd.

Thrombin is a trypsin-like serine protease playing a central role in both hemostasis and thrombosis.[1] Since many cardiovascular diseases such as myocardial infarction, unstable angina, and deep vein thrombosis, are caused by thrombosis, a wide variety of thrombin inhibitors have been synthesized and tested for the prevention of thrombosis and acceleration of thrombolysis in conjunction with thrombolytic agent.[2] Among them, D-Phe-Pro-Arg-H[3] analogs[4] and argatroban[5] are the typical examples of synthetic small molecular thrombin inhibitors. However, these first generation compounds have limitations, in specificity, half-life, and oral bioavailability.[6]

Many trypsin-like serine proteases sharing high sequence homology with thrombin[7] are involved in blood coagulation and fibrinolysis.[8] Selectivity of thrombin inhibitors over these other proteases have been achieved so far by positioning hydrophobic groups into the so-called D- and P-pockets.[4, 5, 9, 10] In comparison to trypsin, each of these proteases has its own insertion sequence in this region.[8] This feature serves as one of the bases of its substrate specificity. On the other hand, in the case of trypsin this region is wide open giving trypsin rather broad substrate/inhibitor specificity. Consequently most compounds claimed as thrombin inhibitors failed to achieve selectivity over trypsin and in some cases even higher inhibition of trypsin than of thrombin are observed.[11] Perhaps the only exceptions are argatroban (~500 times)[5] and napsagatran (~7000 times).[10] Thus we considered trypsin as the prototype among these proteases and the selectivity over trypsin would be one of

the key features of "truely" selective thrombin inhibitors. Here we report a novel strategy of implimenting high selectivity over trypsin in thrombin inhibitors.

Comparing the crystal structures of thrombin and trypsin, it was noted that the thrombin specificity pocket is quite similar to that of trypsin and the only difference was Ala 190 in thrombin replacing Ser 190 in trypsin.[12] It was also asserted that this exchange renders the thrombin specificity pocket slightly bigger and less polar than trypsin.[12] We call this extra space in thrombin as a "hole". In the crystal structure of thrombin-argatroban complex, the guanidino side chain of argatroban adapts a unique orientation in the specificity pocket; one of the guanidino *N* atoms being close to the "hole".[9(b)] On the other hand, in the trypsin-argatroban complex structure, the "hole" does not exist and the guanidino *N* atom makes close interaction with OH of Ser 190.[9(c)] This situation is schematically depicted in Figure 1.

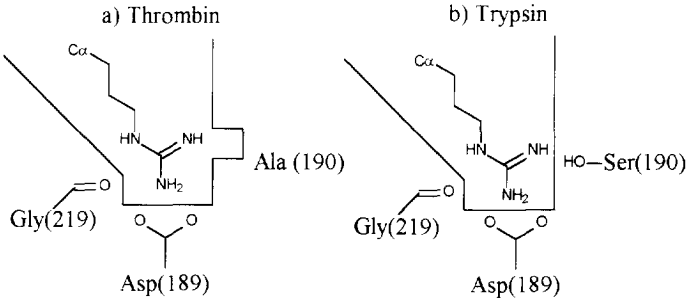
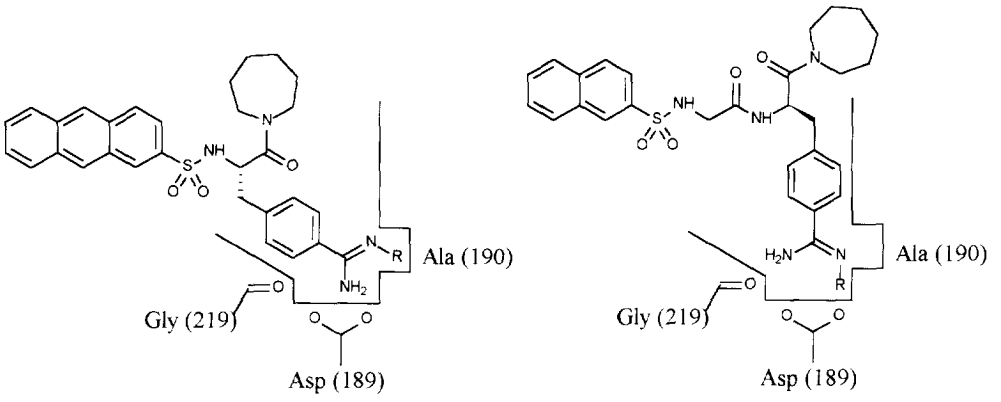


Figure 1. Schematic drawings of the guanidino side chain of argatroban in the active site recognition pockets of thrombin (a) and trypsin (b).

We hypothesize that inhibitors that can fill this "hole" would achieve specificity over trypsin. X-ray structures of thrombin-inhibitor complexes available from the Protein Data Bank (PDB)[13] were examined using a computer graphic system to screen for hits whose simple modification could fill in the "hole". Argatroban would be the first candidate to test this hypothesis. As its synthesis and derivatization is not trivial, we looked for other synthetically amenable candidates. The benzamidine side chain of N_{α}-(4-toluene-sulfonyl)-DL-*para*-amidinophenylalanyl-piperidine (4-TAPAP) adapts a similar orientation as that of argatroban and its amidino group is very close to the "hole" in the crystal structure.[14] It appears that its simple substitution would put the substituent in the "hole" achieving high thrombin/trypsin selectivity. A similar compound, **1a**, and its amidrazone derivative, **1b**, were reported; the latter being 50-fold more potent than the former in thrombin inhibition (Figure 2).[15] The trypsin selectivity of these compounds have not been reported. This scaffold would be a perfect candidate to test our hypothesis. We also examined the crystal structure of N_{α}-(2-naphthyl-sulfonyl-glycyl)-D-*para*-amidinophenylalanyl-piperidine (NAPAP). The benzamidine side chain of NAPAP adapts an orientation quite different from that of 4-TAPAP.[15] If its amidrazone derivative were to maintain the same binding conformation as NAPAP, it would bump into the enzyme. In fact, a NAPAP analog, **2a**, and its amidrazone derivative, **2b**, were reported; the latter being 100 times less potent than the former in thrombin assay (Figure

2).[15] The trypsin selectivity of these compounds have not been reported.

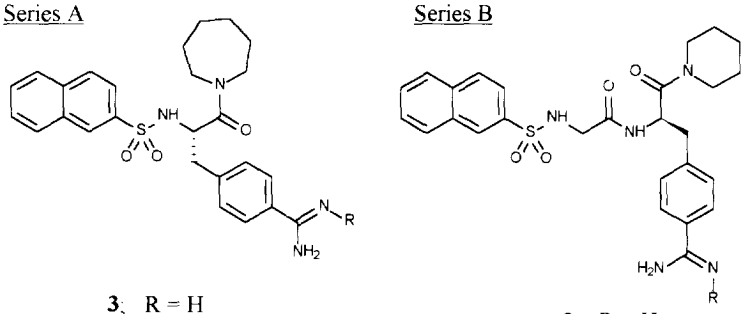


1a; R = H, Ki = 0.05μM
1b; R = NH_2, Ki = 0.001μM

2a; R = H, Ki = 0.02μM
2b; R = NH_2, Ki = 2.7μM

Figure 2. Schematic drawing of putative inhibition modes of the analogs of 4-TAPAP and NAPAP and their amidrazone derivatives in the thrombin specificity pocket.

We have set out to test our design hypothesis and synthesized two series of compounds containing *N*-substituted benzamidine groups. The core structure of Series A is a compromise of 4-TAPAP and **1a**. Although its crystal structure is known, 4-TAPAP is rather a poor thrombin inhibitor (Ki = 1.3 μ M for bovine thrombin).[14] For Series B, NAPAP served as the basis as its X-ray structure is known and it is more potent (Ki = 0.006μM) than **2a**.[14]



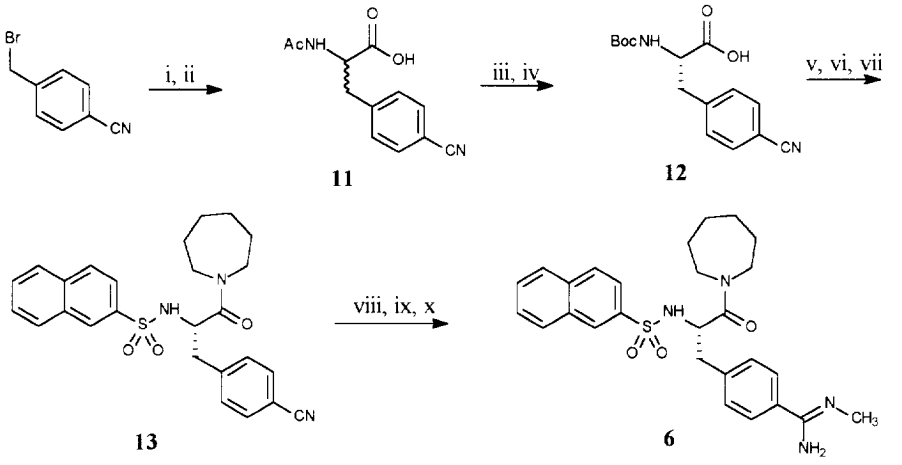
3; R = H
4; R = OH
5; R = NH_2
6; R = CH_3
7; R = CH_2CH_3

8; R = H
9; R = NH_2
10; R = CH_3

Figure 3. Model compounds to test the design hypothesis.

Synthesis

A representative compound **6** was prepared as shown in Scheme 1. Diethyl acetamindomalonate was condensed with α-bromo-*p*-tolunitrile in basic medium. Saponification followed by decarboxylation led to the racemic intermediate **11** (87% yield). Boc-(L)-*p*-cyanophenylalanine (**12**)[16] was obtained by kinetic resolution of **11** using acylase[17] at pH 7 followed by Boc protection of amino group (43% yield).[18] After coupling **12** with hexamethyleneimine using EDC and HOBT, Boc group was deprotected. Sulfonylation with 2-naphthalenesulfonyl chloride gave intermediate **13** in 79% yield. Treatment of **13** with H_2S led to the formation of thioamide in side chain and subsequent treatment of dimethylsulfate and methylamine furnished final compound **6**[19] (67% yield).



Scheme 1. Reagent: (i) $EtO_2CCH(NHCOCH_3)CO_2Et$, KI (0.05eq.), NaOEt, Dioxane, reflux; (ii) aq. NaOH (2eq.), reflux; (iii) acylase; (iv) $(t\text{-}BOC)_2O$, aq. NaOH, Dioxane; (v) EDC, HOBT, DMF, hexamethyleneimine, 4-methylmorpholine; (vi) TFA, CH_2Cl_2; (vii) naphthalene-2-sulfonyl chloride, DMF, 4-methylmorpholine; (viii) H_2S, pyridine, triethylamine; (ix) dimethylsulfate, CH_3CN; (x) CH_3NH_2, methanol.

Results and Discussion

The enzyme inhibition activities were measured by a standard method using bovine α-thrombin and trypsin and the corresponding chromogenic substrates.[20] The results are summarized in Table 1. Introducing NH_2 (**5**) or CH_3 (**6**) group in R position (Figure 3), the thrombin/trypsin selectivity was dramatically increased (20-fold and 70-fold, respectively) as we have hypothesized. Both thrombin and trypsin prefer NH_2 group (**5**) to CH_3 group (**6**) in the position R. The entrance to the "hole" is partially blocked by the OH moiety of Ser 190 in trypsin.[9(c)] In thrombin, the space is sometimes occupied by water molecule as seen in some thrombin-inhibitor complex structures.[9(b), 14] Consequently, we presume that the hole prefers hydrophilic groups such as NH_2 (**5**) to hydrophobic groups such as CH_3 (**6**). This is in sharp contrast to the previous assertion that the thrombin specificity pocket appears less polar.[12] Although OH group (**4**) may fill the "hole", Ki's were increased for both

enzymes. This benzamidoxime species, whose pKa is around 5,[21] may have difficulty in forming a salt bridge with Asp 189. The size of the "hole" is not big enough to accommodate -CH_2CH_3 group in compound 7.

Table 1. Inhibitory effect and specificity of thrombin inhibitors.

Series	Entry[a]	R	Ki (thrombin, μM)	Ki (trypsin, μM)	Ki(trypsin)/Ki(thrombin)
Series A	**3**	H	0.0524	8.65	166
	4	OH	1.140	316.0	277
	5	NH_2	0.00147	3.73	2537
	6	CH_3	0.0455	545.0	11978
	7	CH_2CH_3	13.600	-	-
Series B	**8**	H	0.0065	0.325	50
	9	CH_3	5.37	192.0	36
	10	NH_2	4.32	81.5	19

[a]All entry numbers are consistant with the compounds in Figure 3.

In Series B, slight modification of the benzamidine side chain resulted in loss of thrombin inhibition; **9** and **10** being 800- and 600-fold less potent than **8**, respectively. Trypsin inhibition activity of **9** and **10** is much less potent as well and thus the thrombin/trypsin selectivity is not much different from that of **8**. As depicted in Figure 2 for structurally related compound **2b**, the side chains of both **9** and **10** would bump into the enzyme. An approach more elaborate than simple functionalization should be devised for NAPAP analogs to achieve better thrombin/trypsin selectivity. Here we demonstrated that a simple modification in the P1 side chain of the thrombin inhibitors can greatly improve their selectivity over trypsin. Besides thrombin, factors Xa, XIa, XIIa, activated protein C (aPC), and tPA have alanine residue at the position 190, while trypsin, plasmin, urokinase, factors VIIa, and IXa have Ser 190.[7] In the development of thrombin inhibitors as antithrombotic agents, selectivity over procoagulant proteases is less of concern than the inhibition of anticoagulant protein aPC and other fibrinolytic serine proteases such as plasmin and tPA. Selectivity over these proteases can be readily achieved via optimization of the moieties targeting the so-called D- and P-pockets.[4, 5, 10]

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