Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 12, pp. 1339-1344, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00229-6

DERIVATIVES OF 5-AMIDINE INDOLE AS INHIBITORS OF THROMBIN CATALYTIC ACTIVITY

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Abstract: Substituted 5-amidine indoles were constructed based upon a computational analysis of putative modes of binding to thrombin utilizing coordinates from the crystal structure of **BMS-183,507**-α-thrombin complex. These analogs display competitive kinetics for the inhibition of human α-thrombin. The most potent member of this series **17**, shows marked potency for thrombin with an inhibition constant, K_i of 260 nM.

Introduction

The serine protease thrombin is the product of the blood coagulation cascade that catalyzes the cleavage of fibrinogen to form fibrin and stimulates blood platelets, vascular smooth muscle and endothelium.[1] Most or all of these actions require serine protease catalytic activity. As a result, inhibitors of thrombin catalytic activity have been considered as potential targets for drug design. Much of the recent published work has focused on the development of peptidic molecules often derived from D-Phe-Pro-Arg(H).[2] The poor bioavailibility and short half lifes exhibited by these types of inhibitors are often attributed to the guanidine moiety and/or the peptidic character of the molecules. Various approaches have been devised to prepare an orally active inhibitor of thrombin catalytic activity with acceptable pharmacodynamics consistent with safety and once or twice-daily dosing. We report here our work to devise an inhibitor for thrombin based upon modeling of amidine substituted indoles coupled with structural information derived from the solid state structure of the retro-binding peptide/thrombin complex.[3]

Previously, Tidwell et al.[4] had reported on a number of aromatic amidines as serine protease inhibitors. Some of the most potent members of this class are amidine functionalized indoles. 5-Amidinoindole **1** exhibits unexpected affinity for thrombin when size, number and type of attractive interactions are considered. In addition, this affinity is achieved without an electrophilic moiety so often required for potency in other chemotypes.

We modeled the binding of **1** to the active site region of thrombin and found that this molecule can potentially bind in two different orientations.[5] A common feature in both orientations is the amidinium-Asp (189) interaction in the specificity pocket.[6] We believe this is the most important single protein-inhibitor interaction. The narrow specificity pocket then may accommodate the aromatic moiety in only two orientations. In the first binding mode, the indole nitrogen (N1) forms a hydrogen bond to Ser 214 O. The second orientation has the indole rotated approximately 180 degrees while maintaining the putitive Asp (189) interaction. Consequently, the hydrogen bond to Ser 214 O is lost as the indole nitrogen is now directed toward the solvent and is energetically disfavored by 0.7 kcal/mol relative to the first orientation.

The docked structure of **1** in the first orientation was compared with the crystal structure of the retro-binding peptide **BMS-183,507**/thrombin complex, shown in Figure 1.[3b,7] The phenyl moiety of **BMS-183,507** that occupies the proximal pocket, a region formed by His 57 of the catalytic triad, Tyr 60A and Trp 60D, is a key residue for binding in this series.[8] From an examination of Figure 1, we postulated that lipophilic residues may be appended on the 2-position of indole **1** in order to access the proximal pocket and improve potency. The second phenyl residue of the tripeptide inhibitor lies in the wider lipophilic distal pocket and is also a required moiety for potency. The spacial arrangement of these two putative lipophilic residues suggests that they may be be directly linked with either one or two atoms. Hence the addition of an aromatic moiety binding at the proximal pocket may offer the appropriate molecular framework to introduce additional residues for binding at the distal pocket.

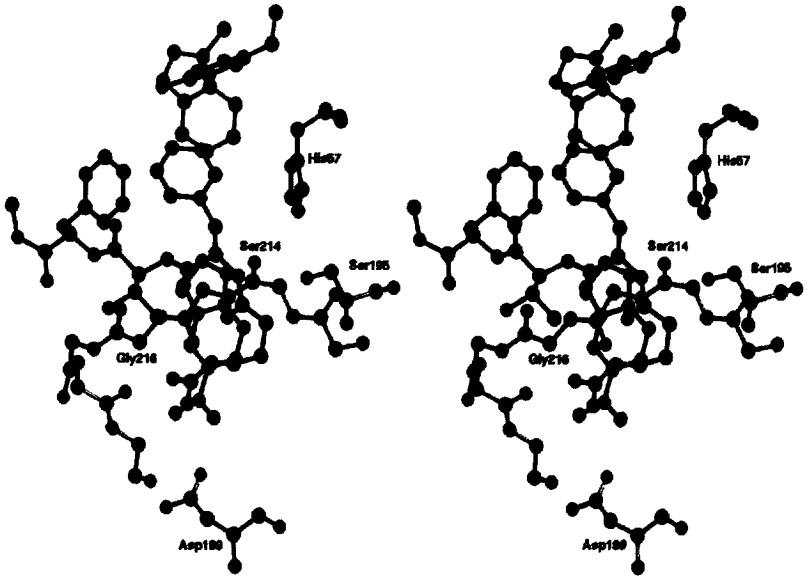
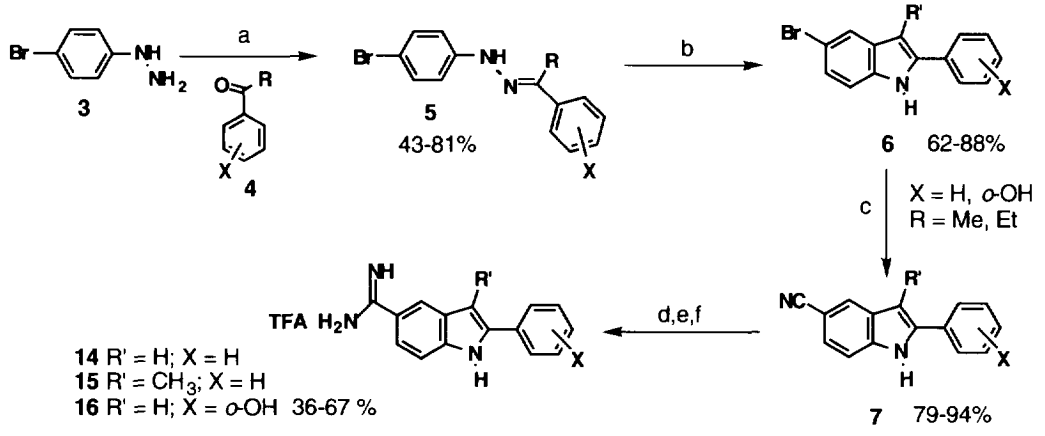


Figure 1. Stereo view comparison of **BMS-183,507** in the crystal structure of **BMS-183,507**-α-thrombin complex superimposed on the computational model for 5-Amidine Indole, **1**.

Synthesis

Two different synthetic strategies were employed to prepare either the 2-phenyl or the 2-benzyl analogs. The former analogs were prepared as outlined in Scheme 1. Hydrazones **5** were prepared from the condensation of hydrazine **3** with the corresponding ketone **4** in refluxing methanol. Polyphosphoric acid was employed to fashion the indole skeleton to give **6**. The nitrile functionality was introduced using cuprous cyanide/quinoline in a sealed tube at 230 °C. The amidine moiety was prepared from the corresponding nitrile *via* a two-step procedure. The intermediate imidoester was prepared by treatment with hydrochloric acid/ethanol, the solvent was removed and the resulting solids treated with saturated ammonia/ethanol to give **14–16**.

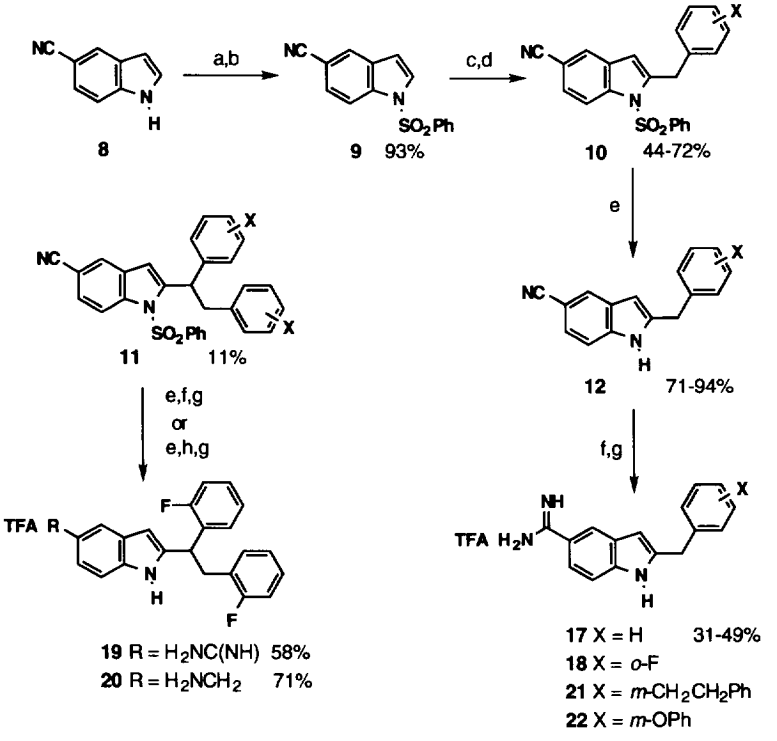
Scheme 1. Synthesis of 2-phenyl indole analogs.



(a) MeOH, **4**, reflux, 10 h; (b) Polyphosphoric acid 120 °C, 3 h; (c) CuCN/quinoline 230 °C, 1 h; (d) HCl/EtOH 0 °C to rt, 10 h; (e) Sat'd NH_3/EtOH in a sealed tube, 80 °C, 24 h; (f) Preparative HPLC purification (CH_3CN/H_2O with 1% TFA).

The 2-benzyl analogs were conveniently prepared by the procedure outlined in Scheme 2. The indole nitrogen was protected as the phenyl sulfonamide **9**. The corresponding 2-lithio analog of **9** was prepared by treatment with lithium diisopropylamide (LDA) and subsequently reacted with the appropriate benzyl bromide to afford **12** in good yield.[9] If excess LDA (1.5 equiv) is employed, the bis-alkylated product **11** is formed. The amidines were prepared by a two-step process. Treatment of the corresponding nitrile with hydrochloric acid/ethanol in a sealed tube at 55 °C gave the intermediate imidoether, removal of solvent and subsequent reaction with ammonia/ethanol gave the crude amidines. Compound **11** was hydrogenated under acidic conditions to give **20**. All final compounds were purified by preparative HPLC and isolated as the TFA salt.

Scheme 2. Synthesis of 2-benzyl indole analogs.

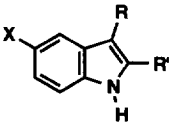


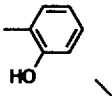
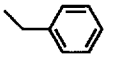
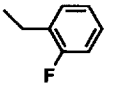
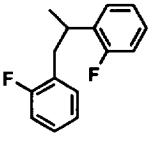
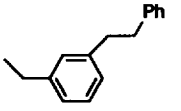
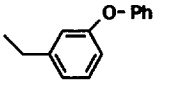
(a) NaH/DMSO; (b) $PhSO_2Cl$/THF; (c) 1.1 equiv LDA, −78 °C warm to 0 °C for 5 min; (d) 1.25 equiv XPhBr −78 °C to rt, 12 h; (e) 1.5 M KOH (EtOH:MeOH:H_2O, 5:5:2), 65 °C, 30 min; (f) Sat'd HCl/EtOH in a sealed tube, 55 °C, 12 h; (g) Sat'd NH_3/EtOH in a sealed tube, 80 °C, 24 h; (h) Pd/C, MeOH, 2.0 equiv 1N HCl, 1 atm H_2, 8h; (i) Preparative HPLC purification (CH_3CN/H_2O with 1% TFA).

Results and Discussion

The 5-amidino indole **1** displays striking affinity for thrombin (22 μM) in spite of its low molecular weight and a lack of an electrophilic residue, see Table 1. Compound **13**, possessing a fused cyclohexyl residue and **14**, the 2-phenyl analog, displayed comparable inhibitory activity. The introduction of a methyl residue at the 3-position of **14** changed the relative planarity of the two aromatic moieties and led to a three-fold loss in activity, compound **15**. Compound **16** with an ortho hydroxy substituent showed similar activity. The 2-benzyl analog **17**, showed a 16-fold improvement over compound **1** with an IC_{50} of 1.4 μM (K_i of 260 nM). Surprisingly, the introduction of an ortho fluorine, compound **18**, led to a 26-fold loss in activity.[10] Introduction of a second ortho-fluorobenzyl moiety **19** led to a modest enhancement in binding relative to **18**, while the corresponding methyl amine analog **20** was four-fold less potent. Compounds **21** and **22** were fashioned in an attempt to enhance activity by providing a lipophilic moiety for binding at the distal pocket region. Both compounds were of comparable activity with the simple benzyl analog, **17**.

Table 1. Inhibition of human α-thrombin catalytic activity.



#	X	R	R'	Thrombin IC_{50} (μM)
1		H	H	22
13	" "	$CH_2CH_2CH_2CH_2$[11]		20
14	" "	H	Ph	19
15	" "	CH_3	Ph	66
16	" "	H		38
17	" "	H		1.4 ***K_i = 260 nM***
18	" "	H		36
19	" "	H		15
20		H	" "	59
21		H		2.4
22	" "	H		2.0

Conclusion

5-Amidino indole, with minimal molecular contacts and without the aid of an electrophilic residue, displays unusual affinity for thrombin. The amidinium-Asp 189 interaction and the formation of a hydrogen bond between the indole N-H and Ser 214, together with lipophilic interactions within the specificity pocket, appear to be largely responsible for the binding affinity. The phenyl moiety of **BMS-183,507** that occupies the proximal pocket is a key residue for binding in this series and this interaction may be advantageously used in the design of new non-peptidic inhibitors. Crystallographic information from the **BMS-183,507**/α-thrombin complex together with the binding model of **1** led us to prepare **17** (K_i = 260 nM), a chemotype that contains this phenyl moiety. Most competitive inhibitors of thrombin presumably interact with the proximal and the distal pocket regions and form hydrogen bonds with Gly 216.[2b] The potency of **17** is more remarkable because the interactions at the distal pocket region and Gly 216 are missing. The development of even more potent and selective inhibitors will necessitate enhancing the inhibitor's affinity for thrombin through appropriate substitutions that effectively take advantage of the unique binding sites proximal to the specificity pocket. Amidine-substituted indoles offer an "anchoring point" for the development of thrombin active site inhibitors through either rational design or a combinatorial approach.

Materials and Methods

Human α-thrombin was either the generous gift of Dr. J.W. Fenton II (NY State Dept. of Health, Albany, NY) or obtained from Sigma Chemical Co. α-Thrombin from either sources had specific activity of greater than 2500 NIH U/mg. The synthetic substrate S-2238 (D-Phe-Pip-Arg pNA) was obtained from Kabi Vitrum.

Kinetic Studies

Thrombin cleavage of the synthetic substrate S-2238 (D-Phe-Pip-Arg pNA) was measured at room temperature (23–25 °C) using a kinetic microplate reader (V_{max} microtiter plate reader, Molecular Devices Corporation). The enzyme reactions (300 μL final volume) contained 145 mM NaCl, 5 mM KCl, 1 mg/mL Polyethylene glycol (PEG-8000), 30 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid, pH 7.4, 0.03 U/mL human α-thrombin and 10 μM S-2238.

The inhibitors were dissolved in water or dimethyl sulfoxide, if required. The concentration of dimethyl sulfoxide used (final concentration 3.3%) had no effect on the enzyme activity. The enzyme was incubated with the inhibitor for three minutes at room temperature prior to starting the reaction with the addition of the substrate. Production of pNA was followed optically at 405 nm. The concentration of compound that inhibited half the enzymatic activity (IC_{50}) was determined using KaleidaGraph from triplicate determinations and each IC_{50} curve was replicated at least four times. The K_i values were determined from the IC_{50} values using relation $K_i = IC_{50}/(1+[S]/Km)$.[12] The K_m for the substrate (s-2238) was experimentally determined to be 2.54 ± 0.3 μM obtained by curve fitting (KaleidaGraph) the enzyme velocity vs. substrate concentration data to the Michaelis-Menten equation.

Acknowledgement

We thank Drs S. D. Kimball and Jack Z. Gougoutas for their many helpful discussions and continued interest.

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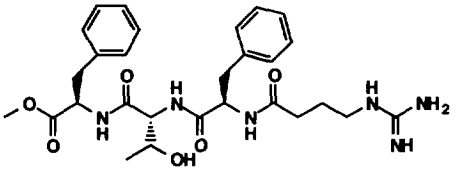
5. The coordinates of α-thrombin were taken from the structure of PPACK-α-thrombin (ref 6a). The molecular model of **1** was constructed using SYBYL (ref 13) and minimized with Macromodel/BatchMin (ref 14) and then docked using DGEOM (ref 15) with hydrogen bond distance contraints between the amidino terminus nitrogens and the carboxyl oxygens of Asp189. Fifty trial DGEOM structures were generated for **1**. These were minimized in the active site of thrombin with BatchMin employing the AMBER (ref 16) 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 minimizations employed a distance dependent dielectric constant of 2r, the electrostatic cutoff was 12 Å, van der Waals cutoff was 7 Å, and the H-bonding cutoff was 4.0 Å. The results were visualized using SYBYL running on a Silicon Graphics 420VGX.

6. (a) Numbering of thrombin residues is based on that for chymotrypsin: Bode, W.; Mayr, I.; Baumann, U.; Huber, R.; Stone, S. R.; Hofsteenge, J. *EMBO J.* **1989**, *8*, 3467. (b) Proximal and Distal pockets as used by: Banner, D. W.; Hadvary, P. *J. Biol. Chem.* **1991**, *266*, 20085.

7. The structure of **BMS-183507**:



8. A lipophilic moiety binding at the proximal pocket region enhances the potency of retro-binding thrombin inhibitors. Results to be published elsewhere.

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10. Based on the SAR for the retro-binding peptide series, introduction of the fluorine atom was expected to enhance activity. These results will be published elsewhere.

11. Compound **13** was derived from cyclohexanone following the synthetic scheme outlined in Figure 2.

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(*Received in USA* 15 *March* 1996; *accepted* 13 *May* 1996)